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Meta-analysis of brain tumor microarray data using Oncomine identifies NRF1, Tfam and Myc co-expressed genes: its implications in the development of childhood brain tumors

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Abstract: The objective of this study was to use Oncomine (a database of DNA microarrays) to identifies NRF1, Tfam and Myc co-expressed genes and their possible implication in childhood brain tumor (CBT) development through controlling mitochondrial biogenesis. A meta-analysis was performed on several microarray studies on brain cancer found within Oncomine. The three main target genes of the meta-analysis were: V-myc myelocytomatosis viral oncogene homolog (avian) (Myc), Tfam: transcription factor A, mitochondrial (mt) (Tfam), and nuclear respiratory factor-1 (NRF1), that are involved in the mitochondrial biogenesis. For the Myc and Tfam meta-analysis we selected 5 studies having co-expression of brain data for Tfam; these same studies were selected for Myc. The meta-analysis included 5 studies with a total of 111 microarrays. A total of 208 co-expressed Myc genes with a significance of 40%+ (significant in 2 of 5 studies) and 206 co-expressed Tfam genes with significance of 40%+ were identified. 9 significant genes overlapped between Myc and Tfam: ALCAM, BMP2, CALCRL, CDH11, DUSP4, EMP1, SMAD3, SNAP23, and UBE2D1. A program called FuncAssociate was used to perform GO Term Enrichment analysis to obtain characteristics of the set of significant genes for both Tfam and Myc. The top GO Term for Myc was 'Phosphoribosylamine-glycine ligase activity'. The function of this GO Term is the Catalysis of the reaction: ATP + 5-phospho-D-ribosylamine + glycine = ADP + phosphate + N1-(5-phospho-D-ribosyl)glycine. Interestingly, this is part of the electron transport chain and thus mitochondrial biogenesis. The top GO Term for Tfam was 'Peptide-aspartate beta-dioxygenase activity'. This functions in the catalysis of the reaction: peptide L-aspartate + 2-oxoglutarate + O2 = peptide 3-hydroxy-L-aspartate + succinate + CO2. We expanded the meta-analysis to include NRF1 which also controls mitochondrial biogenesis via regulating Tfam. Interestingly, this gene has the same promoter recognition sequence as Myc . This meta-analysis, which combined 33 microarrays from two different studies, identified 26 genes co-expressed by all three genes (NRF1, Tfam, and Myc). Of these genes, three were found to be significantly co-expressed in the original meta-analysis of Tfam and myc. They were SMAD3, SNAP23 and UBE2D1. This study was able to identify a set of genes significantly correlated with NRF1, Myc and Tfam that control mitochondrial biogenesis. Furthermore, it identified 9 possible pathway partners of Myc and Tfam and a number of functions enriched in Myc, Tfam, and NRF1 in brain tumors. Since NRF1, Myc and Tfam genes play a large part in both brain development and mitochondrial biogenesis, the identification of co-expressed genes in similar pathways of these three mitochondrial biogenesis controlling genes could be key in elucidating how dysfunction of mitochondrial signaling may be involved in the development of childhood brain tumors.

Keywords: *Brain tumors, NRF1, Tfam, Myc, mitochondrial biogenesis*

1. INTRODUCTION

Mitochondria play a significant role in brain development (Erecinska et al., 2004) and are abundant in brain tissue (Lerman-Sagie et al., 2005). The central nervous system is, after the peripheral nervous system, the second most frequently affected organ in mitochondrial (mt) disorders. Research on mt abnormalities in brain cancer provides some evidence of their possible involvement in childhood brain tumor development. In astrocytic tumors, Kirches et al. (1999) found high sequence variability, as well as a loss of the heteroplasmy present in a polymorphic D-loop of mitochondria, a region thought to function in nucleoid organization (He et al., 2007; Kirches et al., 1999). In 2001, the same group suggested the existence of a common mechanism generating inherited mtDNA mutations and somatic mutations in gliomas, further supporting the idea that mtDNA mutations might be a cause of excess free radical production leading to tumorigenesis (Kirches et al., 2001). A more recent study of 42 cases of malignant gliomas showed alterations in 36% of the cases in the D-loop region, although these alterations did not appear to be associated with increased aggressiveness (Montanini et al., 2005). In a study that analyzed the entire mt genome of 15 cases of medulloblastoma and the cerebrospinal fluid (CSF) of eight of these 15 cases, forty percent of the tumors studied had at least one mt mutation and interestingly, seven of eight of the CSF samples were found to have mtDNA mutations as well (Wong et al., 2003). Malignant glioma is the best-characterized type of brain tumor with respect to mtDNA alterations. The most frequent observation involves changes in the copy number of mtDNA (Liang and Hays, 1996). These studies clearly show that mtDNA alterations are a frequent event in the development and progression of brain tumors and warrant further investigation.

