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Review

Mitochondrial energy metabolism and apoptosis regulation in glioblastoma

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ABSTRACT

Glioblastoma is the most aggressive form of gliomas and is associated with short survival. Recent advancements in molecular genetics resulted in the identification of glioma genomic, epigenomic and transcriptomic hallmarks, and multidimensional data allowed clustering of glioblastomas into molecular subtypes. Parallel with these developments, much scientific attention has been attracted by the exploration of two functional processes linked to mitochondrial regulation. One of these processes involves genomic and mitochondrial gene mutations, mitochondrial protein expression modifications and altered metabolic regulation that define glioblastoma. The second mitochondrially-centered process involves complex molecular interactions and pathways that influence the extrinsic or the intrinsic mechanisms of apoptosis regulation and may underlie the uncontrolled spreading, recurrence and drug resistance of glioblastoma. While the available data are not yet comprehensive, these two complex processes represent important aspects of tumor cell biology, which may provide complementary opportunities for therapeutic manipulations of this highly resistant tumor type.

Contents

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1. Introduction

Gliomas are graded I–IV by the World Health Organization. Glioblastoma (GBM) is the most advanced (grade IV), most aggressive and most frequent form of gliomas (Dunn et al., 2012). Tissue diagnosis of GBM is defined based on histomorphology along with the presence of cell proliferation, necrosis and angiogenesis. The 5-year survival rate is 4.5%, while the 10 year survival rate is 2.7% for patients with GBM (Griguer and Oliva, 2011). The standard of care includes wide surgical resection, irradiation and chemotherapy. The recent addition of the alkylating agent, temozolomide (TMZ) to radiotherapy increased the 2-year survival from 10.4% (radiotherapy alone) to 26.5% (radiotherapy+TMZ), and resulted in a 14.6 month increased median survival (Griguer and Oliva, 2011). Unfortunately, the tumor eventually recurs in all patients, but there are variations in the progression free survival dependent upon the patient's age, gender, extent of surgical removal of pathology, and the tumor biology and genetics, microenvironmental factors and the presence of residual cancer stem cells (CSC). Much effort has been made to better define markers of GBM variability and individual molecular targets for therapy.

At histological level, GBMs are notoriously heterogeneous. The tumor cells are characterized by pleiomorphism, glioblastoma stem cells (GSC) may be present in varying numbers, and primitive neuroectodermal or mesenchymal elements often admix with glial elements (Phillips et al., 2006; Prayson, 2009; de Almeida Sassi et al., 2012). Histological heterogeneity is observed not only among, but also within GBMs. A new definition of inter-tumor heterogeneity was recently proposed based on multi-dimensional molecular (genomic, transcriptomic and epigenomic) studies carried out by The Cancer Genome Atlas Network (TCGA). The TCGA analyses showed clustering of GBMs into four subgroups, namely proneural, neural, classical and mesenchymal, that correlate with biological properties of tumors and measures of clinical outcome (The Cancer Genome Atlas, 2008; Verhaak et al., 2010). Analyses of data from microarray studies identified 38 genes predicting GBM survival (Colman et al., 2010), which could be confined to a 9-gene-expression panel with utility in clinical practice. To provide an even simpler GBM classifying tool, Le Mercier et al. (2012) tested the expressions of EGFR, PDGFRA and p53 proteins by quantitative immunohistochemistry in formalin-fixed, paraffin-embedded clinical specimens. Based on these three markers alone, the separation of two GBM subtypes was feasible: (1) The classical subtype with EGFR+, p53- and PDGFRA- staining; and (2) the proneural subtype with p53+ and/or PDGFRA+ staining. These observations reproduced main points of previous genomic and transcriptomic studies and offered prognostic significance (Verhaak et al., 2010; Le Mercier et al., 2012).

Somatic mutations and expression variations in the EGFR gene received probably the most attention among all molecular markers, since this transmembrane molecule is essential in glioma cell biology while also amenable to therapeutic interventions with tyrosine kinase inhibitors, monoclonal antibodies, EGFR vaccines, and RNA-based therapies (Kalman et al., 2013). Mutations in the EGFR gene in GBM are mainly located in its extracellular domain, and include deletions, insertions and missense mutations. Among them, the EGFRvIII mutant with a deletion involving exons 2–7 is the most frequent pathogenic variant detected in approximately 30% of GBMs. In normal conditions, after the engagement of EGFR and its ligand (*e.g.* the epidermal growth factor, TNFα or amphiregulin), the intracellular tyrosine kinase domain of the receptor activates a cascade of signaling molecules and through that, regulates cell proliferation, survival, apoptosis, migration, and gene transcription (Kalman et al., 2013). The mutated EGFR is constitutively active and contributes to uncontrolled cell proliferation and altered cell biology. Targeting overexpressed wild type or mutated EGFR is an attractive therapeutic approach in those GBMs in which a sufficient proportion of cells are positive for these molecules (Kalman et al., 2013). For both prognostic and therapeutic purposes, intratumor distribution of molecular markers has great clinical relevance (Scottoriva et al., 2013). We recently reviewed the literature concerning the degree and nature of intratumor molecular heterogeneity in the context of tumor behavior and response to therapies and proposed that such evaluation of individual tumors may form a basis for clinical decisions (Eder and Kalman, 2014).

The complexity and interdependence of somatic alterations that define GBM biology is increasingly recognized. We discuss here two pathways involving complex mitochondrial functions linked to tumorigenesis. One of the pathways integrates genomic and mitochondrial (mt)DNA mutations, metabolic changes and tumor formation. The second pathway links altered gene expression and decreased apoptosis together with uncontrolled cell proliferation and tumor formation. Recognizing unique features of mitochondrial genetics, energy metabolism and apoptosis regulation in GBM has the practical relevance of offering new or supplementary targets for therapy.

2. Mitochondrial genetics and function

2.1. *Mitochondrial genetics*

Mitochondria are cytoplasmic organelles bounded by two distinctly different membranes and comprised of four different physical and functional compartments. The inner mitochondrial membrane is highly convoluted to form cristae thereby increasing the surface area and thus the bioenergetic capacity of the organelle. Mitochondria in oxidatively active cells have more extensive cristae. In addition to being the energy- generating center of the cell by synthesizing ATP via

oxidative phosphorylation (OXPHOS), mitochondria perform numerous other important cellular functions, including anabolic/catabolic reactions, metabolic regulation, signal transduction, calcium homeostasis, reactive oxygen species (ROS) generation, redox control and apoptosis.

Cells have variable numbers of mitochondria (ranging from a few to thousands) depending upon their energy needs. In general, the more metabolically active the cell, the greater the number of mitochondria. Mitochondria can exist as single organelles or associate into a complex three-dimensional network. Mitochondria constantly fuse and divide and are actively transported to specific subcellular locations. The size, shape and number of mitochondria are controlled by fission and fusion, vary with cell type and metabolic requirements of the cell, undergo changes during different stages of the cell cycle, and change with physiological/pathological conditions. Thus, mitochondria are individually and collectively dynamic organelles.

Each mitochondrion has between 2 and 10 mtDNA molecules. Mitochondrial DNA is a circular, 16,500 base pair – containing double stranded DNA that encodes 13 OXPHOS protein subunits, 22 tRNAs and two rRNA molecules. There is also a non-coding, so called D-loop region involved in communication with nuclear factors (Kalman et al., 2007). The mammalian mitochondrial proteome comprises ~1700 proteins and all but 13 are encoded by nDNA. All mitochondria in all cells have common functions, however, mitochondria in different cells have different bioenergetic and metabolic capabilities tailored to meet the needs of the cell type in which they reside. In other words, mitochondria are metabolically and energetically heterogeneous.

Each of the OXPHOS enzymes, Complexes I–V, is a multi-subunit enzyme. Complex I is the first and largest electron transport chain (ETC) complex composed of 45 subunits of which only seven subunits are coded by mtDNA while 38 are coded by nuclear (nDNA) genes. Complex II has four subunits that are exclusively coded by nuclear genes. The remaining three enzyme complexes (III–V) are also multi-subunit enzymes with mixed mtDNA/nDNA encoding (Kalman et al., 2007; DiMauro, 2013) (Fig. 1a).

