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DNA-PK is a Potential Molecular Therapeutic Target for Glioblastoma

P. O. Carminati, F. S. Donaires, P. R. D. V. Godoy,
A. P. Montaldi, J. A. Meador, A.S. Balajee,
G. A. Passos and E. T. Sakamoto-Hojo

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1. Introduction

Glioblastoma multiforme is one of the deadliest forms of brain tumor with a median survival of <12 months with a high rate of recurrence. Glioblastoma cells respond poorly to radiotherapy and chemotherapy due to elevated DNA repair efficiency coupled with anti-apoptotic mechanisms [1, 2]. Therefore, development of new therapeutic strategies is absolutely critical either to control or cure the disease. Since glioblastoma cells have inherently elevated levels of DNA damage response (DDR) and DNA repair efficiency, we sought to determine whether or not suppression of key DNA repair factor(s) might be effective in sensitizing the brain tumor cells to chemotherapy. To test our objective, a pair of isogenic glioblastoma cell lines that differ in the functional status of DNA dependent protein kinase (DNA-PK) has been chosen. DNA dependent protein kinase (DNA-PK) belongs to a super family of phosphatidylinositol-3 kinase like kinases (PIKK) which also includes the gene products of ataxia telangiectasia mutated (ATM) and ATM and Rad 3 related (ATR) kinases [3, 4]. DNA-PK is the main component of mammalian NHEJ (non-homologous end joining), and also plays a role as a sensor of DNA damage by phosphorylating several downstream targets [5]. Further, DNA-PK has been demonstrated to be an important component of nucleotide excision repair (NER) pathway, which is chiefly responsible for the repair of bulky and helix distorting lesions induced by ultraviolet radiation (UV) and chemotherapeutic drugs such as cisplatin. To determine whether or not DNA-PK is a potential molecular therapeutic target for glioblastoma, two agents with different modes of action have been chosen for our study: (1) Zebularine and (2) Cisplatin.

Many tumor cells have characteristic epigenetic alterations involving genome wide DNA hypo-methylation and gene specific hyper-methylation at CpG dinucleotide sequences leading to aberrant silencing of critical genes involved in DNA damage response, cell cycle and DNA metabolic processes. Recent studies [6-8] have demonstrated the usefulness of epigenetic targeting in treating many cancer cell types through inhibition of histone deacetylases (HDAC) or DNA methyl transferases (DNMTs). Zebularine is a potent inhibitor of DNMTs and is superior to 5-AzaCR in terms of lower cytotoxicity and increased stability in aqueous solutions [9]. While Zebularine is a DNA-demethylating agent, cisplatin is a DNA cross-linking agent that induces the formation of DNA inter- and intra-strand cross-links. These cross-links are removed preferentially by NER pathway and NER deficient tumor cells are often sensitized to cisplatin treatment. Since DNA-PK plays a critical role in genome surveillance and DNA repair mechanisms, we wished to evaluate the potential of DNA-PK as a molecular therapeutic target for glioblastoma.

2. DNA-PK deficient cells are sensitive to zebularine and cisplatin treatments

2.1. Zebularine sensitizes DNA-PK deficient glioblastoma cells

DNA-PK deficient (MO59J) and proficient (MO59K) glioblastoma cells were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were routinely cultured in OPTI-MEM supplemented with 5% fetal bovine serum, vitamins and antibiotics. Zebularine [1-beta-D-ribofuranosyl)-1, 2-dihydropyrimidin-2-one] was purchased from EMD Biosciences (San Diego, CA, USA). Zebularine was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 100 mM and stored at 4°C. Clonogenic survival and proliferations assays were carried to determine the sensitivity of human gliomas (MO59J and MO59K) to different Zebularine concentrations (0-300 µM). The LC50 value for Zebularine was found to be 245 µM for DNA-PK deficient MO59J cells and at this concentration the survival of MO59K cells was reduced only by 20% [10]. This finding indicates that lack of DNA-PK potentiates the sensitization of gliomas to Zebularine. In contrast to MO59K cells, DNA-PK deficient cells (MO59J) showed a reduction in survival (20%) even at the lowest Zebularine concentration (10 µM). Further, an elevated frequency of polyploid cells observed specifically in MO59J cells after Zebularine treatment pointed out a mitotic checkpoint deficiency. Although depletion of DNMT1 by Zebularine occurred at similar levels in both glioblastoma cell lines, DNA-PK deficient MO59J cells displayed elevated DNA hypo-methylation detected both at genome overall and gene promoter levels. Consistent with increased sensitivity, deoxy-Zebularine adduct level, measured for the first time by us in the genomic DNA, was 3-6 fold higher in DNA-PK deficient MO59J cells relative MO59K cells. Elevated micronuclei frequency was also observed in MO59J cells indicating the impairment of DNA damage response and repair. Collectively, our study suggests that DNA-PK is a potentially useful molecular therapeutic target for gliomas.

