Mol Biol Rep (2012) 39:10373–10382
DOI 10.1007/s11033-012-1916-1

Toxicogenomic activity of gemcitabine in two *TP53*-mutated bladder cancer cell lines: special focus on cell cycle-related genes

Glenda Nicioli da Silva · Elaine Aparecida de Camargo · Daisy Maria Favero Salvadori

Received: 19 July 2012 / Accepted: 1 October 2012 / Published online: 9 October 2012

Abstract Because of its lower toxicity and good tolerability and response, gemcitabine has been described as one of the most highly promising drugs for urinary bladder cancer therapy. Its phosphorylated active-dFdCTP metabolite can incorporate into DNA, causing replication blockage. Additionally, it is known that mutations in the *TP53* gene are related to the high recurrence rate of these neoplasias. Based on these premises, we investigated the effects of gemcitabine on the expression of the cell cycle-related genes in two different *TP53*-mutated bladder transitional carcinoma cell lines–5637 (from a moderate-grade tumor with a *TP53* allele carrying two mutations) and T24 (from an invasive tumor with a *TP53* allele encoding an in-frame deletion). Cell viability and morphology analyses (phase-contrast photomicrographs), Nuclear Division Index and pathway-specific quantitative RT-PCR gene arrays were performed. Treatment with gemcitabine led to the following results: (1) a significant decrease of viable T24 cells after treatment at the highest concentration (3.12 μM) tested; (2) scattered, elongated and vacuolated 5637 and T24 cells; (3) a cytostatic effect in both cell lines; and (4) significant upregulation of the *BRCA1, CCNE1, CDK2, CDK6, CDKN1A, CDKN2B, E2F4, GADD45A, MAD2L2, CCNH, SERTAD1, CDC1,* and *CHEK1* genes. Gemcitabine had distinct toxicogenomic effects in the bladder transitional carcinoma cell lines with two different *TP53* mutations. However, independent of the type of mutation and tumor grade, gemcitabine induced cell cycle arrest; upregulation of DNA repair-related genes, G1/S transition, apoptosis and activation of transcription factors, mainly by upregulation of the *CCNE1, CDKN1A* and *GADD45A* genes.

Keywords Chemotherapy · Gemcitabine · Gene expression profiling · Mutated *TP53* cell lines

Introduction

In most countries of the Western world, bladder cancer is predominantly of the transitional cell carcinoma (TCC), which comprises 90 % of all bladder carcinomas, while 5 % of them are identified as squamous and 2 % as adenocarcinomas [1]. Although men are 3–4 times more likely to develop bladder cancer, women present more often with advanced disease and have a lower chance of survival [2]. About 30 % of TCCs display solid and invasive growth patterns, being locally advanced or metastatic at the time of diagnosis; the other 70 % are confined to the epithelium or subepithelial connective tissue [3]. Urothelial bladder cancers have been reported to have the highest recurrence rate among solid tumors. Their recurrence ranges from 50 % to 70 %, while more than 15 % progress to muscle invasion over a 5-year period [4]. Among these, mutations in the *TP53* gene are the most important alterations frequently found in bladder cancer cells [5].

Chemotherapeutic protocols have been studied extensively with the hope of improving overall survival and treating the cancer. It is known that the characterization of genes associated with tumor sensitivity to antineoplastic agents can play a critical role in the selection of preferable treatments [6]. Based on its lower toxicity and good

Glenda Nicioli da Silva and Elaine Aparecida de Camargo contributed equally to this study.

G. N. da Silva (✉) · E. A. de Camargo · D. M. F. Salvadori
Faculdade de Medicina de Botucatu, Departamento de Patologia, UNESP—Universidade Estadual Paulista, Rubião Junior, Botucatu, SP 18618-000, Brazil
e-mail: nicioli@fmb.unesp.br

tolerability and response, when compared to several chemotherapeutic drugs, gemcitabine has shown promising results when tested alone [7]. In 2006, Bellmut [8] described that gemcitabine is the single most highly promising agent for bladder cancer. Furthermore, clinical data have also indicated that this drug has activity in terms of tumor response and overall survival [9]. Gemcitabine is a deoxycytidine analog, which is phosphorylated to an active dFdCTP metabolite (gemcitabine triphosphate) that incorporates into DNA, causing replication blockage [10], and also into RNA, inhibiting RNA synthesis [11]. Gemcitabine has a molecular weight of 299 Da, which is lower than those of the commonly used intravesical chemotherapeutic agents, such as mitomycin C and doxorubicin. This may enable gemcitabine to penetrate the bladder mucosa with beneficial effects in the treatment of invasive bladder cancers [12].

After incorporation into the cell, gemcitabine can be recognized by the p53 protein [13]. In wild-type *TP53* cells, gemcitabine-induced cellular damage can stimulate p53 expression, resulting in p21 expression and cell cycle arrest, enabling the cell to repair the DNA damage or inducing apoptosis mediated by the *BAX* gene. In cells with a mutated *TP53* phenotype, p53 and p21 cannot be induced, but *BAX* expression can still be found, resulting in apoptosis [14]. Additionally, gemcitabine can inhibit DNA synthesis by causing DNA strand breaks, thereby eliciting a DNA damage response characterized by cell cycle arrest in the G1/S phase [15]. In fact, da Silva et al. [16] have shown that gemcitabine induces G1-phase arrest and triggers apoptosis in bladder cancer cell lines. To explain this mechanism, Toshimitsu et al. [17] have shown that in pancreatic cancer cells, gemcitabine upregulated the gene, a cyclin-dependent kinase inhibitor implicated in the transition from proliferation to a quiescent state. These authors suggest that the elevated expression of *CDKN1B* might cause G1 arrest of cells. Another possible mechanism would include the inhibition of DNA topoisomerases [18]. Therefore, these targets could represent opportunities for chemotherapeutic intervention, contributing to the treatment of cancer [19]. In this direction, high-throughput methods may help to better understand the molecular activities in different cell types, promoting the understanding of the mechanism of action of these drugs [20].

