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Guilherme Castro Lima Silva do Amaral

5-Aza-2CdR Promoted *MGMT* Demethylation and Modified Expression of Different Genes in SCC-9 Cells

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RESUMO

O câncer de cabeça e pescoço é a sexta neoplasia mais frequente no mundo sendo que mais de 90% são do tipo carcinoma escamoso. O carcinoma escamoso oral surge de uma desdiferenciação maligna das células do estrato espinhoso do epitélio da mucosa oral e é geralmente associada ao consumo de cigarro e/ou bebidas alcoólicas. O surgimento dessa neoplasia pode estar associado com a presença de mutações genéticas, bem como, com a presença de eventos epigenéticos, como a metilação do DNA. Na tentativa de reverter a metilação do DNA, a droga desmetilante 5-aza-2'-deoxycitidina (5-aza-2CdR), formulada em 1968, foi inicialmente utilizada no tratamento de leucemias e de síndromes mielodisplásicas. No entanto, o uso dessa substância em tumores sólidos passou a ser liberado em meados de 2015 e, assim, pouco ainda se sabe sobre sua ação no carcinoma oral. A fim de abordar essa questão, investigamos o efeito do agente desmetilante 5-aza-2CdR em células da linhagem SCC-9 como também seu efeito sobre a expressão dos genes supressores tumorais *MGMT, APC* e *BRCA1* e dos oncogenes *cMYC* e *hTERT*. Para isso, células SCC-9 foram tratadas com 5-aza-2CdR na concentração de 0,3μM ou 2μM, durante 24 horas ou 48 horas. Foi realizado o teste MTT para análise da citotoxicidade das doses empregadas e observamos que, em ambos tempos, a concentração de 2μM foi citotóxica. Usando a técnica de análise de melting (MS-HRM), nossos resultados mostraram que 5-aza-2CdR provocou uma desmetilação parcial no promotor do gene *MGMT* após 48 horas de tratamento o que não foi observado para o gene *hTERT* que se manteve hipermetilado. Os demais genes analisados *BRCA1*, *APC* e *cMYC* foram observados originalmente desmetilados na região analisada em células SCC-9. Quanto à transcrição que foi analisada por PCR tempo real, os genes supressores de tumores *MGMT, APC e BRCA1* foi positivamente regulada enquanto a expressão do gene *cMYC* foi negativamente regulada nas células expostas à droga por 48h. Em contrapartida, o gene *hTERT* mostrou uma diminuição da expressão que foi seguida por uma recuperação na expressão no tempo de 48horas. Os resultados também mostraram que a expressão gênica dos genes supressores tumorais sofreu aumento ainda mais significante quando as células foram expostas à dose menor da droga (0,3μM). Dessa forma, concluímos que a aplicação do agente desmetilante 5-aza-2cdr em baixa concentração aumentou a

expressão dos genes supressores tumorais (*MGMT, APC e BRCA1*) e diminuiu a expressão do proto-oncogene cMYC em células de carcinoma espinocelular de oral.

Palavras Chave: câncer oral, 5-aza-2'-deoxicitidina, metilação de DNA, expressão gênica.

ABSTRACT

Head and neck cancer is the sixth most frequent neoplasm in the world, with more than 90% representing squamous cell carcinoma. Oral squamous cell carcinoma arises from a malignant dedifferentiation of epithelium cells generally associated with the consumption of cigarettes and alcoholic beverages. This tumor is characterized by an accumulation of mutations and epigenetic alterations, including DNA methylation. To reverse DNA methylation, 5-aza-2'-deoxycytidine was formulated in 1968, and initially it was used in leukemias and myelodysplastic syndrome treatment. The use in solid tumors was released in 2015 and, therefore, little is known about its action in oral cancer. In order to address this issue, we investigated the effect of demethylating agent 5-aza-2'-deoxycytidine in SCC9 cells as well as the effects on transcription the tumor suppressor genes *MGMT, APC* and *BRCA1* and oncogenes *cMYC* and *hTERT*. SCC-9 cells were exposed to 5-aza-2'-deoxycytidine at concentrations of 0,3μM or 2 μM during 24 hours or 48 hours. After, high resolution melting analysis (MS-HRM) was performed in order to determinate genes methylation pattern. Results showed partial demethylation of the *MGMT* gene after 48 hours of 5-aza-CdR treatment. No difference was observed in *hTERT* gene that remained hypermethylated. The other genes *BRCA1, APC* and *cMYC* were found orinally demethylated in the analyzed region. Tumor suppressor genes *MGMT, APC* and *BRCA1* were up regulated and oncogene *cMYC* was down regulated after 48h of 5-aza-2CdR treatment. Our results also showed that transcription of tumor suppressors genes was significantly increased in cells exposed to lower dose of drug (0,3μM). Thus, we concluded that the low concentration of 5-aza-2cdr demethylating agent increased the expression of tumor suppressor genes *(MGMT, APC and BRCA1)* and decreased *cMYC* proto-oncogene expression in oral squamous cell carcinoma cells.

Key-words: oral cancer, 5-aza-2'-deoxycytidine, DNA methylation, gene expression.

SUMÁRIO

1.INTRODUÇÃO

O câncer de cabeça e pescoço é a sexta neoplasia mais frequente no mundo com alta taxa de mortalidade devido ao diagnóstico tardio. Entre os diferentes tipos, aproximadamente 90% dos casos são de carcinomas de células escamosas (Brocic M, 2009). O carcinoma de células escamosas oral (OSCC) é um câncer com desdiferenciação das células da camada escamosa do epitélio da mucosa oral, sendo mais frequente entre a quinta e sexta décadas de vida. Esse tipo de tumor está associado a fatores de risco como consumo de tabaco na forma de cigarro ou mascável e consumo de bebidas alcoólicas. Em 2012, o projeto GLOBOCAN estimou 300.373 novos casos com uma taxa de incidência global padronizada por idade de 4,0 casos por 100.000 habitantes/ano e uma taxa de mortalidade global de 1,9 mortes por 100.000 habitantes/ano (WHO, 2017). O diagnóstico precoce do câncer de cabeça e pescoço pode melhorar significativamente o prognóstico desta neoplasia (Meng, R., 2015). Além da baixa sobrevida, os pacientes com câncer de cabeça e pescoço podem padecer demais e sentir muita dor durante a doença (Pisani,2002; Molinolo, A,2009). A dor induzida por essa neoplasia limita as funções orofaciais, como a deglutição, a mastigação e a fala, o que resulta em baixa qualidade de vida. A perda das funções do aparelho estomatognático provocada pela dor é uma das maiores preocupações nesses pacientes. Portanto, uma nova abordagem farmacológica é necessária. (Castilho et al., 2017)