2.2. DNA-PK deficient cells are sensitive to cisplatin treatment

The relationship between DNA-PK/Ku activity and cisplatin sensitivity has been observed earlier in gliomas. Gliomas with high expression level of DNA-PK are resistant to cisplatin [11]. In an earlier review, we suggested that DNA-PK inhibition may be a promising strategy for treating glioblastomas [for review see 12]. To determine the role of DNA-PK in conferring cisplatin resistance, MO59J and MO59K cells were treated with cisplatin (5-75 μM ; Sigma Aldrich, USA) for 72 h and assayed for proliferation using a fluorescence based CyQuant assay (Invitrogen, USA). MO59J showed a greater sensitivity to cisplatin compared to MO59K, and displayed a dose dependent decrease in cell proliferation, being reduced to 20% relative to the vehicle (DMSO) treated control cells for the highest drug concentration (75 μM). In contrast, MO59K was sensitive to cisplatin only at high doses (>50 μM). These results clearly indicate that DNA-PK is a critical factor for cellular protection against cisplatin (Figure 1).

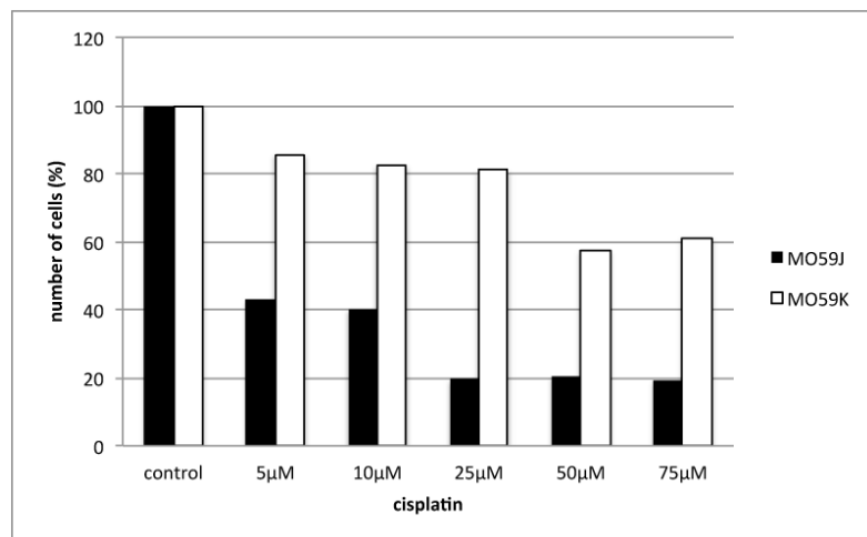


Figure 1. Cell proliferation assay using CyQuant (Invitrogen, USA) in MO59K and MO59J cells treated with cisplatin (5, 10, 25, 50, and 75 μM) for 72 h.

Cell cycle analysis was next performed to verify whether the enhanced cisplatin cytotoxicity of MO59J cells was due to defective cell cycle checkpoints. MO59K and MO59J cells in exponential growth phase were exposed to different concentrations (5 to 25 μM) of cisplatin and the cell cycle profile was analyzed by flow cytometry (24, 48 and 72 h after treatment). While DNA-PK proficient MO59K cells displayed an efficient accumulation of S-phase cells at all the concentrations of cisplatin exposure up to 72 h (Figure 2A), DNA-PK mutant MO59J cells exhibited only a modest S-phase accumulation after 24 h of drug treatment (25 μM cisplatin) (Figure 2B), which was alleviated at later time points (48 and 72 h).