Therefore, based on the fact that gemcitabine is a promising therapy for different types of cancer, because its mechanism of action is not well-known, and because of the importance of *TP53* mutations for bladder cancer, we investigated the effects of gemcitabine on bladder cancer cells carrying different *TP53* mutations. The expression of cell cycle-related genes was analysed. Thus, we evaluated whether gemcitabine activity would be dependent on the *TP53* genetic background of tumor cells.

Materials and methods

Cell lines

The two human bladder TCC cell lines, 5637, with a *TP53* allele carrying two mutations (one at codon 280—Arg>Thr and the other at codon 72—Arg>Pro), obtained from a moderate grade tumor and T24, with a *TP53* allele encoding an in-frame deletion of tyrosine 126, obtained from an invasive tumor, were acquired from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. The cells were maintained as previously described by da Silva et al. [16]. The antineoplastic drug gemcitabine (dFdC, Gemzar) was obtained from Eli Lilly Laboratory (USA). Ultra-pure sterilized water was used for the dilutions.

Experimental design

The cells were seeded into 12-well culture plates (1×10^4 cells/well) for evaluating the cell viability and the morphological changes, into Petri dishes (1×10^6/cells/dish) for evaluating the Nuclear Division Index (NDI) and into 25 cm^3 culture flasks (2×10^6 cells/flask) for the RNA extraction. Twenty-four hours after seeding (T1 = 0 h), the cells were treated with gemcitabine at concentrations of 0.78, 1.56, or 3.12 μM (as defined in previous experiments) [16], during a 24 hour period (T2 = 24 h). Untreated cells were cultured in parallel as a negative control. During T2, the cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1,000 ml H_2O) and collected for morphological, cell viability and gene expression evaluations.

Cell viability, morphology and NDI

Cell viability was evaluated by the trypan blue exclusion test. The assay was performed in triplicate, and the results were represented as the mean ± standard deviation (SD). A phase-contrast microscope was used before and after the gemcitabine treatment for evaluating the morphological changes.

For the NDI assay, the 5637 and T24 cell lines were treated with gemcitabine at concentrations of 0.78, 1.56, or 3.12 μM during a 6-h period. Afterwards, the cells were washed, and cytochalasin B (Sigma-Aldrich) at final concentration of 3 μg/ml was added. Twenty-four hours later, the cells were collected, the suspension was transferred to 15 ml centrifuge tubes and 5 ml of a hypotonic solution (KCl 0.075 M) was added. After a 5 min centrifugation at 1,000 rpm, the cells were fixed with 5 ml of a methanol:acetic acid (5:1) solution plus 0.3 ml of formaldehyde,

and the slides were prepared. The slides were stained with a 5 % Giemsa solution and scored under a light microscope at 400× magnification (2,000 cells/treatment). Cells treated with doxorubicin, at a concentration of 0.4 μg/ml for 2 h, were used as a positive control. The NDI was determined as previously described by Fenech et al. [21]: NDI = M1 + 2 (M2) + 3 (M3) + 4 (M4)/N, where M1–M4 is the number of cells with 1–4 nuclei and N is the total number of viable cells. The assay was performed in duplicate.

RNA extraction

Total RNA was extracted using the Mini RNeasy kit (Qiagen) according to the manufacturer's instructions. The extracted RNA was stored at −80 °C. The integrity and quality of the RNA was evaluated with 2 % denaturing agarose gels and NanoVue equipment (GE Healthcare), respectively.

PCR arrays

For the gene expression evaluation, gemcitabine was used at a concentration of 1.56 μM (this concentration is genotoxic in the comet assay, but not cytotoxic in the trypan blue and XTT tests [16]. The cell cycle pathway PCR Array (PAHS-020A, SA Biosciences) was used for the qRT-PCR. The cDNA was produced using the RT2 First Strand kit (SA Biosciences) according to manufacturer. An aliquot of the diluted first-strand synthesis reaction was added to the SYBR Green/ROX master mix (SA Biosciences) along with nuclease free water according to the PCR array system's user manual. Afterwards, 25 μl of the cDNA/master mix cocktail was placed into each well of the pathway-specific qRT-PCR microplate. Quality controls for the genomic DNA contamination, reverse transcription efficiency, and PCR amplification efficiency were analyzed. The qRT-PCR array data were normalized using the arithmetic mean of five housekeeping genes *B2M*, *HPRT1*, *RPL13A*, *GAPDH* and *ACTB* for the T24 cells and *GAPDH* and *HPRT1* for the 5637 cells. The arrays were all performed in triplicate. The information regarding the biological functions was obtained from FATIGO (http://babelomics.bioinfo.cipf.es/).

Statistical analysis

For statistical analysis of the NDI and cell viability, a one-way ANOVA test was used. For the gene expression analysis, the fold change was used. In this case, the p value was calculated with Student's t test with triplicate values for each gene in the control and treatment groups. A p value <0.05 was considered statistically significant.

Results

The percentages of viable cells in the 5637 cell line after the treatments with gemcitabine at concentrations of 0, 0.78, 1.56, and 3.12 μM were 94.26 ± 2.27, 86.53 ± 12.89, 76.90 ± 15.13, and 77.96 ± 9.82, respectively; for the T24 cells, the percentages were 98.33 ± 2.88, 97.10 ± 5.02, 89.73 ± 3.06, and 86.23 ± 2.76, respectively. A statistically significant decrease in the percentage of viable cells was only detected in the T24 lineage treated with the highest concentration of gemcitabine (3.12 μM). With regard to the NDI, a statistically significant decrease in the number of cells ($p < 0.05$) was observed in both cell lines after treatment with the three concentrations of gemcitabine (Table 1). The phase-contrast photomicrographs of the 5637 and T24 cell lines showed scattered, elongated and vacuolated cells after the gemcitabine treatment. Additionally, a lower number of cells were observed in the gemcitabine-treated cell cultures than in their respective controls (Fig. 1).