Mitochondrial DNA is a highly polymorphic molecule susceptible to a high mutational rate, which is caused by the lack of protective histones, proximity to the site of the production of (mutagenic) ROS and relatively limited DNA repair mechanisms. Biologically neutral (or mildly deleterious) mutations are not subjects of negative selection, and are retained as polymorphisms in the population. Mitochondrial DNA polymorphisms are thus evolutionarily old and present in all mtDNA molecules in a cell (homoplasmic), as opposed to mtDNA pathogenic mutations that often only affect a proportion of mtDNA molecules within a cell (heteroplasmic) and vary in proportion from tissue to tissue.

To date, more than 260 pathogenic mtDNA point mutations and large scale deletions have been associated with human diseases. As mitochondria in the offspring are exclusively derived from the oocyte, mtDNA and mtDNA point mutations are maternally inherited (large scale deletions are typically sporadic). The number of all pathogenic mutations affecting the approximately 1700 nDNA- encoded mitochondrial proteins (including OXPHOS structural and assembly proteins, metabolic enzymes, proteins involved in mtDNA maintenance and replication, protein translation, membrane integrity and dynamics) is estimated to be in the thousands (DiMauro, 2013). These mutations follow Mendelian (autosomal recessive, dominant or X-linked) inheritance (Fig. 1b).

Not only inherited mtDNA mutations may contribute to disease, but also acquired, somatic mutations and deletions. Post-mitotic tissues with the highest energy metabolism such as the brain and muscle are the most prone to the accumulation of somatic mutations/deletions during ageing and (neuro)degenerative disease (Fig. 1c) (Blokhin et al., 2008, Kraytsberg et al., 2006; Bender et al., 2006). When these random somatic mtDNA alterations reach a certain threshold, the cell and tissue will have compromised energy production further contributing to the degenerative pathology. Cancer cells with high metabolic activity, impaired repair mechanisms and increased genomic instability are prone to the accumulation of somatic DNA mutations including mtDNA mutations, while mtDNA mutations are also believed to contribute to cancer genesis and biology (Fig. 1c). The transcriptome profiles of cancer cells, related to the accumulation of somatic mutations in the nuclear genome and to epigenetic alterations, may result in profound changes in the nuclear – mitochondrial intergenomic communications leading to an altered mitochondrial gene expression. Thus, changes in mtDNA and genomic DNA along with altered gene expression profiles contribute to the compromised mitochondrial machineries of energy metabolism and apoptosis regulation (Fig. 1c).

2.2. *Mitochondrial regulation of energy metabolism*

Under aerobic conditions, normal non-proliferative (differentiated) cells predominately utilize glucose to generate pyruvate that is converted to acetyl CoA and metabolized through the citric acid (Krebs or tricarboxylic acid – TCA) cycle in mitochondria. Hydrogen atoms are collected from the oxidation of pyruvate, fatty acids and amino acids in the Krebs cycle into NADH and $FADH_2$ which then donate these reducing equivalents to the ETC for ATP synthesis. As electrons donated by NADH and FADH2 flow down the ETC to oxygen, energy is released and used to translocate H+ out of the matrix into the intermembrane space. This process establishes a transmembrane H+ gradient across the IMM. This proton motive force (PMF) is comprised of a large charge/electrical gradient ($\Delta\Psi$) and a smaller H+ concentration gradient (ΔpH). Mitochondria are now energized and capable of doing work: the PMF can be used to synthesize ATP, accumulate calcium, transport substrates and import proteins. When H+ move back into the matrix by passing through the ATP synthase (Complex V), the energy of the H+ gradient is used to phosphorylate ADP to produce ATP (Kalman et al., 2007). Intermediate metabolic products of mitochondrial metabolism contribute to the synthesis of nucleotides, amino acids and lipids used for cell maintenance and proliferation.

At low oxygen concentrations or when pyruvate oxidation by pyruvate dehydrogenase (PDH) in mitochondria is slowed (as in rapidly proliferating and de-differentiated cells), the rate of glycolysis is very high (Marie and Shinjo, 2011;

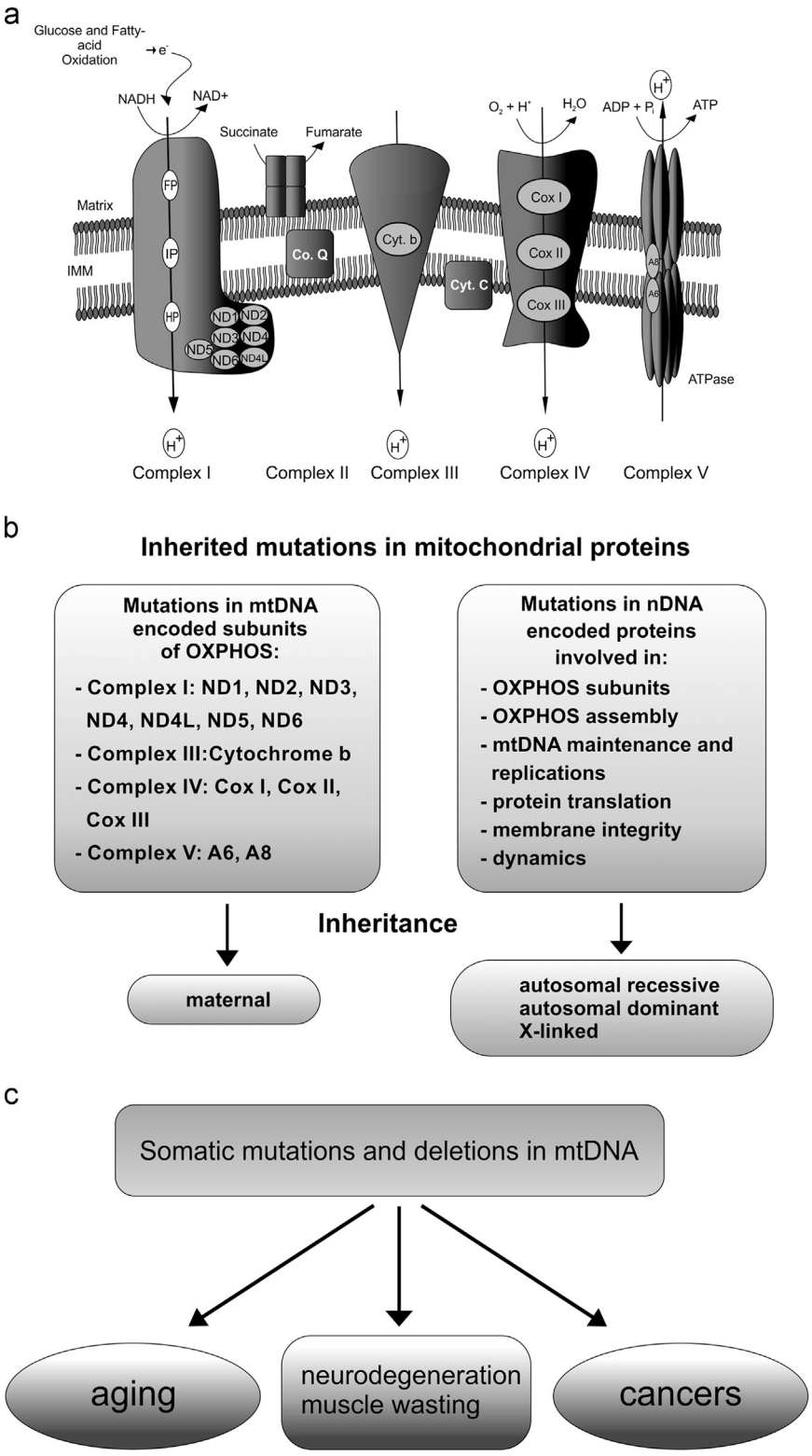

Fig. 1 – Mitochondrial genetics. (a) depicts subunits of OXPHOS enzyme complexes encoded by mtDNA and nDNA (Kalman et al., 2007; DiMauro 2013), (b) highlights that mutations in mitochondrial proteins may be inherited maternally when the affected gene is encoded by mtDNA, or transmitted in autosomal recessive, dominant or X-linked manner when the affected gene is encoded by nDNA, and (c) indicates that acquired somatic mutations in mtDNA contribute to aging, neurodegenerative diseases and muscle weakness, and occur in solid tumors including GBM.