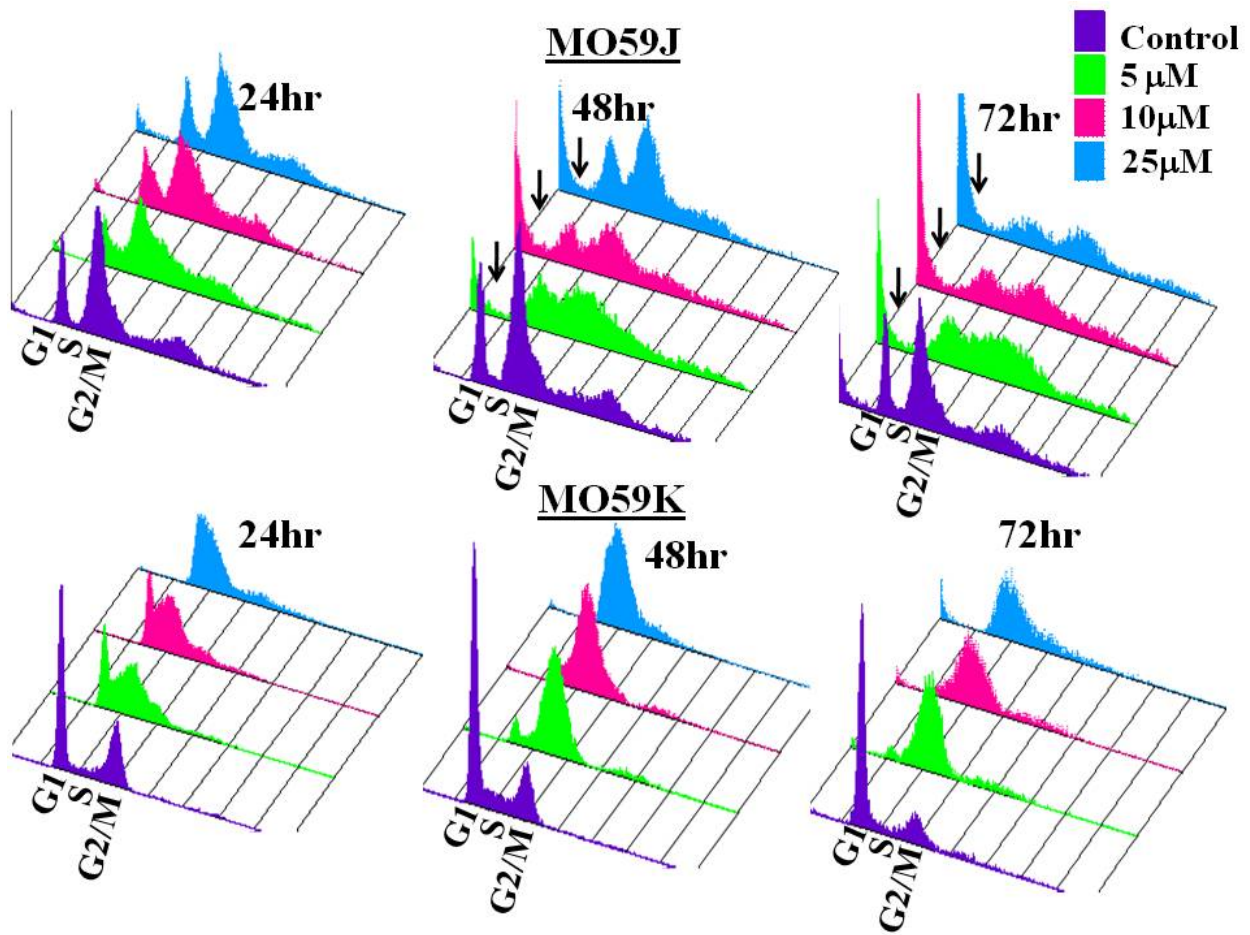


Figure 2. Cell cycle analysis for MO59K and MO59J cells treated with 5, 10 and 25 μM of cisplatin for 24, 48 and 72 h. DMSO was used as control.

Consistent with impaired cell cycle regulation, an increased frequency of apoptosis (43% for 25 μM cisplatin) was observed after cisplatin treatment in MO59J cells; in contrast to MO59K, DNA-PK deficient MO59J cells showed apoptotic death even at 5 μM of cisplatin (Figure 3A,B).

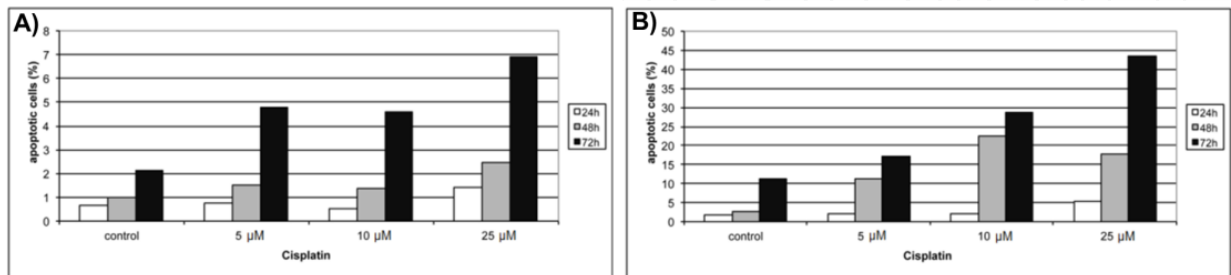


Figure 3. Frequency of apoptotic MO59K (A) and MO59J (B) cells detected by flow cytometry as a subG1 population after exposure to different concentrations of cisplatin (5, 10 and 25 μM). The results were obtained at 24, 48 and 72 h after treatment. 10,000 events were analyzed for each experiment.

Proliferating cell nuclear antigen (PCNA) is recruited to chromatin rapidly after DNA damage and the kinetics of disassembly from the chromatin is considered to reflect the DNA repair efficiency [13, 14]. PCNA expression was evaluated by Western blot in cisplatin- treated MO59K and MO59J cells. The expression of PCNA was increased in a dose-dependent manner in MO59K cells after 24 h of drug treatment, while a constant level of PCNA expression was observed in MO59J cells indicating the lack of S-phase specific PCNA accumulation (Figure 4). Our results from MO59K and MO59J cell lines indicate that DNA-PK is a critical determinant of cell survival after cisplatin treatment and the lack of S-phase arrest (as seen in MO59J cells) is presumably responsible for the increased cell death after drug treatment.

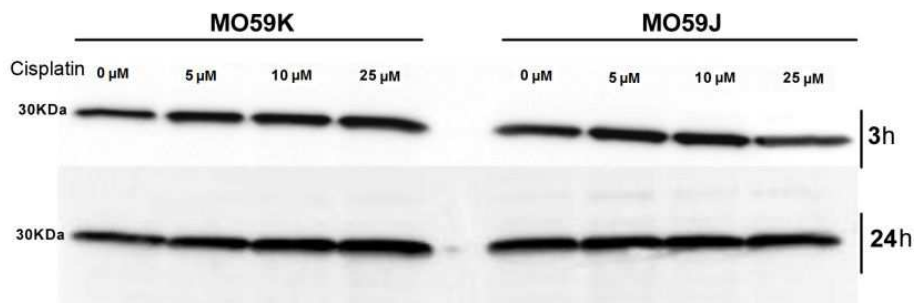


Figure 4. Expression of PCNA protein (30 KDa) analyzed by Western blot using specific antibody. MO59K and MO59J cells were treated with 5, 10 and 25 μM of cisplatin for 3 and 24 h. β-actin was used as control.