Of the 84 genes analyzed, 36 genes in the T24 and 29 genes in the 5637 cells were differentially modulated after the gemcitabine treatment (Tables 2 and 3). The highest changes (fold change >2, $p < 0.05$) were detected for the *BIRC5, BRCA1, CCNE1, CDK2, CDK6, CDK8, CDKN1A, CDKN2B, CUL2, E2F4, GADD45A, HERC5, KPNA2, MAD2L1, MAD2L2, NBN, RAD51, RB1* and *TFDP2* genes in the 5637 cells and for the *BRCA2, CCNE1, CDKN1A, GADD45A, TP53, CCNB1* and *CCNF* genes in the T24 cells. Therefore, the genes that were equally modulated in both cell lines were *CCNE1*, *CDKN1A* and *GADD45A* (fold change >2, $p < 0.05$). Other genes (*BRCA1, CDK2, CDK6, CDKN2B, E2F4, MAD2L2, CCNH, SERTAD1, CDC16, CHEK1*) presented a significant upregulation in the two cell lines ($p < 0.05$), but the fold change for one or both cell lines was less than two (Figs. 2 and 3).

Table 1 The Nuclear Division Index (NDI) for the 5637 and T24 cell lines after the treatments with gemcitabine

Treatments	Concentration (μM)	NDI	
		Mean ± SD	
		5637	T24
Negative control	0	1.96 ± 0.001	1.94 ± 0.004
Gemcitabine	0.78	1.02 ± 0.000*	1.01 ± 0.006*
	1.56	1.01 ± 0.004*	1.01 ± 0.002*
	3.12	1.01 ± 0.002*	1.02 ± 0.006*

* $p < 0.05$, compared to the negative control

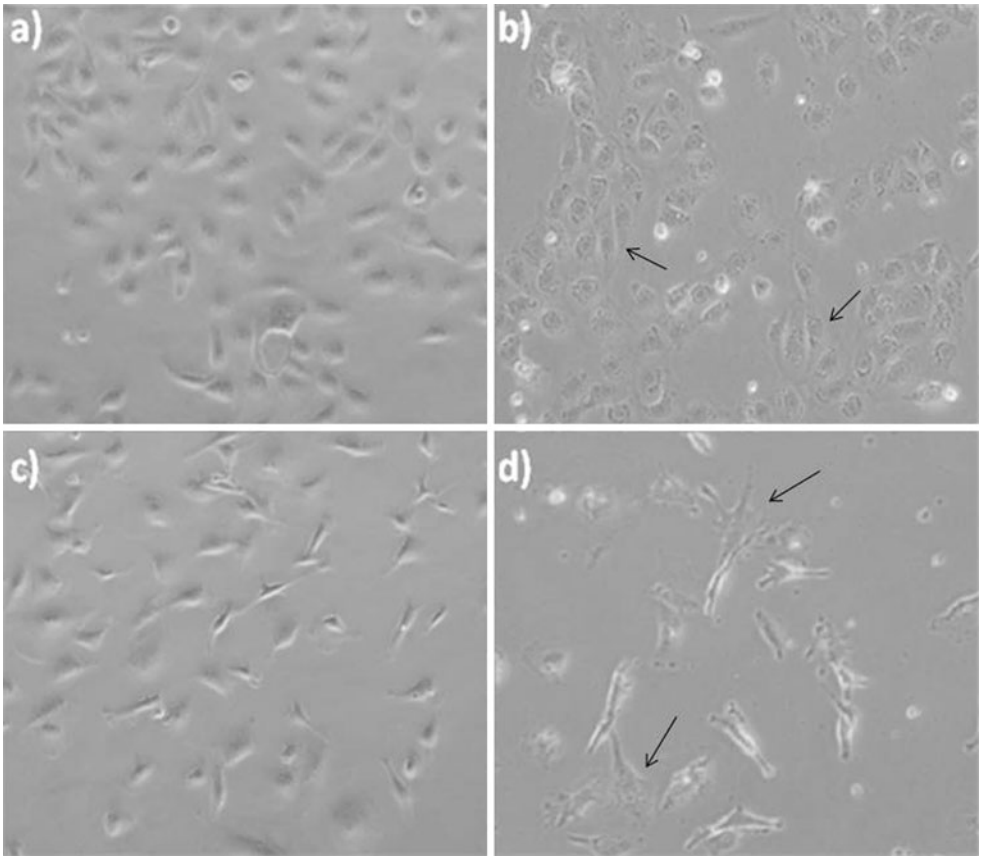

Fig. 1 Photomicrography of two lineages of human bladder transitional carcinoma cells before and after gemcitabine treatment: **a** 5637 cells; **b** 5637 cells after treatment with 1.56 μM gemcitabine; **c** T24 cells; **d** T24 cells after treatment with 1.56 μM gemcitabine. The *arrows* show the elongated and vacuolated cells. Phase-contrast microscope, ×400

Discussion

It is known that mutations in the *TP53* gene are related to the high recurrence rate of urinary bladder cancers [5], and it has been reported that gemcitabine is one of the most highly promising drugs for the treatment of these neoplasias [8]. Therefore, based on these premises, we investigated the effects of gemcitabine on the expression of the cell cycle-related genes in two different *TP53*-mutated bladder transitional carcinoma cell lines. Previously, we have observed that gemcitabine had similar effects in 5637 and T24 cells, i.e., G1-phase arrest and late apoptosis were visualized in both cell lines [16]. Nevertheless, we did not identify which genes might be involved in these pathways. In fact, similar findings had been reported by Fencher et al. [22], showing that gemcitabine-induced apoptosis in bladder cancer cells is independent of the *TP53* status.