O conceito de epigenética foi introduzido por Conrad em 1942 (Easwaran, H, 2014; Waddington, C.H,1942) e se refere às mudanças estáveis e herdáveis nos genes que não alteram a sequência do DNA mas podem modificar a transcrição genética (Bird, A. 2002). As alterações epigenéticas são mecanismos que podem levar à carcinogênese e poderiam servir como possíveis marcadores para detecção precoce, tratamento e avaliação prognóstica para pacientes com câncer (Wang, Z. 2015). Pesquisas apontam que alterações epigenéticas compreendem mudanças críticas envolvidas na iniciação e progressão de cânceres humanos (Baylin e Jones et al., 2016). Essas alterações ocorrem de maneira assistemática que promove vantagem seletiva ao tumor através do silenciamento de genes supressores de tumor ou disfunção em genes de reparo de DNA. Por isso, essas modificações também

contribuem para a plasticidade celular durante a progressão do tumor e a formação de células que o iniciam (Castilho et al., 2017).

Existem diversos eventos classificados como epigenéticos, dentre eles: metilação do DNA, modificações covalentes de histonas, a própria remodelação da cromatina e o efeito dos RNAs não-codificantes sobre o funcionamento do DNA (Esteller,2012; Issa,2017). A metilação do DNA, especificamente, está geralmente associada à diminuição da expressão de alguns genes em tumores, sendo a grande maioria genes classificados como supressores de tumor (Bird, 2010; Kulis e Esteller, 2010). Ela acontece através da adição de radical metil (CH3) na citosina em dinucleotídeos CpG, sendo essa a alteração epigenética mais estudada (Robertson et al., 2005; Baylin et al., 2011). Os dinucleotídeos CpG estão distribuídos pelo genoma de maneira não randômica e se encontram enriquecidos em regiões reguladoras, como os promotores de genes, regiões que recrutam enzimas que darão início à transcrição gênica. Essas regiões enriquecidas em CpG são conhecidas como ilhas CpG e são vistas ordinariamente não metiladas em genes transcricionalmente ativos. A metilação dos dinucleotídeos CpG é realizada por uma família de enzimas chamadas DNA-metiltriltransferases (DNMTs), enzimas capazes de realizar a transferência do grupo metil a partir de um substrato doador para a citosina. Três DNMTs (DNMT1, DNMT3a e DNMTb) são fundamentais para a metilação em mamíferos. A DNMT1, chamada de enzima de manutenção da metilação, está envolvida na restauração do perfil de metilação do DNA parental após a replicação do DNA, garantindo o status de metilação às futuras gerações de células (Li e tal., 1992; kullis et al., 2011). Já as enzimas DNMT3a e DNMT3b realizam a metilação do DNA em dinucleotídeos CpG previamente não metilados, uma situação conhecida como metilação *de novo* (Dawson et al., 2012; Kullis et al., 2011). A metilação do DNA em associação com modificações específicas em histonas são responsáveis pela promoção da compactação da cromatina que consequentemente promove repressão da expressão gênica (Baylin et al., 2011).

A perda da metilação no DNA foi a primeira alteração epigenética caracterizada em tumores benignos e células cancerígenas (Kullis et al., 2011) e tem como alvo várias sequências genômicas, incluindo elementos repetitivos, transposons, dinucleotídeos CpG em íntrons, o que leva a aumento da instabilidade genômica e ativação de proto-oncogenes (Rodriguez,2006; Hatziapostolou et al.,

2011). Em contraste, a hipermetilação das ilhas CpG dos promotores de genes pode contribui com a carcinogênese através do silenciamento de genes supressores de tumor. Regiões promotoras de genes são sítios de recrutamento de enzimas que dão início à transcrição do gene e podem ter por volta de 100-1000 pares de bases de comprimento.

Para reverter a hipermetilação dos genes, a droga 5-aza-2-deoxycitina tem sido utilizada na intenção de reativar genes supressores de tumor. Além disso, tem-se observado que baixa dose de 5-aza-2CdR pode ter efeito antitumoral mais eficaz contra tumores sólidos, reduzindo a frequência de células iniciadoras de câncer (Tsai et al., 2012; Yang et al., 2014; Roulois, D et al., 2015). A inibição da ação das enzimas DNA metiltransferases pela 5-aza-2CdR promoveria senescência ou apoptose das células tumorais (Schnekenburger M, 2011).

A droga 5-aza-CdR é um análogo do nucleosídeo natural 2′-desoxicitidina no qual o carbono na posição 5 da citosina é substituído por nitrogênio. Estudos pré-clínicos em roedores indicaram que 5-aza-CdR é um agente antileucêmico mais potente que a citosina arabinosídeo (ARA-C) (Momparler et al., 2005; Richel et al., 1988). A primeira revisão sobre as propriedades farmacológicas do 5-aza-CdR foi publicada em 1979 (Momparler et al., 1979) e o primeiro ensaio clínico com 5-aza-CdR em pacientes com leucemia aguda foi publicado em 1981 (Rivard et al, 1981). Embora a capacidade de desmetilação única do 5-aza-CdR seja conhecida há muitos anos, sua aprovação para o tratamento de certos tipos de câncer demorou algum tempo, talvez devido à falta de compreensão da importância da epigenética nas alterações de malignidade durante os primeiros anos de desenvolvimento (de Vos et al., 2005). 5-aza-CdR foi aprovado para o tratamento de síndrome mielodisplásica (SMD) em 2006 e tem mostrado bons resultados contra a leucemia mieloide aguda (AML) (de Vos et al., 2005; Jabbour et al 2008). Além disso, baixas doses de 5-aza-CdR foi proposta como sendo mais eficaz no tratamento da SMD, devido à sua ação epigenética, enquanto que doses mais altas mostraram-se muito tóxicas ao organismo e, devido a isso, menos eficientes (Issa et al., 2004).