Therefore, DNA-PK may be an important molecule conferring cisplatin resistance in glioblastoma cells, with potential use as a molecular target, and we also observed that the combined effect of cisplatin and LY294002 (an inhibitor of DNA-PK) caused a significant decrease in cell proliferation and an increase in apoptotic cell death in glioma cell lines [15]. Our studies thus far performed indicate that the specific targeting of DNA-PK by small molecule inhibitors may be an effective strategy for the treatment of gliomas.

3. *In silico* analysis of transcription factors associated with differentially expressed genes in GBM cells

3.1. Introduction

Transcription factors (TFs) regulate gene expression by binding to specific DNA sequences of gene promoter regions. Their expression can be deregulated under certain pathological conditions, such as cancer. Therefore, studies on regulatory elements of the transcription machinery are essential for understanding the mechanisms involved in the regulation of oncogenes and tumor suppressor genes. Additionally, knowledge of the essential functions of TFs, may lead to strategic development of novel chemotherapy regimen for selectively killing cancer cells.

TFs play a critical role in various cellular processes through regulation of genes involved in cell cycle, proliferation, cell differentiation, and apoptosis. It has been suggested that TFs can be potential therapeutic targets for treating the patients with prostate [16] and breast cancer [17]. Recently, *in silico* prediction for regulation of TFs has been effectively utilized for diagnostics and therapeutics of breast cancer [17]. Identification and characterization of activated transcription regulatory elements hold a great promise not only in providing information on neoplastic mechanisms, but also in predicting targets for therapeutic intervention [18]. Although enormous amount of data exist for genes that are differentially expressed in tumor cells, knowledge of TFs associated with these genes is rather limited. Information on TFs may enable us to understand how differentially expressed genes in tumor cells respond to anticancer drugs, as a single or combined treatment with DNA repair inhibitors, which targets DNA repair proteins.

DNA-PK, one of the key components of mammalian NHEJ (non-homologous end joining) repair process, belongs to PI3K-related protein kinase (PIKK) family [19]. Recent studies have projected DNA-PK as a potential molecular target for cancer treatment. Many known PI3K inhibitors such as LY294002, wortmannin and PI-103, inhibit DNA-PK activity with a comparable potency to that observed for PI3K inhibition [20]. LY294002 is a PI3K (phosphatidylinositol-3 kinase) inhibitor, well recognized by its antitumor and pro-apoptotic properties in several cancer cell lines [21]. Although LY294002 alone can inhibit cell proliferation or induce apoptosis in cancer cells, its cytotoxic effects can be further enhanced when combined with radio- or chemotherapy [12, 22]. Consequently, PI3K inhibitors including LY294002 have been investigated as a possible adjuvant for cancer therapy in many tumor models [23-25], and its synergistic effects with other anti-tumoral drugs deserve to be investigated in cancer cells.

Our recent study demonstrated that a combined treatment of cisplatin (5-50 μ M) and LY294002 (50 μ M) for 24 h reduced the survival of U343 GBM cells with elevated apoptotic death at 72 h [15]. In this study, gene expression analysis was also performed using a glass slide microarray containing 4,300 cDNA clones from the human IMAGE Consortium cDNA library [<http://image.llnl.gov/image/>] essentially following the procedures published earlier [26]. Statistical analysis of the results [27] showed that 25 μ M cisplatin alone caused significant changes in the expression of 108 genes (28 up- and 80 down-regulated), while LY294002 alone altered the expression of 33 genes (6 up- and 27 down-regulated); additionally, 274 genes (97 up- and 177 down-regulated) were modulated by the combined treatment of cisplatin and LY294002. The complete gene lists are available at <http://www.rge.fmrp.usp.br/passos/cislygbm/>. The drug combination induced 2.6-fold higher numbers of modulated genes, relative to cisplatin alone, and these genes participate in several biological processes, such as DNA repair, cell death, and cell cycle control/proliferation, metabolism, transcription regulation, and cellular adhesion.

Using the gene sets obtained in the above-mentioned study, we performed an *in silico* analysis of TFs for the differentially expressed genes obtained in the following comparisons: 25 μ M cisplatin *versus* control, and 25 μ M cisplatin plus 50 μ M LY294002 *versus* control; these lists contained 108 and 274 differentially expressed genes (SAM analysis, FDR \leq 0.05), respectively. Among the 274 genes, 97 (35.4%) genes were up-regulated and 177 (64.6%) genes were down-regulated. Both up- and down-regulated genes were submitted separately to *in silico* analysis

through the FatiGO tool, Babelomics v3.2 [28]. For comparisons, the entire list of genes present in the microarray was subjected to analysis (4,300 transcripts). The data were analyzed by the two-tailed Fisher's exact test, with significance threshold for the p-values set at 0.05. Similar procedures were performed for the list of 108 genes (set of differentially expressed genes under 25µM cisplatin treatment). Information regarding the biological functions was obtained at SOURCE (<http://smd.stanford.edu/cgi-bin/source/sourceSearch>), and NCBI AceView (<http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/>).

