RESEARCH ARTICLE

No Relationship between the Amount of DNA Damage and the Level of *hMLH1* and *RASSF1A* Gene Expression in Bladder Cancer Cells Treated with Cisplatin and Gemcitabine

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Abstract

Tumor response to antineoplastic drugs is not always predictable. This is also true for bladder carcinoma, a highly recurrent neoplasia. Currently, the combination of cisplatin and gemcitabine is well accepted as a standard protocol for treating bladder carcinoma. However, in some cases, this treatment protocol causes harmful side effects. Therefore, we investigated the roles of the genes *TP53*, *RASSF1A* (a tumor suppressor gene) and *hMLH1* (a gene involved in the mismatch repair pathway) in cell susceptibility to cisplatin/gemcitabine treatment. Two bladder transitional carcinoma cell (TCC) lines, RT4 (wild-type *TP53*) and 5637 (mutated *TP53*), were used in this study. First, we evaluated whether the genotoxic potential of cisplatin/gemcitabine was dependent on *TP53* status. Then, we evaluated whether the two antineoplastic drugs modulated *RASSF1A* and *hMLH1* expression in the two cell lines. Increased DNA damage was observed in both cell lines after treatment with cisplatin or gemcitabine and with the two drugs simultaneously, as depicted by the comet assay. A lack of *RASSF1A* expression and hypermethylation of its promoter were observed before and after treatment in both cell lines. On the other hand, *hMLH1* downregulation, unrelated to methylation status, was observed in RT4 cells after treatment with cisplatin or with cisplatin and gemcitabine simultaneously (wild-type *TP53*); in 5637 cells, *hMLH1* was upregulated only after treatment with gemcitabine. In conclusion, the three treatment protocols were genotoxic, independent of *TP53* status. However, cisplatin was the most effective, causing the highest level of DNA damage in both wild-type and mutated *TP53* cells. Gemcitabine was the least genotoxic agent in both cell lines. Furthermore, no relationship was observed between the amount of DNA damage and the level of *hMLH1* and *RASSF1A* expression. Therefore, other alternative pathways might be involved in cisplatin and gemcitabine genotoxicity in these two bladder cancer cell lines.

Keywords: DNA damage - DNA repair - gene expression profile - genotoxicity

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Introduction

Bladder cancer is the fourth most common malignancy in the Western world, with a recurrence rate of 60-70%. Urothelial bladder cancers, 90% of which belong to the transitional cell carcinoma (TCC) subtype (Cordon-Cardo, 2008), have a high frequency of mutations in the *TP53* gene (Wolff et al., 2005). *TP53* is a tumor suppressor gene that has a relevant role in the cellular response to various stressors agents, including the response to DNA damage (Basu et al., 2010). After DNA damage occurs, *TP53* promotes the induction of a transient or permanent blockage of cell proliferation or the activation of cell death signaling pathways (Kim et al., 2010). However, while some studies have shown that the presence of normal functional *TP53* in urothelial tumor cells is associated with a good response to chemotherapy and better clinical outcomes (Cote et al., 1997), others have reported that the existence of *TP53* allelic variants and regulators with distinct functionality indicate a complex role for the *TP53* pathway in human neoplasias (Hall et al., 2006). In fact, some authors have shown that mutated *TP53* directly modulates Bcl-2 expression in squamous cell head and neck carcinomas and increases tumor susceptibility to chemotherapy-induced apoptosis (Andrews et al., 2004). To date, the role of mutated *TP53* in bladder tumor sensitivity to antineoplastic drugs has not been described.

Combined chemotherapeutic protocols, including those using methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), have been extensively evaluated in the hope of improving urinary bladder cancer treatments and overall survival rates (Gallagher et al., 2009). However,

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some studies have shown that the combination of gemcitabine and cisplatin may also be successfully used for bladder cancer because it has a similar efficacy and superior safety and tolerability than MVAC (Bellmut et al., 2006). It has been shown that cisplatin and gemcitabine have different genotoxic mechanisms: whereas cisplatin induces irreversible DNA crosslinking and leads to apoptosis (Wang et al., 2005), gemcitabine incorporates itself into DNA, masks chain termination, and causes replication blockage (Toschi et al., 2005).

