Ana Lívia Silva Galbiatti

AVALIAÇÃO DE QUIMIOTERÁPICOS, EXPRESSÃO GÊNICA E QUANTIFICAÇÃO DE PROTEÍNAS EM CARCINOMA DE CABEÇA E PESCOÇO.

Tese apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Doutor no Curso de Pós-graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientadora: Profa. Dra. Eny Maria Goloni Bertollo

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BANCA EXAMINADORA

TESE PARA OBTENÇÃO DO TÍTULO DE DOUTOR

Presidente e Orientadora: Eny Maria Goloni Bertollo

1º Examinador:Profa. Dra Eloiza H. Tajara da Silva

2º Examinador:Prof. Dra. Mariangela T. Ruiz-Cintra

3º Examinador:Prof. Dr. André Lopes Carvalho

4º Examinador:Prof. Dr. Wilson Araujo da Silva Jr.

Suplentes:Rosa S. Oyama e Ana Elizabete Silva

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SUMÁRIO

Dedicatória

A DEUS: *Pela força suprema, pela oportunidade de viver e por sua constante presença em minha vida, guiando meus passos por caminhos que só Ele conhece e colocando soluções onde eu só enxergava problemas.*

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Epígrafe

Quem não valorizar pequenos progressos não conseguirá obter grandes progressos. Os grandes progressos são resultado perseverante da manutenção da força de vontade. Contudo por maior que seja a força de vontade, se a pessoa não tiver sonhos elevados e grandiosos, não conseguirá realizar grandes obras. O sonho faz a mente definir um rumo visando a sua realização e a força de vontade possibilita-nos manter vivo o sonho e perseverar até a sua concretização.

Massaharu Taniguchi

"Aqueles que se sentem satisfeitos sentam-se e nada fazem. Os insatisfeitos são os únicos benfeitores do mundo."

Savage Landor

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LISTA DE ABREVIATURAS E SÍMBOLOS

5,10MTHF	5,10 metilenotetrahidrofolato *(5,10 methylenetetraidrofolate)*
5-FU.	5-fluorouracil *(5-fluorouracil)*
ANOVA	Análise de variância *(Analysis of variance)*
Bcl-2	*B-cell lymphoma 2*
CCP	Câncer de cabeça e pescoço
cDNA	DNA complementar *(Complementary DNA)*
CH3	Grupamento metil
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico *(National Council for Scientific and Technological Development)*
CO_2	Dióxido de Carbono
D –MEN	*Dulbecco Medium*
DHFR	Dihidrofolato redutase *(Diidrofolate reductase)*
DNA	Ácido desoxirribonucleico (Desoxirribonucleic acid)
dTMP	Deoxitimina monofosfato *(Deoxitimine monofosfate)*
dUMP	Deoxiuridina monofosfato *(Deoxiuridine monofosfate)*
FAMERP	Faculdade de Medicina de São José do Rio Preto *(São José do Rio Preto Medical School)*
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo *(São Paulo State Research Foundation)*
FBS	*Fetal Bovine Serum*

FdUMP	*fluorodeoxyuridine monophosphate*
FdUTP	*fluorodeoxyuridine triphosphate*
FITC	*fluorescein isothiocyanate*
FUTP	*fluorouridine triphosphate*
HEP-2	*Human Laryngeal carcinoma cell line*
HN13	*Human tongue carcinoma cell line*
HNC	*Head and neck cancer*
INCA	Instituto Nacional do Câncer
mg	Miligrama
mRNA	Micro ácido ribonucleico
MTHFR	Metileno tetraidrofolato redutase *(Methylenetetrahidrodolate reductase)*
MTX	Methotrexato *(Methotrexate)*
ng/mL	Nanograma/ mililitro
qRT-PCR	*Real-time quantitative PCR*
SLC19A1	Família de carreador de folato (*Solute carrier family 19-folate transporter*)
TNM	Tamanho do tumor, envolvimento de linfonodos e presença de metástase a distância
TYMS	Timidilato sintase (Timidilate synthase)
μM	micromolar
μmol	Micromolar

RESUMO

Introdução: Quimioterápicos antifolato, tais como Methotrexato (MTX) e 5-Fluorouracil (5-FU) agem inibindo enzimas envolvidas na via do folato. Essas enzimas são essenciais para a síntese de DNA e divisão celular. A dose destes quimioterápicos podem alterar níveis de expressão de genes que codificam essas enzimas envolvidas na via do folato e influenciar na resposta ao tratamento. **Objetivos:** Avaliar a relação entre a expressão do RNAm e de proteínas dos genes *MTHFR, DHFR, TYMS* and *SLC19A1* envolvidos no metabolismo do folato em linhagens celulares de câncer oral e câncer de laringe administradas com os quimioterápicos antifolato MTX e 5-FU em diferentes concentrações e em monoterapia. **Materiais e métodos:** Duas linhagens celulares HEP-2 (câncer da laringe) e HN13 (câncer de cavidade oral) foram tratadas com 0,25, 25,0 e 75 mM de MTX e 10 ng / ml, 50 ng / ml e 100 ng / ml de 5 -FU, separadamente, durante 24 horas a 37 °C. Técnicas de Citometria de Fluxo, PCR em tempo real e Western blotting foram realizadas para análise do nível de apoptose, quantificação do RNAm e quantificação das proteínas dos genes, respectivamente. Para análise estatística foi utilizado o teste ANOVA com correção de Bonferroni. P <0,05 foi considerado significante. **Resultados:** O aumento da concentração do quimioterápico MTX foi associado com expressão aumentada dos genes *MTHFR, DHFR, TYMS* e *SLC19A1* na linhagem de câncer de laringe ($p<0,05$) e expressão aumentada dos genes *DHFR* e *SLC19A1* na linhagem de câncer oral ($p<0,05$). A dose mais baixa de MTX foi associada com expressão diminuída do gene SLC19A1 em câncer de laringe ($p<0,05$). O aumento da concentração do quimioterápico 5-FU foi associado com expressão aumentada do gene *DHFR* na linhagem de câncer de laringe ($p<0,05$) e expressão aumentada dos genes *TYMS* e *DHFR* na linhagem de câncer oral ($p<0,05$). A dose mais

baixa de 5-FU foi associada com expressão diminuída do gene SLC19A1. **Conclusão:** Exposição à alta ou baixa dose dos quimioterápicos MTX e 5-FU, em monoterapia, pode modular o nível de expressão de genes envolvidos no metabolismo do folato.

Palavras-chave: Neoplasias de Cabeça e Pescoço, Quimioterapia, Expressão Gênica, ácido fólico, metabolismo.

ABSTRACT

Introduction: Antifolate chemotherapies such as methotrexate (MTX) and 5-fluorouracil (5-FU) act inhibiting enzymes involved in folate pathway. These enzymes are important to DNA synthesis and cell growth. Chemotherapy dose may alter these levels of expression of these genes encoding enzymes involved in folate pathway and to influence in the response to treatment. **Objectives:** To evaluate relationship between mRNA and protein expression levels expression of *MTHFR, DHFR, TYMS* and *SLC19A1* folate metabolic genes in laryngeal and oral cancer cell lines treated with MTX and 5-FU antifolate chemotherapies, separately. **Materials and methods:** HEP-2 (laryngeal cancer) and HN13 (oral cancer) cell lines were treated with 0.25, 25.0, and 75 μM of MTX and 10 ng/ml, 50 ng/ml, and 100 ng/ml of 5-FU, separately, for 24 hours/37°C. Flow Cytometry, Real-time PCR and Western blotting techniques were performed to analyzing the level of apoptosis, quantification of mRNA and quantification of proteins of genes, respectively. ANOVA and Bonferroni's post hoc tests were utilized for statistical analysis. $P<0.05$ was considered significant. **Results:** The higher concentration of MTX chemotherapeutic was associated with increased expression of *MTHFR, DHFR, TYMS* and *SLC19A1* genes in laryngeal cancer cell line ($p <0.05$) and increased expression of *DHFR* and *SLC19A1* genes in oral cancer cell line ($p <0.05$). The lower dose was associated with decreased expression of *SLC19A1* gene laryngeal cancer ($p <0.05$). The higher concentration of 5-FU chemotherapy was associated with increased expression of *DHFR* gene in laryngeal cancer cell line ($p <0.05$) and increased expression of *DHFR* and *TYMS* genes in oral cancer cell line ($p <0.05$). The lower dose of 5-FU was associated with decreased expression of *SLC19A1* gene. **Conclusion:** Exposure to low and high-dose of chemotherapeutics in oral cancer

cell line can modulate the level of expression of genes involved in folate metabolism in laryngeal and oral cancer cell line.

Key Words: Head and Neck Neoplasms, Drug Therapy, Gene Expression, folic acid, metabolism.

1. INTRODUÇÃO

INTRODUÇÃO

O câncer de cabeça e pescoço (CCP) compreende um grupo heterogêneo de tumores classificados por localização: cavidade oral; faringe e laringe. Atualmente ele ocupa a sexta posição na lista de neoplasias mais frequentes no mundo com uma incidência global de 550 mil casos novos por ano. Destes, aproximadamente 300mil correspondem ao câncer de cavidade oral e 157 mil correspondem ao câncer de laringe. (1-4)

No Brasil, as estimativas do Instituto Nacional do câncer (INCA) apontam para os anos de 2014 e 2015 uma incidência de 15.290 mil casos novos de câncer oral, sendo 11.280 em homens e 4.010 em mulheres. Em relação ao câncer de laringe, os dados mostram incidência de 7.640 casos novos de câncer de laringe, sendo 6.870 em homens e 770 em mulheres. Em homens, o câncer de cavidade oral apresenta-se o quinto tipo mais incidente e o câncer de laringe ocupa a sétima posição. (5)

O estabelecimento do planejamento terapêutico e do prognóstico do CCP baseiam-se principalmente em parâmetros clínicos, radiológicos e histopatológicos, os quais consistem no local do tumor primário e no sistema de estadiamento TNM, ou seja, no tamanho do tumor, na presença de metástase em linfonodos cervicais e de metástase a distância. As opções de tratamento para esta doença são cirurgia, radioterapia e quimioterapia, que podem ser utilizados de forma isolada ou combinada, dependendo do grau e do tipo tumoral. (5-9)

A quimioterapia consiste em um tratamento sistêmico que utiliza medicamentos chamados quimioterápicos que impedem a divisão celular, levando a destruição das células tumorais. Este tipo de tratamento pode ser classificado como

curativo, o qual consiste na erradicação completa do tumor, paliativo que consiste no alívio dos sintomas, adjuvante no qual ocorre erradicação de células residuais locais ou circulantes de tratamento prévio e neoadjuvante que é utilizado para erradicação de células tumorais antes da realização da cirurgia. [5]

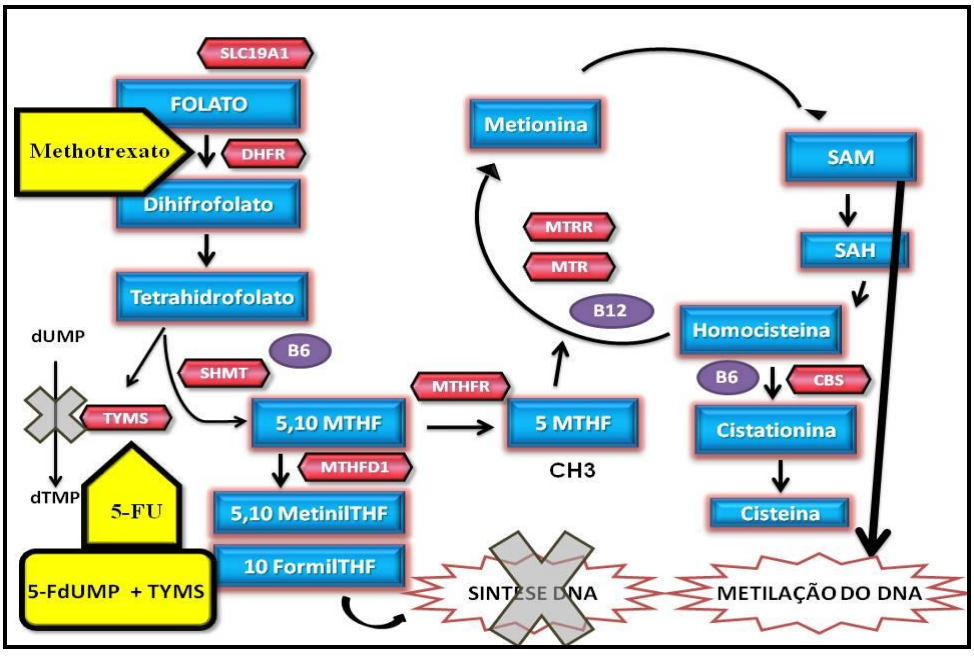
A quimioterapia pode envolver a administração de um único quimioterápico (monoterapia) ou pode ser feita através da administração de mais de um tipo de quimioterápico combinados (Terapia combinada) em um regime pré-determinado de tratamento. Há diversos quimioterápicos com diferentes mecanismos de ação que podem ser utilizados no tratamento do câncer de cabeça e pescoço, tais como cisplatina, cetuximab, docetaxel, gemcitabina, methotrexato (MTX) e 5-fluorouracil (5-FU). [10-14]

Os quimioterápicos MTX e 5-FU são classificados como quimioterápicos antimetabólitos (antifolato) que agem inibindo a síntese e conversão de derivados do folato e, consequentemente, esse bloqueio cessa a divisão celular neoplásica. As células neoplásicas possuem receptores específicos de alta afinidade com folato, ancorados à membrana apical de células epiteliais e por serem células de rápida replicação são também extremamente dependentes de um abundante suprimento de folato, que é responsável por fornecer CH_3 para a síntese de nucleotídeos e para as reações de metilação do DNA. Portanto, o bloqueio de derivados do metabolismo do folato leva a cessação da divisão celular neoplásica, que é altamente dependente do metabolismo do folato para exercer a divisão celular. [15-16]

MTX age na fase S do ciclo celular inibindo a enzima dihidrofolato redutase (DHFR), envolvida no metabolismo do folato. Esta enzima é responsável por converter o folato intracelular no composto tetrahidrofolato e consequentemente em 5,10

metilenotetrahidrofolato (5,10MTHF), que são essenciais para a síntese de purinas e pirimidinas. Essas reações que ocorrem no metabolismo do folato são fundamentais para a divisão e crescimento celular. [17-19] (Quadro 1)

5-FU também age na fase S do ciclo celular inibindo a enzima timidilato sintase (TYMS), envolvida no metabolismo do folato. Essa enzima é responsável por catalisar a transformação de desoxiuridina (dUMP) para desoxitimidina (dTMP), que é a principal reação para a síntese de pirimidinas. O quimioterápico 5-FU quando administrado forma o complexo 5-fluorodeoxiuridilato (5-FdUMP + TYMS) na presença de 5,10 metilenotetrahidrofolato (5,10MTHF), este complexo suprime a conversão de uracila para timina, bloqueando assim a síntese de DNA e divisão celular (Quadro 1). Alterações em outras enzimas envolvidas no metabolismo do folato também podem estar relacionadas com a resposta ao tratamento quimioterápico.[20-21]



Quadro 1. Atuação dos quimioterápicos Methotrexato e 5-Fluorouracil no metabolismo do folato para bloqueio da divisão celular.

Apesar do tratamento com quimioterápicos antifolato apresentarem bons resultados, esses agentes antineoplásicos podem aumentar a toxicidade aguda e efeitos colaterais e tumores apresentando o mesmo estadiamento clínico podem apresentar diferentes respostas em relação ao tratamento. [10, 22-26]

Possíveis causas para toxicidade e resistência clínica aos quimioterápicos antifolato podem estar associadas à diminuição da captação celular, ativação reduzida e alteração da expressão gênica de seus alvos. Genes que codificam enzimas envolvidas no metabolismo do folato, que são alvos dos quimioterápicos antifolato, têm sido investigados como biomarcadores para aperfeiçoar a terapia. Além disso, o efeito citotóxico é dependente de uma interação competitiva com o metabolismo do folato e por essa razão os genes envolvidos nesta via possuem um importante papel na modulação da toxicidade clínica e eficácia desses quimioterápicos.[17,27-36]

Estudos associados à resposta clínica e toxicidade nesta doença são necessários para obter-se uma combinação de fatores prognósticos com parâmetros moleculares, podendo assim trazer benefícios aos pacientes e novas informações sobre estimativa de prognóstico e decisão de tratamentos.

Além disso, fatores prognósticos com maior sensibilidade e especificidade poderiam oferecer certamente subsídios para novas estratégias terapêuticas e, a identificação fármacogenética de quimioterápicos antifolato através da detecção da expressão de genes envolvidos no metabolismo do folato pode fornecer novos protocolos de tratamento onde os pacientes estão bem caracterizados e tratados de maneira uniforme e sistematicamente avaliados para toxicidade e resposta a quimioterápia e assim, podem ser uteis para identificação de subgrupos de pacientes que são predispostos a ter melhores respostas clínicas a quimioterápicos específicos com menor

toxicidade.[30,32]

Os estudos relacionados ao tratamento do câncer com os quimioterápicos MTX e 5-FU e a associação da expressão dos genes metilenotetrahidrofolato redutase (*MTHFR),* dihidrofolato redutase (*DHFR),* timidilato sintase *(TYMS)* e carreador de folato reduzido 1 (*SLC19A1)* envolvidos no metabolismo do folato mostram que a expressão aumentada do gene *DHFR*, responsável por converter compostos essenciais do metabolismo do folato para a síntese de purinas e pirimidinas, pode alterar a resposta ao tratamento com os quimioterápicos antifolato em termos de efetividade e efeitos colaterais em muitos tipos de câncer.[18,37-39] Para o gene *MTHFR*, que é responsável em manter o *pool* de folato intracelular, sua expressão aumentada está associada ao aumento da quimiossensibilidade do MTX e pode aumentar o número de células apoptóticas.[28, 31-32 40]

Dados da literatura relacionados à expressão do gene *TYMS* no tratamento com os quimioterápicos antifolato apresentam resultados contraditórios. Este gene é responsável por converter dUMP em dTMP para a síntese de pirimidinas. Estudos em câncer de laringe e câncer oral confirmaram que a expressão aumentada deste gene está associada com a diminuição da resposta ao tratamento com o quimioterápico 5-FU.[27,29] Diferente desses resutados, Dervieux et al (2006)[40] confirmaram que a expressão aumentada do gene *TYMS* está associada a melhor resposta de tratamento do câncer com o quimioterápico MTX.