3.2. TFs associated with up-regulated genes

Among the predicted TFs associated with up-regulated genes after treatment with cisplatin alone, the most significant ones are AHR ($p = 9.9E-3$, 7.7% of associated genes), HNF4 ($p = 9.6E-3$, 81% of associated genes), NFkappaB ($p = 5.8E-3$, 11.5% of associated genes), MRF2 ($p = 1.9E-3$, 53.8%), and SOX5 ($p = 1.6E-2$, 7.7% of associated genes). These TFs have been linked with distinct biological functions, including differentiation, homeostasis, cell growth, senescence and apoptosis. AHR regulates an array of physiological responses including xenobiotic metabolism, vasculature development, immunosuppression, T-cell differentiation, reproduction, and cell cycle progression [29]. Most genes known to be controlled by HNF4 α are involved in lipid, carbohydrate or amino acid transport and metabolism, indicating a central role in energy homeostasis [30]. MRF2 (ARID5B) plays a vital role in regulating embryonic development, cell growth and differentiation through tissue-specific repression of differentiation-specific gene expression [31, 32]. Aberrant ARID5B expression in developing fetus could halt B-lymphocyte maturation and contribute to leukemogenesis [33]. SOX5 is involved in gene regulation and maintenance of chromatin structure during diverse developmental processes. Recently, SOX5 has been found associated with induced acute cellular senescence [34], while NFkappaB is related to apoptosis evasion in GBM cells [35, 36].

Term	Percentage with term		Enrichment index	p-value
	List #1	List #2		
GATA-3	30.1	16.3	1.85	1.7E-3
FOXP3	60.2	46.0	1.31	8.0E-3
FOXJ2	46.2	32.6	1.42	9.5E-3
C/EBP	18.3	9.8	1.87	1.5E-2
LXR, PXR, CAR COUP, RAR	62.4	54.6	1.14	1.6E-2

Table 1. Transcription factors (Term) associated with up-regulated genes in cisplatin and LY294002 treated U343 cells. "Percentage with term" corresponds to the amount of genes that was associated to each TF in each list: List #1 represents up-regulated genes obtained by statistical analysis, and List #2 represents all the genes present in the microarray slide. "Enrichment index" was calculated for each Term by dividing the percentage in the List #1 by that in the List #2.

For the combined treatment (cisplatin and LY294002), the predicted TFs associated with up-regulated genes showed different TFs (Table 1), indicating that the drug combination induced quantitative and qualitative differences in transcript expression profiles displayed by GBM treated cells.

All the TFs that were associated with up-regulated genes showed enrichment indexes greater than 1 (in the range of 1.14 to 1.87), indicating that these TFs were more specifically associated with the up-regulated genes as compared to the entire array of genes (Table 1). Biological functions of the predicted TFs associated with up- and down-regulated genes after the combined treatment (cisplatin and LY294002) in U343 cells are described below:

GATA-3 (Trans-acting T-cell-specific transcription factor) gene encodes a protein, which belongs to the GATA family of transcription factors. The protein contains two GATA-type zinc fingers and is an important regulator of T-cell development and plays an important role in endothelial cell biology; *GATA-3* is essential in the embryonic development of the parathyroid, auditory system and kidneys, and defects in this gene cause HDR syndrome (hypoparathyroidism with sensorineural deafness and renal dysplasia) [37]. *GATA-3* analysis and the phenotypic spectrum obtained for nine Japanese families with the HDR syndrome suggest that this syndrome is primarily caused by *GATA-3* haploinsufficiency [38]. This TF plays a dual role in transcription regulation in a positive and negative manner. In accordance with this, we found the association of *GATA-3* with up-regulated genes (EI = 1.85) after drug treatment in U343 cells.

Another TF, *FOXP3 (Forkhead box P3)*, a positive regulator of transcription, plays a critical role in the control of immune response. This TF was associated with up-regulated genes (EI = 1.31) in drug-treated U343 cells. Defects in this gene lead to X-linked autoimmunity-immunodeficiency syndrome [39]. In several neoplasias, such as breast [40], ovarian [41], skin [42] and stomach, *FOXP3* was found over-expressed [43], or mutated in prostate cancer [44]. It is well established that tumor cells acquire molecular and biochemical changes which make them potentially vulnerable to the immune system [45]. Alterations in the immune system can be applied for cancer detection and therapies [46]. Interestingly, [47] demonstrated a dependency on functional TP53 for DNA damage induced activation of *FOXP3* in human breast and colon carcinoma cells. However, the precise role of *FOXP3* in drug responses is still unknown.

FOXJ2 (Forkhead box J2) was also associated with up-regulated genes (EI = 1.42); this TF is often described as a positive regulator of transcription [48] and is a member of Forkhead Box TFs, many of which have been reported to participate in tumor migration and invasion. Wang et al. [49] showed that the expression of *FOXJ2* was high in primary breast cancer tissues without lymph nodes metastases; moreover, *FOXJ2* may play a role in maintenance and survival of developing and adult neurons, but its function in the central nervous system is still unclear. Knockdown of this TF in cultured primary astrocytes by siRNA showed that *FOXJ2* played an important role in lipopolysaccharide-induced inflammatory responses [50]. Nonetheless, there is no information in the literature about its function in relation to chemotherapeutic drugs.