Mukherjee et al., 2013). Under all conditions, sustained operation of glycolysis necessitates that NADH generated in the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) reaction be reoxidized. In the presence of oxygen, this is accomplished by one of two systems that shuttle reducing equivalents into the mitochondrion for reoxidation, the malate-aspartate shuttle or the glycerol-3-phosphate dehydrogenase shuttle. Under anaerobic conditions or when PDH is inhibited, NADH reoxidation is instead accomplished by lactate dehydrogenase (LDH), a reaction that converts pyruvate to lactate and reoxidizes NADH thereby recycling NAD+ for G3PDH. Lactate formed by LDH is exported from cells to the extracellular medium. Cytosolic reoxidation of NADH by LDH does not generate ATP and diverts pyruvate away from mitochondria for oxidation. Thus, glycolysis alone produces only two moles of ATP per molecule of glucose oxidized in the phosphoglycerate kinase and pyruvate kinase reactions. By contrast, the complete oxidation of pyruvate to CO2 by the Krebs cycle in the presence of O2 generates 30 mol of ATP per mole glucose oxidized. In the presence of O2 another 4 or 6 mol of ATP/mole glucose are generated depending upon whether cytosolic NADH from G3PDH is reoxidized by the glycerol-3-phosphate dehydrogenase or malate-aspartate shuttle, respectively, for a total of either 34 or 36 mol ATP/mole glucose synthesized by OXPHOS (Marie and Shinjo, 2011) (Fig. 2).

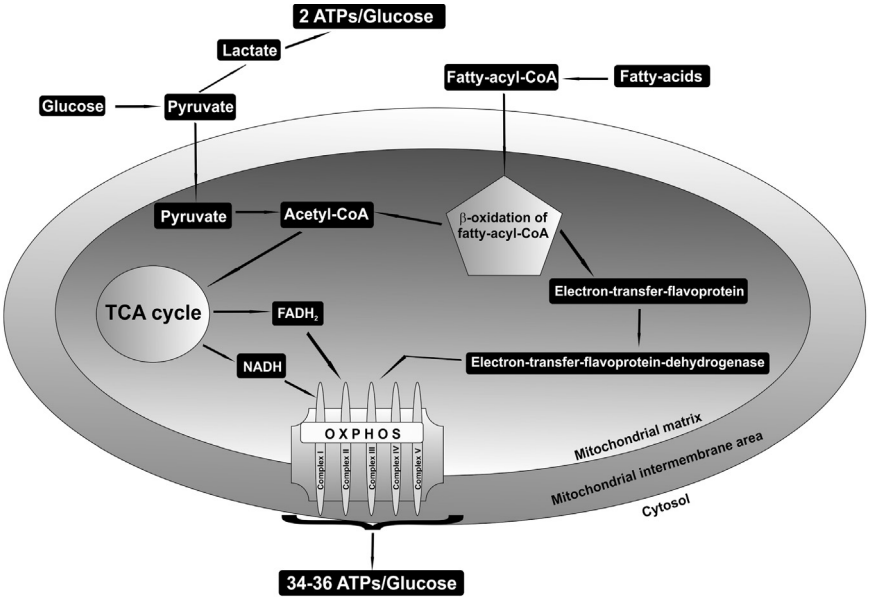

Fig. 2 – Aerobic and anaerobic energy metabolism. It highlights that in a highly simplified manner, main differences of aerobic and anaerobic energy metabolism in normal cells.

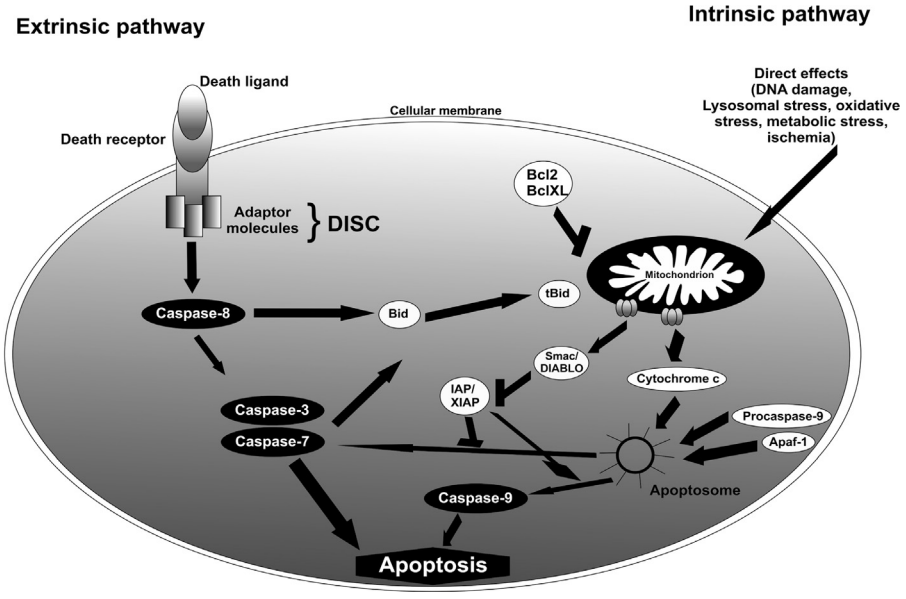

Fig. 3 – Extrinsic and intrinsic pathways of apoptosis. It depicts main elements of the extrinsic and intrinsic pathways of apoptosis regulation.

2.3. *Apoptosis regulation*

The regulation of apoptosis is related to two signaling pathways: (1) an extrinsic (death receptor) pathway and (2) an intrinsic (mitochondrial) pathway (Fig. 3). The death receptor pathway is initiated by ligands that engage receptors of the tumor necrosis factor (TNF) superfamily, among which the TNF-related apoptosis-inducing ligand (TRAIL) receptors are the best characterized. Engagement of TRAIL with its ligand leads to the assembly of the death-inducing signaling complex (DISC) and a sequential activation of cysteine proteases, called caspases. First, the initiator caspases are cleaved and activated, such as caspase-8. Caspase 8 then cleaves and activates the effector (also called executioner) caspases including caspase-9 and -3 (Kalman et al., 2007). The intrinsic mitochondrial pathway of apoptosis can be initiated by many factors including changes in cellular pH, ion concentrations, drop in ATP synthesis, decrease in the mitochondrial transmembrane potential, and upregulation of proapoptotic and downregulation of antiapoptotic components of the B-cell lymphoma 2 (Bcl-2) family proteins. Any of these events may lead to the opening of the mitochondrial permeability transition pore and the release of cytochrome-c and second mitochondria-derived activator of caspase (Smac/DIABLO) from the mitochondrial inter-membrane space into the cytosol. These small molecules in the cytosol trigger the formation of the apoptosome that includes the apoptotic protease activating factor-1 (Apaf-1) (Kalman et al., 2007, Murphy et al., 2013). The assembly of the apoptosome leads to the activation of effector caspase-9 and -3 (Kalman et al., 2007, Cristofanon and Fulda, 2012). Smac/DIABLO in the cytoplasm blocks the X-linked inhibitor of apoptosis protein (XIAP), which inhibit caspases (Murphy et al., 2013). In this manner, the effector caspases get activated and cleave their target proteins involved in cell death. Pro- and antiapoptotic members of Bcl-2 proteins control the release or retention of small molecules from the mitochondrial intermembrane space into the cytoplasm. The Bcl-2 family protein BH3-interacting domain death agonist (Bid) is cleaved by caspase-8 into truncated Bid (tBid) that translocates to the mitochondrial membrane and initiates the permeabilization of the outer mitochondrial membrane. The effector caspase-3 may also cleave Bid into tBid, thereby amplifying the apoptotic processes (Kalman et al., 2007, Cristofanon and Fulda, 2012).

3. Alterations in mitochondrial genetics and function in GBM

3.1. *MtDNA alterations in GBM (Table 1)*

Several studies proposed that variants throughout mtDNA as well as in specific genes, such as ND5, are associated with tumor induction or progression (Polyak et al., 1998; Ohta 2006; He et al., 2010; Shen et al., 2010; Takibuchi et al., 2013). Studies on cybrids carrying specified mtDNA variants after the depletion of the cells' own mtDNA, but having their original nuclear background, showed that a G>A SNP at nucleotide 13,997 and a frame-shift mutation at nucleotide 13,885 (in ND6) were involved in metastatic processes (Imanishi et al., 2011; Griguer and Oliva, 2011). Further cybrid studies suggested that mtNA mutations are involved in the induction of tumorigenesis (Ohta 2006; Mito et al., 2013; Takibuchi et al., 2013). In contrast, numerous studies rejected the idea that certain mitochondrial mutations would cause tumorigenesis (Kirches et al., 2001; Kiebish et al., 2005). Seoane et al. (2011) and Iglesias et al. (2012) argued that mtDNA integrity is essential for tumor cells to survive the increased ROS exposure related to high rates of metabolism and tumorigenesis. In consensus, Khaidakov and Shmookler Reis (2005) proposed that there is an active negative selection against deleterious mtDNA mutations during clonal propagation in tumors. It is also note worthy that pathogenic mtDNA mutations, which cause mitochondrial dysfunction and disease, are not associated with the development of any form of cancer (Ishikawa and Hayashi, 2010). Overall, the literature has been conflicting as to whether mtDNA mutations are causes or consequences of cancer genesis, and this conflict exists also concerning the involvement of mtDNA mutations in GBM genesis (Table 1).