Em relação ao gene *SLC19A1*, que é responsável pelo transporte dos quimioterápicos para o interior das células, os resultados mostram que expressão diminuída do gene está associada a uma redução da resposta ao tratamento com os quimioterápicos MTX e 5-FU. [41-42] Apesar dos reconhecidos avanços

na identificação de genes envolvidos no crescimento, progressão e resistência de tumores aos quimioterápicos MTX e 5-FU, o modo como esses genes participam, interagem e são regulados nesses processos ainda não estão bem esclarecidos no tratamento do câncer de cabeça e pescoço. A falta de entendimento a respeito dos mecanismos de ação e interação entre esses genes tem dificultado o desenvolvimento de estratégias mais efetivas para o tratamento do câncer de cabeça e pescoço. A compreensão de como marcadores moleculares estão associados com resposta terapêutica é indispensável para a escolha do tratamento adequado.

Com base nos dados descritos acima, os objetivos deste trabalho foram avaliar a relação entre a expressão do RNAm e de proteínas dos genes *MTHFR, DHFR, TYMS* e *SLC19A1* envolvidos no metabolismo do folato em linhagens celulares de câncer oral (HN13) e câncer de laringe (HEP-2) administradas com diferentes doses dos quimioterápicos antifolato MTX e 5-FU em monoterapia.

2. MATERIAIS E MÉTODOS

Materiais

Os materiais utilizados nesta pesquisa foram duas linhagens celulares de carcinoma de cabeça e pescoço e os quimioterápicos MTX (Pfizer™) e 5-FU (Calbiochem™):

- Linhagem celular HEP-2 (Carcinoma epidermóide de laringe) ATCC® CCL-23™
- Linhagem celular HN13 (Carcinoma oral: Língua.) Linhagem imortalizada.

Métodos

As linhagens celulares, acondicionadas em criotubos e meio próprio para congelamento, mantidas em tambor de nitrogênio líquido à temperatura de -180°C, foram descongeladas em banho-maria a 37^{o}C por 10 min. e transferidas para placas de cultura de 25 cm de diâmetro (T-25; TPP- Cultilab)

Cultivo celular, aplicação dos quimioterápicos e análise da apoptose

As células da linhagem HEP-2 foram mantidas em frascos de cultura T-25 contendo *Dulbecco's Modified Eagle Medium* (DMEM - Cultilab) suplementado com 10% de soro fetal bovino (Cultilab),1% de solução antibiótica– (100U/ml penicilina, 100µ / 250ng/ml anfotericina - Cultilab), GlutaMAX (Life Technologies), Piruvato de Sódio (Sigma Aldrich) e Aminoácidos não Essenciais (Sigma Aldrich). As células foram mantidas em incubadora sob níveis controlados de temperatura (37°C), umidade e concentração de CO_2 (5%). A contagem celular foi realizada em Câmara de Neubauer e uma concentração de 5 x 10^5 células foi utilizada para os procedimentos.

As células procedentes da linhagem tumoral HN13 (Carcinoma de cavidade oral) foram mantidas em frasco de cultura T-25 (TPP- CULTILAB) contendo Dulbecco's Modified Eagle Medium (DMEM - Gibco by Life Technologies) suplementado com

10% de soro fetal bovino (Gibco by Life Technologies) e 1% de solução antibiótica– (100U/ml penicilina, 100µ / 250ng/ml anfotericina - Gibco by Life Technologies).). As células foram mantidas em incubadora sob níveis controlados de temperatura (37°C), umidade e concentração de CO2 (5%). A contagem celular foi realizada em Câmara de Neubauer e uma concentração de 5×10^5 células foi utilizada para os procedimentos.

O cultivo celular foi monitorado diariamente em microscópio invertido de constraste de fase e o meio de cultura trocado de acordo com o metabolismo das células. Todos os procedimentos envolvendo cultivo celular foram realizados em capela de fluxo laminar.

Após o cultivo celular, as células foram dissociadas (tripsinizadas) utilizando uma solução contendo 0.5g de tripsina e 0.2g de EDTA - Ethylenediamine tetraacetic acid - (Tripsin EDTA solution - Sigma-Aldrich CO, St. Louis, MO, USA) durante 5-10 min. e à temperatura de 37°C. Posteriormente, as células em suspensão foram transferidas para um tubo de centrifugação contendo meio de cultura e centrifugadas a 1500 rpm por 5 minutos para inativar a função enzimática da tripsina por meio do SFB contido no meio de cultura. O sobrenadante resultante foi aspirado e o precipitado de células ressuspendido em novo meio de cultura e distribuído em placa de cultivo contendo seis poços (Six well Plate – Cultilab). Para análise da sensibilidade dos quimioterápicos MTX e 5-FU, as células foram tratadas com três concentrações diferentes de MTX (0,25 µM, 25µM, e 75 µM) de acordo com o estudo de Pai e colaboradores (2009) e com três concentrações diferentes de 5-FU (10 ng/ml, 50ng/ml e 100ng/ml) de acordo com Yoshikawa e colaboradores (2001) em monoterapia por 24 h a 37º. Em cada poço da placa foi adicionado uma concentração do quimioterápico. As concentrações foram estabelecidas com base em cálculos de transformação de unidades de acordo com o

volume da amostra. Estas células foram mantidas por 24 horas em incubadora a 37ºC com 5% de CO_2 para ação do quimioterápico. No dia 1, após tripnização, as células foram colocadas na Câmara de Neubauer. Para determinação do número de células obtido, foi realizada uma diluição 1:10 (v/v) da suspensão celular em tampão fosfato salino (PBS- Gibco). Em seguida, uma alíquota desta solução foi diluída 1:2 (v/v) em solução de azul de Tripano 0,4% para quantificação das células vivas. A solução foi homogeneizada e colocada em um lado da câmara de Neubauer (para contagem com auxílio do microscópio óptico (Nikon Tsi) no aumento de 40X. As células foram contadas nos quatro quadrantes externos da câmara usando o esquema do "L" para que a mesma célula não seja contada duas vezes. Portanto, as células que se encontravam sobre as linhas de baixo e da direita não eram contadas. A equação usada para determinar o número de células por mililitro foi:

$$\mathbf{QC = FD \times 10^4 \times 1ml \times N^o \text{ de células}/4}$$

QC → Quantidade de células por ml

FD → Fator de diluição (40x)

10^4 → Fator de correção da câmara de Neubauer

1ml → Volume da amostra

Nº de células/4 → Média do número de células contadas

Foi utilizado como grupo controle as mesmas células cultivadas sem a aplicação dos quimioterápicos.

Após a aplicação dos quimioterápicos foi realizada a técnica de citometria de fluxo com anticorpo marcado com fluorocromo compatível para análise do nível de apoptose. As células foram colocadas em 10^5 em tubos previamente identificados que foram incubados no escuro com os marcadores específicos para cada ensaio. Ao final,

as células foram ressuspendidas em 400 µl de PBS. A identificação do antígeno foi realizada através do anticorpo monoclonal Bcl-2 (B-cell lymphoma 2: mouse monoclonal IgG 200 µg/ml - Santa Cruz Biotechnology, Inc.) conjugado com o fluorocrômo Fluorescein (sc-7382 FITC - Santa Cruz Biotechnology, Inc.) para confirmação dos resultados obtidos através da contagem manual em câmara de neubauer. As suspensões foram analisadas em citômetro de fluxo FACS calibur (Becton Dickinson Immunocytometry Systems, San José, USA) . A análise das amostras foi realizada utilizando-se o Software CELLQuest (Apple). As células foram visualizadas em gráfico de pontos (dot plot) bidimensional e histogramas e dot plots foram gerados para análise em escala logarítmica. Cada amostra foi lida em triplicata e, de cada uma, foram adquiridos 10.000 eventos (30.000 células).

Extração do RNA das células tratadas e obtenção do cDNA

O RNA total foi extraído da cultura de células tratadas com os quimioterápicos nas diferentes concentrações e da cultura de células controle (sem a aplicação das drogas quimioterápicos), usando o reagente TRIZOL (Invitrogen Life Technologies) de acordo com as instruções do fabricante.

Primeiramente foi retirado o meio de cultura e em seguida acrescentou-se 1000µL de Trizol deixando descansar por 5 minutos à temperatura ambiente. Em seguida, o conteúdo de cada poço da placa foi transferido separadamente para tubos de centrífuga de 15 mL. Adicionou-se 0,2mL de clorofórmio , homogeneizando por inversão e mantendo à temperatura ambiente por 3 minutos; após centrifugação a 12.000g por 15 minutos a 4ºC, a fase aquosa foi transferida para um tubo novo contendo 0,4mL de

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álcool isopropílico e 2µL de glicogênio.

Após 15 minutos à temperatura ambiente, as amostras foram submetidas a centrifugação por 15 minutos a 12.000g à 4ºC. Descartou-se o sobrenadante e adicionamos 1mL de etanol 75% e centrifugamos por 5 minutos a 7.500g a 4ºC descartando novamente o sobrenadante e deixando os tubos escorrendo sobre papel absorvente até secar o pellet. Feito isso, adicionou-se água Depec (Amersham) na quantidade suficiente para diluir o pellet de RNA. Foi realizada a quantificação do RNA através do equipamento Picodrop Pico200^{TM} (Analítica) com concentração de absorbância a 260nm e 280nm.

Para a obtenção do cDNA, utilizou-se o kit high capacity cDNA (Applied Biosystem).As amostras foram tratadas com DNAse para eliminar qualquer resquício de DNA na amostra. Usaram-se as amostras na concentração de 2µg/µL de RNA. Em cada tubo acrescentou-se 1µL de DNase, 2µL de Buffer 10X, levando ao termociclador a 25ºC por 15 minutos. Acrescentou-se 2µL de EDTA (stop solution) e novamente a 65ºC por 10 minutos. Após seguiu-se o uso do kit calculando a quantidade dos reagentes conforme o número de amostras. Essas foram colocadas no termociclador a 25ºC por 10 minutos; 37ºC por 120 minutos e 85ºC por 5 minutos.

Análise da Expressão gênica

A técnica de PCR em tempo real foi realizada para quantificar a expressão dos genes *MTHFR, DHFR, TYMS* e *SLC19A1* envolvidos no metabolismo do folato nas diferentes concentrações testadas dos quimioterápicos e no grupo controle também utilizando placa customizada e sonda Taqman (Kit PCR Master Mix - Applied Biosystems), com concentrações padronizadas pelo fabricante.

O PCR em tempo real foi realizado no equipamento Step One Plus™ Real-Time PCR System (Applied Byosistems) nas condições de 95 º C por 20 segundos, 40 ciclos de desnaturação a 95 º C por 0,3 segundos, anelamento a 60 º C por 30 segundos e extensão a 72 º C por 30 segundos. Todas as reações foram executadas em triplicata para todas as amostras. As concentrações utilizadas para cada amostra foram 1µl de cDNA diluídos em 4 µl de Água E-pure e 5 µl de tampão Taqman master mix. Para controlar as variações na quantidade de amostra, na quantidade e qualidade do RNA usado na reação de transcrição reversa e a eficácia desta reação, em cada amostra foi determinado o nível de expressão (número de cópias) de três genes constitutivos: *Glyceraldehyde 3-phosphate dehydrogenase* (GAPDH), *Hypoxanthine-guanine phosphoribosyltransferase* (HPRT) e β-actina. Os níveis de expressão relativa de genes foram calculados usando o método de delta limiar de ciclo (Ct) de acordo com a fórmula indicada a seguir. Os níveis de expressão dos genes-alvo foram expressos como 2 - (Delta Ct) x 1000 para simplificar os cálculos.

Nível de expressão do gene alvo = 2 - (Delta Ct) x 1000

Delta Ct = (gene alvo Ct de gene alvo - (Média do Ct dos genes de referência)

Para análise estatística utilizou-se o valor da média dos três procedimentos. Os dados foram avaliados através do teste de Análise de Variância simples (RM - One way ANOVA) com pós-teste de Bonferroni através do software do programa estatístico Bioestat (Versão 5.3). Os resultados com $p \leq 0.05$ foram considerados significantes.

Extração das proteínas

Após avaliação da viabilidade celular das células em Citômetro de Fluxo com anticorpo específico, a proteína total foi extraída da cultura de células tratadas com

os quimioterápicos nas diferentes concentrações e da cultura de células controle (sem a aplicação das drogas quimioterápicos) com Tampão RIPA Sigma Aldrich®.

Inicialmente, todo o meio de cultura foi removido dos poços com as diferentes concentrações dos quimioterápicos e com o controle da placa six Wells. Os poços foram lavados com PBS 1X (o procedimento foi realizado duas vezes) e, adicionou-se 1 ml de Tampão RIPA (Sigma Aldrich®) contendo cocktail de inibidores de proteases (EDTA 1M, PMSF 0,1M, DTT 0,1M, Aprotinina 0,1 mg/ml e Leupeptina 0,1 M) em cada poço da placa. As células foram raspadas com rodo de raspagem e transferidas para tubos de centrígufação de 15 mL. Para lise celular, as amostras foram deixadas durante 5 minutos na geladeira. Após, centrifugou-se por 10 minutos a 8.000G. O sobrenadante foi retirado e as proteínas foram quantificadas.

As proteínas foram quantificadas pelo método BCA^{TM} Protein Assay Kit (Thermo Scientific) através do equipamento Picodrop Pico200^{TM} (Analítica) para utilização das concentrações ideais nas reações propostas e uma maior eficiência destas. Após a extração e quantificação das proteínas e para uma melhor análise e observação das quantidades e integridade das proteínas extraídas foi realizada uma eletroforese em gel de poliacrilamida (SDS-PAGE) unidirecional, que evidenciou rastros proteicos. Para a etapa seguinte foi utilizada uma concentração de 50 µg/µl da proteína.

Análise da expressão proteica

As proteínas foram analisadas pela técnica de Western blotting através de detecção por cromógenos e por quimioluminescência (ECL Plus Western BlottingDetectionReagents, Amersham).

Primeiramente o tampão Bolt (Sample Reducing Agent – 10x – Novex – Life Technologies ®) e o tampão LDS Sample Buffer -4x Novex – Life Technologies ®) foram adicionados junto as proteínas de acordo com instruções do fabricante. Em seguida, as amostras com os tampões foram submetidas à temperatura de 70ºC por 10 minutos para desnaturação. Após este procedimento, as proteínas MTHFR, DHFR, TYMS e SLC19A1 foram aplicadas na concentração de 50 ug em Bolt™ NuPAGE® MES 4-12% Bis Tris Plus Mini Gel (Novex by Life Technologies®). O marcador molecular SeeBlue® Pre-Stained Standard (Life Technologies) foi aplicado para identificação dos tamanhos das proteínas. A proteína beta actina foi utilizada como referência nesta técnica. (50 µg/µl)

A corrida foi realizada nas seguintes condições: 140V; 300mA; 45 minutos, seguida de transferência realizada no equipamento iBlot® Dry Blotting System (Invitrogen- Life Technologies) configurada na programação P2 que executa a transferência em 6 minutos para membrana PVDF com poros de 0.2 µm, ideal para proteínas de 20 a 150 kDa.

Após a transferência, a membrana foi colocada na solução de bloqueio (3%BSA em TBS-T) durante 1 hora e 30 minutos. Os anticorpos foram diluídos em solução de 3% BSA em TBS-T na concentração específica estabelecida pelo fabricante e, em seguida, foram adicionados à membrana por um período de 24 horas. Em seguida, os anticorpos foram retirados e realizou-se três lavagens da membrana com solução TBS-T sem BSA. Cada lavagem durou cerca de 15 minutos. Após o procedimento de lavagem,

foi adicionado na membrana o anticorpo secundário IgG (Affinity Purified Antibody Peroxidase Labeled Anti-mouse IgG- KPL®) por 1 hora. Em seguida foi realizada quatro lavagens da membrana com duração de 15 minutos cada com TBS-T sem BSA.

Para finalizar foi adicionado à membrana o reagente ECL™ Select Western Blotting Detection (GE Healthcare) para detecção das proteínas específicas e as proteínas foram reveladas e avaliadas em foto documentador apropriado.

3. ARTIGOS CIENTÍFICOS

3. ARTIGOS CIENTÍFICOS

Os resultados estão apresentados em forma de artigo. No total estão apresentados 04 artigos, três artigos publicados e um a ser submetido para publicação.

Artigo 1: (Short Comunication)

Título: Sensitivity of human laryngeal squamous cell carcinoma HEP-2 to metrotexate chemotherapy.

Autores: Ana Lívia Silva Galbiatti, Heloisa Cristina Caldas, João Armando Padovani Junior, Érika Cristina Pavrino, Eny Maria Goloni-Bertollo.

Periódico: Experimental Oncology, 34: 367-69; 2012.

Artigo 2

Título: Alterations in the expression pattern of *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes after treatment of laryngeal cancer cells with high and low doses of methotrexate

Autores: Ana Lívia Silva Galbiatti, Heloisa Cristina Caldas, Rodrigo Castro, João Armando Padovani Junior, Érika Cristina Pavrino, Eny Maria Goloni-Bertollo.

Periódico: Tumor Biology, 34:3765–3771; 2013

Artigo 3

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

Autores: Ana Lívia Silva Galbiatti, Heloisa Cristina Caldas, Rodrigo Castro, José Victor Maniglia, Érika Cristina Pavrino-Bertelli, Eny Maria Goloni-Bertollo.