Entre os genes que podem estar associados com o surgimento de cancer de cabeça e pescoço, encontramos os genes *MGMT, BRCA1 e BRCA2, C-Myc* e *hTERT. MGMT* desempenha um papel crucial no mecanismo de reparo do dano ao

DNA. O *MGMT* é amplamente expresso em vários tumores e sua função é freqüentemente perdida devido à hipermetilação no promotor. Alguns estudos mostram que a metilação do promotor do gene *MGMT* está intimamente relacionada ao mau prognóstico, metástase e recorrência no carcinoma de cabeça e pescoço (Wong et al.,2011; Misawa et al., 2016; Celebi et al., 2013). Até agora, muitos estudos exploraram a associação entre a metilação aberrante do promotor *MGMT* e o risco de carcinoma de cabeça e pescoço. Fucheng relata em sua meta-análise uma associação entre a metilação do promotor *MGMT* e o risco de carcinoma de cabeça e pescoço. Neste estudo, foram analisados 20 estudos que incluiam 1030 casos e 775 controles. Nele, a frequência de metilação do promotor do *MGMT* no tumor foi de 46,70% e 23,23% no grupo controle. Portanto, o resultado da meta-análise mostrou que hipermetilação do promotor do gene *O6-metilguanina-DNA-metiltransferase* (*MGMT*), o qual é responsável pelo reparo de danos ao DNA, foi associada a um risco aumentado de câncer de cabeça e pescoço (Fucheng Cai,2016; Towle R,2012).

O gene *BRCA1* está associado ao câncer de mama e codificam proteínas que funcionam numa via comum de proteção do genoma, ativando o ponto de controle do ciclo celular e reparando o DNA danificado por recombinação homóloga (Roy R. et al.,2012). Um padrão interessante associado à patogênese do câncer de língua é a super-expressão do gene (*BRCA1*) na leucoplasia e a baixa expressão no carcinoma escamoso de boca (H. H. Vora, 2003). Além disso, o gene *BRCA1* desempenha um papel crucial na patogênese do cancêr de cabeça e pescoço e poderia ser usado independentemente como biomarcador preditivo para esse tipo de câncer (Bhowmik, A. et al., 2016). O gene *APC* classificado como supressor tumoral também apresenta expressão significativamente baixa em 62,5% das linhagens celulares de câncer de cabeça e pescoço (Uesugi et al.,2005).

O gene *c-Myc* é um importante regulador de vários processos biológicos (Brenner et al., 2005). Entre as funções do *c-Myc* estão a capacidade de reprimir a transcrição de inibidores de quinase dependentes de ciclina p21Cip1, p15Ink4b, p27kip1, assim como genes associados à diferenciação celular (Eisenman et al., 2001; Wanzel et., 2003). A função desregulada de *c-Myc* é uma das anormalidades mais comuns em malignidades humanas, incluindo em câncer de cabeça e pescoço. No entanto, a superexpressão desse gene no nível de RNAm, e de proteína variou de 21

a 68% em cânceres de cabeça e pescoço (Field et al., 1989;Riva et al., 1995; Baltaci et al., 2016).

A atividade da telomerase é baixa ou completamente ausente em células somáticas, mas células-tronco e células cancerosas expressam altos níveis dessa enzima para manter o comprimento dos telômeros e evitar a senescência celular (Lewis & Tollefsbol, 2016; Ramlee et al., 2016). O gene *hTERT* (*human telomerase reverse transcriptase)* está localizado no cromossomo 5p15 e sua regulação pode ocorrer em diferentes vias, incluindo a metilação do DNA que desempenha um papel fundamental no controle da transcrição gênica. Além disso, alterações no promotor do gene *hTERT,* que resultam no aumento da expressão, também foram detectadas em uma proporção significativa de pacientes com câncer de cabeça e pescoço (Killela et al., 2013; Vinothkumar et al., 2015; Qu, Y et al., 2014).

Modificações epigenéticas, como a hipermetilação do promotor, podem resultar na desregulação de genes supressores de tumor, levando ao aumento do risco de desenvolvimento de um câncer (Baylin SB, Esteller M, 2001). Tendo em vista a plasticidade epigenética e a possibilidade de se reverter padrões de metilação em regiões previamente desmetiladas, houve aumento do interesse na área da pesquisa por drogas desmetilantes como o 5-aza-2CdR. 5-aza-2CdR se incorpora ao DNA como um aduto e liga covalentemente às DNMTs, bloqueando sua ação e impedindo a metilação da nova fita de DNA no momento da replicação (Schnekenburger M., 2011). Doses altas de 5-aza-2CdR têm se mostrado muito tóxicas para os organismos, levando a desordens hematológicas quando utilizadas clinicamente e também à perda da função desmetilante, justamente a que mais interessa do ponto de vista terapêutico. Assim, a utilização de doses cada vez menos concentradas de 5-aza-CdR têm sido propostas e, aparentemente, essas têm se mostrado até mais eficazes como agente desmetilante (Issa JP, Garcia-Manero, 2004).

O objetivo deste estudo foi avaliar o efeito desmetilante do 5-aza-2-CdR em genes relacionados aos tumores de cabeça e pescoço como também seu efeito sobre a taxa de transcrição desses genes na linhagem celular SCC-9.

2.Artigo

5-Aza-CdR Promotes Partial *MGMT* Demethylation and Modifies Expression of Different Genes in Oral Squamous Cell Carcinoma

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ABSTRACT

Objective: Treatment strategies for Oral Squamous Cell Carcinoma (OSCC) vary based on the stage of diagnosis. Surgery and radiotherapy are options to localized lesions and this protocol is generally successful for stage I patients, while chemotherapy is the mainstays treatment for metastatic OSCC. However, aggressive tumors can relapse, causing frequently the death of patient. Searching for auxiliary therapies could be relevant to patients with poor prognosis. Addressing it, novel treatment protocols using epigenetic drugs that alter the epigenetic profile have emerged as an alternative to control tumor growth and metastasis. We investigated the effect of demethylating agent 5-aza-CdR at the concentrations of 0,3μM and 2μM in the epigenetic profile of SCC9 cells.

Material and Methods: SCC9 cells were treated with 5-Aza-CdR at the concentrations of 0,3μM and 2μM for 24h and 48h. After, DNA methylation of *MGMT, BRCA1, APC*, *c-MYC* and *hTERT* genes were investigated using Methylation Specific-High Resolution Melting technique. RT-PCR and qPCR were performed to analyze genes expression.

Results: 5-Aza-CdR promoted demethylation of *MGMT* gene and modified the mRNA transcription of all analyzed genes. Curiously, treatment of 5-aza-CdR at the concentration of 0,3μM was more efficient than the higher concentration of 2μM in SCC9 cells.