Before analyzing the gene expression signature, we checked the effects of gemcitabine on the cell viability and morphology, and on the NDI. The only significant difference detected between the two cell lines was a decreased viability of the T24 cells just after the exposure to the highest concentration of gemcitabine (3.12 μM). The observed morphological changes (vacuolized, scattered and elongated cells) together with a low cell density in both lineages were suggestive of cell cycle arrest and late apoptosis. The reduced NDIs confirmed that the blockage of cell division is independent of the gemcitabine concentration used. According to Fenech [21], the NDI indicates the proliferation state of the viable cells. Therefore, it can be used as a marker of cytostatic effect. Aydemir et al. [23] also found a decreased rate of cell replication in cultured human lymphocytes after gemcitabine treatment. A possible mechanism to explain this effect is the ability of gemcitabine to incorporate into the DNA strands and inhibit the DNA polymerase activity [24].

In general, toxicogenomic research focuses on the modulation of the gene expression profiling or pathways after the exposure to a toxic agent. If the concentration of an agent induces significant levels of cell death, the transcriptome profiles will be reflective of the dead or dying cells instead of the cellular responses to a lower non-cytotoxic concentration [25]. Therefore, the concentration we used for evaluating the toxicogenomic effect of gemcitabine (1.56 μM)

Table 2 The significantly modulated genes in the T24 cells after the treatment with gemcitabine at 1.56 μM

Ref seq	Symbol	Complete name	Change	*p* value
NM_001184	ATR	Ataxia telangiectasia and Rad3 related	1.76	0.007562
NM_016567	BCCIP	BRCA2 and CDKN1A interacting protein	1.31	0.003766
NM_001168	BIRC5	Baculoviral IAP repeat-containing 5	−1.22	0.031777
NM_007294	BRCA1	Breast cancer 1, early onset	1.42	0.025982
NM_000059	BRCA2	Breast cancer 2, early onset	2.40	0.048404
NM_031966	CCNB1	Cyclin B1	−2.23	0.000693
NM_005190	CCNC	Cyclin C	1.40	0.040830
NM_001238	CCNE1	Cyclin E1	2.28	0.017472
NM_001761	CCNF	Cyclin F	−2.28	0.017938
NM_001239	CCNH	Cyclin H	1.31	0.024728
NM_001240	CCNT1	Cyclin T1	1.61	0.017781
NM_003903	CDC16	Cell division cycle 16 homolog (*S. cerevisiae*)	1.24	0.012541
NM_001786	CDC20	Cell division cycle 20 homolog (*S. cerevisiae*)	−1.44	0.004702
NM_001798	CDK2	Cyclin-dependent kinase 2	1.30	0.003990
NM_000075	CDK4	Cyclin-dependent kinase 4	1.30	0.000370
NM_001259	CDK6	Cyclin-dependent kinase 6	1.55	0.011396
NM_001799	CDK7	Cyclin-dependent kinase 7	1.46	0.008375
NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.20	0.003109
NM_004936	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	1.75	0.002844
NM_001274	CHEK1	CHK1 checkpoint homolog (*S. pombe*)	1.36	0.044549
NM_001827	CKS2	CDC28 protein kinase regulatory subunit 2	−1.50	0.047817
NM_001950	E2F4	E2F transcription factor 4, p107/p130-binding	1.35	0.009312
NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha	3.14	0.003869
NM_005316	GTF2H1	General transcription factor IIH, polypeptide 1, 62 kDa	1.73	0.010079
NM_016426	GTSE1	G-2 and S-phase expressed 1	−1.56	0.010969
NM_004507	HUS1	HUS1 checkpoint homolog (*S. pombe*)	1.53	0.032667
NM_002266	KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	−1.86	0.008039
NM_006341	MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	1.44	0.002508
NM_004526	MCM2	Minichromosome maintenance complex component 2	1.59	0.009932
NM_005914	MCM4	Minichromosome maintenance complex component 4	1.38	0.029714
NM_006739	MCM5	Minichromosome maintenance complex component 5	1.70	0.016861
NM_002431	MNAT1	Menage a trois homolog 1, cyclin H assembly factor	1.22	0.002310
NM_002894	RBBP8	Retinoblastoma binding protein 8	1.69	0.023342
NM_002947	RPA3	Replication protein A3, 14 kDa	1.43	0.030858
NM_013376	SERTAD1	SERTA domain containing 1	1.99	0.002069
NM_000546	TP53	Tumor protein p53	3.34	0.000926

was carefully selected based on the genotoxic (data not shown) and cytotoxic data previously published by our group [16]. The gene arrays initially showed upregulation of the *BRCA1, CCNE1, CDK2, CDK6, CDKN1A, CDKN2B, E2F4, GADD45A, MAD2L2, CCNH, SERTAD1, CDC16,* and *CHEK1* genes in the 5637 and T24 cells after gemcitabine treatment. According to the gene ontology (GO), these genes are primarily involved in the negative regulation of the cell cycle (*BRCA1, CDK2, CDK6, CDKN1A, CDKN2B, GADD45A, MAD2L2, CDC1,* and *CHEK1*), cell cycle arrest (*BRCA1, CDK2, CDKN1A, CDKN2B, GADD45A, MAD2L2, CDC16,* and *CHEK1*), G1/S transition of the mitotic cell cycle (*CCNE1*, *CDK2, CDKN1A, CDKN2B, E2F4,* and *CCNH*), DNA repair (*BRCA1* and *GADD45A*), apoptosis (*BRCA1* and *GADD45A*) and the regulation of transcription (*SERTAD1* and *E2F4*). Several authors have shown that some of these genes can be targets for cancer treatment. Trichostatin A [26] and ellagic acid [27], for example, increase *CDKN1A* expression and promote cell cycle arrest in bladder cancer cell lines; *GADD45A* expression was associated with the efficacy of the treatment with 5-aza-CdR in different pancreatic cancer cell lines [28].