Despite the above evidence, mt abnormalities have generally been considered to be a consequence, rather than the cause of tumorigenesis. However, recent reports argue against this concept and provide support to the idea that mitochondria may control the growth of cancer tissues (Roy et al., 2007a). For example, genetic and sporadic cases of brain tumors (paraganglioma and pheochromocytoma) are caused by mutation of a mt-specific protein, succinate dehydrogenase, a Krebs cycle enzyme (Astuti et al., 2001). Recently, mutations in another Krebs cycle protein, fumarase, have been associated with the development of uterine fibroids, skin leiomyomata and renal cell cancer (Tomlinson et al., 2002). Mutations in these proteins appear to be involved in familial predisposition to benign and malignant tumors, such as malignant phaeochromocytomas and renal cell carcinomas.

Recent studies from our laboratory and many others have suggested that mitochondria may be the source of a signal that regulate the proliferative growth of neurons and non-neuronal cells (Felty and Roy, 2005; Roy et al., 2007a; Roy et al., 2007b). The regulation of mt DNA expression is crucial for mt biogenesis during development and differentiation. The mt genome is transcribed in early embryos. Two mt biogenesis coregulators are: mt transcription factor A (Tfam or mtTFA), a nucleus-encoded molecules governing mt gene expression, and nuclear respiratory factor 1 (NRF1), a nucleus-encoded factor which directs nuclear respiratory gene expression through DNA-binding to respiratory promoters. Interestingly, NRF1 has the same promoter recognition sequence as Myc (Grandori and Eisenman, 1997; Virbasius et al., 1993), a known oncogene found disregulated in many cancers and involved in control of brain development (Grimmer and Weiss, 2006; Oster et al., 2002). Both Myc and NRF1 regulate Tfam, a key transcription factor in the regulation of mt transcription and mtDNA replication (Grimmer and Weiss, 2006; Li et al., 2005; Oster et al., 2002; Scarpulla, 1997). Myc and NRF1 plays a large part in both brain development and mt biogenesis and disregulation of any of these genes could possibly be involved in the development of abnormal mt-nuclear signaling. Therefore, in this study we have performed a meta-analysis to identify co-expressed genes of Myc, Tfam, and NRF1, as identification of their co-expressed genes could be key in elucidating how dysfunction of mt signaling may be involved in the development of childhood brain tumors.

2. METHODS

2.1 Expression analysis of Myc, Tfam, and NRF1 across multiple microarray studies using Oncomine

As an initial investigative path, we chose to perform differential expression analysis on Myc, Tfam, and NRF1 using three separate categories of analysis in Oncomine: Normal vs. Cancer, Survival Status, and Grade. These analyses were performed in order to identify possible relationships among our genes and these classes of analysis. Oncomine processes and normalizes each dataset used in these analyses independently. For the differential expression analysis, they use t-statistics with false discovery rates as a corrected measure of significance (Rhodes et al., 2004). After searching for the gene of interest in Oncomine, we sorted the results based on each class of analysis, noted the significant studies produced, and then created a boxplot with the results of this analysis.