Conclusion: We observed 5-aza-CdR leaded *MGMT* demethylation, upregulated transcription of three important tumor suppressor genes and promoted downregulation of c-Myc.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most frequent and aggressive type of oral cancers, affecting mainly tongue and floor of mouth, followed by gingiva, palatal mucosa, and labial mucosa (Bagan et al., 2010; Li et al., 2018). Alcohol and tobacco consumption are the main and well-established oral cancer risk factors. However, other intrinsic and extrinsic factors can also contribute to oral cancer initiation, such as chronic inflammation (Planello et al., 2016), HPV infection (HPV16 and HPV 18) (Ang et al., 2010), genetic mutations (germline *TP53* gene mutation) (Boyle et al., 1993) and epigenetic changes.

Epigenetics is defined as all meiotically or mitotically heritable changes that can alter the pattern of genes regulation and genes expression without changing DNA sequence (Feinberg, 2007). DNA methylation that takes place in the CpG dinucleotides is the broadly studied epigenetic change. This modification has been recognized as a fundamental mechanism of gene silencing. It represents a covalent transfer of methyl group from S-adenosyl-L-methionine to cytosine preceding guanines (CpG dinucleotides) by the action of DNA methyltransferases (DNMT-1, -3A, -3B) (Hermann et al., 2004a; Hermann et al., 2004b). In the opposite, DNA demethylation depends on the combined function of ten-eleven translocation enzymes (TET1, TET2, and TET3) and thymine-DNA glycosylase (TDG) (Nakashima et al., 2013). CpG dinucleotides tend to be concentrated inside regulatory regions of the genome in a not-random mechanism what originate CpG enriched regions named CpG islands (Bayarsaihan, 2011).

Epigenetic drugs including DNA demethylating agents have been exhaustively investigated in an attempt to reactive the expression of genes that have undergone DNA methylation silencing, including tumor suppressor genes in cancer (Karahoca & Momparler 2013; Castilho et al., 2017). 5-aza-2`-deoxycytidine (5-aza-CdR or Decitabine (trade name Dacogen®)) is a demethylating agent, approved by Food and Drug Administration, that is incorporated into DNA molecule in place of cytosine during DNA replication (S-phase cells) and blocks DNMTs activity by formation of a covalent protein-DNA adduct, triggering genome hypomethylation (Patra et al., 2009; Castilho 2017). Studies have demonstrated that the immortal phenotype

of certain dysplasias could be reversed by treatment with 5-aza-CdR (McGregor et al., 2002). They showed *RAR-β* and *p16* genes could be re-expressed by treatment with 5-aza-CdR in some immortal dysplasias, since gene silencing resulted from promoter hypermethylation and re-expression of *RAR-β* and *p16* represents a key point to turn on cell senescence mechanism. Other study reported the protector role of 5-aza-CdR in preventing oral carcinogenesis induced by 4-nitroquinoline (Tang et al., 2009).

The treatment strategy for patients with OSCC includes commonly surgery and/or radiotherapy (Misawa et al., 2016; Algazi & Gradis 2017). The majority of stage I patients can be cured by this treatment protocol. The current treatment for advanced-stage of OSCC includes cisplatin chemotherapy. However, cisplatin resistance have been observed in many patients, reducing survival and allowing the aggressive tumors relapse, leading death of number of patients since there are no effective alternative treatment in the majority of cases (Viet et al., 2014; Misawa et al., 2016). Novel treatment protocols using epigenetic drugs in combination with conventional therapies could be valuable for patients with poor prognosis (Castilho et al., 2017).

Therefore, the aim of our study was to investigate the effect of two different doses of 5-aza-CdR on DNA methylation pattern and expression of *MGMT, BRCA1, AP1, c-MYC* and *hTERT* genes that play key roles in tumorigenese of oral cancer. Assays were carried on SCC-9 cell, which were derived from squamous cell carcinoma located at tongue of 25-years old man (Rheinwald & Beckett, 1981). SCC-9 cells was chosen for the reason it contains a subpopulation of CD-44^{+} cells, which have been associated with advanced disease, treatment failure, metastases to lymph nodes and worse prognosis (Wu et al. 2013; de Andrade et al., 2017).

MATERIALS AND METHODS

Culture of cells

SCC-9 cells (ATCC® CRL-1629™) were gently provided by Dr. Edgar Graner (Piracicaba Dental School, University of Campinas, Piracicaba, Brazil). Cells were cultured in DMEM/Ham's F12 medium supplemented with 10% fetal bovine

serum, antibiotic solution (100 U/ml penicillin and 100µg/ml streptomycin), and hydrocortisone in the humidified atmosphere (5% CO_2) at 37ºC.

5-aza-CdR treatment

5-Aza-CdR (Sigma-Aldrich) was diluted in PBS at concentration of 1mM. Cells were treated with 0,3µM or 2µM of 5-Aza-CdR (Sigma-Aldrich) during 24h and 48h. 5-aza-CdR was replaced each 24h. Control cells were treated with same amount of PBS and experiments were assayed in triplicate.

MTT assay

Cell viability was assessed by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, Carlsband, CA, USA) as previously described (Mosmann, 1983). Briefly, cells were seeded in 96-well plates at a density of $7{,}5 \times 10^4$ cells/well and allowed to adhere for 24 h. Initially, it was added to the experimental medium of 15µL of MTT solution and incubated for 4h at 37ºC in 5% CO_2. Control wells without cells containing only experimental medium were incubated in parallel with test samples to measure the absorbance background. Afterwards, 100µL of solubilization/stop solution was added to solubilize the formazan product incubating for 1h at 37ºC in 5% CO_2. The precipitated formazan crystals were solubilized in ethanol, and the absorbance was set at 570 nm using a microplate reader. The results were expressed as the percentage of cell viability of control cells (100%) that were treated with the corresponding volume of serum culture medium.

DNA extraction and sodium bisulfite treatment

Total DNA was purified from cells using Quick-DNA Universal kit (Zymo research), following the manufacturer's protocol. Amount of DNA and purity were measured by photometric measurement using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). 1 µg of gDNA was converted by sodium bisulfite using Epitect® Bisulfite kit (Qiagen), according to manufacturer's protocol. Converted DNA was storage at 4ºC until MS-HRM analysis.