Table 3 The significantly modulated genes in the 5637 cells after the treatment with gemcitabine at 1.56 μM

Ref seq	Symbol	Complete name	Change	*p* value
NM_001168	BIRC5	Baculoviral IAP repeat-containing 5	2.70	0.000200
NM_007294	BRCA1	Breast cancer 1, early onset	25.49	0.006730
NM_001238	CCNE1	Cyclin E1	2.81	0.020263
NM_001239	CCNH	Cyclin H	1.30	0.026828
NM_003903	CDC16	Cell division cycle 16 homolog (*S. cerevisiae*)	1.53	0.001316
NM_001786	CDC2	Cell division cycle 2, G1 to S and G2 to M	1.92	0.003047
NM_001798	CDK2	Cyclin-dependent kinase 2	2.20	0.004784
NM_001259	CDK6	Cyclin-dependent kinase 6	3.09	0.042822
NM_001260	CDK8	Cyclin-dependent kinase 8	3.32	0.015924
NM_000389	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	4.69	0.001683
NM_004936	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	2.37	0.027321
NM_001274	CHEK1	CHK1 checkpoint homolog (*S. pombe*)	1.75	0.037759
NM_003592	CUL1	Cullin 1	1.61	0.020450
NM_003591	CUL2	Cullin 2	3.34	0.027972
NM_003590	CUL3	Cullin 3	−1.78	0.004615
NM_001950	E2F4	E2F transcription factor 4, p107/p130-binding	2.01	0.000675
NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha	5.18	0.007580
NM_016323	HERC5	Hect domain and RLD 5	2.62	0.009701
NM_014708	KNTC1	Kinetochore associated 1	1.84	0.007633
NM_002266	KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	4.13	0.008162
NM_002358	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	11.74	0.021659
NM_006341	MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	2.41	0.013582
NM_002485	NBN	Nibrin	2.94	0.046744
NM_002875	RAD51	RAD51 homolog (RecA homolog, *E. coli*) (*S. cerevisiae*)	4.38	0.000381
NM_000321	RB1	Retinoblastoma 1	4.28	0.039761
NM_002894	RBBP8	Retinoblastoma binding protein 8	−1.61	0.022243
NM_005611	RBL2	Retinoblastoma-like 2 (p130)	1.50	0.043466
NM_013376	SERTAD1	SERTA domain containing 1	1.63	0.018925
NM_006286	TFDP2	Transcription factor Dp-2 (E2F dimerization partner 2)	31.32	0.022790

Moreover, inactivation of *CHEK1* appears to contribute to the development of cervical cancer [29], while increased *E2F4* levels following genotoxic stress result in the down-regulation of many mitotic genes and promote a G0-like state [30]. The inactivation of *BRCA1* can be an important therapeutic target in sporadic breast cancers [31].

Three of the modulated genes, *CDKN1A*, *GADD45A* and *CCNE1,* are highlighted because they presented a fold change >2 ($p < 0.05$) in both cell lines. Therefore, their expressions were not associated with the type of *TP53* mutation. The upregulation of *CDKN1A* and *GADD45A* might be respectively, related to the cell cycle arrest and apoptosis, observed in our previous study using the same cell lines [16]. Similarly, a number of authors has reported the induction of *CDKN1A* after gemcitabine treatment in MCF7 and MDA-MB231 cell lines (both from human breast carcinoma) [32]. Classically, the activation of *TP53* gene induces *CDKN1A* mRNA increase, which leads to cell cycle arrest at the G1/S transition. However, it is important to emphasize that, even we have detected a *TP53* overexpression in T24 cells after gemcitabine treatment, no significant alteration was observed in the 5637 cell line. T24 cells harbor *TP53* mutations in the N-terminal transactivation domain which preserve p53 activities such as DNA binding. But in 5637 cells, *TP53* has point mutations at core domain that affect the ability of p53 to bind DNA [33]. Thus, gemcitabine can induce an alternative mechanism of *CDKN1A* activation, disobeying the dogma that DNA damage activates *TP53* gene and triggers *CDKN1A* accumulation, as Soria and Gottifredi [34] have already suggested for some genotoxic agents. With regard to the *CCNE1* upregulation, it has been associated with a poor prognosis and resistance to chemotherapeutic drugs [35]. In fact, we have previously observed that *TP53*-mutated cells are more resistant to treatment with gemcitabine than the wild type [16]. Recently, Jiang et al. [36] explained that