Some data have provided direct evidence that inhibiting the repair of cisplatin-induced DNA lesions plays a critical role in gemcitabine-mediated cytotoxic synergism with cisplatin in MMR-deficient tumor cells (Yang et al., 2000). In fact, it is known that mismatch repair (MMR) genes are usually associated with increased resistance to a variety of chemotherapeutic agents (Fink et al., 1998; Ding et al., 2009). Recently, Tajima et al. (2011) showed that cytotoxicity induced by 5-fluorouracil (5-FU) is dependent on intact DNA MMR and that the MMR complexes provide a hierarchical chemosensitivity for 5-FU cell death, which may have implications for the treatment of patients with certain MMR-deficient tumors. Previously, it has been reported that the loss of MMR proficiency results *in vitro* resistance to a number of clinically important anticancer drugs, including cisplatin (Aebi et al., 1997; Brown et al., 1997). Moreover, *hMLH1* has been related to DNA damage-induced apoptosis by cisplatin by both p53-dependent and -independent mechanisms. This supports the general concept that the DNA damage caused by platinum drugs is recognized by MMR proteins, which leads to the induction of apoptosis. In contrast, in cancer cells with MLH1 deficiency, DNA damage is not sensed by repair proteins, resulting in a reduced apoptotic response and increased cisplatin resistance (Ding et al., 2009). The loss of DNA mismatch repair due to the hypermethylation of the *hMLH1* gene promoter occurs at a high frequency in a number of human tumors (Plumb et al., 2000), and DNA hypermethylation and *hMLH1* silencing have been reported in mammalian cells exposed to gemcitabine (Schafer et al., 2010).

The tumor suppressor gene *RASSF1A* has been included in a gene set to assess DNA methylation in urine sediments for the sensitive/specific detection of bladder cancer (Yu et al., 2007). This gene is epigenetically inactivated during the growth of human urothelial carcinomas (Marsit et al., 2006), although it appears to be an initial event in bladder carcinogenesis (Negraes et al., 2008). The loss of *RASSF1A* or its downstream signaling pathway has been shown to reduce a cell's ability to respond to DNA damage signals (Hamilton et al., 2009). Therefore, the evaluation of *RASSF1A* expression has been recommended in the panels of differentially methylated genes in urinary bladder cancer to maximize the diagnostic coverage of epigenetic markers (Negraes et al., 2008). In fact, the involvement of *RASSF1A* in oncogenic pathways has been recently reported. The *RASSF1A* protein is not only activated by ATM, a major regulator of the DNA damage response, but also activates a number of different pathways (Scrace et al., 2012). Moreover, it has been show that the *RASSF1A* promoter is a target for p53, which was shown to downregulate the transcription of *RASSF1A* by Tian et al. (2011).

Because the role of *TP53* as a predictor of TCC response to chemotherapy remains unclear, we investigated the effects of standard chemotherapies, namely cisplatin, gemcitabine, and the combination of both drugs, on the DNA structure of two TCC cell lines (RT4, which carries the wild-type *TP53*, and 5637, a cell line carrying a mutated version of the gene). The expression levels of *hMLH1* and *RASSF1A* were also evaluated in both cell lines, before and after treatment, to evaluate whether these repair genes are modulated differently according to the *TP53* status.

Materials and Methods

Cell lines and chemicals

The established human TCC cell lines RT4 (wild-type *TP53*) and 5637 (mutated *TP53*) were obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. The RT4 cells, established from a low-grade papillary bladder tumor, carry no mutations in the *TP53* (Cooper et al., 1994). The 5637 cells, obtained from a moderately differentiated tumor, contain a *TP53* allele carrying two mutations: one at codon 280 (Arg>Thr) and the second at codon 72 (Arg>Pro) (Cooper et al., 1994; Sanchez-Carbayo et al., 2007). The cells were cultured according to a previously described protocol (Da Silva et al., 2010). The antineoplastic drugs gemcitabine (dFdC, Gemzar) and cisplatin (CDDP) were obtained from Eli Lilly Laboratory (Eli Lilly and Company, Indianapolis, IN, USA) and Sigma-Aldrich, respectively.

