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Original article

Gene expression profile of 5-fluorouracil metabolic enzymes in laryngeal cancer cell line: Predictive parameters for response to 5-fluorouracil-based chemotherapy

Ana Lívia Silva Galbiatti [a,*], Heloisa Cristina Caldas [b], José Victor Maniglia [c], Érika Cristina Pavarino [a], Eny Maria Goloni-Bertollo [a]

[a] FAMERP- São José do Rio Preto Medical School Genetics and Molecular Biology Research Unit - UPGEM AV Brigadeiro Faria Lima, 5416 São José do Rio Preto, 15090000 Brazil
[b] FAMERP - Laboratory of Experimental Immunology and Transplantation/LITEX, Brazil
[c] FAMERP - Otorhinolaryngology and Head and Neck Surgery Department, Brazil

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ABSTRACT

Background: 5-fluorouracil (5-FU) is an antifolate chemotherapeutic that has become established in many therapeutic regimes, but sensitivity variations and development of resistance are common problems that limit the efficiency of the treatments. Inter-individual variations to 5-FU outcome have been attributed to different expression profiles of genes related to folate metabolism.
Methods: To elucidate the mechanisms of variations to 5-FU outcome, the authors investigated *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* folate genes expression for 5-FU response in laryngeal cancer cell line (Hep-2). Concentrations of 10, 50, and 100 ng/mL of 5-FU chemotherapeutic were added separately in Hep-2 cell line for 24 hours at 37 °C. Cell sensibility was evaluated with fluorescein isothiocyanate (FITC) label Bcl-2 by flow cytometry. The real-time quantitative PCR (qPCR) technique was performed for quantification of gene expression using TaqMan® Gene Expression Assay. ANOVA and Bonferroni's post hoc tests were utilized to statistical analysis.
Results: The numbers of viable Hep-2 cells with 10, 50, and 100 ng/mL concentrations of 5-FU chemotherapy were 15.87, 28.3 and 68.9%, respectively. Statistical analysis showed significant association between control group and increased expression for *TYMS* gene in cells treated with 100 ng/mL/5-FU chemotherapy ($P < 0.05$).
Conclusions: The authors found association between the highest 5-FU dose chemotherapy and increased expression levels for *TYMS* folate gene in laryngeal cancer cell line. Although these experiments were performed *in vitro*, the results suggest that genetic factors are thought to play an important role in drug metabolism and may be useful for predicting treatment outcomes.
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1. Introduction

Head and neck cancers constitute for 12% of all malignancies in the world. Laryngeal cancer is the second most frequent tumor of the head and neck and it is estimated about 151,000 new cases world wild each year and the death incidence for this disease is estimated to be 82 per 100,000 people per year [1,2].

Chemotherapy focused on specific molecular targets and pathways are becoming common for cancer treatments, as antifolate chemotherapeutics that are designed to target key folate-dependent enzymes, therefore leading to inhibition of nucleotide biosynthesis and cell death [3,4]. 5-fluorouracil (5-FU) chemotherapy is an antifolate that may be utilized to head and neck cancer treatment and therapeutic dose range of 20–25 mg hr/L has been consistently shown to yield optimal results in terms of clinical efficacy and safety [5,6]. It achieves its therapeutic efficacy through inhibition of thymidylate synthase enzyme (TYMS), which is essential for DNA synthesis and repair. It causes cell apoptosis and cell cycle arrest by suppressing the cell's ability for synthesizing DNA. It is possible that 5-FU chemotherapy may also influence another enzymes involved in folate pathway. These enzymes are codified by genes that have essential role in folate pathway [7–9].

* Tel.: +55 1732015907; fax: +55 1732015700.
E-mail address: analivia_sg@yahoo.com.br (A.L.S. Galbiatti).

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In recent years, many investigators have been studying and identifying novel genes, mainly genes involved in folate metabolism, that can be associated with resistance and toxicity to 5-FU therapy for many cancer types [10–15], including head and neck cancer [7,16–19]. Such target genes might prove to be therapeutically valuable as new targets for 5-FU chemotherapy or as predictive biomarkers of response and toxicity to 5-FU chemotherapy [7,20,21].

Variability in patient's response to anticancer chemotherapy is a major hurdle in the delivery of optimal efficacy and possible toxicity [14]. Different outcomes for 5-FU chemotherapy can be due to alterations of expression levels of genes involved in 5-FU pathway. To our knowledge, few reports about systematic examination of the expression profiles of genes involved in folate pathway have been published. Therefore, the objective of the present study was to investigate genetic expression profiles of methylenetetrahydrofolate reductase (NAD(P)H) (*MTHFR*), dihydrofolate reductase (*DHFR*), thymidylate synthetase (*TYMS*) and solute carrier family 19 (folate transporter)/member 1 (*SLC19A1*) genes involved in folate metabolism and their association with chemotherapeutic outcome with 5-FU administered with three different concentrations in laryngeal cancer cell line (Hep-2).