Methylation Sensitive-High Resolution Melting (MS-HRM)

Real-time PCR followed by HRM analysis was performed in the LightCycler 480 II thermocycler (Roche, Mannheim, Germany). Primer sets were designed in accordance with guidelines proposed by Wojdacz and collaborators (Table 1). Reaction mixture consisted of 1µL of bisulfite-converted gDNA, 1x LightCycler®480 HRM Master Mix (Roche, Mannheim, Germany), 250nM of each primer, 2mM of $MgCl_2$ in a final volume of 10µl. Cycles parameters were: 1 x 95°C for 10 minutes; 40 x 95°C for 10 seconds, 55°C for 4 seconds and 72°C for 10 seconds. MS-HRM analysis was performed at temperature ramping and fluorescence acquisition settings recommended by manufacturer: 1 minute at 95°C, hold at 70°C for 1 minute (to allow re-annealing of all PCR product), acquisition step ramping from 70°C to 95°C, rising by 0.2°C/second with 25 acquisitions per °C. To estimate the methylation level of each sample, converted fully methylated and fully unmethylated DNA (EpiTect PCR Control DNA Set from Qiagen) were used to prepare dilution series. The dilution series of relevant methylated DNA in a background of unmethylated DNA were prepared at 0, 10, 25, 50, 75 and 100% methylated DNA (providing a standard curve). Standard curve and no template controls were included in each experimental run. In order to compensate for varying starting fluorescence levels MS-HRM data was normalized (Light Cycler 480 II analysis software). The amplicon melting profile of each sample was compared to standard curves. As result samples were classified in a range of methylation equal to 0-10%; 11-25%; 26-50%; 51-75%; 76-100%. Assays were run in quadruplicate.

RT-PCR and qPCR

Total RNA was purified by Trizol reagent (Invitrogen). 1 µg of total highly purified RNA was treated with DNAse, and then 0.5 µg of RNA was used to cDNA synthesis. cDNA synthesis was carried out using Superscript III (Invitrogen), according to manufacturer's instructions. qPCR was carried out in a total of 10 µl, containing 1µl of cDNA, 5 µl of SYBR Green (LightCycler® 480, *Roche* Applied Science), 250 nM of each primer, 2 µl of cDNA and nuclease free H_2O. Relative levels of gene expression were performed using the cycle threshold (Ct) method in reference to GAPDH expression. The graphical representation of results was created using Origin 6.0

software. Primers set are described in Table 2. The relative gene expression was determined using delta-delta Ct method (ΔΔCT). Assays were run in triplicate.

Statistical analysis

Statistical significance of differences was analyzed by Wilcox-Mann-Whitney test at 5% level of significance, using the software Graph Pad Prism version 7.

RESULTS

MTT assay was used to determine SCC-9 cells viability after treatment with 5-aza-CdR. Cytotoxicity effect was seen when cells were incubated with 5-aza-CdR at the concentration of 2μM during 24 and 48 hours. The number of living cells decreased 25% after incubation with that concentration in both experimental times. Cytotoxicity of 5-aza-CdR at the concentration of 0,3μM was not significant (Figure 1). After, the effect of 5-aza-CdR on demethylation of *MGMT, hTERT, BRCA1, APC,* and c-*MYC* genes was evaluated by real-time PCR followed by HRM analysis. *MGMT* gene was found originally partially methylated (50% methylation) in SCC9 cells. We observed a 25% methylation reduction in the *MGMT* gene for both 5-aza-CdR concentrations 48h after treatment (Figure 2A). It means that after 48h 5-aza-CdR promoted the same demethylating effect on *MGMT* regardless concentration. *hTERT* gene was originally partially methylated (50% methylation) and maintained the same percentage of methylation even after 48h of 5-aza-CdR treatment (Figure 2B). *c-MYC, BRCA1* and *APC* genes were originally demethylated in SCC-9 cells.

MGMT, *c-MYC* and *hTERT* expression suffered significant changes when compared to control 24h after treatment and mRNA expression of all investigated genes was significantly changed after 48h (Figure 3A and 3B).

MGMT expression increased 1.74-fold at concentration of 0,3μM24h after treatment (p=0.02). Curiously, no effect was found on *MGMT* expression after 24h at concentration of 2μM (Figure 3A). We observed significant *MGMT* upregulation of 2.46-fold and 2.14-fold at concentration of 0,3μMand 2 μM, respectively, after 48h treatment (p=0.02) (Figure 3B). Similar results were found in the other tumor suppressor genes *BRCA1 and APC* after 48h treatment. *BRCA1* expression was

increased 5.15-fold and 3.20-fold at concentration of 0,3μMand 2 μM, respectively (p=0.02) (Figure 3B). *APC* mRNA level was increased 2.46-fold and 1.98-fold at concentration of 0,3μMand 2 μM, respectively (p=0.02) (Figure 3B). Use of 5-aza-CdR at 0,3μMstimulated higher mRNA levels of *MGMT, BRCA1 and APC* tumor suppressor genes than concentration of 2 μM in both experimental times.

c-MYC gene expression was decreased 1.06-fold and 1.11-fold at the concentration of 0,3μM and 2μM 5-aza-CdR, respectively, after 24h treatment (p=0.02) (Figure 3A). The effect was slightly more pronounced after 48h treatment when mRNA was decreased 1.44-fold and 1.55-fold at the concentrations of 0,3μM and 2μM 5-aza-CdR, respectively, ($p<0.05$) (Figure 3B).

The 5-aza-CdR effects on *hTERT* gene varied according to treatment time. We found downregulation of *hTERT* gene after 24h treatment. Gene expression decreased 3.33-fold and 6.7-fold at concentration of 0,3μM and 2μM 5-aza-CdR, respectively (p=0.02) (Figure 3A). In contrast, *hTERT* gene was upregulated after 48h treatment. Gene expression increased 2.05-fold at the concentration of 0,3μM 5-aza-CdR ($p<0.05$) (Figure 3B). At the concentration of 2μM 5-aza-CdR we also observed upregulation of *hTERT* gene but this change was not statistically significant.

DISCUSSION

Compiled evidence have demonstrated the importance of epigenetic changes on oral carcinogenesis, cancer progression and acquired therapy resistance. Besides the well-known roles of the epigenetics modifications such as DNA methylation, histone modifications and non-coding RNAs in regulating gene transcription, epigenetics can also allow cancer progression by the selection of cancer-initiating cells/cancer stem cells, which have the ability to self-renew and promote heterogeneous clones of neoplastic cells expansion, including therapy resistant clones (Castilho et al., 2017). The associated use of epigenetic drugs such as the demethylating agent 5-aza-CdR could be an attempt to re-sensitize OSCC.

Hypomethylation of oncogenes is related to cell proliferation and it is commonly observed in OSCC (Castilho et al. 2017) as well other cancer types. However, DNA

hypermethylation of tumor suppressor genes that are responsible for triggering cell apoptosis is also seen in cancer (D'Souza & Saranath 2015; Ovchinnikov et al., 2012). *MGMT, BRCA1, APC, RASSF1, MLH1,* and *p16* are some examples of tumor suppressor genes frequently altered by DNA methylation in OSCC samples (Misawa et al., 2016; Mascolo et al. 2012; Gasche & Goel 2012; Lim et al. 2016).