Experimental design

To investigate the level of DNA damage by the comet assay, cells were seeded into 12-well culture plates (1×10^4cells/well). For qRT-PCR and methylation analyses, 5,637 cells were seeded into 25cm^2 culture flasks (1×10^6cells/flask) in RPMI medium (Sigma-Aldrich, Inc, St Louis, MO, USA) or, for the RT4 cells, in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Inc, St Louis, MO, USA). Twenty-four hours after seeding, the cells were treated with 1.0μM cisplatin, 1.56μM gemcitabine, or with both drugs simultaneously (1.0μM cisplatin+1.56μM gemcitabine), at 37°C. These drug concentrations were chosen because they decrease tumor cell proliferation (Da Silva et al., 2010). Therefore, the genetic background related to the cell toxicity mechanisms could be evaluated. Untreated cells were used as negative controls. After 24h, the cells were washed with Hank's solution (0.4g KCl, 0.06g $KH2PO_4$, 0.04g $Na2HPO_4$, 0.35 g $NaHCO_3$, 1g glucose, and 8g NaCl in 1,000ml H_2O) and collected (T=24h) for the comet assay, gene expression, and methylated status analyses of the *RASSF1A* and *hMLH1* genes. Next, fresh medium was added, the cells were incubated for an additional 24 hours and then collected (T=48h) for the same analyses (gene expression and methylated status). These two time points (T=24h and T=48h) were chosen because the cells have different proliferation time and different apoptosis rates (Da Silva et al., 2010).

Comet assay

After cisplatin treatment, cells were incubated with 10μM H_2O_2, which introduces a number of strand breaks into the DNA and allows for the identification of cross-linking damage by the assay (Blasiak et al., 1999; Heringova et al., 2006; Shimabukuro et al., 2011). Briefly, 10μl of cells was added to 100μl 0.5% low-melting-point agarose at 37ºC. This mixture was layered onto precoated slides with 1.5% standard agarose and covered with a coverslip. The agarose was allowed to solidify at 4ºC, and then the coverslip was gently removed. Then, the slides were immersed in a lysis solution (2.5M NaCl; 100mM EDTA; 10mM Tris-HCl buffer pH=10; 1% sodium sarcosinate; 1% triton X-100; and 10% DMSO) overnight at 4ºC and then incubated in an alkaline buffer (0.3mM NaOH and 1mM EDTA; pH>13) for 20 minutes to allow for DNA unwinding and alkali-labile site expression. Electrophoresis was conducted in the same alkaline buffer at 4°C for 20 minutes at 25V (0.86V cm^{-1}) and 300mA. After electrophoresis, the slides were neutralized in 0.4M Tris-HCl (pH=7.5) solution for 15 min, fixed with absolute ethanol, and stored at room temperature until analysis. All of these steps were conducted in the dark to prevent any additional DNA damage. The slides were stained with SYBR Gold (1:10,000, Invitrogen, Grand Island, NY, USA) immediately before analysis. Cell viability was assessed by the Trypan Blue (0.4%, Sigma-Aldrich, Inc, St Louis, MO, USA) exclusion test and was never below 90%. As positive controls, RT4 and 5637 cells were treated with 14μM methyl methanesulfonate (MMS; Sigma-Aldrich, Inc, St Louis, MO, USA). A total of 150 randomly selected nucleoids per treatment were analyzed under 400× magnification with a fluorescence microscope connected to an image analysis system (Comet Assay II, Perceptive Instruments, Suffolk, Haverhill, UK). The comet assay was performed according to the protocol described by Tice et al. (2000) with some modifications. Tail intensity (% DNA in tail) was used to estimate DNA damage (Tice et al., 2000). The slides were prepared in duplicate for each of three independent experiments.

