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DNA damage response in patients with pediatric Acute Lymphoid Leukemia during induction therapy

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ABSTRACT

Predicting the individual response to chemotherapy is a crucial challenge in cancer treatment. DNA damage caused by antitumor therapies evokes different repair mechanisms responses, such as Nucleotide Excision Repair (NER), whose components are being studied as prognosis biomarkers and target therapies. However, few reports have addressed DNA damages in pediatric Acute Lymphoid Leukemia (ALL). Hence, we conducted an observational follow-up study with pediatric patients to assess DNA damage (by Comet Assay) and gene expression from NER pathway during chemotherapy induction. Bone marrow samples from diagnosis, 15th(D15) and 35th (D35) days of the treatment were collected from 28 patients with ALL. There was no increase in damage index. However, there was a reduction of cells with low damages on D35 compared with diagnosis. NER pathway expression remained the same, however, in a single patient, a significant decrease was observed, maybe due to silencing or downregulation of repair pathways. DNA damage levels and repair may influence the clinical outcome, being involved in drug resistance and risk of relapse. In pediatric ALL, we analyzed for the first time DNA damage and repair behavior in BM samples. Monitoring patient's outcomes will help to access the implication of our findings in survival and relapse rates.

1. Introduction

In developed countries, cancer is the second cause of death. Fortunately, survival has increased thanks to the development of new therapies and the fact of taking into account individual genetic differences [1]. Acute Lymphoid Leukemia (ALL) is the most frequent neoplasia during childhood [2]. The treatment is based on the risk stratification, because a body of evidence supports that the analysis of individual clinical features brings great benefits regarding survival and reduction of morbidity. Although the high cure rates, still 30% of patients relapse from the disease [2], maybe due to variable degrees of chemotherapy resistance during treatment.

DNA integrity is critical for proper cellular function and proliferation [3]. Most antitumor therapies damage tumor cell DNA, and studying DNA damage responses has become an important aspect on patients' outcome [1]. DNA Damage response (DDR) is a collective term for the different intra and inter-cellular signaling events and enzyme activities that result from the induction and detection of DNA damage. If DNA repair is not possible or suboptimal, leading

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to genomic instability, DDR can also be responsible for downstream cell fate decisions, such as cell death or senescence [4,5].

Various chemotherapies used in leukemia protocols damages DNA, such as methotrexate, daunorubicin [6], cyclophosphamide [1], and, also, glucocorticoids [7]. DNA Damage Response (DDR) has been investigated as a therapy for cancer [8], and it is known that different forms of DNA damage evoke different repair mechanisms responses [9]. The Nucleotide Excision Repair (NER) pathway deals with modified nucleotides that distort the structure of the double helix and is the pathway that primarily deals with UV-induced damage and platinum drugs [10]. Components of this pathway are being studied as prognosis biomarkers and targeted therapies in cancer [11].

Predicting the individual response to chemotherapy is a crucial challenge in cancer treatment, especially concerning personalized medicine. Beyond that practical and cost-effective techniques are needed. However, when it comes to ALL, few reports have addressed those DNA damages caused by chemotherapy, the response to the damages and consequently outcomes of the treatment. Hence, the main purpose of the present study is to assess DNA damage and gene expression from NER pathway in bone marrow cells from ALL pediatric patients during chemotherapy induction.

2. Methods

An observational follow-up study was performed. The sample consisted of pediatric patients admitted to the Pediatric Oncology Unit of Clinical Hospital from Porto Alegre from January 2013 to December 2015. Patients of both genders were included, diagnosed with ALL between 0 and 18 years of age. After application of the Consent and Informed Term, bone marrow (BM) samples were collected by clinical staff at three pre-defined stages from induction therapy: diagnosis (D0), after 15 days of treatment (D15) and after 35 days of treatment (D35). Clinical and laboratory variables were obtained by medical records. The project was approved by hospital's Ethics Committee (GPPG Number 130023).