Periódico: Biomedicine & Pharmacotherapy, 68(5):515-9, 2014

Artigo 4

Título: Exposure dose of Methotrexate and 5-Fluorouracil chemotherapies can modulate expression of genes involved in folate pathway in oral cancer cells in vitro.

Autores: Ana Lívia Silva Galbiatti, João Armando Padovani-Junior, José Victor Maniglia, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo.

ARTIGO 1

Exp Oncol 2012
34, 4, 367–369

Experimental ONCOLOGY

SENSITIVITY OF HUMAN LARYNGEAL SQUAMOUS CELL CARCINOMA HEP-2 TO METROTEXATE CHEMOTERAPY

A.L.S. Galbiatti[1,], H. C. Caldas[2], J. A. Padovani Junior[3], E.C. Pavarino[1], E.M. Goloni-Bertollo[1,*]*

[1]*Genetics and Molecular Biology Research Unit, Upgem, FAMERP, São José do Rio Preto Medical School, Brazil*

[2]*Laboratory of Experimental Immunology and Transplantation, Litex, FAMERP, São José do Rio Preto Medical School, Brazil,*

[3]*Otorhinolaryngology and Head and Neck Surgery Department of University São José do Rio Preto Medical School, FAMERP, Brazil*

Aim: **Methotrexate (MTX) is an antifolate agent that acts inhibiting purine and pyrimidine synthesis. The objective of the study was to evaluate the viability of Hep-2 human laryngeal cancer cells to the treatment with MTX chemotherapy *in vitro*. *Methods:* Cultured Hep-2 cells were treated with 0.25, 25.0 and 75 µM MTX for 24 h, and their viability was evaluated with Bcl-2-FITC antibody in flow cytometry. *Results:* The numbers of viable Hep-2 cells after 24 h treatment with 0.25, 25.0 and 75.0 uM MTX were 85.43%, 22.46% and 8.42%, respectively ($p < 0.05$). Therefore, MTX possesses a dose-dependent effect on viability of Hep-2 cells *in vitro*. *Conclusion:* The highest MTX concentration is associated with highest tumor cell sensitivity of human laryngeal cancer cells of Hep-2 line.**

Key Words: **cell line, laryngeal cancer, methotrexate, dose-response relationship, flow cytometry.**

Head and neck cancer (HNC) includes tumors of pharynx, oral cavity and larynx. The treatment of these tumors may be surgery, radiotherapy and chemotherapy [1–3]. Methotrexate (2,4-diamino,N10-methylpteroyl glutamic acid) (MTX) is an antiproliferative and immunosuppressive chemotherapeutic agent widely used against a broad spectrum of diseases, including HNC [4, 5]. It acts via inhibition of the synthesis and conversion of folate derivatives responsible for providing methyl groups for the nucleotides synthesis and DNA methylation reactions [6–13]. Although chemotherapy presents good results, tumors may develop resistance to antifolate agents. A number of factors are critical for a favorable clinical outcome for MTX therapy, in particular acute toxicity, side effects, and drug resistance development [5, 14–16]. The current study was undertaken to evaluate *in vitro* the human larynx squamous cell carcinoma Hep-2 cell line sensitivity to the MTX treatment. The cell line was cultured in Dulbecco Medium (D-MEN 00068 medium, Cultilab), supplemented with 10% fetal bovine serum (FBS, Cultilab), 2 µM glutamine (Cultilab), 100 U/ml of penicillin, 100 U/ml of streptomycin, 1 µM sodium pyruvate (Sigma–Aldrich) and 1 µM non-essential amino acid (Sigma–Aldrich) in a humidified 5% CO_2/95% air atmosphere at 37 °C. MTX concentrations of 0.25 µM, 25 µM, and 75 µM were calculated according to Pai et al. [13]. Hep-2 cells were incubated with the mentioned MTX concentrations for 24 h, while the control cells were cultured in MTX-free medium. Cell viability was measured by flow cytometry (FACS caliber- Becton Dickinson Immunocytometry Systems, San José, USA) with double staining with fluorescein isothiocyanate (FITC)/Bcl-2 according to manufacturer's manual (Santa Cruz Biotechnology, Inc). Each experiment was performed in triplicate. The normal distribution of the samples was verified with Normality tests (Shapiro — Wilk's test and Kolmogorov — Smirnov test). Statistical analysis was performed by nonparametric methods based upon the comparison between the groups. The effects for MTX concentrations in the cell viability were evaluated independently by Kruskal-Wallis test (Control group x 0,25 uM MTX concentration / Control group x 25 uM MTX concentration / Control group x 75 uM MTX concentration). For comparison of the variables between groups exposed with MTX and free-MTX group we used the Mann — Whitney test. The Spearman correlation degree between variables of interest was calculated by Spearman test.

The results of flow cytometry analysis with FITC/Bcl-2 double staining showed that 92.6% of control cells were vital, while the numbers of viable Hep-2 cells after 24 h treatment with 0.25; 25.0 and 75.0 uM MTX were 85.43; 22.46 and 8.42%, respectively (Figure, Table) ($p < 0.05$). The Shapiro — Wilk's test indicated that there was a normal distribution for the groups ($p = 0.180$). The Kolmogorov — Smirnov test confirmed that all samples presented significance level of 5% for groups (K-S = 0.383; $p = 0.008$) (Table). The Kruskal-Wallis test indicated a significant effect of MTX in the cell viability ($H = 9.00$, $p = 0.003$). The value of Mann — Whitney test showed significant results (p= 0.0304). The Spearman correlation between frequencies of cells exposed with MTX and unexposed showed an interaction between these cells (r = 0.50). Our study confirmed that cells were more sensitive and became less resistant to the MTX chemotherapy as dose was increasing. Moreover, there was a correlation between cells exposed frequencies with MTX and unexposed cells; literature

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*Correspondence: E-mail: analivia_sg@yahoo.com.br; eny.goloni@famerp.br

Abbreviations used: HNC – head and neck cancer; MTX – methotrexate.

data show MTX concentration correlates with drug therapeutic efficacy [17]. These results suggest that MTX has substantial antiproliferative activity and is used effectively as a chemotherapy agent in the treatment for solid-organ neoplasms and the treatment is more efficient when the dose is increased. However, the pilot study of Pai et al. [13] that evaluated the sensibility of oral cancer cells to MTX *in vitro* and its association with clinical response to MTX in oral cancer showed that there is differential sensitivity to MTX among the various tumor cells in the *in vitro* assay, and these data had significant correlation when compared with clinical outcome for 7 out of 10 patients. MTX is an antimetabolite, analogous to folate, that competitively inhibits diidrofolatoredutase (DHFR) enzyme activity, essential for nucleotides purines and thymidylic acid biosynthesis, interfering with DNA synthesis. [10, 18, 19]. Although we found that MTX treatment is highly effective in HNC cells, data confirm that high-dose MTX schemes may arrest normal epidermal cell proliferation and cause direct cell toxicity [20]. Toxicity is increased by folic acid deficiency or by medications such as barbiturates and nitrofurantoin, which impair folic acid absorption [10, 20]. However, it has been documented that folic acid (1 to 5 mg/day) supplementation helps to prevent MTX associated toxicities and concomitant use of either folic acid with methotrexate has no impact on the therapeutic efficacy of MTX in multiple clinical trials and meta-analyses [10, 21].

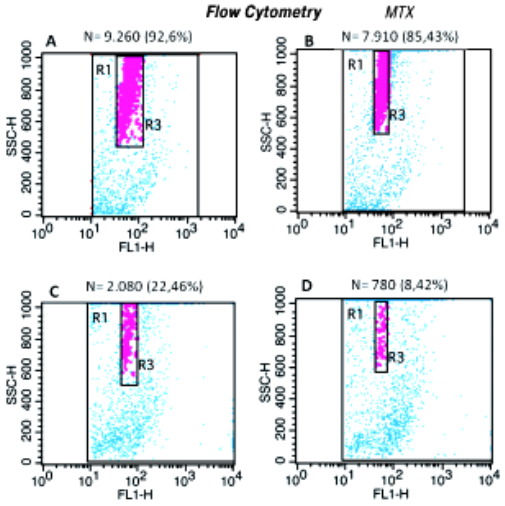

Figure. Flow cytometry analysis of Hep-2 cells treated with 0.25, 25 and 75 mM MTX (*b*, *c*, *d* respectively) and control cells (*a*). The cells in the "R1" block are cells non-viable and cells in the "R3" are viable. Cell viability was evaluated by double staining with fluorescein isothiocyanate (FITC) label Bcl-2 (100: sc-509); Pink cells are viable, blue cells — non-viable

Table. Viability of Hep 2 cells treated with MTX for 24 h

MTX dose Concentration	Viable cells, M ± SD
Control group	9,260 ± 50
0.25 mM	7,910 ± 26*
25 mM	2,080 ± 44*
75 mM	780 ± 30*

* The difference is significant compared to the control ($p < 0.05$).

Research about chemosensitivity is important to screen new therapeutic agents, identify patterns of sensibility for different tumor types, to select chemotherapy regimens to individual patients and improvement in life quality [22]. We conclude that the highest MTX concentration is associated with highest tumor cells sensibility; as a consequence, the knowledge of the drug sensibility can do significant impact in decision-making and treatment.

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REFERENCES

1. Haddad R, Annino D, Tishler RB. Multidisciplinary approach to cancer treatment: focus on head and neck cancer. Dent Clin N Am 2008; **52**: 1–17.

2. Marcu LG, Yeoh E. A review of risk factors and genetic alterations in head and neck. Clin Oncol 2009; **135**: 1303–14.

3. Ignoffo RJ, Rosenbaum EH. What happens in chemotherapy. In: Ko AH, Dollinger M, Rosenbaum EH, eds. Everyone's Guide to Cancer Therapy, Revised 5th Edition. Kansas City, MS: Andrew McMeel Publishing; 2008.

4. Moura JA, Valduga CJ, Tavares ER, ***et al***. Novel formulation of a methotrexate derivative with a lipid nanoemulsion. Int J Nanomedicine 2011; **6**: 2285– 95.

5. American Cancer Society. Methotrexate. 2012 (www.cancer.org)

6. Allegra CJ. Antifolates: the next millenium. Semin Oncol 1999; **26**: 1–2.

7. James SJ, Pogribny IP, Pogribna M, ***et al***. Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis. J Nutr 2003; **133**: 3740–7.

8. Jackman AL, Theti DS, Gibbs DD. Antifolates targeted specifically to the folate receptor. Adv Drug Deliv Rev 2004; **56**: 1111–25.

9. Nzila A, Ward SA, Marsh K, ***et al***. Comparative folate metabolism in humans and malaria parasites (part I): pointers for malaria treatment from cancer chemotherapy. Trends Parasitol 2005; **21**: 292–8.

10. Tian H, Cronstein BN. Understanding the mechanisms of action of methotrexate. Bulle NYU Hospital Joint Dis 2007; **65**: 168-73.

11. Guardiola E, Peyrade F, Chaigneau L, ***et al***. Results of a randomized phase II study comparing docetaxel with methotrexate in patients with recurrent head and neck cancer. Eur J Cancer 2004; **40**: 2071–6.

12. Katori H, Tsukuda M, Taguchi T. Analysis of efficacy and toxicity of chemotherapy with cisplatin, 5-fluorouracil, methotrexate and leucovorin (PFML) and radiotherapy in the treatment of locally advanced squamous cell carcinoma of the head and neck. Cancer Chemother Pharmacol 2007; **59**: 789–94.

13. Pai RB, Lalitha RM, Pai SB, ***et al***. Analysis of *in vitro* and *in vivo* sensitivity of oral cancer cells to methotrexate. Exp Oncol 2009; **31**: 118–20.

14. Banerjee D, Mayer-Kuckuk P, Capiaux G, ***et al.*** Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. Biochim Biophys Acta 2002; **1587**: 164–73.

15. Forastiere AA, Goepfert H, Maor M, ***et al.*** Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003; **349**: 2091–8.

16. Yao M, Epstein JB, Modi BJ, ***et al***. Current surgical treatment of squamous cell carcinoma of the head and neck. Oral Oncol 2007; **43**: 213–23.

17. Chan ES, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis Res 2002; **4**: 266–73.

18. Morales C, Garcia MJ, Ribas M, ***et al***. Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells. Mol Cancer Ther 2009; **8**: 424–32.

19. Lansiaux A. Antimetabolites. Bull Cancer 2011; **98**: 1263-74.

20. Tazi I, Madani A, Zafad S, ***et al.*** Methotrexate-induced toxic epidermal necrolysis: A case report. Int J Med Sci 2009; **1**: 99–101.

21. Cronstein BN. Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. Pharmacol Rev 2005; **57**: 163–72.

22. Blumenthal RD, Goldenberg DM. Methods and goals for the use of *in vitro* and *in vivo* chemosensitivity testing. Mol Biotechnol 2007; **35**: 185–97.

ARTIGO 2

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RESEARCH ARTICLE

Alterations in the expression pattern of *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes after treatment of laryngeal cancer cells with high and low doses of methotrexate

Ana Lívia Silva Galbiatti · Rodrigo Castro · Heloisa Cristina Caldas · João Armando Padovani Jr · Érika Cristina Pavarino · Eny Maria Goloni-Bertollo

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Abstract Inter-individual variations to methotrexate (MTX) outcome have been attributed to different expression profiles of genes related to folate metabolism. To elucidate the mechanisms of variations to MTX outcome, we investigated *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* gene expression profiles by quantifying the mRNA level of the genes involved in folate metabolism to MTX response in laryngeal cancer cell line (HEP-2). For this, three different concentrations of MTX (0.25, 25, and 75 μmol) were added separately in HEP-2 cell line for 24 h at 37 °C. Apoptotis quantification was evaluated with fluorescein isothiocyanate-labeled Bcl-2 by flow cytometry. Real-time quantitative PCR technique was performed by quantification of gene expression with TaqMan® Gene Expression Assay. ANOVA and Bonferroni's post hoc tests were utilized for statistical analysis. The results showed that the numbers of apoptotic HEP-2 cells with 0.25, 25.0, and 75.0-μmol of MTX were 14.57, 77.54, and 91.58 %, respectively. We found that the expression levels for *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes were increased in cells with 75.0-μmol of MTX ($p<0.05$). Moreover, *SLC19A1* gene presented lower expression in cells treated with 0.25 μmol of MTX ($p<0.05$). In conclusion, our data suggest that *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes present increased expression after the highest application of MTX dose in laryngeal cancer cell line. Furthermore, *SLC19A1* gene also presents decreased expression after the lowest application of MTX dose in laryngeal cancer cell line. Significant alterations of expression of these studied genes in cell culture model may give support for studies in clinical practice and predict interesting and often novel mechanisms of resistance of MTX chemotherapy.

Keywords Antifolate · Chemotherapy treatment · Gene expression · Methotrexate · Cell-line · Laryngeal carcinoma

A. L. S. Galbiatti (✉) · R. Castro · É. C. Pavarino · E. M. Goloni-Bertollo (✉)
Genetics and Molecular Biology Research Unit (UPGEM), São José do Rio Preto Medical School (FAMERP), Avenida Brigadeiro Faria Lima, n.5416, São José do Rio Preto, SP, Brazil
e-mail: analivia_sg@yahoo.com.br
e-mail: eny.goloni@famerp.br

H. C. Caldas
Laboratory of Experimental Immunology and Transplantation (LITEX), São José do Rio Preto Medical School (FAMERP), Avenida Brigadeiro Faria Lima, n.5416, São José do Rio Preto, SP, Brazil

J. A. Padovani Jr
Otorhinolaryngology and Head and Neck Surgery Department, São José do Rio Preto Medical School (FAMERP), Avenida Brigadeiro Faria Lima, n.5416, São José do Rio Preto, SP, Brazil

Introduction

Laryngeal carcinoma is the second most common head and neck squamous carcinoma, and its definite cause has not been determined yet [1]. Some risk factors such as tobacco consumption and human papilloma virus infections may be associated to the development of the disease [2, 3]. The option of treatment is mainly based on clinical, radiological, and pathological parameters, and may consist of mutilating surgery, radiotherapy and/or chemotherapy. Chemotherapy has been widely accepted as a treatment modality for head and neck cancer tumors, including laryngeal cancer [4].

In the past 65 years, antifolate chemotherapies targeting folate metabolism played a pivotal role in cancer therapeutic treatment [5]. This chemotherapy modality disrupts cellular proliferation by blocking and inhibiting key folate dependent

enzymes, thereby leading to the inhibition of methylation and nucleotide biosynthesis and consequently cell death [6].

Methotrexate (MTX) is an antifolate chemotherapy currently used as an integral component of chemotherapeutic regimens that show efficiency in several treatments for cancer, including laryngeal cancer. The concentration utilized in clinical routine for MTX is 15 to 30 mg doses daily for a 5-day course. The physician-recommended dose is taken weekly and depends on each disease and patient. [7–11]. Despite more than 65 years after MTX was introduced for cancer therapy, several topics related to MTX still remain unclear. Research has focused to study the mechanism of action of MTX, especially the mechanism behind why some patients have intrinsic resistance to this chemotherapeutic drug, and if the effectiveness of MTX can be increased. It may be determined by individual genetic differences [7, 12, 13].

It is known that MTX blocks the synthesis of purines and pyrimidines by inhibiting a group of key enzymes that are codified by genes involved in folate metabolism [14, 15]. Alterations in the expression levels of genes involved in this pathway may contribute to the pharmacokinetic and pharmacodynamic MTX pathways, which could affect the drug clinical activity, causing toxicity, resistance, or increased effectiveness. Variability in the clinical course and outcomes to antineoplastic treatment lead us to search for new biological markers that allow better understand on the behavior of the cancer development and their treatment outcomes [14, 16, 17].