2.2 Meta-analysis and Enrichment Analysis using Oncomine and FuncAssociate

We chose to use meta-analysis to identify possible novel partners of Myc, Tfam, and NRF1 in brain tumor tissues. To accomplish this goal, we explored the co-expression data for these genes in several Oncomine studies on brain tumors, as this co-expression data can show if proteins may be in same pathway (e.g. both coregulated together, or one directly affecting the other). While Oncomine provides a meta-analysis function, our meta-analysis differs from the one allowed by Oncomine in that it combines gene lists from Oncomine studies and then sorts them for meta-analysis in Excel. This analysis follows the technique first described by Wilson and Giguere 2007 (Wilson and Giguere, 2007). GO Term Enrichment Analysis was then performed to identify any functional relevance of significant genes of the meta-analysis. A step-by-step workflow of these analyses was:

1. Searched for studies with co-expression data for brain on both Myc and Tfam using the Oncomine.
2. Chose several studies to perform meta-analysis of the co-expression data for these genes. We specifically wanted study overlap and similar analyses in the studies selected for the meta-analysis. We then chose the top 500 genes that showed differential co-expression with Myc and NRF1 and removed any repetitive genes.
3. Used Excel to analyze which genes were overlapped.
4. Performed co-expression analysis on these genes using Oncomine; Genes listed in the top 500 of 2 of 5 studies were considered significant.
5. Compared significance lists for each gene to produce possible novel partner genes.
6. Performed GO Term Enrichment analysis to identify potentially overrepresented functions of our gene lists.
7. Performed co-expression analysis and GO Term Enrichment analysis on Myc, Tfam, and NRF1 in same manner as above and compared results.

3. RESULTS AND DISCUSSION

3.1 Differential-expression analysis of Myc, Tfam and NRF1 using Oncomine microarray data

Before conducting the meta-analysis, we chose to investigate individual expression patterns of our genes within select classes of analysis of brain tumors in Oncomine. This analysis provided some substantial preliminary evidence as to the significance of our target genes (Myc, Tfam, and NRF1) in the possible development of brain tumors. The results that across several studies, each of our target genes was overexpressed in different classes of analysis, including normal vs. cancer (i.e. Myc is overexpressed in cancer vs. brain tissue), survival status (i.e. Myc is overexpressed in patients who died from brain cancer vs. those who survived), and grade (i.e. Myc overexpression increases as grade of brain cancer increases) (Figures 1-3 include data on Myc; Figures 4-6 include data on Tfam; and Figures 7-9 include data on NRF1). Note: Y-axis units for these boxplots are normalized expression values (standard deviations above or below the median per array.

3.2 Tfam and Myc meta-analysis

Five studies using analysis which compared Tfam expression data for normal brain tissue to cancerous brain tissue were selected for the initial anlaysis; these same studies were selected for Myc. The total microarrays in the meta-analysis of Tfam and Myc were: Tfam - five studies with a total of 111 microarrays; Myc - same five studies as Tfam with a total of 111 microarrays. In total, there were 208 co-expressed Myc genes with significance of 40%+ (2+ of 5 studies) and 206 co-expressed Tfam genes with significance of 40%+. Nine significant genes overlapped between Myc and Tfam. They were: ALCAM, BMP2, CALCRL, CDH11, DUSP4, EMP1, SMAD3, SNAP23, and UBE2D1.

3.3 Myc, Tfam, and NRF1 Meta-analysis

A search of Oncomine for brain studies which included Myc, Tfam, and NRF1 in the analysis found only two studies using normal vs. cancer analysis that were common among all three genes. Therefore microarrays from these two studies (33 total) were combined for meta-analysis to identify a list of genes co-expressed among all three genes. Initial results identified 26 genes co-expressed in NRF1, Tfam, and Myc. Table 1 lists these identified genes' symbols. Three of these 26 genes were common among the 9 identified as significantly co-expressed in the meta-analysis performed on Myc and Tfam above. These three genes were SMAD3, SNAP23 and UBE2D1.

Table 1. List of common co-expressed genes for Myc, Tfam, and NRF1

CARD10	DBN1	PDGFRA	SPARC
CDC2	E2S1	PTRF	SULF1
CDKN2A	FER	RBPMS	TM4SF1
CITED1	GALNT10	SERPINB2	TPM2
COL11A1	IRS1	SERPINH1	UBE2D1
COL4A2	MNAT1	SMAD3	
CSPG4	NROB1	SNAP23	

One of these genes, SMAD3, presents an interesting scenario where our genes of interest are possibly involved as downstream

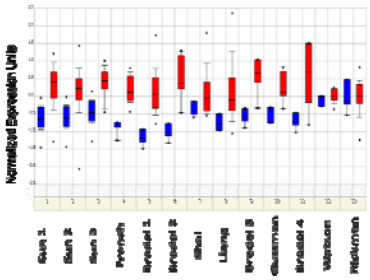

Figure 1. MYC expression in brain tumors vs. normal brain. Studies showing significant overexpression of MYC in brain tumors were Sun 1 (P=7.4E-12), Sun 2 (P=1E-11), Sun 3 (P = 4.9E-10), French (P=6.8E-8), Bredel 1 (P=2.2E-5), Bredel 2 (P=1.1E-4), Shai (P=7.3E-4), Liang (P=.004), Bredel 3 (P=.006), Gutmann (P=.015), and Bredel 4 (P=.016). Normal brain is denoted in blue; brain tumors are red.