2. Materials and methods

2.1. Cell line

Human laryngeal carcinoma cell line Hep-2 was utilized for this study. Hep-2 cell line has been described to originate from tumours produced in irradiated-cortisonised weanling rats after injection of epidermoid carcinoma tissue isolated from the larynx of a 56-year-old male. Hep-2 cell was cultured in Minimum Essential Medium Eagle Medium (D-MEN 00068 medium/Cultilab) supplemented with 10% fetal bovine serum (FBS–Cultilab), 2 mM glutamine (Cultilab), 100 U/mL of penicillin, 100 U/mL of streptomycin, 1 mM sodium pyruvate (Sigma–Aldrich) and 1 mM non-essential amino acid (Sigma–Aldrich) at 37.0 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells plated in tissue culture dishes and grown in mid-log phase with viability > 95% were used in our experiments.

2.2. Drug preparation and apoptosis analysis

The chemotherapy utilized was 5-FU anticancer agent. Cells were plated in six-well culture plates at a density of 1×10^5/well and incubated separately with three different concentrations of 5-FU for 24 hours (10, 50 and 100 ng/mL). The control cells were the cell line with drug-free medium. After 24 hours incubation with 5-FU, cells were collected, washed with phosphate buffered saline (PBS), and then suspended in 100 uL cell pellets.

The rate of apoptotic and living cells was evaluated by Bcl-2 (100: sc-509) fluorescein isothiocyanate FITC/PI assay according to the manufacturer's manual (Santa Cruz Biotechnology, Inc). Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Cells were analyzed in a flow cytometer FACS calibur (Becton Dickinson Immunocytometry Systems, San José, USA). The sample analysis was performed using the CELLQuest software (Apple). The procedure was performed in triplicate to expose erroneous data points and excessive random variations.

2.3. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA of each 5-FU application was extracted from Hep-2 cell line using Trizol® Reagent Kit (Invitrogen) according to the manufacturer's instructions. RNA concentration was adjusted to 50 ng/μL using a Picodrop® Equipment. Reverse transcription was then carried out at 95 °C for 20 seconds and 40 cycles of denaturation at 95 °C for 0,3 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds, with 2 μg of total RNA with random primers according to high capacity cDNA kit (Applied Biosystem®) manufacturer's protocol.

2.4. Taqman gene expression assay

Gene expression was measured by quantitative real time (qRT-PCR) using a StepOnePlus™ Equipament (Applied Biosystems). The PCR reaction condition was 40 cycles of 20 s at 94 °C, 0.3 s at annealing temperature, and 60 °C for 30 s. 10 μL reaction mix contained the following components with final concentration: 2 × TaqMan® Gene Expression Master Mix, 20 × TaqMan® Gene Expression Assay and 2 μL of cDNA sample solution (50 ng) (Applied Biosystems). Gene-especific primers were used for quantification of one-carbon metabolizing gene expressions in TaqMan® Custom Array Plate, which included triplicated wells of one reference genes and *MTHFR, DHFR, TYMS* and *SLC19A1* folate genes. Relative mRNA expression levels were calculated against quantified data on the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All reactions were run on triplicate for four samples. Melting curves were checked to validate the PCR specificity. The gene expression levels were normalized with β-actin and GAPDH reference genes. Relative gene expression levels were calculated using the delta threshold cycle (Ct) method according to the mathematical formula shown below. The expression levels of target genes were expressed as 2 − (delta Ct) × 1000 to simplify the calculation.

$$\text{Expression level of target gene} = 2 - (\text{Delta Ct}) \times 1000$$

$$\text{Delta Ct} = \text{Ct of target gene} - (\text{Mean Ct of } \beta - \text{actin and } GAPDH \text{ genes})$$

2.5. Statistical analysis

Statistical analyses was performed using Bioestat software program–Version 5.3 View software package. Mean Ct values of triplicate measurements were used for analysis. These data were evaluated by one-way RM analysis of variance (ANOVA) to assess *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* gene expression in three different 5-FU concentrations against expression of genes in the control cells (no 5-FU). Bonferroni's post hoc test was used to determine the p-value at each concentration *vs.* control. Results with $P \leq 0.05$ were considered statistically significant.

3. Results

Flow cytometry analysis with Bcl-2 FITC staining showed that 3.6% of cells in the control group were apoptotic cells. After administration of 10, 50 and 100 ng/mL of 5-FU, the apoptotic cells were 15.87, 28.3 and 68.9%, respectively (Fig. 1).

Expression levels for *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* genes were higher in cells administered 100 ng/mL of 5-FU chemotherapy (Fig. 2). Statistical analysis also showed significant association between control group and increased expression of *TYMS* gene in cells treated with 100 ng/mL of 5-FU chemotherapy ($P < 0.05$) (Table 1).

4. Discussion

The results of the current study showed that the number of apoptotic cells in the control group was significantly lower than a

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