Similar to other cancers, GBM cells carry mtDNA mutations preferentially in the D-loop and protein coding regions (Kirches et al., 2001; Carew and Huang 2002; Polyak et al., 1998). The D-loop region of these tumors contains not only nucleotide transitions but also new length variations in repeat regions reflecting instability. Both synonymous and missense mutations were detected in coding regions. These mutations may occur in an early stage of glioma genesis and often reach a homoplasmic state (meaning that all mtDNA molecules in a cell carry the same mutant sequence) (Kirches et al., 2001). Since 89% of base substitutions in GBM were found at sites highly polymorphic in normal tissues, the authors proposed that the same mechanisms that generate mtDNA polymorphisms during phylogenesis may be enhanced in gliomas and result in somatic mutations (Kirches et al., 2001). In addition, Kirches et al. (2002) also reported the lack of accumulation of somatic mtDNA variants in low grade gliomas. Furthermore, Kiebish and Seyfried (2005) detected no pathogenic mtDNA mutations in either induced or spontaneous mouse brain tumors. Altogether, these findings argue against the role of somatic mtDNA mutations in the development of GBM.

However, there are also data that conflict with this conclusion. A T->C transition at nucleotide 14,634 in the ND6 subunit of Complex I was detected in hypoxia resistant but not in hypoxia sensitive GBM cell lines (DeHaan et al., 2004). Computational molecular analyses of the wild type and mutated ND6 3D protein structures predicted that the T14,634C mutation alters the structure and orientation of the trans-membrane helices of the ND6 protein, which represent proton channels. Therefore, the T14,634C mutation may have functional significance.

Using next generation sequencing and high resolution melt analyzes, Yeung et al. (2014) reported that both coding and non-coding region mtDNA mutations accumulate in GBM when compared to the non-cancerous part of the corresponding brain tissue, and distinct sets of mtDNA variants may be detected in the tumor and normal sample cohorts. Interestingly, no somatic variants were detected in the *ND1* gene (NADH dehydrogenase-1) and within the 22 mtDNA encoded

Table 1 – Alterations in mitochondrial genes, proteins and energy metabolism in GBM.

Alterations in GBM	Effect	References
mtDNA D-loop and coding region mutations	Occur in early stage of glioma genesis, may affect growth and metabolism	Kirches et al 2001, Carew and Huang 2002 and Polyak et al 1998
mtDNA T14,634C in ND6 subunit of Complex I	Alters the structure of the ND6 protein, confers hypoxia resistance	DeHaan et al 2004
Mutations in ND4 or ND6 subunits	Shift tumor metabolism towards glycolysis	Yeung et al 2014
IMP2	Supports OXPHOS maintenance	Janiszewszka et al. (2012)
IDH1 mutations (common R132) (predominantly found in secondary GBMs)	Reduce the binding of isocitrate (loss of function)	Yan et al., (2009), Zhao et al. (2009), Krell et al. (2011); Preusser et al. (2011)
	Lead to decreased amounts of aKG and NADPH (loss of function)	
	Produce R(-)-2HG	Ward et al. (2012)
	Promote reduction of αKG to D-2HG (gain of function)	Dang et al. (2009)
	Promote oxidation of NADPH to $NADP^+$ (gain of function)	
	Cause DNA hypermethylation	Turcan et al. (2012)
IDH2 mutations (common R172 and R140) (predominantly found in secondary GBMs)	Reduce the binding of isocitrate (loss of function)	Yan et al. (2009), Zhao et al. (2009) and Krell et al. (2011)
	Lead to decreased amounts of αKG and NADPH (loss of function)	
	Produce R(-)-2HG	Ward et al. (2012)
	Promote reduction of αKG to D-2HG (gain of function)	Dang et al. (2009)
	Promote oxidation of NADPH to $NADP^+$ (gain of function)	
Shift in PKM isoforms - favoring enhanced PKM2	Shifts metabolism from OXPHOS to aerobic glycolysis and enhance tumor aggressiveness	Mukherjee et al. (2013)
Elevated PDK1, EGFR, and HIF-1a caused by hypoxia	Promote aerobic glycolysis and enhance tumor aggressiveness	Velpula et al. (2013)
PDK activation	PDH inhibition leading to enhanced glycolysis and enhanced tumor growth	Michelakis et al. (2010), Sutendra and Michelakis 2013
EGFRvIII mutant form	Mitochondrially located EGFRvIII decrease glucose dependency	Cvrljevic et al. (2011)
	Enhance OXPHOS	
EGFR and EGFRvIII translocate into the mitochondia	Protect from mitochondria-mediated apoptotic death	Cao et al. (2011)
Mutations in EGFR, EGFRvIII, PTEN, PDGFRα, p53, pAkt, pErk, Enk4a/ARF	Enhanced glycolysis as well as glucose oxidation in complex metabolic processes to support tumor growth	Marin-Valencia et al. (2012)
Enhanced cytochrome c oxidase activity	Facilitates a metabolic switch from glycolysis to OXPHOS	Griguer et al. (2013)

Summarizes some of the known alterations in mitochondrial genes and proteins linked to altered energy metabolism in GBM. Abbreviations: mtDNA – mitochondrial DNA; IDH1 and IDH2 – isocitrate dehydogenase 1 and 2; EGFR – epidermal growth factor receptor; PKM – pyruvate kinase M isoform; PDH – pyruvate dehydrogenase; PDK – pyruvate dehydrogenase kinase; HIF1a – hypoxia inducible factor 1α.

tRNA genes in the two sample cohorts. In GBM, mutations occurred with the highest frequencies in the D-loop and the origin of light strand replication regions. ND6 was the most susceptible to, while ND4 had the highest frequency of mutations in the coding regions. Both ND4 and ND6 are mtDNA encoded subunits of Complex I, suggesting that Complex I may be a new target for pharmaceutical intervention. It is also note worthy that certain sets of acquired mtDNA variants were pathology-related even though many mutated regions overlapped between affected and non-affected specimens. Mutations within the origin of light strand replication, *COX II* and *ND4L* genes appeared to be GBM specific, while variants in the *ND2* and *ND3* genes as well as in the *ATP6*, *ATP8* and *COX III* genes appeared to be specific for the normal samples. When GBM cells were depleted to various degrees of their mtDNA and transplanted into immune deficient mice, mtDNA mutations, similar to the ones in the original tumor and in other GBMs, occurred during tumor growth and preferentially within the ND4 and ND6 regions. These mtDNA mutations negatively influenced the ETC, shifted tumor metabolism towards glycolysis and promoted cell proliferation, lending further support to the hypothesis that mtDNA mutations do have a pathogenic role in GBM development and maintenance (Yeung et al., 2014).

Marucci et al. (2013) studied 10 GBM cell lines that had mono- and multinucleated tumor cells with abundant cytoplasms due to enlarged and increased numbers of mitochondria (designated as oncocytic characteristics). Nine of these ten cell lines also showed increased mtDNA copy numbers. When patients with oncocytic GBM were compared to those

with non-oncocytic GBM, a trend for longer survival was observed. This finding was unexpected, since the oncocytic phenotype had previously been associated with mtDNA mutations and impaired OXPHOS triggering compensatory mitochondrial proliferation and a worse tumor outcome (Iommarini et al., 2013). However, the study by Marucci et al. (2013) suggests that the oncocytic GBM phenotype may be less proliferative and aggressive and mtDNA sequences should be further studied in these particular GBM types.