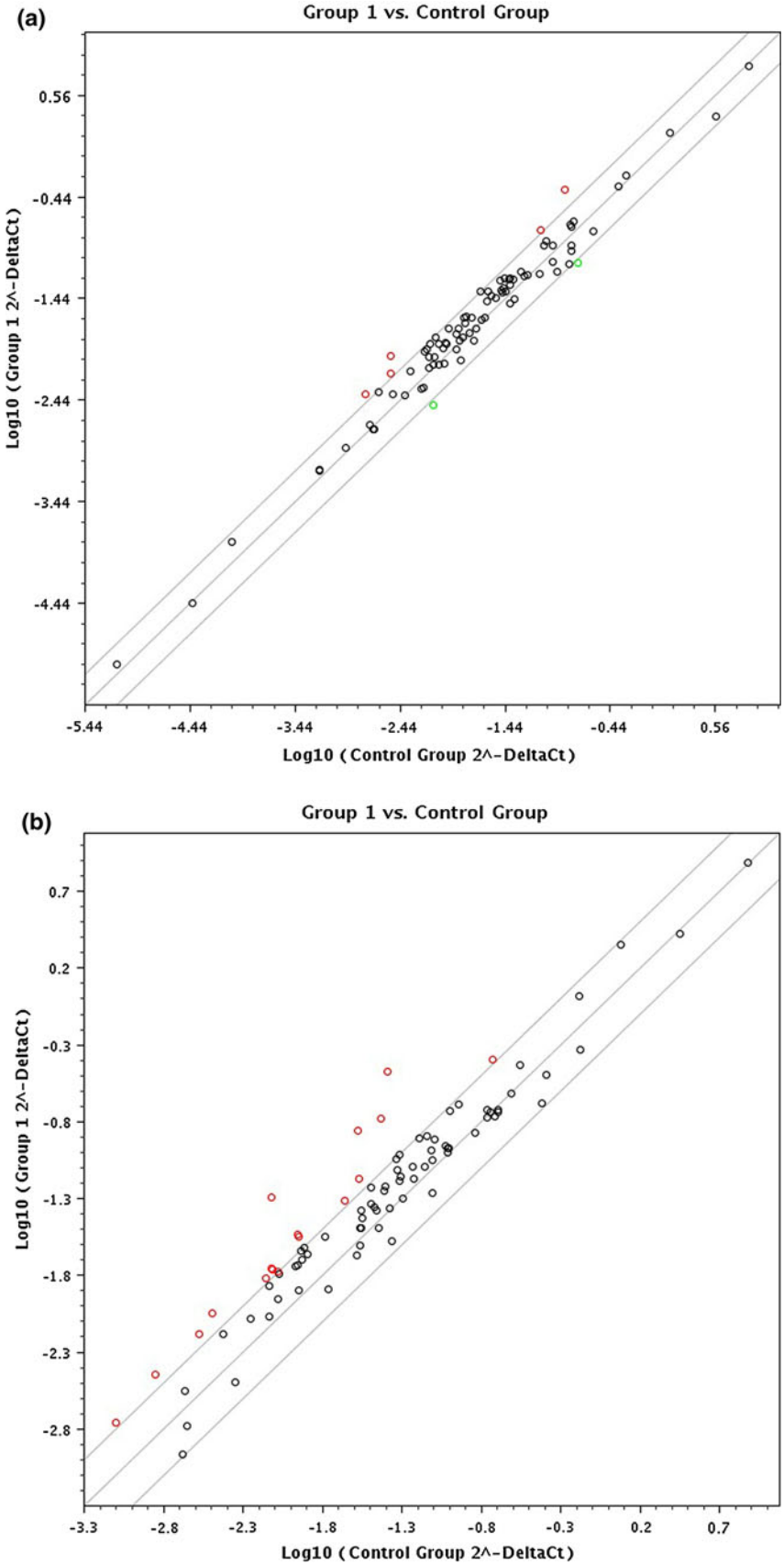

Fig. 2 *Scatter plots* representing all of the significantly modulated genes in the T24 and 5637 cells, respectively. Each *dot* represents one gene; the *dots* above the superior and below the inferior *lines* represent the highest changes of gene expression, fold change >2 or <2, respectively

Fig. 3 *Venn diagram* showing the upregulated genes in both the T24 and 5637 cell lines

TP53 dysfunction compromises the nuclear export of wild-type *BRCA1*, thus characterizing the mechanism to increase cellular resistance to DNA damage in sporadic breast cancer. As far as we know, there are no data in literature about the modulation of the *CCNE1* after gemcitabine treatment.

On the contrary, differential gene expression (with fold change >2) was also detected between the two cell lines. The *BIRC5, CDK6, CUL2, HERC5, KPNA2, MAD2L1, NBN, RAD51, RB1,* and *TFDP2* genes were significantly modulated in the 5637 cells, while *CCNB1, CCNF* and *TP53* were significantly modulated only in the T24 cells. Although most of these genes are associated with cell cycle regulation [37–39], their modulation was not solely responsible for the cell cycle arrest detected in the NDI experiments, since it occurred in both cell lines. Thus, the divergence between the gene expression signature in 5637 and T24 cell lines probably reflects the complexity of *TP53* activity for regulating apoptosis and cell proliferation. Obviously, we cannot also rule out the possibility that other genes might be related to the observed differences in cell responses. In fact, *TP53* is only one component of the giant surveillance network whose efficiency is modulated by many other elements, including other signaling pathways [40]. In a recent study using gene networks, we have described interactions among *TP53* and several other genes, suggesting that other pathways might be associated with the malignant phenotype and chemotherapeutical response [41]. Furthermore, the results of this present study have showed different gene expression patterns after gemcitabine treatment, what suggest that combined evaluation of several genes may be required to finally predict gemcitabine sensitivity. We must remind, however, that not only differences in the gene signature were found in the present study. Some genes were similar and could explain the efficacy of treatment, independently of the genetic background of the cell lines analyzed. Therefore, *TP53* status may not be related to some cell response to gemcitabine treatment.

To T24 cells, regarding the downregulation of the cyclin B1 (*CCNB1)* and cyclin F (*CCNF)* genes, they could be associated with a positive response to chemotherapy, as was observed in our study. Several authors have related the upregulation of these genes to the recurrence of the tumor [42, 43]. The increased risk for a sporadic benign meningioma recurrence has already been identified in cases with elevated expression of *CCNB1* [42]. Furthermore, *CCNF* upregulation is characterized by reduced apoptosis and a more aggressive growth phenotype in HCT116 colorectal cancer cells that are resistant to the chemotherapeutic drug 5-fluorouracil [43].