DNA and RNA extraction

According to the manufacturer's instructions, RNA and DNA were extracted using the mirVana kit (Ambion, Applied Biosystems, Foster City, CA, USA) and the Illustra Tissue and cells genomic prep mini spin kit (GE Healthcare, UK), respectively. Extracted DNA and RNA were stored at -80°C, and their integrity was evaluated through electrophoresis on a 2% agarose gel and on a 2% denaturing agarose gel, respectively, under standard conditions.

Methylation-specific polymerase chain reaction (MSP-PCR)

DNA was treated with sodium bisulfite using the methylSEQr kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was suspended in 10 µl of sterile water and stored at -4°C. The methylation patterns of the *RASSF1A* and *hMLH1* promoter regions were evaluated with an MSP-PCR approach (Burbee et al., 2001; Kim et al., 2010). For each gene, previously described primers (Burbee et al., 2001; Kim et al., 2010) specific for the methylated and unmethylated sequences were used. For the amplification of *RASSF1A*, we used 2.5mM $MgCl_2$, 200μM dNTP, 0.2μM of each primer and 1U DNA polymerase (GE Healthcare, Germany); for the amplification of *hMLH1*, we used 2.5mM Master Mix (Promega Corporation, USA) and 0.2μM of each primer. The amplified products were visualized after electrophoresis on a 2% agarose gel. Water blanks were included in each assay as negative controls. As positive control for the methylated alleles, DNA from the lymphocytes of healthy volunteers was treated with SssI methyltransferase (New England Biolabs, Beverly, MA, USA) and then exposed to bisulfite modification (Negraes et al., 2008). For *RASSF1A*, blood cell DNA from healthy volunteers was exposed to bisulfite modification and used as a control for the unmethylated sequence (Park et al., 2012). Each MSP-PCR was repeated at least once to confirm the results. The visual analyses (the presence or absence of amplification for the methylated and unmethylated sequences) were conducted based on the methods of Dulaimi et al. (2004).

Gene expression data by quantitative real-time PCR

The differential expression of *hMLH1* and *RASSF1A* was evaluated by real time-PCR with the TaqMan system (Applied Biosystems, Foster City, CA, USA). First, 1μg RNA from each cell line was reverse transcribed using 6μl of random hexamer primer (10×), 6μl reaction buffer (10×), 2.5μl dNTP (25×), and 3μl MultiScribe reverse transcriptase (50 U/μl) (High Capacity, Applied Biosystems, Foster City, CA, USA). The reactions were incubated at 25°C for 10 min, then at 37ºC for 2h, and finally at 4ºC until the samples were transferred to -20°C, where they were stored until use. For quantitative Real-Time PCR, each tube contained 2μl cDNA template, 5μl 2× TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.5μl 20× primers/probe (Assays-on-Demand gene expression products, Applied Biosystems, Foster City, CA, USA). β-actin was used for normalization. The PCR program consisted of the following steps: 2 min at 50ºC; 10 min at 95ºC; and 40 cycles of 15 sec at 95ºC and 1 min at 60ºC. Fluorescence data were collected during each annealing/extension step. The reactions were performed with an Applied Biosystems 7500 FAST Real-Time PCR System and SDS version 1.2.3 software (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems, Foster City, CA, USA). In all PCR reactions, a negative (no template) control was processed as a routine quality control for the assay. The assays were performed in duplicate for each of the two independent experiments.

Standard curve and data analysis

To generate standard curves, serial dilutions of cDNA from RT4 and 5637 control cells (samples were pooled together) were used. To *RASSF1A* gene, standard curves were also constructed using cDNA from a pool of blood cells expressing *RASSF1A* from healthy volunteers; a single sample of cDNA was used as a reaction control

in all plates tested. The smallest cDNA standard dilution was assigned a relative value of 100, and the other three dilutions were correspondingly assigned values of 20, 4.0, and 0.8. The relative concentrations of *hMLH1* and *RASSF1A* were determined by the relative standard curve method after normalization with β-actin with SDS version 1.2.3 software (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems, Foster City, CA, USA) (Pagliarone et al., 2009; Orsatti et al., 2010).