While mtDNA transcription is tightly linked to mtDNA replication, OXPHOS function and energy production requires transcription. Embryonic stem cells (ESC) are highly proliferative, require much energy but strictly regulate and maintain a low copy of mtDNA during cell differentiation by synchronizing cell differentiation and mtDNA replication (Dickinson et al., 2013). Dickinson et al. (2013) investigated a well characterized GBM cell line, HSR-GBM1 that expresses neural stem cell (NSC) makers (NESTIN, MUSASHI1 and prominin 1 [CD133]), and differentiates *in vitro* into neurons and astrocytes. The question was raised whether or not GBM cells can modulate their mtDNA copy numbers and chromosomal gene expression during differentiation similar to that of human NSCs. The study revealed that during differentiation, the multipotent GBM cells did not follow the same strict regulation observed in NSCs regarding the coordination among mtDNA copy numbers, patterns of gene expression and increased OXPHOS. Partial depletion of mtDNA rescued mtDNA replication and promoted cell differentiation in the GBM cell line, while prolonged depletion lead to impaired mtDNA replication, reduced cell proliferation and enhanced expression of early developmental and pro-survival markers. When GBM lines with depleted mtDNA were transferred into immune deficient mice, significantly longer time was required to form tumors compared with non-depleted cells, and there was a correlation between mtDNA depletion and the number of tumors formed. The tumors derived from mtDNA depleted GBM lines recovered their mtDNA copy numbers during tumor formation. These observations emphasize the importance of mtDNA in the initiation and maintenance of GBM tumorigenesis (Dickinson et al., 2013).

How the communication occurs between the nuclear and mitochondrial genome has been investigated mostly in *in vitro* conditions. Janiszewska et al. (2012) showed that insulin-like growth factor 2 mRNA-binding protein 2 (IMP2, IGF2BP2) regulates OXPHOS in primary GBM sphere cultures (gliomaspheres), an *in vitro* model for cancer stem cell (CSC). In this system, IMP2 binds multiple mRNAs that encode OXPHOS subunits and interacts with Complex I proteins. Depletion of IMP2 decreased the rate of oxygen consumption and both Complexes I and IV activity in the gliomaspheres, which lead to impaired clonogenicity *in vitro* and decreased tumorigenicity *in vivo*. Inhibition of OXPHOS also eliminated clonogenicity in the tumor cells. The authors proposed that gliomaspheres depend on mitochondrial OXPHOS for their energy production and survival. IMP2 supports OXPHOS maintenance by transporting OXPHOS subunit-encoding mRNAs to mitochondria and contributes to the assembly of Complexes I and IV.

Altogether, these studies suggest the importance of altered mtDNA and mtDNA expression regulation in defining development and biology of cancer cells including CSCs, influencing tumor clonogenicity and controlling outcomes in GBM. However, the available data are neither unequivocal nor comprehensive. Further studies are needed to better understand the roles of mtDNA mutations and expression regulation in GBM development, metabolism and possibly chemosensitivity or resistance.

3.2. *Alterations in mitochondrial energy metabolism in GBM (Table 1)*

In normal cells, the rate of glycolysis and lactate production decrease with the introduction of oxygen, a metabolic response known as the Pasteur effect. By contrast, malignant cells take up and use 5–10 times more glucose and convert it to lactate even though oxygen is present and the cells may have normal rates of respiration and OXPHOS. This aberrant Pasteur effect is known as the Warburg effect (Warburg, 1956). Although rapidly proliferating normal cells perform aerobic glycolysis, they stop proliferating and revert to OXPHOS when glucose becomes limiting. However, aerobic glycolysis in cancer cells can be many fold higher than in normal cells and consumes large amounts of glucose (Oudard et al., 1996; Dickinson et al., 2013). The many metabolic intermediates generated through glucose catabolism via glycolysis and the pentose phosphate pathway provide necessary biosynthetic intermediates required for synthesis of nucleotides, nonessential amino acids and fatty acids thereby sustaining high rates of cell proliferation (Marie and Shinjo, 2011; Dickinson et al., 2013). Increased glycolysis does not cause cancer but does confer a growth advantage allowing malignant cells to survive and grow rapidly, especially when oxygen availability is limited as it may be in solid tumors. This metabolic reprogramming results from acquired gene mutations and expression alterations.

Mutations in subunits B,C and D of succinate dehydrogenase (SDH) and fumarate hydratase (FH), key enzymes of the TCA cycle in mitochondria, lead to the development of tumors such as paragangliomas, pheochromocytomas and renal cell carcinoma (Bayley and Devilee, 2010). SDHAF2 (SDH5), another nuclear encoded mitochondrial protein required for flavination of SDH is also known as a paraganglioma-related tumor suppressor gene. Mutations in isocitrate dehydrogenase (IDH), including cytosolic IDH1 and mitochondrial IDH2, have been detected in several solid and hematological tumor types (Mardis et al., 2009; Dang et al., 2009; Bayley and Devilee, 2010).

The best studied gene mutations in GBM are the IDH mutations. IDHs catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG) and the reverse reductive carboxylation of αKG to isocitrate. IDH1 is localized to the cytoplasm and peroxisomes, while IDH2 and IDH3 are localized to mitochondria. IDH1 and IDH2 are both $NADP^+$-dependent, homodimeric enzymes that share a high degree of sequence homology and show similar protein structures (Krell et al., 2011). IDH3 is a heterodimer composed of two α, one β and one γ chains, – is NAD^+ dependent and is strongly allosterically regulated by ATP, citrate, calcium and NADH/NAD+. IDH1 and

IDH2 play protective roles in cells by generating NADPH to maintain GSH and serve as electron donor in numerous biosynthetic reactions, including lipid biosynthesis. By contrast, IDH3 plays a central role in the Krebs cycle and catabolism by generating NADH for ATP synthesis by the ETC. The occurrence of IDH1 mutations was revealed by a genome-wide sequence analysis in 12% of GBM in 2008 (Parsons et al., 2008), followed by the discovery of IDH2 mutations in gliomas a year later (Yan et al., 2009). Importantly, IDH1 and IDH2 mutations were frequently found in grade II-III gliomas and 70–75% of secondary GBMs, but only rarely in about 5% of primary GBMs (Krell et al., 2011). These mutations occur more frequently in younger patients with GBM and are associated with better outcome (Yan et al., 2009; Hartmann et al., 2009; Krell et al., 2011). The most common IDH1 mutation affects R132, while the IDH2 mutations affect R172 and R140 (R172 in IDH2 is analogous to R132 in IDH1; these arginine sites represent binding sites for isocitrate) (Preusser et al., 2011). The aforementioned mutations reduce the binding of isocitrate to IDH1 or IDH2, and result in decreased amounts of αKG and NADPH. This loss of function likely is the result of a dominant negative effect exerted by the heterodimers which form between the mutated and wild type proteins, and the decreased αKG may result in decreased activity of the αKG-dependent HIF prolyl-hydroxylases (Yan et al., 2009; Zhao et al., 2009; Krell et al., 2011). Mutant IDH1 and IDH2; however, also gain a new (neomorphic) enzymatic activity that favors the reduction of αKG to D-2-hydroxyglutarate (D-2HG) while oxidizing NADPH to $NADP^+$. D-2HG can be detected in mutant GBM cells as well as the extracellular medium (Dang et al., 2009). Excessive 2HG may also result from mutations in the D and L-2-hydroxyglutarate dehydrogenase enzymes (D2HGDH, L2HGDH). To sort out if IDH mutations are critical for tumor formation or the accumulation of 2HG, Krell et al. (2011) screened 47 GBM specimens for the presence of mutations in IDH, D2HGDH and L2HGDH genes. The study revealed the presence of R132 mutation in IDH1 in 12% of samples, but no mutations were detected in the other IDH or 2HGDH genes. The authors concluded that both loss of function of IDH and accumulation of 2HG are likely required for tumorigenesis (Krell et al., 2011). Ward et al. (2012) identified additional mutations in IDH enzymes in various tumors, and proposed that IDH mutations may contribute to cancer through either the neomorphic R enantiomer 2HG production (due to the catalytic activity of the new mutant enzymes) or to reduced wild-type enzymatic activity.

Metabolic consequences of IDH mutations raise the possibility that *in vivo* identification of these mutants may be achieved by the detection of the elevated oncometabolites. IDH1 mutants, however, not only modify cell metabolism but also have an effect on the epigenome (Turcan et al., 2012). Analyses of the epigenome in a set of intermediate-grade gliomas revealed a CpG island methylator phenotype that is dependent on the presence of IDH mutants. The introduction of mutant IDH1 into primary human astrocytes altered certain histone marks and induced extensive DNA hypermethylation. Epigenetic changes associated with the IDH mutations activated important gene expression programs that were characteristic of the CpG methylation-positive proneural but not of other GBM subtypes, and were predictive of better survival. These observations show that IDH mutations may act through several pathways in GBM: exert loss of function for isocitrate binding and αKG synthesis, lead to accumulation of the oncometabolite 2HG, and cause hypermethylation of DNA, altogether indicating a complex interdependence of genomic, epigenomic and metabolic changes in GBM.