Literature data shows that alterations in the expression levels of genes involved in folate metabolism in patients treated with MTX antifolate chemotherapy may be related to sensitivity and resistance [17–24]. Therefore, we investigated methylenetetrahydrofolate reductase (NAD(P)H) (*MTHFR*), dihydrofolate reductase (*DHFR*), thymidylate synthetase (*TYMS*), and solute carrier family 19 (folate transporter)/member 1 (*SLC19A1*) folate gene expression profiles in laryngeal cancer cell line (HEP-2) administered with three different concentrations of MTX chemotherapy.

Materials and methods

Cell line

HEP-2 cell line (laryngeal carcinoma) was obtained from the Laboratory of Medical Molecular Markers and Bioinformatics (Profa. Dra Eloiza Helena Tajara). HEP-2 cell line has been described to originate from tumors which were produced in irradiated cortisonized weanling rats after the injection of epidermoid carcinoma tissue isolated from the larynx of a 56-year-old male human.

The cells were plated in tissue culture dishes and grown in minimum essential medium eagle medium (D-MEN 00068 medium; Cultilab) containing 10 % fetal bovine serum (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. The cells in a mid-log phase with viability >95 % were used in experiments.

Drug preparation

MTX chemotherapy was provided by the Oncology Department, Hospital de Base, São José do Rio Preto. The cells were plated in six-well culture plates at a density of 1×10^5/well and incubated with three different concentrations: 0.25, 25, and 75 μmol of MTX for 24 h at 37 °C and harvested by trypsinization, according to Pai et al. [20]. The control cells were the cell line with drug-free medium.

Flow cytometry and apoptotis quantification

After the 24-h MTX incubation, the determination of viable and apoptotic cells was evaluated by double staining with fluorescein isothiocyanate (FITC)-label Bcl-2 (100:sc-509) according to manufacturer's manual (Santa Cruz Biotechnology, Inc). The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Within 1 h, the cells were analyzed with flow cytometer FACS calibur (Becton Dickinson Immunocytometry Systems, San José, CA, USA). The analysis of the sample was performed using the CELLQuest software (Apple).

RNA extraction, cDNA generation, and qRT-PCR

Total RNA was extracted from cell culture with TRIzol® Reagent, according to manufacturer's instructions. RNA concentrations were measured using Picodrop® equipment and stored at −70 °C. For cDNA synthesis, 2 μg of total RNA was used with random primers according to *High capacity cDNA* kit (Applied Biosystem®) manufacturer's protocol.

Real-time quantitative PCR (qRT-PCR) technique was performed for quantification of gene expression by StepOnePlus™ equipment (Applied Biosystems) with cycling condition of 95 °C for 20 s, 40 cycles of denaturation at 95 °C for 0.3 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. TaqMan® Gene Expression Assay was pre-optimized PCR primer and probe sets for qRT-PCR was formulated at 20× concentration. Gene-specific primers were used for quantification of one-carbon metabolizing gene expressions in TaqMan® Custom Array Plate, which included triplicated wells of two reference genes and four target genes (Table 1). The reaction mix contained TaqMan® Fast Advanced Master Mix with final concentration. All reactions

Table 1 Reference and target genes evaluated in the study

Category	Gene name	Assay ID
Reference genes		
ACTB	*Actin beta*	Hs01060665_g1
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	Hs02758991_g1
Target genes		
MTHFR	*Methylenetetrahydrofolate reductase (NAD(P)H)*	Hs00195560_m1
DHFR	*Dihydrofolate reductase*	Hs00758822_s1
TYMS	*Thymidylate synthetase*	Hs00426586_m1
SLC19A1	*Solute carrier family 19 (folate transporter), member 1-reduced folate carrier 1*	Hs00953344_m1

were run in triplicate for the 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 the target genes were expressed as $2^{-(\text{Delta Ct})} \times 1{,}000$ to simplify the calculation.

$$\text{Expression level of target gene} = 2^{-(\text{Delta Ct})} \times 1{,}000$$

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

Statistical analysis

The mean Ct values of triplicate measurements were used for analysis. These data were evaluated by one-way RM analysis of variance (ANOVA) to assess the *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* gene expression in three different MTX concentrations against the expression of genes in the control cells (No MTX). Bonferroni's post hoc test was used to determine the p value of each concentration vs. control. BioEstat software program (version 5.3) was utilized for statistical analysis. The results were considered to be statistically significant at $p \leq 0.05$.

Results

The results for flow cytometry analysis with Bcl-2/FITC staining showed that 7.4 % of cells in the control group were apoptotic cells. Regarding the administration of 0.25, 25.0, and 75.0 μmol of MTX, the apoptotic cells were 14.57, 77.54, and 91.58 %, respectively (Fig. 1).

We found that the expression levels for *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes were increased in cells treated with 75 μmol of MTX chemotherapy. The *SLC19A1* gene also presented decreased expression in cells treated with 0.25 μmol of MTX chemotherapy (Fig. 2). There was significant association between the control group and increased expression of all genes evaluated in the 75 μmol MTX treatment group (*MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes; $p \leq 0.05$). Our results also showed significant association of decreased *SLC19A1* expression gene in cells treated with 0.25 μmol MTX ($p \leq 0.05$) (Table 2)

Discussion

Our findings confirmed that HEP-2 cell line administered with 75 μmol of MTX chemotherapy presents increased expression levels for *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes when compared to HEP-2 cell line without MTX treatment. Furthermore, our results also confirmed decreased expression for *SLC19A1* gene in HEP-2 cell line administered with 0.25 μmol of MTX chemotherapy. The study of Lee et al. [25], which evaluated the expression profiles of the genes involved in folate metabolism in normal individuals found that these genes were expressed in lymphocytes at moderate levels.

Folate metabolism is involved in both DNA synthesis and methylation, and impacts both genetic and epigenetic processes in the development of the disease. DNA synthesis is essential for cell division, and DNA methylation is responsible for gene expression control, chromatin structure stability, and maintenance of genomic stability [26]. It is known that MTX acts by inhibiting folate-dependent enzymes and thus blocks DNA synthesis by acting on the S phase of the cell cycle [14, 15]. The basic principle of MTX therapeutic efficacy is due to the inhibition of *DHFR*, a key enzyme in the folate metabolism. MTX also inhibits *TYMS*, *MTHFR*, and transport of reduced folates into the cell occurring by the *SLC19A1* gene. The inhibition of key enzymes involved in folate metabolism results to the interruption of DNA replication and cell death [27].

Studies have found varying degrees of success in head and neck cancer patients treated with MTX chemotherapy as

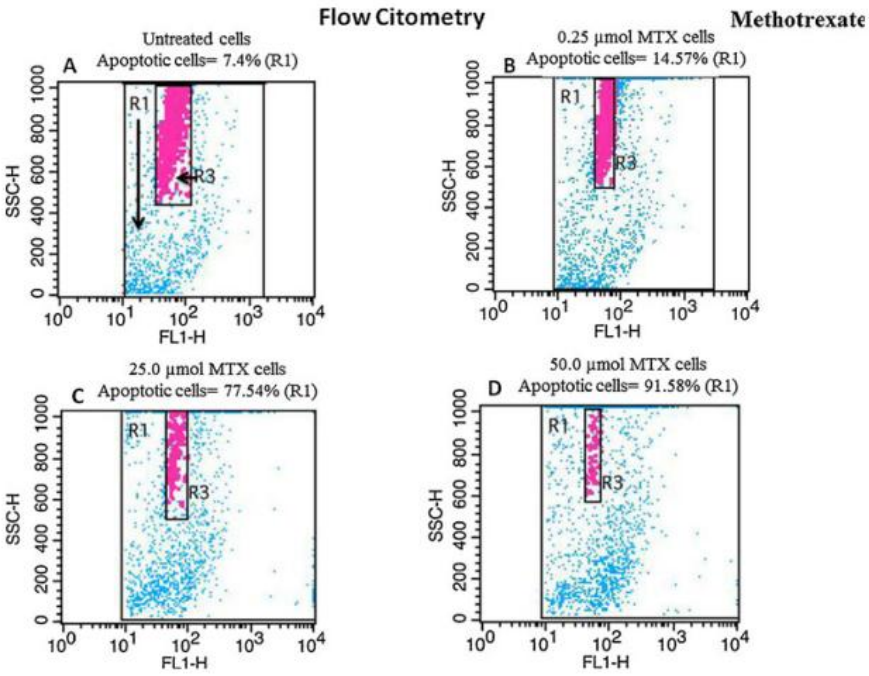

Fig. 1 Flow cytometry photodocumentation of cells from cell line HEP-2 treated with MTX chemotherapy showing apoptotic and viable cells. **a** Cells without treatment with chemotherapy (control), **b** cells treated with a concentration of 0.25 µmol MTX, **c** cells treated with 25.0 µmol concentration of MTX and **d** cells treated with a concentration of 75.0 µmol MTX. *R1* denotes apoptotic cells; *R3* represents the viable cells

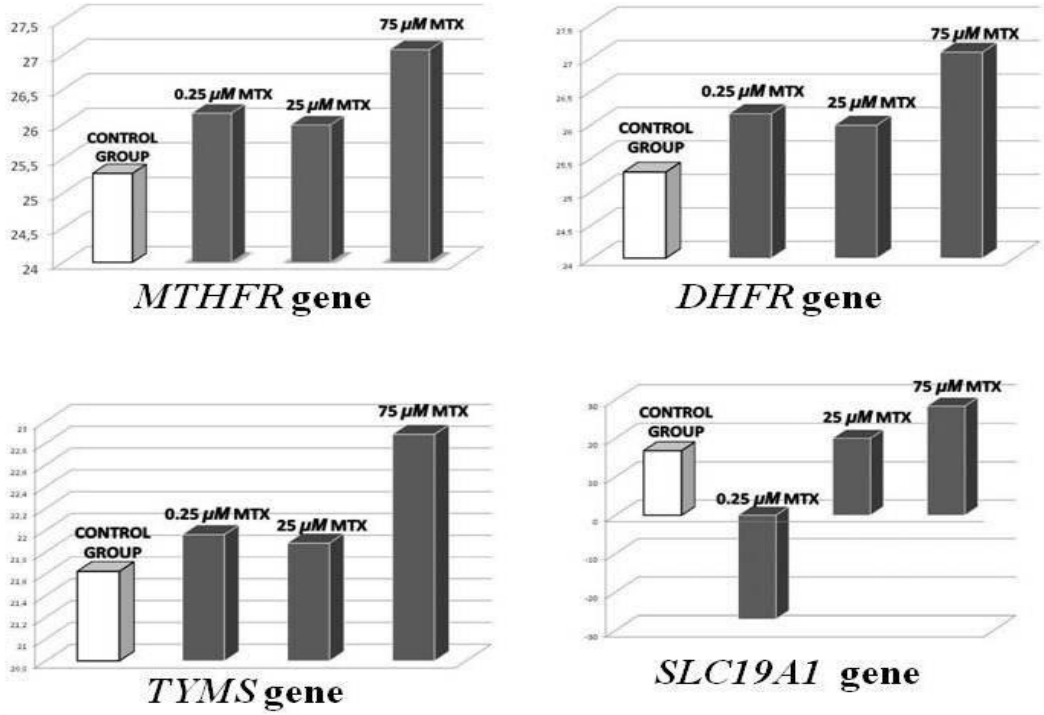

Fig. 2 Quantitative gene expression of *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* genes in HEP-2 cell line treated with three different concentrations of MTX (*black*) and cell line without MTX application (*white*)

Table 2 ANOVA test for *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* gene expressions in three different MTX concentrations against the expression of the genes in the control cells (no application of MTX)

Sample	Bonferroni difference	*F* ratio	*p* Value
MTHFR			
0.25 μmol MTX	0.87	310.11	ns
25 μmol MTX	0.70	107.83	ns
75 μmol MTX	1.79	755.31	<0.05
DHFR			
0.25 μmol MTX	0.45	186.66	ns
25 μmol MTX	0.24	69.18	ns
75 μmol MTX	1.33	3002.16	<0.05
TYMS			
0.25 μmol MTX	0.34	25.41	ns
25 μmol MTX	0.25	10.86	ns
75 μmol MTX	1.25	298.68	<0.05
SLC19A1			
0.25 μmol MTX	49.9	183.3806	<0.05
25 μmol MTX	1.08	129.41	ns
75 μmol MTX	1.78	136.559	<0.05

ns not significant

a single agent or in combination, and the varying degree depends on the dose received by patients and may lead to resistance mechanisms and collateral effects due to alterations in the genetic expression levels of the genes involved in folate pathway [17–20, 28].

DHFR enzyme, codified by the *DHFR* gene, catalyzes the reduction of DHF to THF, an essential cofactor for the synthesis of thymidylate monophosphate (dTMP) which is involved in DNA biosynthesis and cell replication [29]. MTX blocks the regeneration of THF from DHF by competitively inhibiting *DHFR* activity. If there is an alteration in the *DHFR* expression, the DNA may become unstable, leading to abnormal cell proliferation as well as to many pathogeneses [30].

In our research, we confirmed that there was an increased expression of *DHFR* in cancer cells treated with higher MTX concentration. According to the studies of Sowers et al. [31], Serra et al. [32], and Askari and Krajinovic [22], the increased expression of *DHFR* and *THF* accumulation can contradict a cytotoxic effect of MTX, thereby reducing treatment efficacy in patients who have cancer. Alterations in the *DHFR* genetic expression that may lead to sensitivity to MTX can also be due to genetic polymorphisms such *DHFR* C829T that seems to be related with increased *DHFR* expression and consequently reduced sensitivity to MTX [22, 33].

Regarding the *TYMS* gene, we found that there was an increased expression for this gene in cancer cells treated with higher MTX concentration. Data shows that genetic alterations in *TYMS* gene may lead to increased expression for this gene when the treatment is realized with high-dose MTX and may be related to toxicity [28]. *TYMS* is a folate-dependent enzyme codified by the *TYMS* gene, which catalyzes the reductive methylation of deoxyuridylate (dUMP) to thymidylate (dTMP) that results in the accumulation of dihydrofolate and depletion of cellular folates, thereby playing a central role in DNA synthesis and repair by serving as the primary intracellular source of dTMP [27, 34].

In relation to *MTHFR* gene, studies show that increased *MTHFR* expression is associated with increased chemosensitivity to MTX [17, 21], as our study found increased expression for this gene in cancer cells treated with higher MTX concentration. *MTHFR* enzyme that is codified by the *MTHFR* gene serves as a source of carbon units to the conversion of dUMP to dTMP by *TYMS* [12].

MTHFR is also involved in MTX activity by modulating the intracellular pool of folates. Alterations of the *MTHFR* activity can affect the cellular concentrations of folate, which can change sensitivity to MTX. Indeed, the exact biological effect of *MTHFR* increased expression is not completely defined [35, 36], although data show that there are two genetic polymorphisms in *MTHFR* gene (C677T and A1298C), which seem to be associated with alterations in *MTHFR* expression level, may interfere with both antitumor activity of the chemotherapy, and is related to toxicity [36, 37]. A study of Robien et al. [38] showed an association between decreased expression of *MTHFR* and greater response to MTX-related toxicity, consequently increasing the expression of *MTHFR* which may influence the MTX toxicity mainly if MTX chemotherapy is utilized in high doses.

With respect to the *SLC19A1* gene that encodes the *SLC19A1* enzyme which is involved in the absorption and transport of folate to the interior of the cells, we found in our study an increased expression level in cells treated with 75-μmol of MTX chemotherapy and decreased expression of *SLC19A1* gene in HEP-2 cell line treated with 0.25 μmol of MTX [5]. Data confirms that differences in the regulation of *SLC19A1* gene may be important in determining the sensitivity of tumors to both low-dose and high-dose MTX therapy [39].

The *SLC19A1* gene is the major transporter of antifolate chemotherapies, and the effectiveness of chemotherapy is linked to levels and activity of SLC19A1 [40]. Studies showed that after acute exposure to low-dose MTX, the levels of *SLC19A1* expression were decreased, resulting in impaired transport of MTX by *SLC19A1* into cancer cells; our study confirmed decreased expression of *SLC19A1* gene in HEP-2 cell line treated with lower concentrations of MTX [32, 39, 40]. The decreased expression for *SLC19A1* leads to decreased intracellular MTX concentration and may be involved in the MTX resistance [24].

In our research, we also found an increased expression of the *SLC19A1* gene in laryngeal cancer cells treated with higher concentration of MTX chemotherapy. It is possible that higher dose of chemotherapy is related to more production of *SLC19A1* that is responsible for MTX transport. Increased expression for *SLC19A1* gene was also detected in breast cancer cell lines treated with MTX chemotherapy. However, the MTX dose administered in this cell was normal, not high dose. There are no studies that have showed association between high doses of MTX and increased expression for *SLC19A1* gene. Data shows that the increased *SLC19A1* expression would lead to the inactivation of various tumor suppressor genes like p16, p15 and/or p53, resulting in increased sensitivity to MTX [23].

Chemotherapy may result in late and long-term side effects, and investigations show that genetic variation is associated with drug response, keeping the promise of individualized treatment to maximize efficacy and minimize toxicity [21]. Cell line that presents alterations in the genetic expression of genes involved in folate metabolism may serve as a useful model to investigate the regulation of the genetic expression. The determination of different patterns of genetic expression may to help identify reliable prognostic markers. Identifying biomarkers for treatment response and toxicity to MTX chemotherapy is critically important to advance the field of personalized medicine in cancer.

In conclusion, our data suggests that *MTHFR*, *DHFR*, *TYMS*, and *SLC19A1* genes present increased expression after the highest application of MTX dose in laryngeal cancer cell line. Moreover, *SLC19A1* gene also presents low expression after the lowest application of MTX dose in laryngeal cancer cell line. Significant alterations of expression of these studied genes in cell culture model may give support for studies in clinical practice and predict interesting and often novel mechanisms of resistance of MTX chemotherapy.

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Conflicts of interest None.