Several genes were significantly upregulated in only the 5637 cells, including those related to cyclin-dependent kinases (*CDK* 6 and 8) and apoptosis inhibition (*BIRC5*). The upregulation of *CDK6*, *CDK8* and *BIRC5* have already been associated with cancer [44, 45]. However, it is important to notice that the balance between cell proliferation and cell death involves several mechanisms and that, sometimes, the signal for apoptosis surpasses the cell cycle signals. In fact, *RB1,* a tumor suppressor gene, and *CDKN2B,* which is involved in the negative regulation of the cell cycle, were also upregulated.

In conclusion, independent of the site of the *TP53* mutation or the tumor grade, gemcitabine was cytostatic and modulated several molecular pathways, including DNA repair, G1/S transition, apoptosis and transcription factors. The identification of gemcitabine-responsive genes provides insight into its anti-cancer mechanisms and can be used in the clinical setting to predict chemotherapeutical responses. Further studies would be necessary to confirm whether the genes identified in the present study are associated with other types of cell growth inhibition after gemcitabine treatment.

Acknowledgments This study was supported by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Cordon-Cardo C (2008) Molecular alterations associated with bladder cancer initiation and progression. Scand J Urol Nephrol 218:154–165
2. Fajkovic H, Halpern JA, Cha EK, Bahadori A, Chromecki TF, Karakiewicz PI, Breinl E, Merseburger AS, Shariat SF (2011) Impact of gender on bladder cancer incidence, staging, and prognosis. World J Urol 29:457–463
3. Cheng L, Zhang S, MacLennan GT, Williamson SR, Lopez-Beltrn A, Montironi R (2011) FRCPath and IFCAP. Bladder cancer: translating molecular genetic insights into clinical practice. Human Pathol 42:455–481
4. Sylvester RJ, van der Meijden APM, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DWW (2006) Predicting recurrence and progression in individual patients with stage TaT1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol 49:466–477
5. Nishiyama H, Watanabe J, Ogawa O (2008) p53 and chemosensitivity in bladder cancer. Int J Clin Oncol 13:282–286
6. Fujita H, Ohuchida K, Mizumoto K, Itaba S, Ito T, Nakata K, Yu J, Kayashima T, Souzaki R, Tajiri T, Manabe T, Ohtsuka T, Tanaka M (2010) Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy. Neoplasia 12:807–817
7. Castagneto B, Zai S, Marenco D, Bertetto O, Repetto L, Scaltriti L, Mencoboni M, Ferraris V, Botta M (2004) Single-agent gemcitabine in previously untreated elderly patients with advanced bladder carcinoma: response to treatment and correlation with the comprehensive geriatric assessment. Oncology 67:27–32
8. Bellmut J, Albiol S, de Olano AR, Pujadas J, Maroto P (2006) On behalf the Spanish Oncology Genitourinary Group (SOGUG). Gemcitabine in the treatment of advanced transitional cell carcinoma of the urothelium. Ann Oncol 17:113–117
9. Shelley MD, Cleves A, Wilt TJ, Mason MD (2011) Gemcitabine chemotherapy for the treatment of metastatic bladder carcinoma. BJU Int 108:168–179
10. Toschi L, Finocchiaro G, Gioia V (2005) Role of gemcitabine in cancer therapy? Future Oncol 1:7–17
11. Ruiz Van Haperen VWT, Veerman G, Vermoken JB, Peters GJ (1993) 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA from tumor cell lines. Biochem Pharmacol 46:762–766
12. Gontero P, Frea B (2006) Actual experience and future development of gemcitabine in superficial bladder cancer. Ann Oncol 17:123–128
13. Achanta G, Pelicano H, Feng L, Plunkett W (2001) Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. Cancer Res 61: 8723–8729
14. Bergman AM, Pinedo HM, Peters GJ (2002) Determinants of resistance to 2′,2′- difluorodeoxycytidine (gemcitabine). Drug Resist Updat 5:19–33
15. Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR, Dumontet C (2002) Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int J Cancer 97: 439–445
16. da Silva GN, Marcondes JPC, Camargo EA, Sakamoto-Hojo ET, Passos GA, Salvadori DMF (2010) Cell cycle arrest and apoptosis in TP53 subtypes of bladder carcinoma cell lines treated with cisplatin and gemcitabine. Exp Med Biol 235:814–824
17. Toshimitsu H, Iizuka N, Yamamoto K, Kawauchi S, Oga A, Furuya T, Oka M, Sasaki K (2006) Molecular features linked to the growth-inhibitory effects of gemcitabine on human pancreatic cancer cells. Oncol Rep 16:1285–1291
18. Pourquier P, Gioffre C, Kohlhagen G, Urasaki Y, Goldwasser F, Hertel LW (2002) Gemcitabine (20,20-difluoro-20-deoxycytidine), an antimetabolite that poisons topoisomerase I. Clin Cancer Res 8:2499–2504
19. Pearce HL, Miller MA (2005) The evolution of cancer research and drug discovery at Lilly, Research Laboratories. Adv Enzyme Regul 45:229–255
20. Coppée J-Y (2008) Do DNA microarrays have their future behind them? Microbes Infect 10:1067–1071
21. Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2:1084–1104
22. Fencher G, Perabo FGE, Schmidt DH, Haase L, Ludwig E, Schueller H, Blatter J, Muller C, Albers P (2003) Preclinical evaluation of a radiosensitizing effect of gemcitabine in p53 mutant and p53 wild type bladder cancer cells. Urology 61: 468–473
23. Aydemir N, Celikler S, Bilaloglu R (2005) In vitro genotoxic effect of the anticancer drug gemcitabine in human lymphocytes. Mutat Res 582:35–41
24. Jiang HT, Hickey RJ, Abdel-Aziz W, Malkas LH (2000) Effects of gemcitabine and araC on in vitro DNA synthesis mediated by the human breast cell DNA synthesome. Cancer Chemother Pharmacol 45:320–328
25. Attene-Ramos MS, Nava GM, Muellner MG, Wagner ED, Plewa MJ, Gaskins HR (2010) DNA damage and toxicogenomic analyses of Hydrogen Sulfide in human intestinal epithelial FHs 74 Int cells. Environ Mol Mutagen 51:304–314
26. Li G-C, Zhang X, Pan T-J, Chen Z, Ye Z-Q (2006) Histone deacetylase inhibitor trichostatin A inhibits the growth of bladder cancer cells through induction of p21waf1 and G1 cell cycle arrest. Int J Urol 13:581–586
27. Li T-M, Chen G-W, Su C-C, Lin J-G, Yeh C-C, Cheng K-C, Chung J-G (2005) Ellagic Acid Induced p53/p21 Expression, G1 Arrest and Apoptosis in Human Bladder Cancer T24 Cells. Anticancer Res 25:971–980
28. Missiaglia E, Donadelli M, Palmieri M, Crnogorac-Jurcevic T, Scarpa A, Lemoine NR (2005) Growth delay of human pancreatic cancer cells by methylase inhibitor 5-aza-2′-deoxycytidine treatment is associated with activation of the interferon signalling pathway. Oncogene 24:199–211
29. Mazumder D, Mitra S, Singh RK, Dutta S, Roy A, Mondal RK, Basu PS, Roychoudhury S, Panda CK (2011) Inactivation of CHEK1 and EI24 is associated with the development of invasive cervical carcinoma: clinical and prognostic implications. Int J Cancer 129:1859–1871
30. Pleska D, Crosby ME, Gupta D, Almasan A (2007) E2F4 function in G2: maintaining G2-arrest to prevent mitotic entry with damaged DNA. Cell Cycle 6:1147–1152
31. Galizia E, Giorgetti G, Piccinini G, Santinelli A, Loretelli C, Bianchi F, Gagliardini D, Carbonari G, Pisa E, Belvederesi L, Bracci R, Ferretti C, Corradini F (2010) BRCA1 expression in triple negative sporadic breast cancers. Anal Quant Cytol Histol 32:24–29
32. Hernandes-Vargas HH, Rodrigues-Pinilla SM, Julian-Tendero M, Sanchez-Rovira P, Cuevas C, Anton A, Rios MJ, Palacios J, Moreno Bueno G (2007) Gene expression profiling of breast