Statistical analysis

Statistical analyses were performed with SAS software, v.9.1.3 (Statistical Analysis System, SAS Institute, Cary, NC, USA). The qRT-PCR results were compared with a factorial analysis adjusted to the Tukey-Kramer Test. For the data obtained in the comet assay, a factorial analysis based on gamma distribution was used. A comparison between both cell lines regarding the interference of *TP53* on the amount of DNA damage was also made using the Tukey-Kramer Test. The p value<0.05 was considered statistically significant.

Results

DNA damage (comet assay)

Table 1 shows the amount of DNA damage induced by cisplatin (+H_2O_2) and gemcitabine monotherapies, by the combined treatment with the two drugs, and data from negative and positive controls (untreated cells and MMS treated cells, respectively). The three treatment regimens significantly damaged DNA in both the RT4 (wt *TP53*) and 5637 (mutated *TP53*) cell lines compared with the negative control. In RT4, both cisplatin and gemcitabine had a similar effect, they increased DNA damage (p<0.01), but only gemcitabine was significantly different from the combined treatment (cisplatin+gemcitabine). On the other hand, in the mutated *TP53* cells, gemcitabine and the combined treatment induced a similar increase in DNA damage (p<0.01), whereas cisplatin alone caused higher degree of damage than gemcitabine and cisplatin+gemcitabine (p<0.05). No significant difference was detected between the two cell lines after treatments.

***RASSF1A** and **hMLH1** expression*

RASSF1A expression was not detectable in either cell line, regardless of *TP53* status, the sampling time or the antineoplastic protocol used (data not shown). Cisplatin monotherapy and the combination of cisplatin and gemcitabine induced *hMLH1*downregulation in RT4 cells that were sampled 24 hours (relative values T=24h – control: 1.47±0.12; cisplatin: 1.10±0.08; gemcitabine: 1.16±0.05; cisplatin plus gemcitabine: 0.89±0.08) after treatment. However, the mRNA levels returned to baseline values 48 hours after treatment (relative values T=48h - control: 0.89±0.05; cisplatin 1.18±0.05; gemcitabine 1.05±0.00; cisplatin plus gemcitabine: 1.21±0.12). In contrast, in mutated *TP53* (5637) cells, *hMLH1* was upregulated after gemcitabine treatment (relative values T=24h – control: 0.56±0.06; cisplatin: 0.81±0.09; gemcitabine: 1.26±0.15; cisplatin plus gemcitabine: 0.73±0.08), although it returned to the control level at T=48h (relative values T=48h - control: 0.73±0.10; cisplatin: 1.13±0.14; gemcitabine: 0.85±0.15; cisplatin

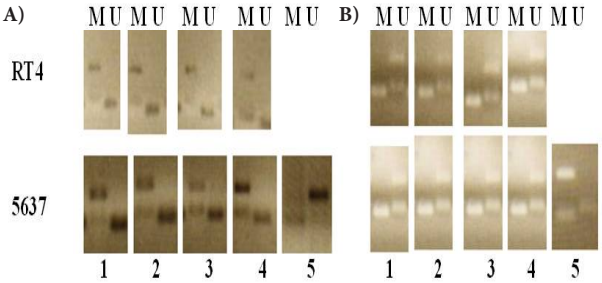

Figure 2. A) *RASSF1A* and B) *hMLH1*, Methylation Profiles at 24h in RT4 and 5637 Cells. M=methylated, U=unmethylated. 1-Control (untreated cells); 2-Cisplatin treatment; 3-Gemcitabine treatment; 4-Combination treatment; 5-DNA from healthy volunteers with no methylation at the *RASSF1A* gene was used as a control for unmethylated alleles. DNA from the lymphocytes of healthy volunteers, treated with SssI methyltransferase, was used as a control for methylated alleles (*hMLH1* and *RASSF1A*)