In this study 5-aza-CdR at the concentrations of 0,3μM was cytotoxic to 20% of cells while 2μM was cytotoxic to 25% of cell, regardless how long cells were treated with 5-aza-CdR (24 or 48 hours). Low dose and/or intense therapy with 5-aza-CdR have been applied in order to reduce the proliferative potential of tumors. Therefore, it is possible to find in the literature studies that have used concentrations of 5-aza-CdR from 0.05μM up to 100μM (Karahoca & Momparler 2013). In our study SCC9 cells were treated with 2μM and the lower dose of 0,3μM based on results of recent reports, which worked with similar concentrations (Koutsimpelas et al. 2012; Roulois et al. 2016).

Experiments were performed in order to investigate the 5-aza-CdR treatment effect on genes that play different roles within oral cancer. A panel containing three important tumor suppressor genes (*MGMT, BRCA1, APC*) and also two oncogenes (*c-MYC, hTERT*) was elected based on data of SCC9 mRNA expression from Cancer Cell Line Encyclopedia (CCLE) and previous reports (Supić et al. 2009; Noorlag et al. 2014; Ribeiro et al. 2018). *MGMT* (O-6-methylguanine DNA methyltransferase) represents a conserved gene throughout the evolution (Grombacher et al., 1996), located at 10q26 chromosome, and a key regulator of the base excision repair pathway, helping in the DNA adducts removal process (Gerson 2004). *MGMT* promoter hypermethylation has been found in association to OSCC in different populations, including Japanese and North Americans and is negatively correlated with overall disease-free survival (Jayaprakash et al 2017). *MGMT* was seen partially methylated in SCC9 cells what means half of the cells (50%) contain the gene analyzed region hypermethylated. After 5-aza-CdR treatments SCC9 methylation decreased 25%, regardless of drug concentration. Gene expression was upregulated after 24h and 48h of 5-aza-CdR treatments and, curiously, a more pronounced effect on mRNA transcription was observed when the lower concentration of 0,3μM was applied.

BRCA1 and *APC* were found demethylated in SCC9 and, consequently, no direct effect of 5-aza-CdR on these genes methylation pattern could be found. *BRCA1* gene (breast cancer susceptibility gene 1) is located at 17q21 chromosome and was discovered more than two decades ago. Important biological functions have been addressed to this gene, including mRNA splicing processing, non-coding RNA biogenesis, cell-cycle checkpoint control (Zhao et al., 2017). Also, BRCA1 protein forms a complex in association to BARD1 (BRCA1-BARD1), which takes part in the repair of DNA double-stranded breaks by homologous recombination (Zhao et al., 2017). *APC* gene (adenomatous polyposis coli gene) is located at 5q21 chromosome and encodes a protein that plays a key role in the Wnt signalling pathway, cell cycle control and apoptosis (Chen et al., 2013). Although *BRCA1* and *APC* were originally demethylated in SCC9, 5-aza-CrR was able to lead to an indirect effect on *BRCA1* and *APC* expression, promoting its upregulation. These results are probably due 5-aza-CdR demethylating effects over transcription factor(s) gene(s) that acting upstream in *BRCA1* and *APC* activation pathway. Again, 5-aza-CdR at the concentration of 0,3μM had a higher effect on mRNA levels than 2μM after 48h, increasing more than 5-fold *BRCA1* gene transcription.

c-MYC gene is located at 8p24 chromosome and represents an important regulator of several biological processes (Brenner et al., 2005). Among *c-MYC* functions are the ability to repress transcription of cyclin-dependent kinase inhibitors *p21Cip1, p15Ink4b, p27kip1* as well as genes associated to cellular differentiation (Eisenman et al., 2001; Wanzel et al., 2003). Although 5-aza-CdR had not altered *c-Myc* methylation pattern (at least the CpG sites contained in the HRM analyzed region), its transcription was downregulated after 24h and 48h of treatment, with no statistical difference between the concentrations of 0,3μM or 2μM.

Telomerase activity is low or completely absent in somatic cells but stem cells and cancer cells express high levels of this enzyme in order to maintain the telomeres length and to avoid cell senescence (Lewis & Tollefsbol, 2016; Ramlee et al., 2016). *hTERT* gene (human telomerase reverse transcriptase) is located at 5p15 chromosome and its regulation can occur at different avenues, including DNA methylation that plays a key role in gene transcription control. The core promoter of *hTERT* contains motifs that bind activator, and regulator or repressor factors and these motifs are distributed in three different promoter areas that can show different

methylation pattern (Lewis & Tollefsbol, 2016; Avin et al., 2016). Actively transcribed *hTERT* is demethylated at the TSS region [-150 to +150] but it is generally found methylated at the further upstream sequence [-650 to -400] and [-400 to -150] (Avin et al., 2016). The upstream promoter is methylated in cancer cells in order to avoid binding of repressor such as WT1 and CTCF factor (Avin et al., 2016). Demethylation by 5-aza-CdR restores the binding ability of CTCF to *hTERT* motifs which shows that DNA methylation represents a strategy to prevent repressor binding (Lewis & Tollefsbol, 2016). We observed reduced levels of *hTERT* transcription in SCC9 cells after 24h of 5-aza-CdR treatment. We hypothesize that 5-aza-CdR downregulated *hTERT* transcription in reason of the passenger hypomethylation promoted, which leads to repressors binding to upstream promoter. However, *hTERT* expression was restored after 48h, probably due to the epigenetic plasticity of cells, that disabled *hTERT* upstream promoter by re-methylation but maintained TSS region demethylated, what is advantageous to tumor growth.

It is not clear how the hypomethylation response at different doses correlates with gene activation. A hypothesis would be that higher concentrations of 5-aza-CdR could induce serious DNA damage or promote disadvantageous cytotoxic effects, which disturbs ability in promoting DNA demethylation. At higher doses, 5-aza-CdR can increase gene expression of some hypermethylated genes regardless additional effects on methylation (Quin et al 2015). The clinical use of 5-aza-CdR for epigenetic therapy is most effective when low-dose of demethylating drug regimens are practiced and until better results have been obtained by a combination of 5-aza-CdR to carboplatin or cisplatin (Tsai et al., 2012; Viet et al., 2014; Quin et al 2015).