References

1. Chu EA, Kim YJ. Laryngeal cancer: diagnosis and preoperative work-up. Otolaryngol Clin North Am. 2008;41(4):673–95.
2. Sapkota A, Hsu CC, Zaridze D, Shangina O, Szeszenia-Dabrowska N, Mates D, et al. Dietary risk factors for squamous cell carcinoma of the upper aerodigestive tract in central and eastern Europe. Cancer Causes Control. 2008;19(10):1161–70.
3. Torrente MC, Rodrigo JP, Haigentz Jr M, Dikkers FG, Rinaldo A, Takes RP, et al. Human papillomavirus infections in laryngeal cancer. Head & Neck. 2011;33(4):581–6.
4. Lorch JH, Posner MR, Wirth LJ, Haddad RI. Induction chemotherapy in locally advanced head and neck cancer: a new standard of care? Hematol Oncol Clin North Am. 2008;22(6):1155–63.
5. Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resist Updat. 2012;15(4):183–210.
6. Visentin M, Zhao R, Goldman ID. The antifolates. Hematol Oncol Clin North Am. 2012;26(3):629–48.
7. Taguchi T, Tsukuda M, Mikami Y, Matsuda H, Horiuchi C, Yoshida T, et al. Concurrent chemoradiotherapy with cisplatin, 5-fluorouracil, methotrexate, and leucovorin in patients with advanced resectable squamous cell carcinoma of the larynx and hypopharynx. Acta Otolaryngol. 2006;126(4):408–13.
8. Doe J. Methotrexate. Mylan Pharmaceuticals Inc. 2012. http://www.drugs.com/pro/methotrexate.html. Accessed 11 Jun 2013.
9. Kraljevic Pavelic S, Cacev T, Kralj M. A dual role of *p21*$^{waf1/cip1}$ gene in apoptosis of HEp-2 treated with cisplatin or methotrexate. Cancer Gene Ther. 2008;15(9):576–90.
10. Brockstein BE. Management of recurrent head and neck cancer: recent progress and future directions. Drugs. 2011;71(12):1551–9.
11. Price KA, Cohen EE. Current treatment options for metastatic head and neck cancer. Curr Treat Options Oncol. 2012;13(1):35–46.
12. Bertino JR. Cancer research: from folate antagonism to molecular targets. Best Pract Res Clin Haematol. 2009;22:577–82.
13. Sáez-Ayala M, Fernández-Pérez MP, Montenegro MF, Sánchez-del-Campo L, Chazarra S, Piñero-Madrona A, et al. Melanoma coordinates general and cell-specific mechanisms to promote methotrexate resistance. Exp Cell Res. 2012;318(10):1146–59.
14. Morales C, Garcia MJ, Ribas M, Miro R, Muñoz M, Caldas C, et al. Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells. Mol Cancer Ther. 2009;8:424–32.
15. Moura JA, Valduga CJ, Tavares ER, Kretzer IF, Maria DA, Maranhão RC. Novel formulation of a methotrexate derivative with a lipid nanoemulsion. Int J Nanomedicine. 2011;6:2285–95.
16. Hider SL, Bruce IN, Thomson W. The pharmacogenetics of methotrexate. Rheumatology. 2007;46:1520–4.
17. Katori H, Tsukuda M, Taguchi T. Analysis of efficacy and toxicity of chemotherapy with cisplatin, 5-fluorouracil, methotrexate and leucovorin (PFML) and radiotherapy in the treatment of locally advanced squamous cell carcinoma of the head and neck. Cancer Chemother Pharmacol. 2007;59:789–94.
18. Celtikci B, Leclerc D, Lawrance AK, Deng L, Friedman HC, Krupenko NI, et al. Altered expression of methylenetetrahydrofolate reductase modifies response to methotrexate in mice. Pharmacogenet Genomics. 2008;18:577–89.
19. Specenier PM, Vermorken JB. Current concepts for the management of head and neck cancer: chemotherapy. Oral Oncol. 2008;45:409–15.
20. Pai RB, Lalitha RM, Pai SB, Kumaraswamy SV, Lalitha N, Bhargava MK. Analysis of in vitro and in vivo sensitivity of oral cancer cells to methotrexate. Exp Oncol. 2009;31:118–20.
21. Celtikci B, Lawrance AK, Wu Q, Rozen R. Methotrexate-induced apoptosis is enhanced by altered expression of methylenetetrahydrofolate reductase. Anticancer Drugs. 2009;20(9):787–93.
22. Askari BS, Krajinovic M. Dihydrofolate reductase gene variations in susceptibility to disease and treatment outcomes. Curr Genomics. 2010;11(8):578–83.

23. Erčulj N, Kotnik BF, Debeljak M, Jazbec J, Dolžan V. Influence of folate pathway polymorphisms on high-dose methotrexate-related toxicity and survival in childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2012;53(6):1096–104.
24. Obuchi W, Ohtsuki S, Uchida Y, Ohmine K, Yamori T, Terasaki T. Identification of transporters associated with etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics. Mol Pharmacol. 2013;83(2):490–500.
25. Lee YL, Xu X, Wallenstein S, Chen J. Gene expression profiles of the one-carbon metabolism pathway. J Genet Genomics. 2009;36:277–82.
26. Linhart HG, Troen A, Bell GW, Cantu E, Chao WH, Moran E, et al. Folate deficiency induces genomic uracil misincorporation and hypomethylation but does not increase DNA point mutations. Gastroenterology. 2009;136:227–35.
27. Bertino JR, Gorlick R. Mechanisms of methotrexate resistance in osteosarcoma. Clin Cancer Res. 1999;5:621–7.
28. Guardiola E, Peyrade F, Chaigneau L, et al. Results of a randomized phase II study comparing docetaxel with methotrexate in patients with recurrent head and neck cancer. Eur J Cancer. 2004;40:2071–6.
29. Abali EE, Ercikan E, Skacel NE, Celikkaya H, Hsieh YC. Regulation of human dihydrofolate reductase activity and expression. Vitam Horm. 2008;79:267–92.
30. Parle-McDermott A, Pangilinan F, Mills JL, Kirke PN, Gibney ER, Troendle J, et al. The 19-bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR) may decrease rather than increase risk for spina bifida in the Irish population. Am J Med Genet A. 2007;143:1174–80.
31. Sowers R, Toguchida J, Qin J, Meyers PA, Healey JH, Huvos A, et al. mRNA expression levels of E2F transcription factors correlate with dihydrofolate reductase, reduced folate carrier, and thymidylate synthase mRNA expression in osteosarcoma. Mol Cancer Ther. 2003;2(6):535–41.
32. Serra M, Reverter-Branchat G, Maurici D, Benini S, Shen JN, Chano T, et al. Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells. Ann Oncol. 2004;15:151–60.
33. Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GS, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. Proc Natl Acad Sci USA. 2007;104(33):13513–8.
34. Trinh BN, Ong CN, Coetzee GA, Yu MC, Laird PW. Thymidylate synthase: a novel genetic determinant of plasma homocysteine and folate levels. Hum Genet. 2002;111:299–302.
35. Kremer JM. Toward a better understanding of methotrexate. Arthritis Rheum. 2004;50:1370–82.
36. Chiusolo P, Reddiconto G, Farina G, Mannocci A, Fiorini A, Palladino M, et al. MTHFR polymorphisms' influence on outcome and toxicity in acute lymphoblastic leukemia patients. Leuk Res. 2007;31:1669–74.
37. Mattia E, Toffoli G. C677T and A1298C MTHFR polymorphisms, a challenge for antifolate and fluoropyrimidine-based therapy personalization. Eur J Cancer. 2009;45:1333–51.
38. Robien K, Boynton A, Ulrich CM. Pharmacogenetics of folate-related drug targets in cancer treatment. Pharmacogenomics. 2005;6:673–89.
39. Ma D, Huang H, Moscow JA. Down-regulation of reduced folate carrier gene (RFC1) expression after exposure to methotrexate in ZR-75-1 breast cancer cells. Biochem Biophys Res Commun. 2000;279(3):891–7.
40. Abdel-Haleem AM, El-Zeiry MI, Mahran LG, Abou-Aisha K, Rady MH, Rohde J, et al. Expression of RFC/SLC19A1 is associated with tumor type in bladder cancer patients. PLoS One. 2011;6(7):e21820.

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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.

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.

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

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

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

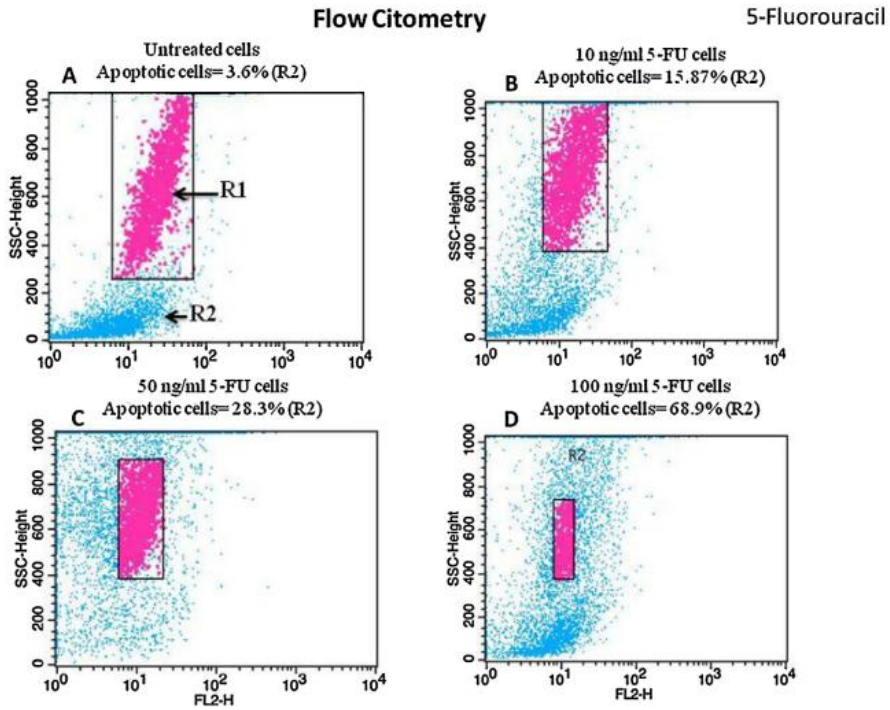

Fig. 1. Flow cytometry photodocumentation of HEP-2 cell line treated with 5-FU chemotherapy showing apoptotic and viable cells. A. Cells without treatment with chemotherapy (control). B. Cells treated with a concentration of 10 ng -5-FU. C. Cells treated with 50 ng concentration of 5-FU. D. Cells treated with a concentration of 100 ng -5-FU. R1 denotes apoptotic cells; R2 represents the viable cells.

group of cells treated with 5-FU chemotherapy. Regarding to cells treated with 10, 50 and 100 ng/mL of 5-FU, the number of apoptotic cells increased as the dose was increased. Cells treated with 100 ng/mL concentration had the most apoptotic cells. This chemotherapy is widely utilized as treatment for solid tumors, such laryngeal carcinoma, but its mechanism of action is not fully understood. It is believed that after administration, 5-FU is converted intracellularly to three active metabolites that disrupt

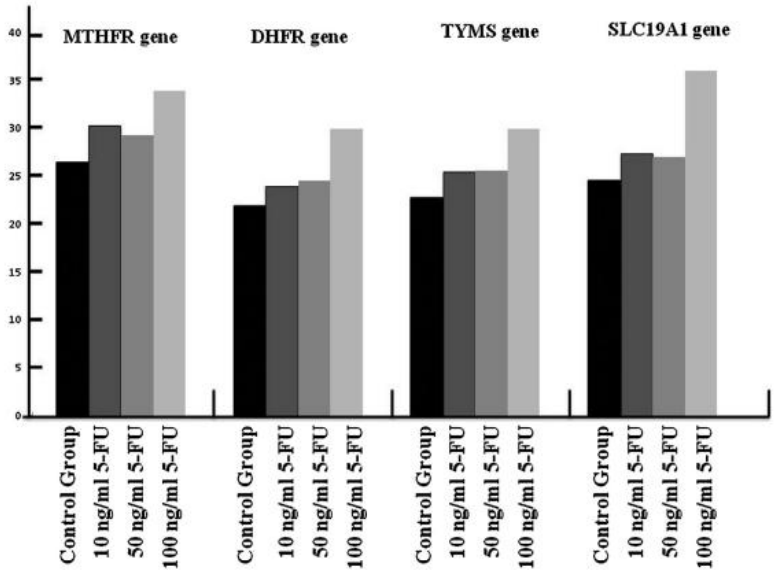

Fig. 2. Quantitative gene expression of *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* genes in HEP-2 cell line treated with three different concentrations of 5-FU and cell line without 5-FU application.

Table 1
ANOVA test: *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* gene expression in three different 5-FU concentrations against expression of the genes in control cells (no application 5-FU).

Sample (ng/mL)	Bonferroni diference	*F*-ratio	*P*-value
	MTHFR		
10	3.88	1056.4311	ns
50	2.78	1733.7322	ns
100	7.41	22,713.3605	ns
	DHFR		
10	2.08	308.36	ns
50	2.68	491.8	ns
100	8.06	4712.41	ns
	TYMS		
10	2.77	1295.8937	ns
50	2.82	1251.6734	ns
100	46.90	469.6407	< 0.05
	SLC19A1		
10	2.80	351.4392	ns
50	2.40	259.2960	ns
100	7.06	1911.6980	ns

RNA and DNA synthesis: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) [22,23].

Regarding to disruption of RNA synthesis, 5-FU is modified by orotate phosphoribosyl transferase enzyme (OPRT) to form dFUMP, which is then converted to FUTP. FUTP is incorporated into cellular RNA, resulting in RNA dysfunction. For disruption of DNA synthesis, 5-FU already transformed in dFUMP suppresses thymidylate synthase (TYMS), an important enzyme for DNA replication and cell growth. It forms covalent ternary complexes with 5,10-methylenetetrahydrofolate (CH2THF) that leads reduction of deoxythymidine triphosphate (dTTP) levels and high levels of dUTP, and as consequence dUTP become misincorporated into DNA in locations where dTTP should be incorporated during replication. Therefore, it results in DNA damage, inhibition of DNA synthesis and cell death [7,24,25]. In the present study was confirmed that the more high the dose of 5-FU chemotherapeutic the more effective it is. Indeed the development of resistance and sensitivity variations are common problems that limit the efficiency of 5-FU chemotherapy treatment, as resistance to 5-FU treatment and toxicity [5,6,25,26].

It is known 5-FU dosage may influence toxicity and drug efficacy [6]. It may be due to intratumoral gene expression and activities of several enzymes related to 5-FU metabolism that have been shown to correlate with drug response leading to resistance to treatment and an increase or decrease in collateral effects in cancer treatment, as the effects of 5-FU are closely related to the activity of its metabolic and associated enzymes. Such target genes might prove to be therapeutically valuable as new for 5-FU chemotherapy or as predictive biomarkers of response and toxicity to 5-FU chemotherapy [11–19,21,27–29].

The present study is the first to determine the expression levels of *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* in a laryngeal cancer cell line given 5-FU in order to identify if alterations in expression levels of these genes may be involved in drug sensitivity according to dose administered and it was found expression levels of the evaluated genes were higher in cells given 100 ng/mL of 5-FU than another concentrations (10 and 50 ng/ml). According to Lee et al. (2009) [20], expression of *MTHFR*, *DHFR*, *TYMS* and *SLC19A1* genes presents normal levels in healthy individuals. However, our results confirmed altered levels of *TYMS* gene in laryngeal cancer cell line treated with high doses of 5-FU chemotherapeutic. *TYMS* gene showed significant association between increased expression and the highest concentration of 5-FU administered when compared to Hep-2 cell line without 5-FU. Increased expression of *TYMS* possibly decreases the inhibitory effect of 5-FU on the DNA synthesis. Studies suggested that increased expression of the *TYMS* gene in tumor cells might decrease efficiency of 5-FU and lead to drug resistance in head and neck cancer treatment [7,16,17,19].

Furthermore, mRNA increased expression level of *TYMS* was also significantly correlated with non-response to 5-FU and poor prognosis in esophageal carcinoma cell lines and primary cancer cells isolated from malignant ascites, according our results in laryngeal cancer cell line [11,13]. Different from that, the study of Yamashita et al (2009) [27] that investigated expression of folate genes in oral squamous cell carcinoma tissues of patients treated with neoadjuvant therapy with 5-FU and a non-treated group indicated that *TYMS* gene expression was not significantly altered by the neoadjuvant therapy using 5-FU and irradiation. Sameshima et al. (2008) [12] also did not found the association between 5-FU treatment and mRNA expressions of some 5-FU related genes in patients with colorectal carcinoma.

It was confirmed mRNA level of *TYMS* gene correlates with the enzymatic activity and increased expression of *TYMS* mRNA and its protein is linked to resistance to 5-FU, both *in vitro* and *in vivo* [14,30]. Increased expression of *TYMS* gene probably lead to alteration in the methylation of dUMP to dTMP and consequently can alter DNA synthesis and repair leading to resistance to 5-FU treatment. These data may offer additional base for individualized cancer chemotherapy based on the 5-FU-related molecular characteristics in patients with laryngeal cancer. However, little data exist on systematic examination of expression profiles of one-carbon metabolizing genes in population studies. In conclusion, the present study confirms significant association between the highest 5-FU dose chemotherapy and *TYMS* mRNA expression increased levels in laryngeal cancer cell line. Although these experiments were performed in cell lines, the results suggest that assessing *TYMS* mRNA expression would be useful in predicting 5-FU sensitivity of laryngeal cancer patients before treatment.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN. Int J Cancer 2010;127(12):2893–917.
[2] Woźniak A, Szyfter K, Szyfter W, Florek E. Head and neck cancer – history. Przegl Lek 2012;69(10):1079–83.
[3] Nowak F, Soria JC, Calvo F. Tumour molecular profiling for deciding therapy-the French initiative. Nat Rev Clin Oncol 2012;9:479–86.
[4] Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resist Updat 2012;15:183–210.
[5] Saif MW, Choma A, Salamone SJ, Chu E. Pharmacokinetically guided dose adjustment of 5-fluorouracil: a rational approach to improving therapeutic outcomes. J Natl Cancer Inst 2009;101(22):1543–52.
[6] Yang CG, Ciccolini J, Blesius A, Dahan L, Bagarry-Liegey D, Brunet C, et al. DPD-based adaptive dosing of 5-FU in patients with head and neck cancer: impact on treatment efficacy and toxicity. Cancer Chemother Pharmacol 2011;67(1):49–56.
[7] Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 2003;3(5):330–8.
[8] Vinod BS, Antony J, Nair HH, Puliyappadamba VT, Saikia M, Shyam Narayanan S, et al. Mechanistic evaluation of the signaling events regulating curcumin-mediated chemosensitization of breast cancer cells to 5-fluorouracil. Cell Death Dis 2013;4:505.