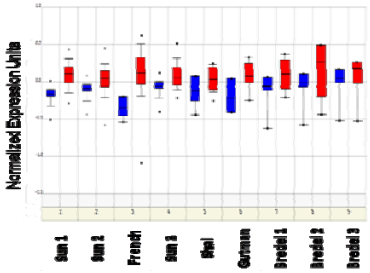

Figure 4. Tfam expression in brain tumors vs. normal brain. Studies showing significant overexpression of Tfam in bt are Sun 1 (P=2E-11), Sun 2 (P=1.1E-5), French (P=1.2E-5) and Sun 3 (P=4.6E-4). Normal brain samples are denoted in blue; brain tumors are red.

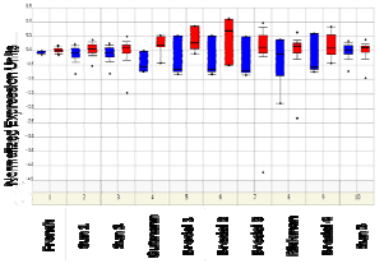

Figure 7. NRF1 expression in brain tumors vs. normal brain. Studies showing significant overexpression of NRF1 in bt are French (P=.002), Sun 1 (P=.007), and Sun 2 (P=.0444). Normal brain is denoted in blue; Brain tumors are red.

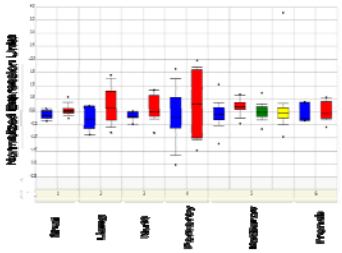

Figure 2. MYC expression in brain tumors by prognosis. Studies showing significant overexpression of MYC with bad prognosis were Shai (P=0.008), French 2 (P=.003) and Liang (P=.034). Alive patients are denoted by blue boxplots; Patients who died are red; Patients without disease relapse are green; and patients with disease relapse are yellow.

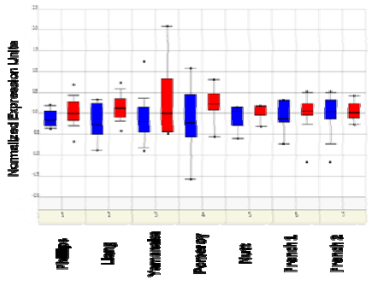

Figure 5. Tfam expression in brain tumors by prognosis. 1 of 7 studies showed significant overexpression of Tfam in dead vs. alive patients [Phillips (P=.037)]. Patients who survived are denoted in blue, patients who died are red.

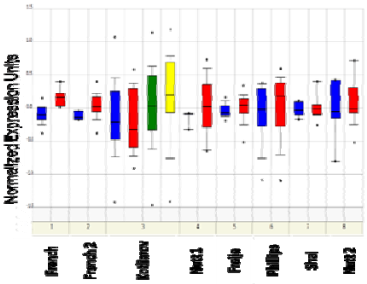

Figure 8. NRF1 expression in brain tumors by prognosis. NRF1 was significantly overexpressed in brain tumor in French 1 (P=4.4E-5), French 2 (P=.003), and Kotliarov (P=.003). Patients who survived are blue; Patients who died are red; No disease relapse are green; and Disease relapse are yellow.

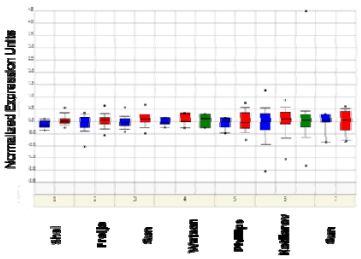

Figure 3. MYC expression in brain tumors by grade. 1 of 7 studies showed significant overexpression of MYC as grade increased in bt [Shai (P=.018)]. Lower grade tumors are denoted in blue, and higher grade tumors are red and green, with green being the highest grade category for the Watson and Kotliarov studies.