C/EBP (CCAAT/enhancer-binding protein) family plays important roles in many processes such as cell differentiation, metabolism, and development. *C/EBP (CEBPA)* gene, CCAAT/enhancer

binding protein alpha, involved in cell cycle arrest [51], and also plays a role in DNA damage response dependent on TP53 [52]. The protein acts as a dominant-negative inhibitor by forming hetero-dimers with other C/EBP members, such as C/EBP and LAP (*liver activator protein*), and preventing their DNA binding activity. Another member, GADD153 (CHOP), is a critical initiator of ER stress-induced apoptosis, and its knockdown prevents perturbations in the *AKT (protein kinase B)/FOXO3a (forkhead box, class O, 3a)* pathway in response to ER stress [53]. C/EBP β , a member of a subfamily of basic leucine zipper (bZIP) protein family, play a potential role in the proliferation and invasion of glioma, being considered as a novel molecular target for therapy, as well as a predictive marker for survival of patients with glioma [54]. In the present study, C/EBP showed an association with up-regulated genes (EI = 1.87), suggesting a possible role in DNA damage responses under drug treatment, and most probably, via AKT pathway, which is a target for LY294002.

Three TFs (*LXR, PXR* and *CAR*) were related to the same percentage (62.4%) of up-regulated genes, presenting EI = 1.14. The *liver X receptors (LXR)*, *LXRA* and *LXRB*, comprise a subfamily of the nuclear receptor superfamily and are key regulators of macrophage function, controlling transcriptional programs involved in lipid homeostasis and inflammation. Polymorphism of *LXR* gene is associated with obesity since this gene is involved in cellular lipid metabolic process [55]. *PXR*, also named *NR1I2 (Nuclear receptor subfamily 1, group I, member 2)* is a TF that acts in both positive and negative regulation of transcription, as well as in xenobiotic transport and metabolic process, in addition to exogenous drug catabolic process; this gene seems to be also implicated in breast cancer development [56, 57]. Similarly to *PXR*, *CAR (NR1I3, Nuclear receptor subfamily 1, group I, member 3)* is also a key regulator of xenobiotic and endobiotic metabolism [58]. Identification of TFs related to liver and xenobiotic metabolism regulating the over-expressed genes suggests that these genes may play a role in drug responses. However, this interesting possibility needs further validation.

The foregoing account demonstrates that all the TFs that are associated with up-regulated genes after combined treatment (cisplatin and LY294002) are regulators of important biological processes, such as cell differentiation, metabolism, development, and immune response, but the literature is still scarce regarding the role of above mentioned TFs in cellular response mechanisms to chemotherapeutic drugs. In spite of this, the increment observed in the number of associated genes (in terms of EI index, which ranged from 1.14 to 1.87) tends to suggest the involvement of these TFs in the outcome of cellular response to combined treatment.

3.3. TFs associated with down-regulated genes

Similar to the prediction of TFs associated with up-regulated genes, we performed the same kind of analysis for determining the TFs associated with down-regulated genes. None of the TF was found associated with down-regulated genes after treatment with cisplatin alone. However, for the combined treatment (cisplatin and LY294002), six TFs were predicted (Table 2). All TFs displayed enrichment indexes greater than 1 (ranging from 1.10 to 1.80), indicating that the enrichment was greater in the list of down-regulated genes (list #1) in comparison to all the genes (list #2) included in the array.

Term	Percentage with term		Enrichment index	p-value
	List #1	List #2		
<i>GATA-3</i>	23.6	16.3	1.45	2.2E-2
<i>Evi-1</i>	50	38.1	1.31	3.9E-2
<i>HNF-1</i>	90.2	84.0	1.10	4.8E-2
<i>USF1</i>	26.4	20.1	1.31	5.1E-2
<i>CDX</i>	82.2	67.3	1.22	5.6E-2
<i>AP-1</i>	27.6	15.5	1.80	5.6E-2

Table 2. Transcription factors (Term) associated to down-regulated genes in treated U343 cells (cisplatin combined to LY294002) vs. control. "Percentage with term" corresponds to the amount of genes that was associated to each TF in each list: list #1 represents the down-regulated genes obtained in the statistical analysis, and list #2 represents all genes present in the microarray slide. An "Enrichment index" was calculated for each Term by dividing the percentage in the list #1 by that in the list #2.

GATA-3, as already described above, was found associated with both up- and down-regulated genes. This could be due to the dual activity of this TF, which acts as a positive or negative regulator of transcription. *GATA-3* also participates in the negative regulation of cell proliferation process, which can be triggered as a response to drug treatments. Accordingly, we found *GATA-3* association with down-regulated genes (EI = 1.45) after drug treatment in U343 cells.

EVI-1, also known as *RUNX1* (*Runt-related transcription factor 1*), is a positive regulator of transcription related to peripheral nervous system, neuron development, hematopoietic stem cell proliferation, and angiogenesis, among other functions. Due to its role in hematopoietic and nervous systems, deregulation of this TF results in hematological disorders [59-62] and central nervous system diseases [63]. Recently, Satoh et al. [64] reported that the C-terminal deletion of *RUNX1* attenuates DNA-damage repair responses in hematopoietic stem/progenitor cells, and additionally, they found that *RUNX1* directly regulates the transcription of *GADD45A* gene, which is involved in cell cycle arrest. Association of *EVI-1* with down-regulated genes (EI = 1.31) presumably indicates an impaired function of this TF in U343 cells, since it is expected to positively regulate the gene targets.