[9] Dar UK, Khan I, Javed M, Ali M, Hyder SW, Murad S, et al. Development of 99mTc-5-fluorouracil as a potential tumor diagnostic agent. Pak J Pharm Sci 2013;26(2):333–7.
[10] Cho HJ, Park YS, Kang WK, Kim JW, Lee SY. Thymidylate synthase (TYMS) and dihydropyrimidine dehydrogenase (DPYD) polymorphisms in the Korean population for prediction of 5-fluorouracil-associated toxicity. Ther Drug Monit 2007;29(2):190–6.
[11] Ando T, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Sugito N, et al. Relationship between expression of 5-fluorouracil metabolic enzymes and 5-fluorouracil sensitivity in esophageal carcinoma cell lines. Dis Esophagus 2008;21(1):15–20.
[12] Sameshima S, Tomozawa S, Kojima M, Koketsu S, Motegi K, Horikoshi H, et al. 5-fluorouracil-related gene expression in primary sites and hepatic metastases of colorectal carcinomas. Anticancer Res 2008;28(3A):1477–81.
[13] Wang T, Wang L, Qian X, Yu L, Ding Y, Liu B. Relationship between gene expression of 5-fluorouracil metabolic enzymes and 5-fluorouracil sensitivity in primary cancer cells isolated from malignant ascites. Cancer Invest 2011;29(2):130–6.
[14] Muhale FA, Wetmore BA, Thomas RS, McLeod HL. Systems pharmacology assessment of the 5-fluorouracil pathway. Pharmacogenomics 2011;12(3):341–50.
[15] Hayashi R, Ishii Y, Ochiai H, Matsunaga A, Endo T, Hasegawa H, et al. Suppression of heat shock protein 27 expression promotes 5-fluorouracil sensitivity in colon cancer cells in a xenograft model. Oncol Rep 2012;28(4):1269–74.
[16] Kawano K, Goto H, Kanda T, Yanagisawa S. Predictive value of immunohistochemical thymidylate synthase expression for histological response to tegafur/uracil (UFT) in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2003;32:633–7.
[17] Harada K, Kawashima Y, Yoshida H, Sato M. Thymidylate synthase expression in oral squamous cell carcinoma predicts response to S-1. Oncol Rep 2006;15:1417–23.
[18] Yoshitomi I, Kawasaki G, Yanamoto S, Mizuno A. Orotate phosphoribosil transferase mRNA expression in oral squamous cell carcinoma and its relationship with the dihydropyrimidine dehydrogenase expression and the clinical effect of 5-fluorouracil. Oral Oncol 2006;42:880–7.
[19] Aubry K, Labourey JL, Bessède JP, Tubiana-Mathieu N, Rigaud M. Expression levels of thymidylate synthase, thymidylate phosphorylase and dihydropyrimidine dehydrogenase in head and neck squamous cell carcinoma: preliminary study. Clin Med Oncol 2008;2:27–35.
[20] Lee YL, Xu X, Wallenstein S, Chen J. Gene expression profiles of the one-carbon metabolism pathway. J Genet Genomics 2009;36(5):277–82.
[21] Scartozzi M, Maccaroni E, Giampieri R, Pistelli M, Bittoni A, Del Prete M, et al. 5-Fluorouracil pharmacogenomics: still rocking after all these years? Pharmacogenomics 2011;12(2):251–65.
[22] Marin-Vicente C, Lyutvinskiy Y, Romans Fuertes P, Zubarev RA, Visa N. The effects of 5-fluorouracil on the proteome of colon cancer cells. J Proteome Res 2013;12(4):1969–79.
[23] Benasso M. Induction chemotherapy for squamous cell head and neck cancer: a neverending story? Oral Oncol 2013;49(8):747–52.
[24] Peters GJ, Backus HH, Freemantle S, et al. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta 2002;1587:194–205.
[25] Zhang N, Yin Y, Xu SJ, Chen WS. 5-fluorouracil: mechanisms of resistance and reversal strategies. Molecules 2008;13(8):1551–69.
[26] Wyatt MD, Wilson III DM. Participation of DNA repair in the response to 5-fluorouracil. Cellular Mol Life Sci 2009;66(5):788–99.
[27] Yin NY, Xu SJ, Chen WS. 5-fluorouracil: mechanisms of resistance and reversal strategies. Molecules 2008;13(8):1551–69.
[28] Lin JT, Lai GM, Chang TH, Liu MT, Bi CP, Wang JW, et al. Chemotherapy with modified docetaxel, cisplatin, and 5-fluorouracil in patients with metastatic head and neck cancer. Adv Ther 2012;29(1):71–7.
[29] Yin W, Wang P, Wang X, Song W, Cui X, Yu H, et al. Identification of microRNAs and mRNAs associated with multidrug resistance of human laryngeal cancer Hep-2 cells. Braz J Med Biol Res 2013;46(6):546–54.
[30] Yamashita T, Toida M, Kato K, Long NK, Miyazaki Y, Asaka Y, et al. The effect of neoadjuvant therapy on the 5-fluorouracil metabolic and relative enzymes of oral squamous cell carcinoma. Oncol Rep 2009;3:501–7.

ARTIGO 4

Exposure dose of Methotrexate and 5-Fluorouracil chemotherapies can modulate expression of genes involved in folate pathway in oral cancer cells *in vitro*.

Ana Lívia Silva Galbiatti[1], João Armando Padovani-Junior[2], José Victor Maniglia[2], Érika Cristina Pavarino[3], Eny Maria Goloni-Bertollo[3].

1 Master - Genetics and Molecular Biology Research Unit - UPGEM, FAMERP- São José do Rio Preto Medical School – Brazil.

2 Doctorate - Otorhinolaryngology and Head and Neck Surgery Department of University São José do Rio Preto Medical School – FAMERP, Brazil.

4 PhD - Genetics and Molecular Biology Research Unit - UPGEM, FAMERP- São José do Rio Preto Medical School – Brazil.

Corresponding author: Eny Maria Goloni Bertollo

UPGEM, FAMERP (bloco U6); Avenida Brigadeiro Faria Lima, n.° 5416

São José do Rio Preto – SP, Brazil. CEP: 15.090-000

Phone: +55 17 3201-5720

E-mail: eny.goloni@famerp.br

Abstract

Introduction: Antifolate chemotherapies as Methotrexate (MTX) and 5-Fluorouracil (5-FU) act inhibiting enzymes involved in folate pathway. The enzymes are important to DNA synthesis and cell growth. Exposure to dose of these chemotherapeutics can alter genes that codifying these enzymes and to influence in effectiveness and resistance to treatment. **Objectives:** To evaluate relationship between mRNA and protein expression levels expression of *MTHFR, DHFR, TYMS* and *SLC19A1* folate metabolic genes in oral cancer cell line treated with MTX and 5-FU antifolate chemotherapies in different doses, separately. **Materials and methods**: Oral cavity cancer cell line (HN13) was treated with 0.25, 25.0, and 75 μM of MTX and 10 ng/ml, 50 ng/ml, and 100 ng/ml of 5-FU, separately, for 24 hours/37°C. Flow Cytometry, Real-time PCR and Western blotting techniques were performed. ANOVA and Bonferroni's post hoc tests were utilized for statistical analysis. $P<0.05$ was considered significant. **Results:** Increased concentration of MTX chemotherapy was associated with increased expression of *DHFR* and *SLC19A1* genes in oral cancer cell line ($p <0.05$). Increased concentration of 5-FU chemotherapy was associated with increased expression of *DHFR* and *TYMS* genes in oral cancer cell line ($p <0.05$). The lower dose of 5-FU was associated with decreased expression of *SLC19A1* gene. **Conclusion:** Exposure to low and high-dose of MTX and 5-FU chemotherapeutics in oral cancer cell line can modulate the level of expression of genes involved in folate metabolism.

Introduction

Oral cancer accounts about 300,373 cases annually worldwide and the latest data showed that there was a total of 97,919 deaths for this disease. This devastating disease still presents low survival due to recurrence of disease and uncontrollable persistency of tumor. (Jemal., 2011; Globocan., 2012; Siegel et al., 2014, Foundation OC.,2014)

The choice of treatment for the disease depends of clinical, radiological and histopathological parameters, which consists in local of tumor, size of the primary tumor, presence of lymph node loco-regional and distant metastasis (TNM classification), tumor depth and presence of lymphovascular invasion. Patients with unresectable disease or those who are unfit for surgery receive radiation treatment with or without concurrent chemotherapy or only chemotherapy. (Woolgar., 2006; Ignoffo et al., 2008; Licitra et al., 2008; Kakria et al., 2014) There are several chemotherapies that can be utilized for oral cancer treatment, such as methotrexate (MTX) and 5-fluorouracil (5FU) that are classified as antifolate chemotherapies. They act inhibiting the purine and pyrimidine pathways by blocking enzymes involved in folate metabolism that are essential to DNA synthesis and cell growth. (Yang et al., 2011; Price and Cohen et al., 2012)

Although antifolate chemotherapies present positive therapeutic results, studies have shown that these antifolate agents increase acute toxicity and side effects, and tumors may exhibit resistance to these agents. Moreover, tumors exhibiting the same clinical stage can demonstrate different patterns of growth leading to persistence of tumoral growth and failure in chemotherapy treatment (Thomas et al., 2005; Pignon et

al., 2009; Price and Cohen., 2012).

It is believed that inter-individual variability in antifolate chemotherapies response and toxicity in cancer treatment can be due to genetic expression of target genes that codifying enzymes involved in folate pathway. Some studies show dose exposition of chemotherapeutics in cancer treatment can induce different responses in cancer treatment and resistance through alteration of expression of some genes involved in the metabolism of chemotherapeutic, including MTX and 5-FU. This may explain why chemotherapy regimens have not increased efficacy.(Yoshikawa et al., 2001; Kawano et al., 2004; Psyrri &DiMaio., 2008; Aubry et al., 2008; Mattia and Toffoli., 2009; Chuan Chuang et al., 2012; Bhosle et al., 2013; Galbiatti et al., 2013; Galbiatti et al., 2014).

In order we carried out this study to investigate the effect of high and low dose of MTX and 5-FU antifolate chemotherapeutics in the expression of mRNA and protein of *MTHF, DHFR, TYMS* and *SLC19A1* folate genes genes in oral cancer cell line.

Materials and methods

Cell line and Anticancer agents

It was utilized HN13 cell line derived of a tongue squamous cell carcinoma that was immortalized. Cell line was maintained Minimum Essential Medium Eagle Medium (D-MEN 00068 medium/ Cultilab) supplemented with 10% heat-inactivated fetal bovine serum and a cocktail of penicillin and streptomycin (Gibco; Invitrogen) at 37 °C in 5% carbon dioxide. Cells growing exponentially were harvested when a confluence of 90% was achieved.

MTX and 5-FU chemotherapies were plated in six-well culture plates at a density of 1×10^5/well. HN13 cell lines were incubated separately with three different concentrations of MTX chemotherapeutic: 0.25micromole (µmol), 25µmol, and 75µmol for 24 hours at 37 °C according to Pai et al., (2009). 2) and three different concentrations of 5-FU chemotherapeutic: 10 ng/ml, 50 ng/ml and 100 ng/ml for 24 hours at 37 °C according to Yoshikawa et al., (2001). Control cells were the cell line with drug-free medium.

Flow Cytometry (FCM) and apoptotis quantification

After 24-h chemotherapies incubation, the determination of viable and apoptotic cells was evaluated by double staining with fluorescein isothiocyanate (FITC) label Bcl-.2 (100: sc-509) according to manufacturer's manual (Santa Cruz Biotechnology, Inc). Cells were gently vortexed and incubated for 15 min in room temperature in the dark Within 1 h, cells were analyzed with flow cytometer FACS calibur (Becton Dickinson Immunocytometry Systems, San José, USA). The sample analysis was performed using the CELLQuest software (Apple).

RNA Extration, cDNA generation and qRT-PCR analysis

The RNA isolation was performed using Trizol (Invitrogen) according to manufacturers' manuals. The RNA concentration was adjusted to 2 ug using a Picodrop® Equipament. For cDNA synthesis 2 ug of total RNA was used with random primers according to High capacity cDNA kit (Applied Biosystem®) manufacturer's protocol.

Gene expression in all samples was measured by quantitative RT-PCR (qRT-PCR) with StepOnePlus™ Equipament (Applied Biosystems). A polymerase chain reaction (PCR) was performed in a 20-μL reaction mixture containing 10 μL of Taqman Universal PCR Master Mix (Applied Biosystems), each primer at 80 nmol/L, 2 nmol/L probe and 2 μL of cDNA sample solution. Thermal cycling conditions comprised an initial denaturation step at 95°C for 20 s, 40 cycles of denaturation at 95ºC for 0,3 seconds, annealing at 60°C for 1 min and extension at 72ºC for 30 seconds. TaqMan® Gene Expression Assay was pre-optimized PCR primer and probe sets for qRT-PCR formulated at 20X concentration.

Gene-especific primers were used for quantification of one-carbon metabolizing gene expressions in TaqMan® Custom Array Plate, which included triplicated wells of two reference genes (b-actin and Glyceraldehyde-3-phosphate dehydrogenase - GAPDH) and 4 target genes (*MTHFR, DHFR, TYMS* and *SLC19A1*). The reaction mix contained the following components with final concentration: TaqMan® Fast Advanced Master Mix. All reactions were run in triplicate for four samples. Melting curves were checked to validate the PCR specificity. 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 mathematical formula shown below. The expression levels of target genes were expressed as 2-(Delta Ct) x 1,000 to simplify the calculation.

Expression level of target gene = 2-(Delta Ct) x 1,000

Delta Ct= Ct of target gene – (Mean Ct of β-actin and GAPDH genes)

Western Blot Analysis

Western blot analysis was used to verify the distribution of MTHFR, DHFR, TYMS and SLC19A1 proteins. It was utilized beta actin protein as control of reaction. Proteins were extracted from 1×10^5 cells treated with chemotherapeutics in different concentrations with 1 mL RIPA buffer (Sigma Aldrich®) according manufacturer's instructions. They were quantified by BCA™ Protein Assay Kit (Thermo Scientific) method by Picodrop Pico200™ equipment (Analytical) for use in the reactions of the proposed optimal concentrations and higher efficiency.

Dilution factors for different antibodies were prepared according to manufacturer. The specific procedure was: Firstly we add Bolt (Sample Reducing Agent - 10x - Novex - Life Technologies ®) and buffer LDS Sample Buffer-4x Novex - Life Technologies ®) in the proteins. The samples were placed to a temperature of 70 ° C for 10 minutes for denaturation. After this procedure, the proteins were applied at a concentration of 100 ug Bolt™ in MES NuPAGE 4-12% Bis ® Plus Mini Tris Gel (Novex ® by Life Technologies). The voltage was 140V; 300mA for 45 minutes. The molecular marker SeeBlue Pre-Stained Standard ® (Life Technologies) was applied for identification of size of proteins. After, the transfer was realized with iBlot Dry Blotting System (Invitrogen-Life Technologies®) that executes the transfer in 6 minutes to PVDF membrane. The membran was blocked with 3% BSA in TBS-T for 1 hour and 30 minutes. The antibodies were diluted in a solution of 3% BSA in TBS-T at the specified concentration established by the manufacturer, then were added to the membrane for a period of 24 hours. After, the antibodies were removed and held by three washes (15 minutes) of the membrane with TBS-T solution without BSA.. After the washing procedure, was added to the membrane IgG secondary antibody (Peroxidase Labeled

Antibody Affinity Purified Anti-Mouse IgG-KPL ®) for 1 hour. Then the membrane with four washes of 15 minutes each with TBS-T was performed without BSA. To finish was added to the membrane reagent ECL™ Select Western Blotting Detection (GE Healthcare) for detection of specific proteins. The membrane was visualized in appropriated photo documentation system.

Statistical analysis

Statistical analysis was performed using Bioestat software program–Version 5.3 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 chemotherapeutics concentrations against expression of genes in control cells (no chemotherapeutic application). 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.

RESULTS

The results for Flow Cytometry analysis that show the percentile of apoptotic cells level of HN13 cell line during 24 h of exposure to MTX and 5-FU separately are described in Figure 1. The induction of apoptotic cells for treatment of 0.25 μM, 25 μM, and 75 μM of MTX was 24.4%, 75.5% and 94.4% respectively. For treatment with 10, 50 and 100 ng/mL of 5-FU, the percentile of apoptotic cells was 19.8%, 38.12% and 68.69%, respectively.