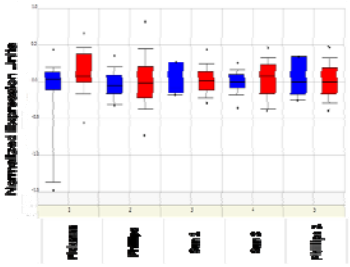

Figure 6. Tfam expression in brain tumors by grade. 1 of 7 studies approached significance for overexpression of Tfam as grade increased in brain tumors [Rickman (P=0.074)]. Lower grade tumors are denoted in blue; Higher grade tumors are denoted

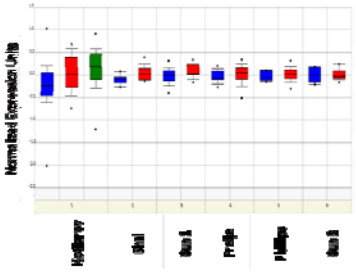

Figure 9. NRF1 expression in brain tumor (bt) by grade. NRF1 was significantly overexpressed as grade increased in bt in Kotliarov (P=7.9E-7) and Shai (P=.018). Lower grade bt are denoted in blue; higher grade bt are red and green, with green being the highest grade for the Kotliarov study.

targets in the TFG-β signaling pathway. This pathway, which includes SMAD3 as a major signaling molecule, has been implicated in several cancers (Bierie and Moses, 2006) and includes Myc as a known target gene (Yagi et al., 2002). Phosphorylation of SMAD3 by TGF-β allows for migration of SMAD3 to the nucleus where it can function as a transcription factor (Weinberg, 2007). However, many of these downstream targets are unknown (2006), though one study indicates TGF-β's possible involvement in DNA damage-induced apoptosis through promotion of a SMAD3-BRCA1 complex that reduces DNA repair (Dubrovska et al., 2005). Interruption of this pathway leads to the inability of TGF-β to regulate cell proliferation. Interaction of Myc with Myc-interacting zinc-finger protein 1 (Miz-1) prevents repression of cyclin dependent kinase inhibitors, allowing for cell proliferation, a process which may allow normal cells to evade TGF-β's natural inhibitory signal and continue on a path towards tumorigenesis (Seoane et al., 2001). Research on TBF-β signaling and brain tumors does implicate its involvement in a large percentage of tumors, as Forkhead Box G1 (FOXG1), a theorized repressor of TGF-α induced expression of p21cip1 and cellular growth which interacts with NOTCH signaling, shows copy gain between 2 and 21 fold in 93% of MB (Adesina et al., 2007).

Evidence also points to SMAD3's ability to interact with proteins that regulate cytochrome c release from the mitochondria (Wildey et al., 2003), a process important to apoptosis, as mitochondria's intermembrane space acts as a storage site for numerous proapoptotic proteins, including cytochrome c and apoptosis-inducing factor (AIF). This pathway to apoptosis involves Bcl-2 family proteins (e.g. Bax, Bid) promoting cytochrome c release through binding to the outer mitochondrial membrane, leading to release of other proteins involved in apoptosis, such as AIF, and Smac/Diablo (Weinberg, 2007). While research on the ERK signaling pathway shows that H_2O_2-induced apoptosis in glioma cells may be initiated upstream of the mitochondria (Lee et al., 2005), a study investigating exposure of neuroblastoma cells to thimerosal, an organomercury compound used in vaccines, found that apoptosis was induced through the cytochrome c/capsase mt cascade described above (Humphrey et al., 2005). Finally, research on the proapoptotic Apoptosis Related Protein in TGF-β Signaling Pathway (ARTS) in astrocytic tumors supports survival and grade dependent mt-mediated apoptosis in brain tumors (Gottfried et al., 2004).