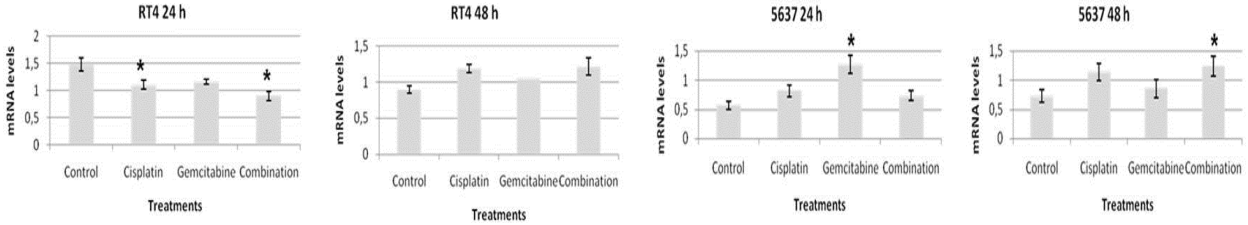

Figure 1. *hMLH1* Gene Expression Relative Values in Two Bladder Transitional Carcinoma Cell Lines (RT4 and 5637) Treated with Cisplatin (1.0μM), Gemcitabine (1.56μM), or with Both Drugs Simultaneously. All of the results were normalized to β-actin and expressed as arbitrary units relative to the value of 1.0. The data are expressed as the average and standard deviation of two biological replicates (*p<0.05)

Table 1. Chemically Induced DNA Damage (tail intensity) in RT4 and 5637 Bladder Carcinoma Cell Lines (the means±S.D.)

Cell line	Negative control[1]	Positive control[2]	Cisplatin (1.0μM)	Gemcitabine (1.56 μM)	Cisplatin+gemcitabine
RT4	35.54±30.50	90.19±12.11*	69.63±32.13*,a,b	57.28±36.57*,a	72.53±30.22*,b
5637	47.68±32.95	88.39±23.87*	83.74±22.46*,a	66.95±28.43*,b	67.52±34.20*,b

*p<0.01, compared to the negative control; a,bdifferent letters means significant differences among the treatments (p<0.05); [1]No treatment; [2]Methyl methanesulphonate (14 μM)

plus gemcitabine: 1.24±0.17). In addition, the combined protocol (cisplatin+gemcitabine) caused *hMLH1* upregulation no earlier than 48 hours following treatment. No change was detected after cisplatin treatment (Figure 1).

***RASSF1A** and **hMLH1** methylation patterns*

There was no change in the methylation patterns of the promoter regions of either *RASSF1A* or *hMLH1*, regardless of the cell line, *TP53* status, or the antineoplastic protocol used (Figure 2).

Discussion

In recent years, some key DNA repair proteins that belong to different DNA repair pathways have been described as acting through a dual function in DNA damage sensing/repair and apoptosis. These proteins include ATM, ATR, BRCA1, XPD, p53, MSH2, MLH1, DNA-PK (Bernstein et al., 2002). It has been well established that *TP53* is a key tumor suppressor gene that integrates multiple stress signals into an appropriate cellular response (Basu et al., 2010). Therefore, to determine the relationship between *TP53* status and the tumor response to chemotherapeutic agents, we analyzed the genotoxic effect of cisplatin and gemcitabine, and the combination of the two drugs, in two human bladder cancer cell lines, with and without *TP53* mutations.