The link between gene expression alteration, metabolic changes and tumorigenicity is further exemplified by the changes in pyruvate kinase M isoforms (PKM) that control aerobic glycolysis (Christofk et al., 2008). PKM is one of three PKs (PKM, PKL, PKM) (Imamura et al., 1986), and only PKM is associated with cancer. The two, PKM1 and PKM2 isoforms are due to alternative splicing (Noguchi et al., 1986). The PKM1 protein is constitutively active and converts phosphoenolpyruvate (PEP) to pyruvate that is transported to mitochondria for oxidation in the TCA cycle (Mazurek et al., 2001). PKM1 is expressed in most normal adult tissues, but its highest levels are measured in muscle and brain, tissues with the highest rates of OXPHOS befitting their high energy demands. In contrast, PKM2 is primarily expressed in embryonic tissues and cancer cells (Noguchi et al., 1986; Guminska et al., 1997). PKM2 can exist as tetrameric and dimeric forms. The dimeric form has a low affinity for PEP, converts pyruvate to lactate and, unlike PKM1, is allosterically regulated by fructose 1,6 diphosphate, the upstream product of the phosphofructokinase 1 reaction (Gupta, 2010). Consequently, glycolysis is low, the intermediates of glucose metabolism accumulate in tumors, and these intermediates are used to generate the nucleotides and amino acids needed for tumor cell proliferation (Wolf et al., 2010). The maintenance of tumor growth, thus, involves a shift in PKM isoform expression favoring PKM2 and a shift in metabolism from OXPHOS to aerobic glycolysis.

Another study investigated the expression of PKM isoforms and activity as well as the effects of their expression alterations in grade I–IV gliomas (Mukherjee et al., 2013). PKM1 expression and PK activity was low in all glioma cell lines. Low grade gliomas had modestly increased mRNA and protein expression of PKM2, while GBM tumors had significantly increased expression of these molecules. Maintaining low levels of PKM1 expression and low PK activity appeared to be a key to the aggressiveness of GBM, as overexpression of PKM1 slowed GBM proliferation. Interestingly, shRNA knockdown of PKM2 resulted in not only low expression of the molecule and low activity of PK, but also slowed GBM growth. These observations indicate that the grade of gliomas is correlated with PKM isoform expression, and low PK activity and expression of PKM2 are prerequisites for GBM growth (Mukherjee et al., 2013). Further examples of gene expression changes implicate phosphoinositide 3-kinase/protein kinase, MYC, and HIF-1 in brain tumors (Marie and Shinjo, 2011).

Velpula et al. (2013) observed that the expression levels of pyruvate dehydrogenase kinase (PDK1), EGFR, and HIF-1a were elevated in their clinical GBM specimens, and reasoned that hypoxia contributes to the aggressiveness of GBM by promoting aerobic glycolysis. Hypoxia enhances PDK1, EGFR and HIF1α expression, which are not independent of each other. PDK1 expression is regulated by HIF1α and hypoxia-induced PDK1 expression may promote EGFR activation. These molecular interactions in the hypoxic tumor region

may represent a feed-forward loop that can underlie tumor progression. In fact, when siRNA lowered PDK1 and EGFR expression and activation, HIF-1α expression also decreased, and caused a shift from aerobic glycolysis to OXPHOS, which then attenuated GBM proliferation (Velpula et al., 2013).

Multiple studies have demonstrated that the activation of PDK in many different cancer types inhibits pyruvate dehydrogenase (PDH), the enzyme complex responsible for conversion of pyruvate to acetyl coenzyme A (the substrate for the TCA cycle) in the mitochondrial matrix. Phosphorylation and inhibition of PDH by PDK thus diverts pyruvate away from mitochondrial oxidation and favors conversion of pyruvate to lactate in the cytoplasm, thereby enhancing glycolysis. Inhibition of PDK by either siRNA or dichloroacetate (DCA), would shift the metabolism from glycolysis to glucose oxidation, and likely also attenuate the inhibition of mitochondrial apoptosis and reduce tumor cell proliferation (Michelakis et al., 2010; Sutendra and Michelakis, 2013). It was postulated that such a shift in tumors would increase intermediates of the citric acid cycle, mitochondrial ROS, and activate the tumor suppressor p53, nuclear factor of activated T cells and HIF1α. All these changes would suppress tumor growth, angiogenesis and viability. Since DCA-mediated inhibition of PDK enhances mitochondrial function and reduces tumor growth, metabolic manipulations of GBM by PDK inhibitors are promising strategies in the treatments of gliomas (Sutendra and Michelakis, 2013).

Cvrljevic et al. (2011) studied mitochondrial localization and roles of EGFRvIII in the distinct metabolic patterns of GBM. In gliomas with EGFRvIII, there was an intracellular pool of receptor molecules with mannose glycosylation, a sign of delayed processing primarily in the Golgi complex, but also in mitochondria. Activation of a Src family kinase increased, inhibition of it decreased the mitochondrially located EGFRvIII. The effect of Src on EGFRvIII appeared to be mediated by the phosphorylation of EGFRvIII at position Y845. When the phosphorylated EGFRvIII was expressed in a glioma cell line, the molecule was not located to mitochondria, even when coexpressed with constitutive Src. However, low level of glucose enhanced mitochondrial localization of the phosphorylated mutant receptor and under this condition, the EGFRvIII positive cells showed increased proliferation and survival. Mitochondrial EGFRvIII decreased glucose dependency and enhanced OXPHOS. The metabolic consequences of mitochondrially localized EGFRvIII may explain yet another tumorigenic effect of EGFRvIII (in addition to its proliferative signaling) and shed light on as to how the mutant receptor may contribute to resistance to targeted therapy (Cvrljevic et al., 2011).

Bertagnolli et al. (2013) used the method of singular value decomposition (SVD) to investigate mRNA transcript length distributions in brain tumors. mRNA transcripts involved in protein synthesis and mitochondrial metabolism were significantly shorter than typical both in human and yeast, and particularly shorter than those involved in glucose metabolism. Analyzing the TCGA transcriptome data revealed that GBM maintains shorter transcripts involved in protein synthesis and metabolisms, but suppresses longer transcripts involved in glucose metabolism and brain activity. The authors proposed the existence of a mechanism that differentially regulates metabolism in a transcript length-dependent manner in tumor and normal cells.

The above data highlight the links between single mutations and gene expression alterations as well as complex patterns of gene expression alterations in tumors. Single gene sequence or expression level changes may also be associated with complex patterns of metabolic reprogramming in a tumor to support its biological properties and survival. In this line, the study by Chae et al. (2013) showed that in a tumor's mitochondria, Heat Shock Protein 90 (Hsp90) controls protein folding and central metabolic networks such as the TCA cycle, fatty acid oxidation, amino acid synthesis, cellular redox status and OXPHOS. By binding and stabilizing the Complex II subunit SDH-B of OXPHOS, mitochondrial Hsp90 contributes to maintaining cellular respiration under low-nutrient conditions, and to HIF1α-mediated tumorigenesis in patients carrying SDH-B mutations. Thus, Hsp90-controlled protein folding and homeostasis in mitochondria regulate a complex tumor cell metabolism.

As indicated above, several studies in cultured tumor cell lines with various genomic alterations established that tumorigenic mutations may alter cell proliferation and cause a metabolic shift from oxidative metabolism towards glycolysis (Ramanathan et al., 2005). Since tumor cell lines *in vitro* acquire further genomic and gene expression alterations (Lee et al., 2006) and lack the metabolic support from the normal tissue microenvironment (Zhang et al., 2012), interpretation of observations made in *in vitro* cell cultures involves limitations. In fact, using ^{13}C-isotope-labeled substrates, Maher et al., (2012) showed that the metabolic profiles of human brain tumors also include glucose oxidation *in vivo*. To further explore *in vivo* characteristics of the GBM metabolic profiles, Marin-Valencia et al. (2012) implanted three surgically removed human GBM tumors with various sets of mutations (EGFR, EGFRvIII, PTEN, PDGFRα, p53, pAkt, pErk,Enk4a/ARF) into the brains of NOD-SCID mice (human orthotopic tumor model). The animals were injected with ^{13}C-labeled substrates, which revealed complex metabolic patterns and accumulation of glutamine within the tumors but not in the surrounding normal brains. The patterns in the orthotopic tumors were also different from that observed in *in vitro* GBM cell lines. As expected, the tumors showed enhanced glycolysis. However, these tumors also utilized glucose for multiple and complex metabolic processes including glucose oxidation via pyruvate dehydrogenase and citric acid cycle, anaplerosis (replenishing citric acid cycle intermediates), glutamate synthesis and other biochemical activities. Thus, this *in vivo* study has established that GBM tumors not only depend on glycolysis but also utilize glucose for oxidation as well as other biochemical pathways for their growth and high energy metabolism.