3.4. GO TERM Enrichment Analysis

A program called FuncAssociate was used to perform GO Term Enrichment analysis to obtain characteristics of the set of significant genes from our meta-analyses. This analysis provides a list of gene functions which are over-represented in a gene set. The 208 genes and 206 genes found co-expressed in 2 or more studies on Myc and Tfam respectively, were first used to perform this analysis. The top four results of this analysis on both Myc and Tfam are shown in Table 2. The top GO Term for Myc was 'Phosphoribosylamine-glycine ligase activity' which catalyses the reaction: ATP+5-phospho-D-ribosylamine +glycine=ADP+phosphate+N1-(5phospho -D-ribosyl) glycinami. Interestingly, this is part of the electron transport chain and thus mt biogenesis. This is significant given that mitochondria are one of the most significant producers of reactive oxygen species (ROS) in the injured brain (Robertson et al., 2006), and evidence points to the possible involvement of mt respiratory chain defects in development of tumors. While a direct link is not established, mtDNA mutations and

Table 2. Top results of enrichment analysis for co-expressed genes.

Study	N	X	LOD	P-adj	GO Attribute
Myc	5	5	3.259	<0.001	phosphoribosylamine-glycine ligase activity
	98	8657	0.415	<0.001	protein binding
	53	3332	0.502	<0.001	developmental process
	11	109	1.296	<0.001	antigen processing and presentation of peptide/polysaccharide
Tfam	7	15	2.171	<0.001	peptide-aspartate beta-dioxygenase activity
	160	19574	0.400	<0.001	cellular process
	32	1604	0.578	<0.001	system development
	163	20371	0.395	<0.001	binding
NRF1 (Dong)	112	3599	0.337	<0.001	multicellular organismal process
	25	355	0.674	<0.001	cation channel activity
	77	2282	0.356	<0.001	multicellular organismal development
	26	429	0.604	0.001	metal ion transmembrane transporter activity
NRF1 (Guttmann)	220	8319	0.354	<0.001	protein binding
	172	6107	0.347	<0.001	biological regulation
	18	103	1.134	<0.001	antigen processing and presentation of peptide antigen
	106	3204	0.382	<0.001	developmental process
Myc, Tfam, and NRF1	14	3204	0.954	<0.001	developmental process
	8	818	1.187	<0.001	regulation of developmental process
	7	582	1.262	<0.001	regulation of catalytic activity
	2	2	3.756	0.001	senescence

N: # of genes in query with attribute; X: number of genes overall with attribute; LOD: The logarithm (base 10) of the odds ratio; positive and negative values indicate over- and underrepresentation respectively; P-adj: adjusted P-value

increased oxidative stress have been observed in various types of cancer cells in several independent studies (Carew and Huang, 2002). Additionally, experiments at the single cell level show biochemical defects of progeny cells correlate with accumulation of mtDNA mutations in parent cells, indicating that point mutations have a considerable effect on mt function (Taylor et al., 2003). Interestingly, cell-types, like the spiral ganglion neurons of the inner ear, show greater susceptibility to accumulation of mtDNA mutations (Kujoth et al., 2005).

As only 2 studies were used to identify genes co-expressed in NRF1, we chose to run each of the two studies' co-expressed gene lists (468 genes from Dong and 453 genes from Guttman) separately in FuncAssociate in order to obtain evidence of which processes may be significantly overrepresented in NRF1 co-expressed genes in brain tumors. Results of this analysis are found in Table 2. Additionally, a FuncAssociate analysis of the 26 genes identified as commonly co-expressed across the two studies for Myc, Tfam, and NRF1 was performed. Results are provided in Table 2. Interestingly, results from these final FuncAssociate analyses show cell development and differentiation as some of the major cellular processes that were over-represented among the genes co-expressed by both NRF1 alone and by the genes co-expressed mutually by Myc, Tfam, and NRF1, lending support to our concept that deregulation of these genes may contribute in the development of brain tumor.

This study was able to identify a set of genes significantly correlated with Myc, Tfam and NRF1 in brain tissue. Furthermore, it identified 9 possible pathway partners of Myc and Tfam, 3 of which were also found to be co-expressed with NRF1 as well. Additionally, a number of functions enriched in Myc, Tfam, and NRF1 in brain tissue were identified, and the relation of these processes to the mitochondria and cell development and differentiation seem to fit a hypothesis of early predisposition to brain tumors through deregulation of cellular pathways involving Myc, Tfam, and NRF1.

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