In conclusion, we observed 5-aza-CdR leaded to *MGMT* demethylation, upregulated transcription of three important tumor suppressor genes and promoted down regulation of c-Myc. This drug is potentially an alternative for patients with aggressive tumors relapse or non-operable oral cancer if combined with conventional therapies.

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CONFLICTS OF INTEREST: None to declare

AUTHOR CONTRIBUTION

This work was designed and draft by Ana Paula de Souza. Guilherme C.S.L. do Amaral, Gabriell Borgato and Dieila Giomo cultivated SCC9 cells and analyzed data. Guilherme C.S.L. do Amaral, Aline Planello, and Gustavo Narvaes performed MS-HRM and qPCR assays.

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Figure 1

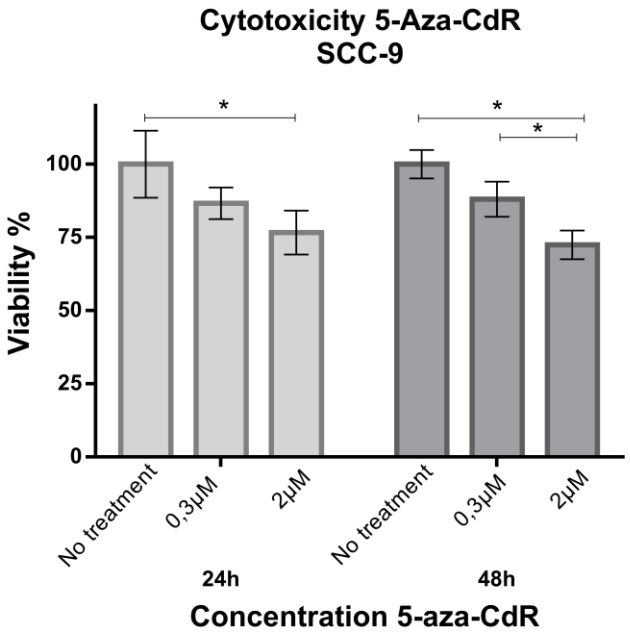
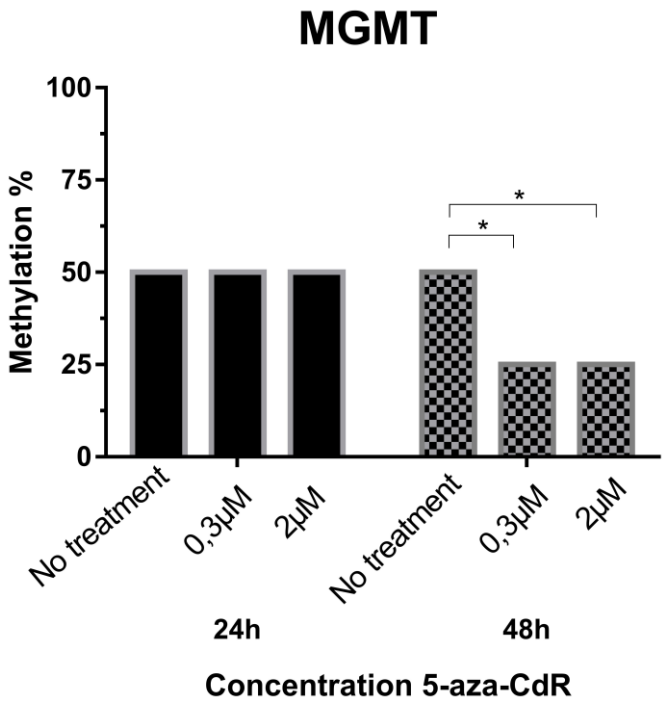


Figure 2

(A)



(B)

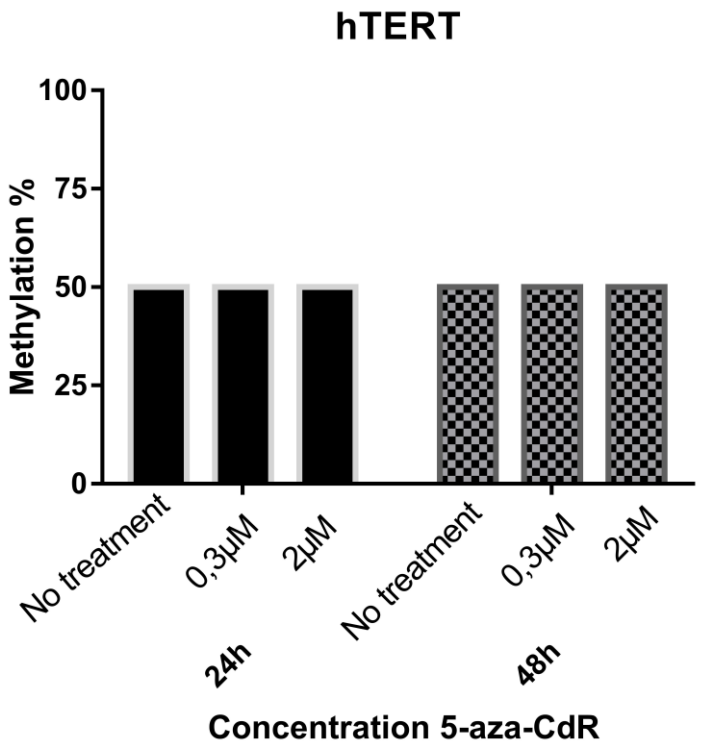
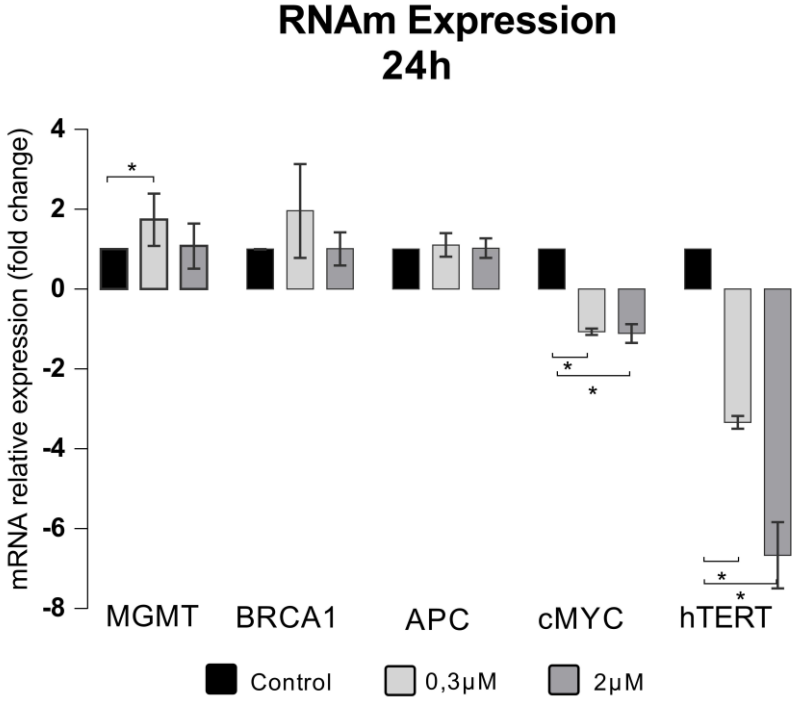
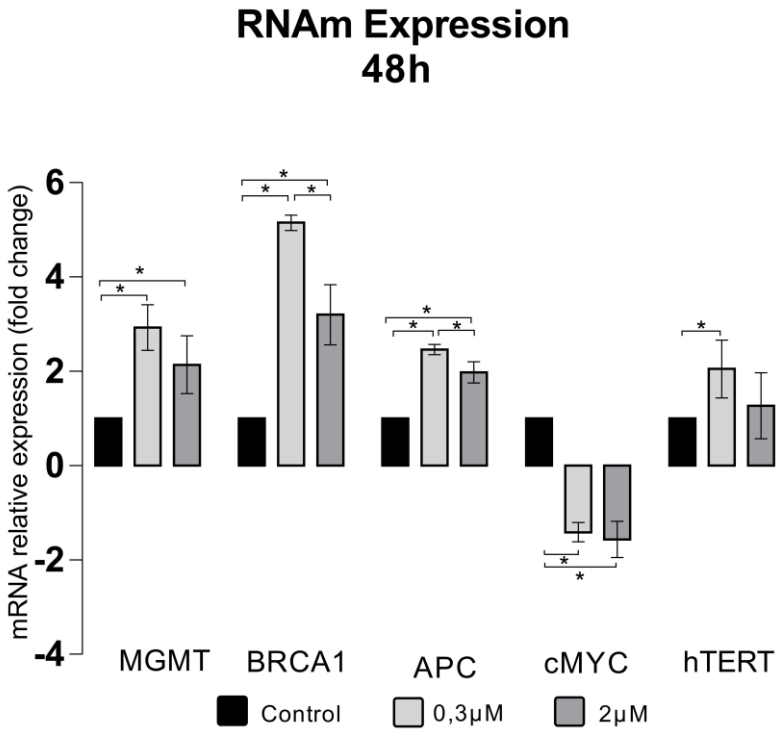