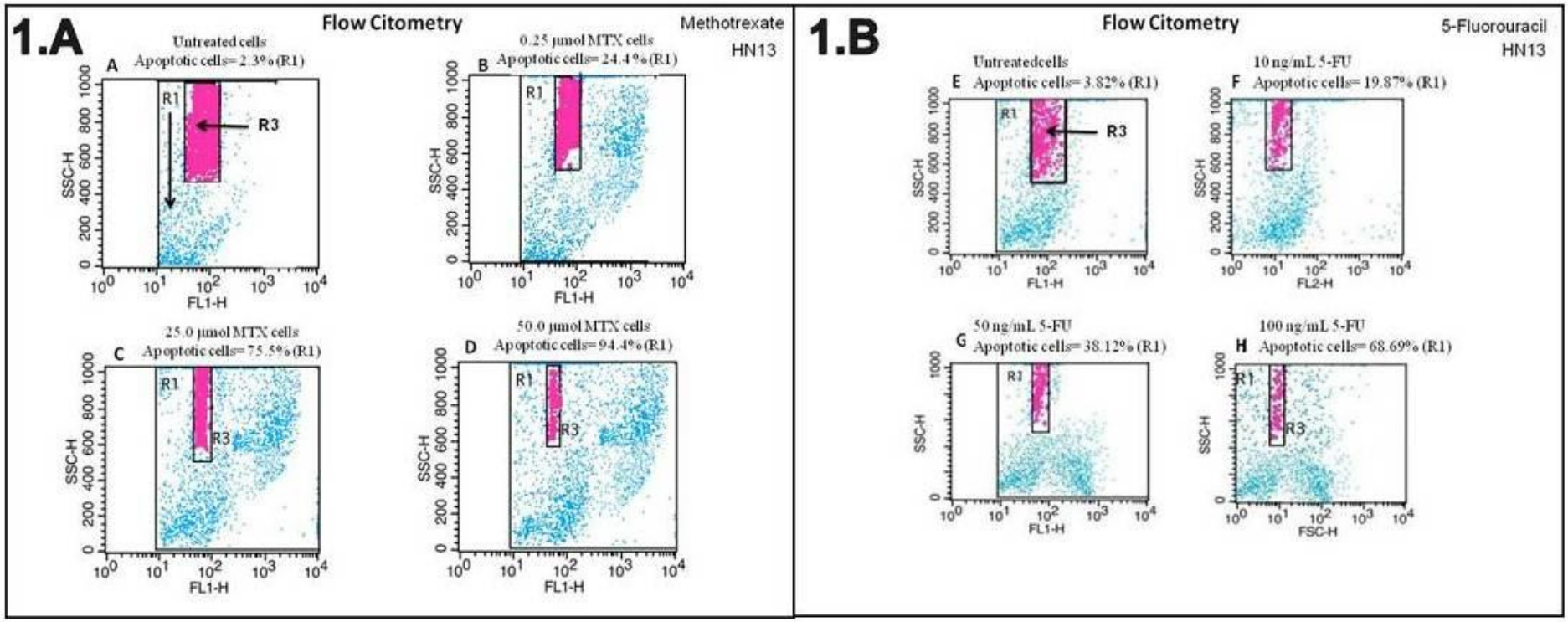

Figure 1.A. Flow cytometry photodocumentation of HN13 cell line treated with MTX chemotherapy showing apoptotic and viable cells. A) cells without treatment with chemotherapy (control), B) Cells treated with a concentration of 0.25 µM MTX, C) Cells treated with 25.0 µM concentration of MTX and D) Cells treated with a concentration of 75,0 µM MTX.

Figure 1.B. Flow cytometry photodocumentation HN13 cell line treated with 5-FU chemotherapy showing apoptotic and viable cells. A. Cells without treatment with chemotherapy (control). B. Cells treated with a concentration of 10 ng -5-FU. C. Cells treated with 50 ng concentration of 5-FU. D. Cells treated with a concentration of 100 ng 5-FU. R1 denotes apoptotic cells; R3 represents viable cells.

Regarding to protein and gene expression, as shown in Figure 2, treatment with 75 μM of MTX chemoterapeutic was associated with altered expression of *DHFR* gene (ANOVA with post hoc Bonferroni: $p<0.05$) and *SLC19A1* gene (ANOVA with post hoc Bonferroni: $p<0.05$). Treatment with 5-FU chemoterapeutic showed association with concentration of 100 ng and altered expression of *TYMS* gene (ANOVA with post hoc Bonferroni: $p<0.05$) and *DHFR* gene (ANOVA with post hoc Bonferroni: $p<0.05$), as shown in Figure 3.

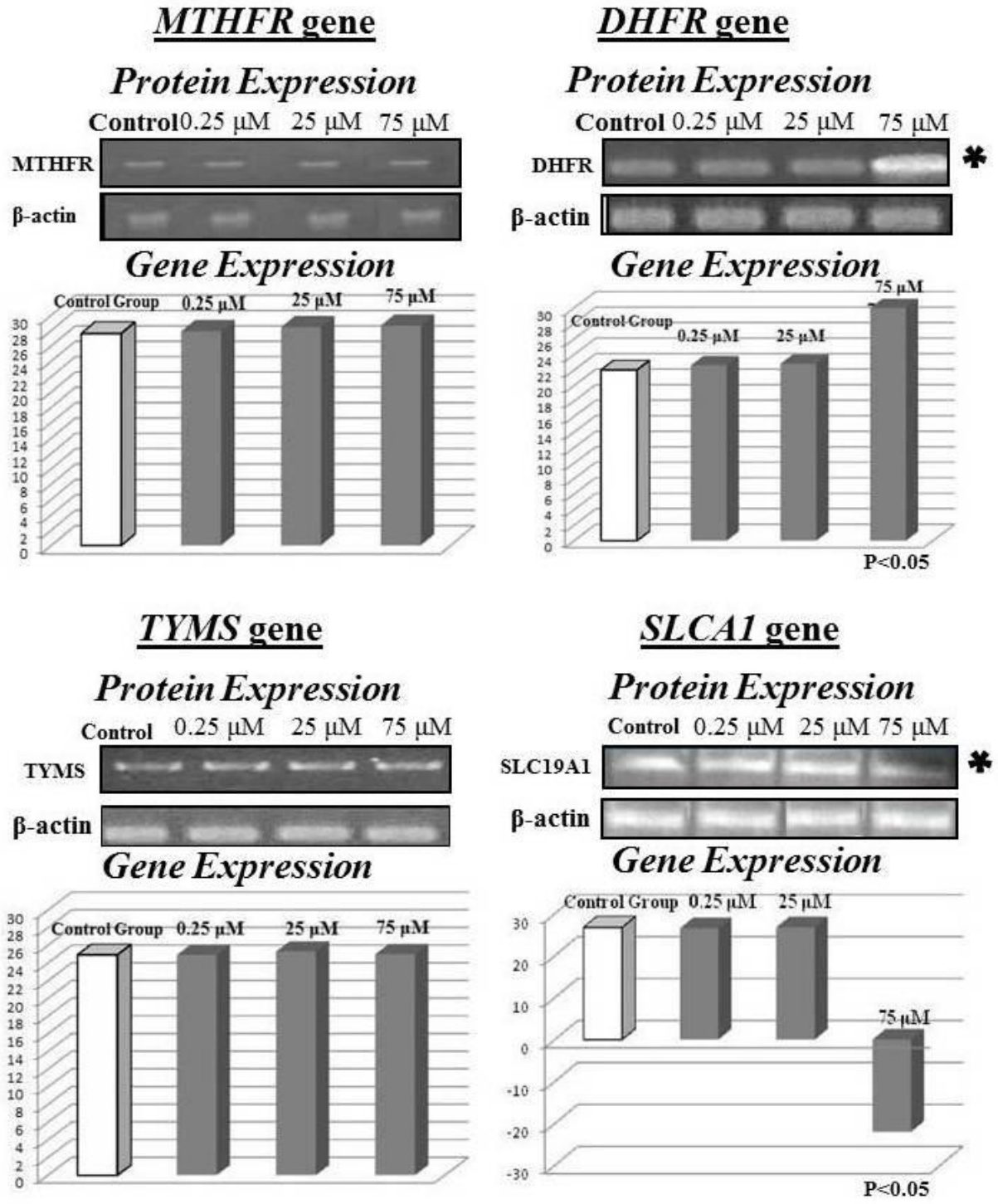

Figure 2. Quantitative protein and mRNA gene expression of *MTHFR, DHFR, TYMS* and *SLC19A1* genes in HN13 cell line treated with three different concentrations of MTX (black) and cell line without MTX application (White).

✱ **P<0.05**

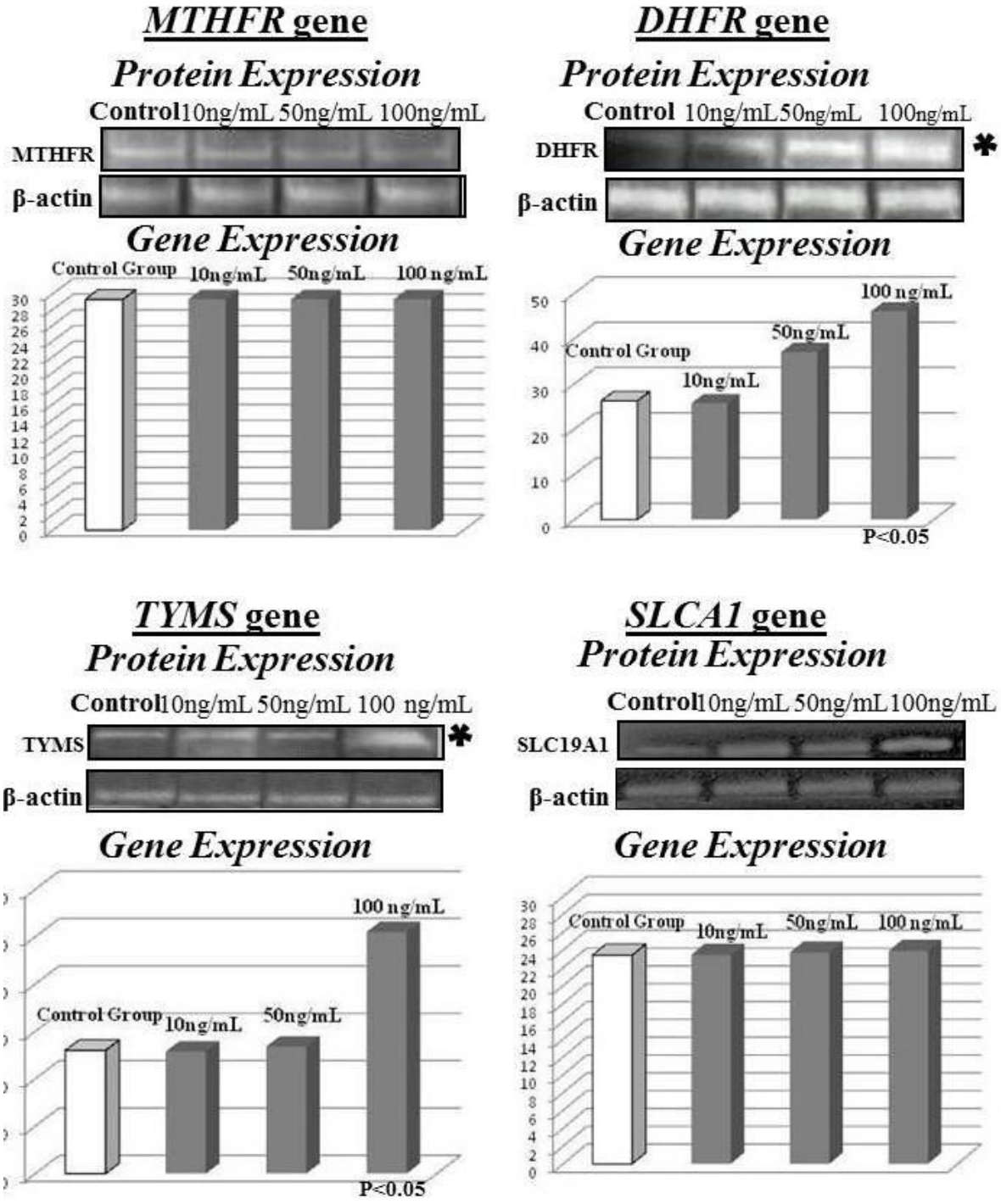

Figure 3. Quantitative protein and mRNA gene expression of *MTHFR, DHFR, TYMS* and *SLC19A1* genes in HN13 cell line treated with three different concentrations of 5-FU (black) and cell line without 5-FU application (White).

✱ **P<0.05**

DISCUSSION

Antifolate chemotherapy for HNC treatment still presents many problems such drug resistance and no-effectiveness of treatment in certain patients. The fact can be explained due to genetic factors. For this reason, predictive genetic molecular biomarkers may help select patients who will response for determined chemoterapy with better effectiveness and without toxicity through personalized therapeutic regimens. (Bertino., 2009; Jessri et al., 2014)

Our study confirmed that higher concentration of MTX chemotherapy in oral cancer cell line presented higher number of apoptotic cells. Moreover, the higher concentration of MTX led to increased expression of *DHFR* protein and gene and decreased expression of *SLC19A1* protein and gene. Studies have reported potential folate genes that may be associated with toxicity, ineffectiveness or better response to MTX. In addition, the dosage of MTX can alter the folate pathway and the activity of chemotherapeutic in cancer cells, as we found in the present study. (Panetta et al., 2010; Erčulj et al., 2012; Galbiatti et al., 2013).

This antifolate chemotherapy acts on several folate key enzymes including dihydrofolate reductase (DHFR). MTX inactives this enzyme that is responsible to catalyzes the conversion of folate in tetrahidrofolate, an essential cofactor for the synthesis of pyrimidine synthesis. DHFR also is responsible to convert folate in 5,10MTHFR, cofactor important to purine synthesis. Therefore, MTX cause depletion of tetrahydrofolate cofactors that are required for DNA and RNA syntheses and cell growth. (Abali et al., 2008; Morales et al., 2009)

According our results, Askari and Krajinovic (2010) showed that increased expression of *DHFR* gene is associated with individual's predisposition to respond to the treatment with MTX in terms of effectiveness and drug side effects and, the study of Saes Ayala et al (2011) confirmed increase of expression of *DHFR* gene is associated with many types of cancer cells in response to MTX treatment. Our previous study also showed that MTX treatment with higher dose is associated with increased expression of *DHFR* gene in larynx cancer. (Galbiatti et al., 2013) MTX dose probably induce increased *DHFR* gene expression by interfering in the process of DHFR regulation.

Probably, as shown in our study and according literature data, increased expression in *DHFR* gene induces high concentration of folate wich is converted to tetrahydrofolate and 5,10 MTHF which are essential product to cell division leading to increased effectiveness to MTX. (Hider et al., 2007; Assarafi., 2007; Patiño-García et al., 2008; Askari and Krajinovic., 2010). The hypothesis is that due to increasing dose of MTX, the organism understands that is necessary to increase the expression of *DHFR* gene for action of MTX. Once the chemotherapeutic inhibits DHFR enzyme which is encoded by the *DHFR* gene and this inhibition leads to block of cell division. Therefore, the higher dose of chemotherapy, more enzyme probably will be encoded to the chemotherapeutic perform the effect.

The present study also confirmed that higher dose of MTX lead to decreased expression of *SLC19A1* gene. This gene is responsible to codify SLC19A1 enzyme, which to carrier intracellular folate and antifolate chemotherapies into cells. Expression decreased levels of *SLC19A1* gene may result in less intracellular transport of MTX with a consequent reduction of effectiveness and cytotoxicity. (Assaraf., 2007; Abdell-

Hallem et al., 2011). However, in higher concentrations of MTX, despite the decreased expression reduce the transport of MTX, as there is high dose of chemotherapeutic, it is possible there is another mechanism of MTX transport, but more studies are necessary to confirm this hypothesis.

Regarding to 5-FU chemotherapy, our results confirmed that higher concentration of 5-FU chemotherapy presented higher number of apoptotic cells. We also found that the higher dose of 5-FU led to increase of expression of *DHFR* and *TYMS* genes in oral cancer cell line. 5-FU chemotherapy is a commonly utilized for head and neck cancer treatment.

This chemotherapeutic is an inhibitor of TYMS, which is an important enzyme for DNA synthesis responsible to catalyze deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) for DNA synthesis. A metabolite of 5-FU, 5fluorodeoxyuridylate (5-FdUMP), forms a stable covalent ternary complex with TYMS in the presence of 5,10- methylenetetrahydrofolate folate cofactor. The complex formation suppresses the conversion of uracil to thymidine, blocking DNA synthesis and cell growth. (Zhang et al., 2008; Lima et al., 2013). Studies have suggested poorer clinical response to 5-FU-based chemotherapy showing increased expression levels of *TYMS* in cancer.(Fukui et al., 2008; Aubruy et al., 2008; Wang et al., 2011; Sasako et al, 2014; Galbiatti et al., 2014)

Our previous study showed association between 5-FU chemotherapy and increased expression of *TYMS* gene in larynx cancer cell line, according our present study in oral cancer. (Galbiatti et al., 2014) We thought that increased higher dose of 5-FU induce the increase of expression of *TYMS* gene because this gene forms a complex with 5-FU (5-FdUMP) suppressing the translation mRNA of and protein of *TYMS* gene

Therefore, if there is more 5-FU concentration, probably the organism will synthetize more *TYMS* to bind with 5-FU chemotherapeutic. Once the target of 5-FU chemotherapeutic is TYMS enzyme that is codified by *TYMS* gene.

The results of present study also showed association of increased expression of *DHFR* gene and 5-FU treatment with higher dose in oral cancer cell line *DHFR* is essential to pyrimidine and purine synthesis, it converts tetrahydrofolate to 5,10 MTHF, and increased expression can be related with high levels of tetrahydrofolate and 5,10 MTHF which cofactors that are required for DNA and RNA synthesis. (Hider et al., 2007; Assarafi., 2007; Patiño-García et al., 2008; Askari and Krajinovic., 2010) It is thought that despite *DHFR* gene is not involved directly with 5-FU chemotherapeutic action, it is an important enzyme of folate pathway and probably as 5-FU inhibits *TYMS* enzyme, also involved in folate pathway will be no conversion of dUMP to dTMP for pyrimidine synthesis and, consequently it is possible an uncontrolled in folate pathway. Maybe there is increase expression of *DHFR* to try control the folate pathway and block of cellular division. More studies are necessary to confirm these data.