HNF-1 is also named *HNF1A* (*Hepatocyte Nuclear Factor 1 Alpha*) and *TCF-1* (*Transcription Factor 1*). This TF, previously related to diseases such as diabetes and neoplasias [65-70], encodes a protein involved in the WNT signaling pathway [71]. The activation of this pathway has been pointed as one of the main causes of colon cancer; in addition, it has been reported that *HNF1A* plays an important role in transduction of this pathway in the intestine [72]. Furthermore, Roth et al. [73] demonstrated alterations in the regulation of WNT signaling pathway in glioma cells. Inhibition of genes participating in the WNT pathway promote blockage of tumor growth [74]. This is compatible with reduction in cell survival after drug treatment in GBM cells which may be most likely due to down-regulation of several genes regulated by *HNF1A*.

USF1 (*Upstream transcription factor 1*), another TF predicted in our analysis, is a gene with known functions in cellular responses under stress conditions, such as UV and hypoxia. In

breast tumors, a role for *USF1* in regulating the expression of *estrogen receptor alpha (ERalpha)* has been demonstrated [75]. Recently, Baron et al. [76] demonstrated a role for USF-1 in maintaining genome integrity in response to UV-induced DNA photolesions. Additionally, using a mouse knock out model, the authors showed that the loss of USF-1 compromises DNA repair, suggesting that USF-1 may play a similar role in human cells. However, in the present study, USF1 was found associated with down-regulated genes (EI = 1.31) in U343 and the role of USF1 remains to be investigated in tumor cells.

CDX represents a gene family of *caudal-related homeobox* TFs, composed of the following members: *CDX1*, *CDX2*, and *CDX4*. All these members participate in the regulation of intestine-specific gene expression, and also in anterior-posterior pattern specification and blood vessel development. These TFs have been found associated with several neoplasias, including esophageal [77, 78], gallbladder [79], liver [80], colorectal [81-83], and pancreatic [84], but their role in drug responses is still unknown.

AP-1 (presented EI = 1.8) is a TF complex formed by proteins of the JUN family dimerized with members of the *FOS* gene family, which consists of 4 members (*FOS*, *FOSB*, *FOSL1*, and *FOSL2*) that encode leucine zipper proteins. All these genes are oncogenes with established roles in neoplastic cell transformation [85-87], prostatic [88], colorectal [89, 90], liver [91, 92], stomach [93], and breast cancers [94]. Interestingly, Hamdi et al. [95] demonstrated the roles of AP-1 components, ATF3 and FRA1 (*FOSL1*), in JNK- and ERK-dependent cell cycle arrest and apoptosis after cisplatin or UV-light treatments, by activating both JNK and ERK pathways in human glioblastoma cells lacking functional TP53. Recently, Meise et al. [96] showed that *FOSL1 (FRA1)* was up-regulated in glioblastoma cells following nimustine (ACNU) treatment. Thus, it becomes apparent that the AP-1 complex also participates in drug responses, and could be activated by combined treatment of cisplatin and LY294002 in GBM cells.

Therefore, TFs associated with down-regulated genes are critical participants in several biological processes such as neuron development, WNT signaling pathway, cellular response under stress conditions, and neoplastic cell transformation. It seems that all these TFs work in a concerted manner to regulate several genes leading to initiation of cellular responses after the combined treatment of cisplatin and LY294002 in GBM cells. Since most of the TFs predicted by us were not included in the gene arrays, expression of these TFs after drug treatment could not be verified. Further experiments are certainly warranted to understand their concerted roles in understanding the cellular response mechanisms to cisplatin and LY294002.

TFs and cis-regulatory elements are considered key components for transcription regulation [97]. Specific TFs can be involved in the control of co-expressed genes [98], and these genes tend to participate in related pathways, for example, cell cycle control and apoptosis [99, 100]. Thus, search for the common TFs that control the transcription of a specific group of genes such as those involved in DNA repair or replication may provide biomarkers, which can be potential targets for therapeutic intervention [101]. Interestingly, some of the predicted TFs were recently reported to be associated with DNA repair/DNA damage responses in human cells, but few were reported in GBM. Nevertheless, the TFs predicted by *in silico* analysis described above may be useful for understanding how the transcription machinery responds

to chemotherapeutic agents in GBM cells. Further studies are required to get insights regarding the role of TFs identified by us in modulating the drug response of GBM cells.

3.4. Signaling pathways involved in drug responses

In addition, data obtained on differentially expressed genes between treated (cisplatin combined to LY294002) and control U343 cells were submitted to a pathway analysis, using tools available in the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [102]. The KEGG pathway analysis was selected and pathways presenting p-values ≤ 0.05 were chosen.

Signaling pathway analysis was performed by means of tools available in DAVID, as previously described. This analysis allowed the identification of common biological pathways mapped in the KEGG pathway database, in which the up-regulated genes in treated U343 cells are involved. Three pathways were significantly associated with up regulated genes (Table 3), among which two are related to the inositol molecule (inositol phosphate metabolism and phosphatidylinositol signaling system); three genes (*PIK3C3*, *PIP5K1B*, *OCRL*) were included as participants of both pathways. The *TP53* signaling pathway was also associated which included three important genes: *ATR*, *CCNG1* and *SESN1*. These results are consistent with earlier studies demonstrating the activation of these signaling pathways under drug treatments [103-105].