Griguer et al. (2013) examined the correlation between spectrophotometrically determined activity values of mitochondrial cytochrome c oxidase in the tumors and survival of 84 patients with GBM. High enzyme activity values detected in 30% of patients were proven to be prognostic factors for short progression-free and overall survival. Low tumor cytochrome c oxidase activity was associated with a median survival time of 14.3 months, while high enzyme activity was associated with 6.3 months. As cytochrome c oxidase

facilitates a metabolic switch from glycolysis to OXPHOS, elevated cytochrome c oxidase activity in GBM likely contributes to short survival and poor outcome through defining this tumorigenic metabolic switch in high grade gliomas. Therefore, cytochrome c oxidase activity may be considered as a metabolic predictor of prognosis and treatment response in patients with GBM (Griguer et al. (2013)). This is an interesting set of observations as enhanced mitochondrial activity is most frequently associated with decreased tumorigenesis.

Arismendi-Morillo et al. (2012) investigated activated mitochondria in invadopodia of GBM by using transmission electron and immunofluorescent microscopy. The morphological characteristics (dense matrix configuration) of mitochondria in *in situ* tumors suggested their activated state and their frequent association with the smooth endoplasmic reticulum in filopodia protruding into the extracellular matrix. Likewise, these structures were detected in invadopodia, which were associated with microblood vessels. In *in vitro* glioma cell lines, similar observations were made indicating that these structures (filopodia and invadopodia with activated mitochondria) are involved in the invasion process, and may be future targets of blocking GBM cell mobility and invasiveness.

The unique dependence of tumors on aerobic glycolysis that confers proliferative advantage, resistance to apoptosis and even increased angiogenesis, raised the hypothesis, that GBM may be therapeutically targeted by focusing on their biochemical pathways and energy metabolism. One potential approach could be using a ketogenic diet that would provide useful energy source for normal neurons and glial cells with active mitochondria capable of oxidizing ketone bodies, but metabolically starve malignant glioma cells with a strong dependence on glucose and glycolysis. Such a diet has been proven feasible and safe in humans (Champ et al., 2014). To explore further the ketogenic approach, Chang et al. (2013) assessed the expression levels of key enzymes involved in ketolytic and glycolytic processes in grade III and IV gliomas by using quantitative immunohistochemistry. The rate limiting mitochondrial ketolytic enzymes (3-oxoacid CoA transferase-OXCT1 and 3-hydroxybutyrate dehydrogenase 1-BDH1) were simultaneously low or very low in most GBMs and a fifth in anaplastic gliomas, whereas at least one of the glycolytic enzymes was positive in 13 of these 17 GBMs and in all 5 anaplastic gliomas. Cytosolic BDH2 and mitochondrial acetyl-CoA acetyltransferase 1 were positive in most of the tumors. The authors conclude that the genetically heterogeneous gliomas express various levels of ketolytic and glycolytic enzymes. It is very likely that patients with low expression levels of ketolytic enzymes could most benefit from a ketogenic diet (Chang et al., 2013).

While our survey primarily focuses on the links among gene alterations, metabolic changes and tumor genesis in GBM, it is important to point out that this scope is somewhat limited and a broader array of metabolic changes occur in cancer cells which differentiate them from their normal counterparts. In addition to the increases in glycolysis and the pentose phosphate pathway discussed earlier, cancer cells undergo reprogramming of lipid and protein metabolism, which cannot be fully covered here. However, we call attention to a key cellular energy sensor, AMPK (5'-AMP-activated protein kinase), that upon activation stimulates catabolic processes such as glycolysis and fatty acid oxidation and inhibits anabolic processes such as lipid, glycogen and protein synthesis (Mihaylova and Shaw, 2011; Hardie et al., 2012). Activation of AMPK leads to phosphorylation and thereby inhibition of acetyl-CoA carboxylase (ACC1 and ACC2), resulting in decreased levels of malonyl-CoA, a potent inhibitor of carnitine palmitoyltransferase I (CPTI) and a necessary precursor for fatty-acid biosynthesis (McGarry et al., 1977). Increased activity of CPTI promotes mitochondrial beta-oxidation by facilitating uptake of fatty-acids into the matrix, a response that leads to increased ATP production by oxidative phosphorylation. AMPK activation is also associated with an inhibition of protein synthesis by inhibiting mammalian target of rapamycin (mTOR) (Tokunaga et al., 2004). AMPK, a kinase that regulates metabolism and cell growth in response to the cellular energy status, is considered a tumor suppressor for cancer cells. Indeed, activation of AMPK as occurs with metformin and AICAR are pharmacological approaches being explored as anti-cancer therapies because rapid and sustained cell proliferation as occurs in cancer require lipid synthesis for membrane biosynthesis and protein synthesis (Inoki et al., 2012; Dandapani and Hardie, 2013).

Altogether, these studies highlighted the link between genomic mutations, gene expression alterations and metabolic processes, revealed the complexity of interrelated metabolic pathways and suggested that intervention within these circuits may represent new targets for controlling growth and metastatic properties of GBM.

3.3. *Alterations in apoptosis regulation in GBM*

As cancers evade apoptosis regulation, targeting either the extrinsic or the intrinsic apoptotic pathway (Fig. 3) may represent new therapeutic strategies. Proapoptotic agonists of the TRAIL receptor not only activate the extrinsic apoptotic pathway but also trigger the intrinsic death program more in cancer than in normal cells (Moretto and Hotte, 2009). This approach appeared to be a useful strategy in GBM, but turned out that glioma cells are either *a priori* resistant or quickly develop resistance to TRAIL targeting apoptosis induction. Upregulation of the antiapoptotic Bcl-2 and Bcl-2-like 2 (Bcl-XL) and downregulation of the proapoptotic Bcl-2-associated X protein (Bax) have been detected in recurrent GBM, explaining at least in part, resistance of GBM to TRAIL-directed treatments (Krakstad and Chekenya, 2010).

Recently, small molecules have been developed to antagonize antiapoptotic Bcl-2 family members. Such molecules are the Bcl-2 homology domain 3 (BH3)-mimetics with structural or functional similarities to BH3-only proteins. One of the best-characterized BH3 mimetics is ABT-737 that binds to Bcl-2, Bcl-XL and Bcl-w. ABT-737 exerts antitumor activity *in vitro* in GBM (Tagscherer et al., 2008). Cristofanon and Fulda, 2012 investigated the combined use of ABT-737 and TRAIL to target the intrinsic and extrinsic pathways of apoptosis in five GBM cell lines. The study revealed that ABT-737 and TRAIL synergistically induced apoptosis.

ABT-737 and TRAIL cooperated in cleaving Bid into tBid, leading to the accumulation of tBid at mitochondrial membranes, which appeared to be the key element of their synergistic actions. tBid accumulation was accompanied by the activation of Bax, loss of mitochondrial membrane potential, release of cytochrome-c and Smac to the cytosol and caspase activation. The key role of Bid was also confirmed in Bid knockdown cells, where the ABT-737- and TRAIL-mediated Bax activation as well as apoptosis was decreased. Caspase-3 silencing reduced ABT-737- and TRAIL-induced Bid cleavage and apoptosis, underscoring the role of a caspase-3-driven, mitochondrial feedback loop in Bid processing. As ABT-737 enhanced TRAIL-triggered apoptosis in both primary GBM cell cultures derived from clinical tumor specimens, and in an *in vivo* GBM model, this approach appeared very promising in targeting the apoptosis pathways in GBM (Cristofanon and Fulda, 2012).