Cisplatin is one of the most potent antitumor agents and has the ability to induce DNA crosslinking and apoptosis, particularly in rapidly replicating cells (Wang et al., 2005; Shimabukuro et al., 2011). Similarly to other platinum compounds, cisplatin forms DNA adducts that are difficult to repair and ultimately interfere with replication and transcription, contributing to its antitumor efficacy (Kosmider et al., 2005). Our data demonstrated that this drug caused significant DNA damage in grade 1 (RT4) and grade 2 (5637) bladder tumor cell lines, independent of the *TP53* status of the cell lines. Similarly, a recent study showed that *TP53* mutation does not confer prognostic value and does not necessarily influence the tumor response to various chemotherapy protocols, including cisplatin, methotrexate, and vinblastine monotherapies (Stadler et al., 2011). However, it must be considered that these findings can be tumor specific because glioma cell lines, with different *TP53* statuses, have opposing responses to methylating or chloroethylating agents (Batista et al., 2007). Furthermore, gliomas with a *TP53* mutation have been shown to be significantly more sensitive to apoptosis induced by UV light, despite their resistance to temozolomide (Batista et al., 2009). Additionally, it has been demonstrated that XPC protein (a DNA damage recognition protein that activates NER process) plays a key role in the severity of bladder tumors and in the response to cisplatin treatment (Chen et al., 2007; Xu et al., 2011). A decreased DNA repair capability and apoptotic rate after cisplatin treatment were also detected in the HT1197 bladder cell line that has a low level of XPC protein (Chen et al., 2007). The restoration of XPA levels through a cDNA expression vector increases cisplatin-induced apoptosis and p53 and p73 responses. These data suggest that XPC protein may act as one of the upstream activators of the p53 protein-mediated cisplatin-induced cellular responses. Our data also demonstrated that gemcitabine had genotoxic effects regardless of the status of *TP53* because the two cell lines evaluated displayed an increased amount of DNA damage after treatment. These findings are in accordance with those obtained in the lung cancer cell line A549, in which a relationship between gemcitabine genotoxicity and *TP53* status was not observed (Paulwels et al., 2005). Previously, we reported that the gemcitabine activity on cell cycle arrest was not influenced by *TP53* status, although the apoptosis indexes have been different in the RT4 (wild-type *TP53*) and 5637 (mutated *TP53*) cell lines (Da Silva et al., 2010).

On the other hand, in spite of the genotoxicity of the combined treatment (cisplatin plus gemcitabine) in both wild-type (RT4) and *TP53*-mutated cells (5637), we detected significantly lower levels of DNA damage in mutated cells simultaneously treated with the two drugs than in those treated with cisplatin alone. These findings suggest that *TP53*-mutated TCC cells are more resistant to DNA damage inducted by combined chemotherapy than the wild-type *TP53* cells and corroborate our previous study in which a higher percentage of clonogenic survival was detected in the high-grade *TP53*-mutated cells (Da Silva et al., 2010).

Some investigators have suggested that the synergism between cisplatin and gemcitabine involves homologous recombination (HR) and, to a lesser extent, nucleotide excision repair (NER) (Crul et al., 2005). The incorporation of gemcitabine into the gaps resulting from the removal of cisplatin-induced platinum-DNA adducts may explain the roles of HR and NER in the synergism between the two drugs. Additionally, when cisplatin interstrand-DNA adducts are repaired by HR, two guanines on opposite strands are excised and can be replaced by two cytidines that in turn have to compete with gemcitabine. Nevertheless, the authors conclude that base excision repair, NER, HR, and non-homologous end joining cannot efficiently modulate the effects of gemcitabine on DNA (Crul et al., 2005). Herein, we investigated whether the expression of *hMLH1* (DNA mismatch repair gene) was modulated in response to cisplatin, gemcitabine or by their combined treatment. Our data suggested that the increase in DNA damage was not associated with modulations in *hMLH1* expression because the increased levels of damage induced by gemcitabine or cisplatin in the RT4 and 5636 cell lines, respectively, did not occur simultaneously with *hMLH1* mRNA modulation.