KEGG term	Genes*	p-value
hsa00562: Inositol phosphate metabolism	<i>PIK3C3</i> , <i>PIP5K1B</i> , <i>OCRL</i>	3.3E-2
hsa04115: TP53 signaling pathway	<i>ATR</i> , <i>CCNG1</i> , <i>SESN1</i>	5.0E-2
hsa04070: Phosphatidylinositol signaling system	<i>PIK3C3</i> , <i>PIP5K1B</i> , <i>OCRL</i>	5.0E-2

* Genes participating in each pathway

Table 3. Cellular signaling pathways associated with up-regulated genes in treated U343 cells (cisplatin combined to LY294002), as analyzed by tools available in DAVID.

The *PIK3C3* gene (*Phosphoinositide-3-kinase, class 3*) participates in the innate immune response and cell cycle. This gene also plays an essential role in regulating functional autophagy and is crucial for normal liver and heart function [106]. *PIP5K1B* (*Phosphatidylinositol-4-phosphate 5-kinase, type I, beta*) is involved in the biosynthesis of phosphatidylinositol 4,5-bisphosphate. The *OCRL* gene (*Oculocerebrorenal syndrome of Lowe*) encodes a phosphatase enzyme with a demonstrated role in actin polymerization, and it is an important participant in the trans-Golgi network. Mutations in this gene cause oculocerebrorenal syndrome of Lowe and also Dent disease [107].

The protein encoded by the *ATR* gene (*Ataxia telangiectasia and Rad3 related*) act in the cell cycle checkpoint and is required for cell cycle arrest and DNA damage repair in response to DNA damage. The *ATR* gene belongs to the PI3/PI4-kinase family, as well as ATM and DNA-PK,

and is known as playing an important role in cellular response to cisplatin [108-110]. The aberrantly activated antiapoptotic phosphatidylinositol-3-kinase (PI3K)/Akt signaling is induced by cisplatin and restricts the efficiency of chemotherapy [49]. The *CCNG1* gene (*cyclin G1*) plays a role in cell cycle and growth, brain development and G2/M checkpoint, acting in the negative regulation of apoptotic process. Altered regulation of this gene was observed in breast cancer [111], and leukemia [112]. *SESN1* (*Sestrin 1*) is responsible for the negative regulation of cell proliferation, acting in cell cycle arrest in response to DNA damage stimulus. This gene was found differentially induced by genotoxic stress (UV, gamma-irradiation and cytotoxic drugs) in a TP53-dependent manner, presenting properties common to the GADD family of growth arrest and DNA damage-inducible stress-response genes [113].

Therefore, we found relevant pathways related to TP53 (cell cycle arrest and DNA repair) in response to DNA damage, as well as to the inositol phosphate metabolism and signaling. Furthermore, several predicted TFs associated with modulated genes participate in "basal vital processes", and these processes include the inositol metabolism and signaling, highlighting the importance of these pathways in cellular response mechanisms after drug treatment. However, it is not known whether the observed sensitivity of GBM to cisplatin and LY294002 is due to disruption of some of the above-mentioned vital processes through inhibition of PI3K dependent signaling pathways. A likely possibility that needs consideration is that several sub-pathways can be inactivated/activated due to loss of major tumor suppressor genes such as PTEN, which negatively regulates PI3K/AKT pathways [114] in TP53 proficient U343 GBM cell line. [115]. Furthermore, TP53 pathway, which has been extensively studied in cancer, was also involved in U343 cell line after drug exposure.

3.5. Conclusion

Identification of TFs associated with a set of differentially expressed genes is an interesting approach that may allow to interpret mechanisms triggered in a cellular milieu after a given treatment condition. In the context of cancer research, differential expression of genes and pathways in response to drug treatment may either result in tumor growth reduction or cell killing.

Beside the relevance of our findings, some methodological limitations should be mentioned regarding *in silico* prediction of TFs. Numerous TF binding sites exist for each of the gene targets and it is hard to predict which binding site is critical for transcriptional regulation. It is possible that a considerable fraction of these binding sites are nonfunctional and may constitute biological noise [116]. Other choices, such as ChIP experiments, may overcome this concern by detecting indirect TF-DNA interactions through protein/protein interaction [117]. Even though most of the predicted TFs we identified in our study have not been specifically shown to be associated with GBM in previous studies, they can be considered as potential predictors for evaluating the effectiveness of drug response in GBM. Further verification and validation of the TFs involved in GBM may provide useful information for developing novel therapeutic strategies for brain tumors.

Author details

P. O. Carminati¹, F. S. Donaires¹, P. R. D. V. Godoy¹, A. P. Montaldi¹, J. A. Meador², A.S. Balajee², G. A. Passos³ and E. T. Sakamoto-Hojo^{1,4}

1 Department of Genetics, Faculty of Medicine of Ribeirão Preto, São Paulo University (USP), Ribeirão Preto, SP, Brazil

2 Center for Radiological Research, Department of Radiation Oncology, College of Physicians and Surgeons, Columbia University, New York, NY, USA

3 Department of Genetics, Faculty of Medicine and Faculty of Dentistry, University of São Paulo, Ribeirão Preto, SP, Brazil.

4 Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, São Paulo University (USP), Ribeirão Preto, SP, Brazil

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