cancer cells in response to gemcitabine: nK-kB pathway activation as a potential mechanism of resistance. Breast Cancer Res Treat 102:157–172
33. Cordon-Cardo C, Dalbagni G, Saez GT, Oliva MR, Zhang ZF, Rosai J, Reuter VE, Pellicer A (1994) p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. Int J Cancer 56:347–353
34. Soria G, Gottifredi V (2010) PCNA-coupled p21 degradation after DNA damage: the exception that confirms the rule? DNA Repair 9:358–364
35. Lockwood WW, Stack D, Morris T, Grehan D, O'Keane C, Stewart GL, Cumiskey J, Lam WL, Squire JA, Thomas DM, O'Sullivan MJ (2011) Cyclin E1 is amplified and overexpressed in osteosarcoma. J Mol Diagn 13:289–296
36. Jiang J, Yang ES, Jian G (2011) Susceptibility to DNA damage p53-dependent BRCA1 nuclear export controls cellular. Cancer Res 71:5546–5557
37. Beukelaers P, Vandenbosch R, Caron N, Nguyen L, Belachew S, Moonen G, Kiyokawa H, Barbacid M, Santamaria D, Malgrange B (2011) Cdk6-dependent regulation of G(1) length controls adult neurogenesis. Stem Cells 29:713–724
38. Liu J, Vasudevan S, Kipreos ET (2004) CUL-2 and ZYG-11 promote meiotic anaphase II and the proper placement of the anterior-posterior axis in C. elegans. Development 131:3513–3525
39. Redwood AB, Gonzalez-Suarez I, Gonzalo S (2011) Regulating the levels of key factors in cell cycle and DNA repair: new pathways revealed by lamins. Cell Cycle 10:3652–3657
40. Soussi T, Wiman KG (2007) Shaping genetic alterations in human cancer: the p53 mutation paradigm. Cancer Cell 12:303–312
41. da Silva GN, Evangelista AF, Magalhães DA, Macedo C, Búfalo MC, Sakamoto-Hojo ET, Passos GA, Salvadori DM (2011) Expression of genes related to apoptosis, cell cycle and signaling pathways are independent of TP53 status in urinary bladder cancer cells. Mol Biol Rep 38:4159–4170
42. Bie L, Zhao G, Ju Y, Zhang B (2011) Integrative genomic analysis identifies CCNB1 and CDC2 as candidate genes associated with meningioma recurrence. Cancer Genet 204:536–540
43. de Angelis PM, Fjell B, Kravik KL, Haug T, Tunheim SH, Reichelt W, Beigi M, Clausen OP, Galteland E, Stokke T (2004) Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. Int J Oncol 24:1279–1288
44. Di Stefano AB, Iovino F, Lombardo Y, Eterno V, Höger T, Dieli F, Stassi G, Todaro M (2010) Survivin is regulated by interleukin-4 in colon cancer stem cells. J Cell Physiol 225:261–555
45. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL (2011) Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 11:558–572