In 2006, Nadin et al. (2006) reported a decrease in *hMLH1* protein expression in the lymphocytes of cancer patients after polychemotherapy. In fact, previously published data showed that Adriamycin (a crosslinking agent) reversibly inhibited human mismatch repair *in vitro* at low micromolar concentrations by interacting with the MMR pathway through a mechanism distinct from the manner by which covalent DNA lesions are processed. This inhibition apparently resulted from the ability of the intercalated drug to prevent mismatch binding. Thus, the authors suggested that the MMR inhibition might

be due to a physical distortion of the DNA helix that is promoted by Adriamycin (Larson et al., 2001). Similarly, our data demonstrated *hMLH1* downregulation in RT4 cells 24h after treatment with cisplatin. Therefore, these data may suggest that the decreased level of hMHL1 mRNA in RT4 cells could be the consequence of the lack of recognition of DNA lesions promoted by cisplatin. However, we also detected *hMLH1* upregulation in 5637 cells after gemcitabine treatment. Recently, LaConti et al. (2011) observed that gemcitabine treatment decreases the levels of miR-155 (whose target is *hMLH1* mRNA) in the pancreatic cancer tissues of transgenic animals. Furthermore, the authors reported an inverse relationship between miR-155 and *hMLH1* expression. Thus, we might perhaps hypothesize that *hMLH1* upregulation after gemcitabine treatment in 5637 cells could be related to post-transcriptional regulation mediated by microRNAs. Nevertheless, this upregulation was not observed in RT4 cells, demonstrating that this effect might also be related to *TP53* status. Interestingly, a previous study conducted by Suzuki et al. (2009) demonstrated the role of the *TP53* gene in facilitating the processing of primary miRNAs to precursor miRNAs through an interaction with the Drosha processing complex through association with the DEAD-box RNA helicase p68.

The downregulation of the *hMLH1* gene was also observed in *TP53* wild-type TCC cells after the cells were treated with a combination of cisplatin and gemcitabine. A previous study showed that the downregulation of MMR results in the reduction of the cytotoxic synergism of the two agents (Yang et al., 2000). It is conceivable that gemcitabine may act as an NER inhibitor for DNA damage induced by cisplatin and that NER inhibition may be an important mechanism through which gemcitabine mediates cytotoxic synergism with cisplatin. However, it is unlikely that gemcitabine-mediated DNA repair inhibition results in increased DNA platination and adduct retention (Yang et al., 2000). Furthermore, *hMLH1* activity could also result in the incorporation of gemcitabine nucleotide analogues into cells that are not involved in DNA replication (Kufe et al., 1984), promoting late apoptosis, as observed in a previous study (Da Silva et al., 2010).

Finally, our data showed promoter hypermethylation and undetectable *RASSF1A* expression in both the RT4 and 5637 cell lines, regardless of the treatment protocols. In fact, *RASSF1A* is known to be epigenetically inactivated during human bladder carcinogenesis (Marsit et al., 2006; Negraes et al., 2008), and this epigenetic event is associated with tumor aggressiveness (Phé et al., 2009). Therefore, according to some authors, *RASSF1A* promoter hypermethylation may be a reliable predictor of tumor progression in bladder cancer and could be used to distinguish between patients with the initial disease that will ultimately require more aggressive treatment than those at a low risk of disease progression and patients who would need local treatment and less intensive surveillance (Phé et al., 2009). Additionally, previously published data also indicated a relationship between *TP53* and *RASSF1A* (Song et al., 2008). *TP53* is regulated by a number of additional pathways that may be redundant to the *RASSF1A* pathway, which would make the cells less sensitive to *RASSF1A* loss (Brooks et al., 2003; Chen et al., 2005). In a previous study, we found that cells arrested in the G1 phase after treatment with gemcitabine and with the combination of cisplatin and gemcitabine and that there was an increased number of cells in S phase after treatment with cisplatin, independent of *TP53* status (Da Silva et al., 2010). Therefore, an association between *RASSF1A* /*TP53* and changes in the cell cycle might not be established.

In conclusion, the three treatment protocols were genotoxic, independent of *TP53* status, and cisplatin was the most genotoxic and gemcitabine was the least genotoxic compound in either cell lines. Treating cells with these drugs induced small changes in *hMLH1* expression. Thus, the lack of relationship detected between the amount of DNA damage and *hMLH1* and *RASSF1A* expression suggested that alternative pathways may be involved in the genotoxicity of cisplatin and gemcitabine in RT4 and 5627 bladder cancer cell lines.

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