Tchoghandjian et al. (2013) studied the effects of a Smac mimetic, BV6 that antagonizes inhibitors of apoptosis proteins (IAP). IAPs can regulate pathways other than apoptosis, and BV6 appears to influence IAPs involved in the non-apoptotic pathways. The study reveals that BV6 stimulates migration and invasion of GBM derived tumor cell lines through non-canonical nuclear factor-κB (NF-κB) signaling and a TNFα/TNFR1 autocrine/paracrine loop. In primary GBM cell cultures, BV6 also promoted cell elongation, migration and invasion at concentrations that did not affect viability and proliferation. In an *in vivo* chicken chorioallantoic membrane model, BV6 even increased tumor cell growth. BV6 also increased mRNA levels of NF-κB target genes such as TNFα, IL-8, MCP-1, CxCR4 and MMP9 involved in the regulation of cell migration. In addition, BV6 caused degradation of several IAP proteins, accumulation of NIK, generation and nuclear translocation of p52 and increased NF-κB DNA-binding accompanied by increased transcription of target genes. NF-κB DNA-binding subunits included p50, p52 and RelB pointing to the activation of non-canonical NF-κB pathways. The BV6-triggered effects on cell elongation, migration and invasion could be abrogated by the overexpression of IκB, a repressor of NF-κB. Silencing of NIK by RNA-interference inhibited the non-canonical NF-κB signaling and led to decreased BV6-induced cell elongation, migration and invasion as well as suppressed the upregulation of NF-κB target genes. Inhibition of the BV6-stimulted TNFα/TNFR1 autocrine/paracrine loop by a TNFα-specific antibody or by the knockdown of TNFR1 also blocked the BV6-induced cell elongation, migration and invasion. The elucidation of this regulatory loop, in which a Smac-mimetic, BV6 plays key regulatory roles on cell migration and invasion via NF-κB signaling, may have significant therapeutic importance (Tchoghandjian et al., 2013).

In the previous sections, we discussed the roles of the transmembrane EGFR in mediating signals from extracellular ligands through multiple signaling molecules to the nucleus and influencing tumor cell metabolism when translocated into cytoplasmic organelles including the mitochondrion. Cao et al. (2011) also showed that both EGFR and EGFRvIII translocate to mitochondria. The apoptosis inducer staurosporine and anisomycin, and an EGFR kinase inhibitor enhanced mitochondrial translocation of both receptors, which in turn, contributed to drug and apoptosis resistance. These data highlighted that there is a strong relation between mitochondrial translocation of EGFR/EGFRvIII and regulation of apoptosis (Cao et al., 2011).

Murphy et al. (2013) used a mathematical program based on system biology to predict susceptibility to apoptosis in GBM cell lines and resected tumors. First, the investigators quantified proapoptotic proteins including procaspase-3, procaspase-9, Smac and Apaf-1, and antiapoptotic protein XIAP. Then, the results were entered in the APOPTO-CELL program to predict susceptibility of cells to undergo caspase activation. Cells surviving or undergoing apoptosis upon temozolomide treatment could be distinguished with high accuracy in 10 lines out of 11 analyzed. The program was also able to stratify patients according to their progression-free survival times and identified tumor cells capable of caspase activation in 16 of 21 patients with GBM. This study suggests that a system biology-based mathematical program such as APOPTO-CELL may have clinical utility, since it predicts capability to apoptosis and response to chemotherapy with high accuracy (Murphy et al., 2013).

The complexity of regulation of apoptosis is indicated by the study of Zhang et al. (2014). This study investigated the effects of nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1), which is a member of the transforming growth factor-β (TGF-β) superfamily and acts through many opposing pathways in cancer while also behaves as a tumor suppressor gene in GBM. Overexpression of NAG-1 induced intrinsic mitochondrial mechanism of apoptosis in several but not all investigated GBM tumor cell lines. NAG-1 induced phosphorylation of PI3K/Akt and Smad2/3 in all apoptosis-prone GBM lines, but not Smad3 phosphorylation in the two (A172 and LN-229) cell lines that were resistant to NAG-1 induced apoptosis and showed very low Smad3 expression. In addition, NAG-1 induced direct interactions between Akt and Smad3 in GBM cells. PI3K inhibitors facilitated NAG-1-induced GBM cell apoptosis, whereas siRNA to Smad2 and Smad3 decreased the rate of apoptosis. In the two apoptosis-resistant GBM cell lines (A172 and LN-229), elevation of Smad3 expression led to NAG-1-induced apoptosis. The authors conclude, that PI3K/Akt and Smad-mediated signaling pathways exert opposing effects on NAG-1-induced apoptosis in GBM (Zhang et al., 2014).

Dendritic cell factor 1 (DCF1), a cell membrane protein has been implicated in the differentiation of neuronal stem cells. However, overexpression of DCF1 can inhibit cell proliferation, migration and invasion, while also promote apoptosis in a GBM cell line U251 (Xie et al., 2014). Deletions in the DCF1 functional region revealed that the full molecule was necessary for apoptosis induction. When U251 overexpressing DCF1 was transplanted in an immune deficient murine recipient, the transplant showed significantly reduced tumorigenic potential. DCF1 induced apoptosis was associated with ultrastructural abnormalities such as mitochondrial swelling and damage to the cristae, and reduced mitochondrial membrane potential, and activation of caspase-3. Silencing of the endogeneous DCF1 in the GBM cell line was related to hypermethylation in the *dcf1* promoter, suggesting a strong correlation between *dcf1* silencing and tumorigenicity. Thus, DCF1 may be a new target for manipulation in the treatment of GBM (Xie et al., 2014).

Several experimental cytostatic drugs are being developed to selectively enhance apoptosis in high grade gliomas. Zanotto-Filho et al. (2012) studied a proteosome inhibitor, MG132 in GBM cell lines. Irrespective of the mutational status of tumor suppressor genes p53 and PTEN, MG132 elicited selective toxicity and anti-proliferative potential in GBM lines but not in astrocytes. MG132 caused cell cycle arrest in G2/M phase and induced apoptosis in tumor cells. The enhanced apoptotic process was accompanied by caspase-3 activation, chromatin condensation, formation of sub-G1 apoptotic cells as well as mitochondrial depolarization, decreased mitochondrial Bcl2 xL (an antiapoptotic protein) expression, and activation of JNK and p38, and inhibition of NFκB and PI3K/Akt survival pathways. Treatment of cells with a mitochondrial permeability transition pore inhibitor or JNK1/2 and p38 inhibitors significantly reduced cell death. MG132 not only behaved as a selective proapoptotic molecule in highly malignant glioma cells, but also enhanced the effects of other cytostatic drugs used in the treatment of patients with cancer (Zanotto-Filho et al., 2012).

Quercetin, a naturally occurring flavonoid, also induces cell cycle arrest and apoptosis in cancer. Kim et al. (2013) studied quercetin in U373MG glioblastoma cells and found that the drug exerted anti-proliferative effect in a concentration-dependent manner. U373MG cells were arrested in G1 phase, and exhibited signs of apoptosis with fragmented nuclei, decreased mitochondrial membrane potential, activation of caspases and degradation of the poly(ADP-ribose) polymerase protein. In addition, JNK was activated and expression of p53 increased, followed by a translocation of p53 to mitochondria, release of cytochrome c from mitochondria to the cytosol. In addition, quercetin induced autophagy. Inhibition of autophagy by chloroquine augmented apoptosis suggesting a fine balance between the processes of autophagy and apoptosis, controlled by quercetin, at least in U373MG cells (Kim et al., 2013).

Altogether, these studies call attention to several potential therapeutic targets within the extrinsic and intrinsic apoptotic pathways in GBM, and point to complex interacting molecular links that directly or indirectly influence cell survival and apoptosis.

4. Conclusion

GBM is one of the most aggressive human cancers that largely resists most currently employed therapeutic approaches. Although establishing the clinical diagnosis using patient history, physical exam, brain imaging and tumor histology play the most important roles, the characterization of tumor biology has been increasingly extended to the molecular level in the last decade. Parallel with achievements in genomics, epigenomics and transcriptomics, the metabolic characterization of GBM has also been greatly advanced, although the temporal evolutions and individual patterns of GBM metabolomics remain to be determined. By linking somatic nuclear/mtDNA mutations and gene expression alterations with complex changes in (1) cell metabolism and (2) apoptosis regulation, we arranged recent observations into two mitochondrially centered functional groups defining GBM. The multifactorial and increasingly tumorigenic shifts observed over time in conserved metabolic and apoptosis regulatory processes indicate extremely complex mechanisms which may explain, at least in part, the difficulties in overcoming these resilient tumor cell types. The current data are enlightening but much work remains to be done to identify those key molecular drivers that may be clinically useful tumor targets. The current level of understanding indicates that the two discussed mitochondrial processes are unique determinants of GBM biology and likely will play important roles in future clinical monitoring and therapeutic management.

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