Our goal was to determine how MTX and 5-FU can influence genetic variation in folate pathway and response in oral cancer cell lines. We conclude that the dose of MTX and 5-FU antifolate chemotherapeutic can modulate expression of genes involved in folate pathway in oral cancer cell line.

References

Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011; 61:69.

Globocan2012. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64:9.

Foundation OC. Oral Cancer Facts, 2014. Available at: http://oralcancerfoundation.org/facts/index.htm. Asia Pac J Clin Oncol. 2014 Aug 17. doi: 10.1111/ajco.12261. [Epub ahead of print]

Woolgar J. Histopathological prognosticators in oral and oropharyngeal Squamous cell carcinoma, Oral Oncol 2006; 42: 229–39.

Ignoffo RJ, Rosenbaum EH. What happens in chemotherapy. In: Ko AH, Dollinger M, Rosenbaum EH, eds. Everyone's Guide to Cancer Therapy, Revised 5th Edition. Kansas City, MS: Andrew McMeel Publishing; 2008.

Licitra L, Locati LD, Bossi P. Optimizing approaches to head and neck cancer. Metastatic head and neck cancer: new options. Ann Oncol. 2008; 7.

Kakria A, Rawat S, Bhutani R, Gupta G, Devnani B, Wahi IK, et al. Retrospective analysis of treatment outcomes following reirradiation in locoregionally recurrent head and neck cancer patients: A single institutional study. Asia Pac J Clin Oncol. 2014 Aug 17. doi: 10.1111/ajco.12261. [Epub ahead of print].

Yang CG, Ciccolini J, Blesius A, Dahan L, Bagarry-Liegey D, Brunet C et al. DPD-based adaptive dosing of 5-FU in patients with head and neck cancer: impact on treatment efficacy and toxicity. Cancer Chemother Pharmacol. 2011;67(1):49-56

Price KA, Cohen EE. Current treatment options for metastatic head and neck cancer. Curr Treat Options Oncol 2012;13(1):35–46.

Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. Int J Exp Pathol 2005; 86:347-63

Pignon JP, le Maître A, Maillard E, Bourhis J, on behalf of the MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-

NC): An update on 93 randomised trials and 17,346 patients. Radiother Oncol 2009; 92: 4–14.

Yoshikawa R, Kusunoki M, Yanagi H, Noda M, Furuyama JI, Yamamura T, Hashimoto-Tamaoki T. Dual antitumor effects of 5-fluorouracil on the cell cycle in colorectal carcinoma cells: a novel target mechanism concept for pharmacokinetic modulating chemotherapy. Cancer Res. 2001; 61:1029-37.

Kawano K, Goto H, Kanda T, Yanagisawa S. Predictive value of immunohistochemical thymidylate synthase expression for histological response to Tegafur/Uracil (UFT) in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2003; 32(6):633-7.

Psyrri A, DiMaio D. Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol 2008; 5: 24-31.

Aubry K, Labourey JL, Bessède JP, Tubiana-Mathieu N, Rigaud M.Expression levels of thymidylate synthase, thymidylate phosphorylase and dihydropyrimidine dehydrogenase in head and neck squamous cell carcinoma: preliminary study. Clin MedOncol.2008;2:27-35.

Mattia E e Toffoli G. C677T and A1298C MTHFR polymorphisms, a challenge for antifolate and fluoropyrimidine-based therapy personalization. Europ. J. of Cancer. 2009; 45: 1333-51.

Chuang JC, Sheu GT, Wang PC, Liao FT, Liu WS, Huang CF, Tseng MH, Wu MF. Docetaxel and 5-fluorouracil induce human p53 tumor suppressor gene transcription via a short sequence at core promoter element. Toxicol In Vitro. 2012 Aug;26(5):678-85.

Bhosle J, Kiakos K, Porter AC, Wu J, Makris A, Hartley JA, Hochhauser D. Treatment with gefitinib or lapatinib induces drug resistance through downregulation of topoisomerase IIαexpression. Mol Cancer Ther. 2013 Dec;12(12):2897-908.

Galbiatti AL, Castro R, Caldas HC, Padovani JA Jr, Pavarino EC, Goloni-Bertollo EM. Alterations in the expression pattern of MTHFR, DHFR, TYMS, and SLC19A1genes after treatment of laryngeal cancer cells with high and low doses of methotrexate. Tumour Biol. 2013 34(6):3765-71

Galbiatti AL, Caldas HC, Maniglia JV, Pavarino EC, Goloni-Bertollo EM. Gene expression profile of 5-fluorouracil metabolic enzymes in laryngeal cancer cell line:

Predictive parameters for response to 5-fluorouracil-based chemotherapy. Biomed Pharmacother. 2014;68(5):515-9

Pai RB, Lalitha RM, Pai1 SB, Kumaraswamy SV, Lalitha N, Bhargava MK. Analysis of in vitro and in vivo sensitivity of oral cancer cells to methotrexate. Exp Oncol 2009; 31:118–20.

Bertino JR. Cancer research: from folate antagonism to molecular targets. Best Pract Res Clin Haematol. 2009;22(4):577-82

Jessri M, Farah CS. Next generation sequencing and its application in deciphering head and neck cancer. Oral Oncol. 2014;50(4):247-53.

Panetta JC, Sparreboom A, Pui CH, Relling MV, Evans WE. Modeling mechanisms of in vivo variability in methotrexate accumulation and folate pathway inhibition in acute lymphoblastic leukemia cells. PLoS Comput Biol. 2010 Dec 2;6(12)

Erčulj N, Kotnik BF, Debeljak M, Jazbec J, Dolžan V. Influence of folate pathway polymorphisms on high-dose methotrexate-related toxicity and survival in childhood acute lymphoblastic leukemia. Leuk Lymphoma. 2012;53(6):1096–104.

Abali EE, Ercikan E, Skacel NE, Celikkaya H, Hsieh YC. Regulation of human dihydrofolate reductase activity and expression.Vitam Horm. 2008;79:267–92.

Morales C, García MJ, Ribas M, Miró R, Muñoz M, Caldas C, Peinado MA. Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells. Mol Cancer Ther. 2009;8(2):424-32

Askari BS, Krajinovic M. Dihydrofolate reductase gene variations in susceptibility to disease and treatment outcomes. Curr Genomics. 2010;11(8):578-83

Sáez-Ayala M1, Fernández-Pérez MP, Montenegro MF, Sánchez-del-Campo L, Chazarra S, Piñero-Madrona A, Cabezas-Herrera J, Rodríguez-López JN. Melanoma coordinates general and cell-specific mechanisms to promote methotrexate resistance. Exp Cell Res. 2012; 10;318(10):1146-59

Hider SL, Bruce IN and W. Thomson. The pharmacogenetics of methotrexate. Rheumatology 2007;46:1520–1524

Assaraf YG. Molecular basis of antifolate resistance. Cancer Metastasis Rev 2007;26:153-81.

Patiño-García A, Zalacaín M, Marrodán L, San-Julián M, Sierrasesúmaga L. Methotrexate in pediatric osteosarcoma: response and toxicity in relation to genetic

polymorphisms and dihydrofolate reductase and reduced folate carrier 1 expression. J Pediatr. 2009;154(5):688-93.

Abdel-Haleem AM, El-Zeiry MI, Mahran LG, Abou-Aisha K, Rady MH, Rohde J, Mostageer M, Spahn-Langguth H. Expression of RFC/SLC19A1 is associated with tumor type in bladder cancer patients. PLoS One. 2011;6(7):e21820.

Zhang N, Yin Y, Xu SJ, Chen WS. 5-fluorouracil: mechanisms of resistance and reversal strategies. Molecules 2008;13(8):1551–69

Lima A, Azevedo R, Sousa H, Seabra V, Medeiros R. Current approaches for TYMS polymorphisms and their importance in molecular epidemiology and pharmacogenetics. Pharmacogenomics. 2013;14(11):1337-51.

Fukui Y, Oka T, Nagayama S, Danenberg PV, Danenberg KD, Fukushima M. Thymidylate synthase, dihydropyrimidine dehydrogenase,orotate phosphoribosyltransferase mRNA and protein expression levels in solid tumors in large scale population analysis. Int J Mol Med. 2008;22(6):709-16.

Wang T, Wang L, Qian X, Yu L, Ding Y, Liu B. Relationship between gene expression of 5-fluorouracil metabolic enzymes and 5-fluorouracil sensitivity in primary cancer cells isolated from malignant ascites. Cancer Invest. 2011;29(2):130-6.

Sasako M, Terashima M, Ichikawa W, Ochiai A, Kitada K, Kurahashi I, Sakuramoto S, Katai H, Sano T, Imamura H. Impact of the expression of thymidylate synthase and dihydropyrimidine dehydrogenase genes on survival in stage II/III gastric cancer. Gastric Cancer. 2014 Aug 12. [Epub ahead of print]

4. CONCLUSÕES

Conclusões

O aumento da concentração do quimioterápico MTX está associado com expressão aumentada dos genes *MTHFR, DHFR, TYMS* e *SLC19A1* na linhagem de câncer de laringe; expressão aumentada do gene *DHFR* e expressão diminuída do gene *SLC19A1* na linhagem de câncer oral ($p<0,05$). A dose mais baixa de MTX está associada com expressão diminuída do gene *SLC19A1* em câncer de laringe. O aumento da concentração do quimioterápico 5-FU está associado com expressão aumentada do gene *DHFR* na linhagem de câncer de laringe ($p<0,05$) e expressão aumentada dos genes *TYMS* e *DHFR* na linhagem de câncer oral ($p<0,05$). Portanto, a dose dos quimioterápicos MTX e 5-FU em linhagens de câncer de laringe e câncer oral pode modular o nível de expressão de genes envolvidos no metabolismo do folato.

5. REFERÊNCIAS BIBLIOGRÁFICAS

Referências Biblográficas

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin 2011; 61:69.

2.Globocan 2012. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx,

3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64:9.

4.Foundation OC. Oral Cancer Facts, 2014. Disponível em: http://oralcancerfoundation.org/facts/index.htm. *Asia Pac J Clin Oncol*. 2014 Aug 17. doi: 10.1111/ajco.12261. [Epub ahead of print]

5. Instituto Nacional do Câncer – INCA. Disponível em www.inca.gov.br

6. Woolgar J. Histopathological prognosticators in oral and oropharyngeal Squamous cell carcinoma, *Oral Oncol* 2006; 42: 229–39.

7. Ignoffo RJ, Rosenbaum EH. What happens in chemotherapy. In: Ko AH, Dollinger M, Rosenbaum EH, eds. Everyone's Guide to Cancer Therapy, Revised 5th Edition. Kansas City, MS: Andrew McMeel Publishing; 2008.

8. Licitra L, Locati LD, Bossi P. Optimizing approaches to head and neck cancer. Metastatic head and neck cancer: new options. *Ann Oncol.* 2008; 7.

9. Kakria A, Rawat S, Bhutani R, Gupta G, Devnani B, Wahi IK, et al. Retrospective analysis of treatment outcomes following reirradiation in locoregionally recurrent head and neck cancer patients: A single institutional study. *Asia Pac J Clin Oncol*. 2014 Aug 17. doi: 10.1111/ajco.12261. [Epub ahead of print]

10. Price KA, Cohen EE. Current treatment options for metastatic head and neck cancer. *Curr Treat Options Oncol* 2012;13(1):35–46.

11. Kuehr T; Thaler J. Chemotherapy Protocols 2013 – Current Protocols and "Target Therapies". Sandoz Oncology., 2013.

12. Wu CF, Chang KP, Huang CJ, Chen CM, Chen CY, Steve Lin CL. Continuous intra-arterial chemotherapy for downstaging locally advanced oral commissure carcinoma. Head Neck. 2014; 36(7):1027-33

13. Minicucci EM, da Silva GN, Salvadori DM. Relationship between head and neck cancer therapy and some genetic endpoints. World J Clin Oncol. 2014; 10;5(2):93-102.

14. Marvaretta M Stevenson, MD. Head and Neck Cancer Treatment Protocols. Medscape., 2014. Available at: http:// http://emedicine.medscape.com/article/2006216-overview

15. Visentin M, Zhao R, Goldman ID. The antifolates. Hematol Oncol Clin North Am. 2012; 26(3):629-648.

16. Gonen N, Assaraf YG. Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance. Drug Resist Updat 2012; 15(4):183-210.

17. Bertino JR. Cancer research: from folate antagonism to molecular targets. Best Pract Res Clin Haematol. 2009;22(4):577-82

18. Morales C, García MJ, Ribas M, Miró R, Muñoz M, Caldas C, Peinado MA. Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells. Mol Cancer Ther. 2009;8(2):424-32

19. Moura JA, Valduga CJ, Tavares ER, Kretzer IF, Maria DA, Maranhão RC. Novel formulation of a methotrexate derivative with a lipid nanoemulsion. Int J Nanomedicine. 2011; 6:2285-95.

20. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3(5):330-8.

21. Zhang N, Yin Y, Xu SJ, Chen WS. 5-fluorouracil: mechanisms of resistance and reversal strategies. Molecules 2008;13(8):1551–69.

22. Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol* 2005; 86:347-63.

23. Taguchi T, Tsukuda M, Mikami Y, Matsuda H, Horiuchi C, Yoshida T, Nishimura G, Ishitoya JI and Katori H. Concurrent chemoradiotherapy with cisplatin, 5-fluorouracil, methotrexate, and leucovorin in patients with advanced respectable squamous cell carcinoma of the laryngeal and hypopharynx. Acta Oto-Laryngologica, 2006; 126: 408-13.

24. Pai RB, Lalitha RM, Pai1 SB, Kumaraswamy SV, Lalitha N, Bhargava MK. Analysis of in vitro and in vivo sensitivity of oral cancer cells to methotrexate. Exp Oncol 2009; 31:118–20.

25. Pignon JP, le Maître A, Maillard E, Bourhis J, on behalf of the MACH-NC Collaborative Group. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): An update on 93 randomised trials and 17,346 patients. *Radiother Oncol* 2009; 92: 4–14.

26. Faivre S, Albert S, Raymond E. Induction chemotherapy challenges for head and neck cancer. Lancet Oncol. 2013;14(3):188-9

.

27. Kawano K, Goto H, Kanda T, Yanagisawa S. Predictive value of immunohistochemical thymidylate synthase expression for histological response to Tegafur/Uracil (UFT) in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg*. 2003; 32(6):633-7.

28. Chiusolo P, Reddiconto G, Farina G, et al. MTHFR polymorphisms' influence on outcome and toxicity in acute lymphoblastic leukemia patients. *Leuk Res* 2007; 31:1669–74.

29. Aubry K, Labourey JL, Bessède JP, Tubiana-Mathieu N, Rigaud M.Expression levels of thymidylate synthase, thymidylate phosphorylase and dihydropyrimidine dehydrogenase in head and neck squamous cell carcinoma: preliminary study. *Clin Med Oncol.* 2008;2:27-35.

30. Psyrri A, DiMaio D. Human papillomavirus in cervical and head-and-neck cancer. Nat *Clin Pract Oncol* 2008; 5: 24-31.

31. Celtikci B, Lawrance AK, Wu Q, Rozen R. Askari krajinov. Methotrexate-induced apoptosis is enhanced by altered expression of methylenetetrahydrofolate reductase. *Anticancer Drugs*. 2009;20(9):787-93.

32. Mattia L; Toffoli G. C677T and A1298C MTHFR polymorphisms, a challenge for antifolate and fluoropyrimidine-based therapy personalization. Europ. J. of Câncer. 2009; 45: 1333-51.

33. Chen WW, Lin CC, Huang TC, Cheng AL, Yeh KH, Hsu CH. Prognostic factors of metastatic or recurrent esophageal squamous cell carcinoma in patients receiving three-drug combination chemotherapy. *Anticancer Res*. 2013;33(9):4123-8.

34. Galbiatti AL, Castro R, Caldas HC, Padovani JA Jr, Pavarino EC, Goloni-Bertollo EM. Alterations in the expression pattern of MTHFR, DHFR, TYMS, and SLC19A1 genes after treatment of laryngeal cancer cells with high and low doses of methotrexate. *Tumour Biol*. 2013 34(6):3765-71.

35. Galbiatti AL, Caldas HC, Maniglia JV, Pavarino EC, Goloni-Bertollo EM. Gene expression profile of 5-fluorouracil metabolic enzymes in laryngeal cancer cell line: Predictive parameters for response to 5-fluorouracil-based chemotherapy. *Biomed Pharmacother.* 2014;68(5):515-9

36. Jessri M, Farah CS. Next generation sequencing and its application in deciphering head and neck cancer. *Oral Oncol.* 2014;50(4):247-53.

37. Askari BS, Krajinovic M. Dihydrofolate reductase gene variations in susceptibility to disease and treatment outcomes. *Curr Genomics.* 2010;11(8):578-83

38. Hider SL, Bruce IN and W. Thomson. The pharmacogenetics of methotrexate. *Rheumatology* 2007;46:1520–1524

39. Assaraf YG. Molecular basis of antifolate resistance. *Cancer Metastasis Rev* 2007;26:153-81.

40. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. *Arthritis Rheum.* 2006;54(10):3095-103.

41. Obuchi W, Ohtsuki S, Uchida Y, Ohmine K, Yamori T, Terasaki T. Identification of transporters associated with Etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics. *Mol Pharmacol.* 2013;83(2):490-500.

42. Abdel-Haleem AM, El-Zeiry MI, Mahran LG, Abou-Aisha K, Rady MH, Rohde J, Mostageer M, Spahn-Langguth H. Expression of RFC/SLC19A1 is associated with tumor type in bladder cancer patients. *PLoS One.* 2011;6(7):e21820.