2.1. DNA damage evaluation

DNA damage was measured using the Comet Assay protocol as described by Singht et al. [12]. The assay itself was carried out as described for *in vivo* samples [13,14]. All procedures subsequent to bone marrow collection were carried out so as to prevent interference from light. One hundred μL of biological solution (7 μL of bone marrow + 93 μL of low-melting point agarose 0.007%) were spread onto regular agarose precoated slides, covered with cover slips and placed into a cold tray. Once samples have solidified, cover slips were removed and the slides left to stand in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100 and 10% DMSO are added) for 1 or 2 days, under refrigeration. Excess fluid was removed from each slide, and all slides were placed in an electrophoresis tank, to which a basic solution (300 mM NaOH, 1 mM EDTA, pH > 13) was added. Slides were left to stand in this solution for 20 min to enable unwinding of DNA and expression of alkali-labile sites and single-strand breaks. Electrophoresis was then run for 20 min at 25 V, 300 mA and 0.9 V/cm. Slides were removed from the electrophoresis tank, washed three times in neutralizing solution (0.4 M Tris, pH 7.5), rinsed three times with distilled water and left to dry at room temperature. Slides were then fixed and silver-stained as described by Nadin et al. [15]. For assessment of DNA damage, 100 cells per sample were examined under light microscopy (x200 magnification). Cells were scored on a scale of 0 (no migration) to 4 (maximal migration) according to tail intensity (dimensions and shape). Therefore, the total sum of damage scores for a sample of 100 cells (the damage index) ranged from 0 (no migration in any cell) to 400 (maximal migration in all cells).

Table 1
Gene sequence.

Gene	Gene sequence
β-actin	
Forward Sequence	GAGACCTTCAACACCCCAG
Reverse Sequence	GCTACAGCTTCACCAGCAG
ERCC1	
Forward Sequence	GCTGGCTAAGATGTGTATCCTGG
Reverse Sequence	ATCAGGAGGTCCGCTGGTTTCT
CSA	
Forward Sequence	GCAGTTTCCTGGTCTCCACGTT
Reverse Sequence	CAAACATCCTGATGCTCTTCTCAC
CSB	
Forward Sequence	CGTTGCCTGTGTTTATGGAGCAG
Reverse Sequence	CTGACTTCATTCTCCGCAGTAGG
XPA	
Forward Sequence	GAAGTCCGACAGGAAAACCGAG
Reverse Sequence	GATGAACAATCGTCTCCCTTTTCC
XPC	
Forward Sequence	TTGTCGTGGAGAAGCGGTCTAC
Reverse Sequence	CTTCTCCAAGCCTCACCACTCT
XPD	
Forward Sequence	TCACCGACCTTGCTGACTTCTC
Reverse Sequence	GTTCTGTCGTCAAAGGGCTCGA

2.2. Expression of nucleotide excision repair genes

Expression of genes from Nucleotide Excision Repair (NER) pathway was accessed by Real-Time PCR. Total RNA from BM was extracted using PureLink R RNA Mini Kit (Life Technologies) according to manufacturer's instructions. All steps of the procedure were conducted under RNase-free conditions with caution to avoid contamination. The concentration of total RNA (ng/μL) was determined at an absorbance of 260 nm (A260) using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and was used to calculate the total RNA yield. Total RNA purity was assessed by measuring the A260/280 ratio. Samples with an A260/A280 ratio of at least 1.8 were considered of sufficient quality for further analysis. Then, 50 ng of total RNA from each patient sample was converted to cDNA using the superscript TM III First-Strand Synthesis supermix (Invitrogen, São Paulo, Brazil) with a mix of random hexamers in a 20 μL reaction volume. Real-time PCR was used to test target genes. The reaction was performed at 95 °C for 20s, followed by 40 cycles of 95 °C for 30s, 60 °C for 30s, and 72 °C for 35 s [16]. β-actin was selected as the internal reference. A negative control was included in each PCR set. Results data are shown as percentage changes relative to β-actin and were calculated by 2-ΔΔCt method. The sequence of the primers is shown in Table 1.

2.3. Statistical analysis

Statistical analysis was performed using SPSS software version 18.0 (IBM Corp., Armonk, NY, USA). Variables with normal distribution were expressed as mean and standard deviation and with abnormal distribution, as median and interquartile range. Non-parametric tests, such as Friedman Test and Mann-Whitney, were used to comparison between groups. P values under 0.05 were considered significant. Graphs were performed using GraphPad version 6.0 (GraphPad Software, San Diego, CA, USA).