hTERT
100
75
50
25
0
Methylation %
No treatment
0,3µM
2µM
No treatment
0,3µM
2µM
24h
48h
Concentration 5-aza-CdR

Figure 3

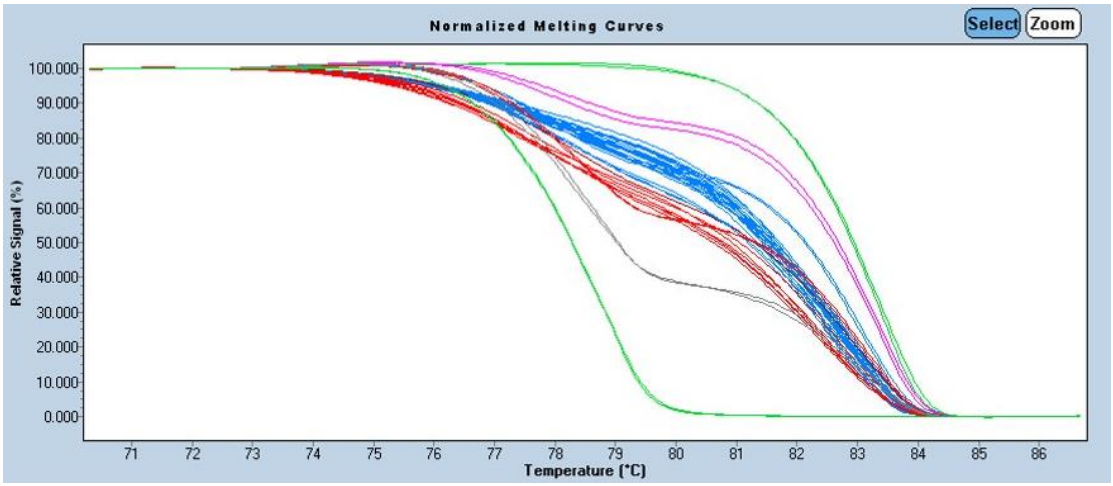
(A)


RNAm Expression
24h
mRNA relative expression (fold change)
4
2
0
-2
-4
-6
-8
MGMT
BRCA1
APC
cMYC
hTERT
Control
0,3µM
2µM

(B)

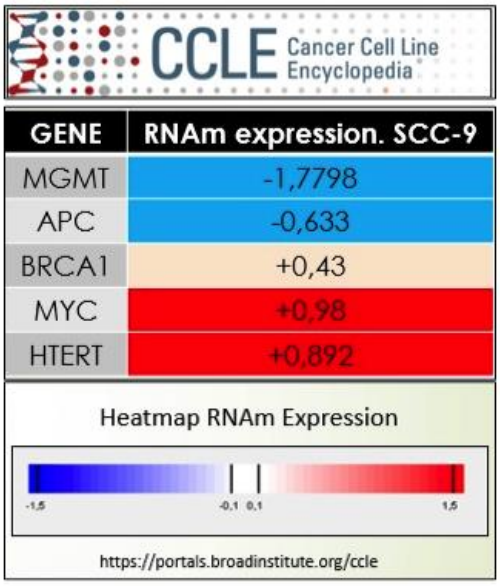


Supplementary Figure 1



100% METHYLATED
75% METHYLATED
50% METHYLATED (SCC9 Control)
25% METHYLATED (SCC9 5-aza-CdR)
10% METHYLATED
100% UNMETHYLATED

Supplementary Figure 2



Figures Legend

Figure 1. Effects of 5-aza-CdR on cell viability. Semi-confluent layers of SCC9 cells were challenged with 5-aza-CdR at concentration of 0,3μM or 2μM in culture medium containing 10 % FBS. Cellular viability was assessed by MTT reduction assay and results were expressed as a percentage of control cell viability (100%) and represented as mean ± SD of three independent experiments. No treatment cells (control) were treated with PBS.

Figure 2. MGMT and hTERT gene methylation. (A) MS-HRM analysis showed MGMT gene is partially (50%) methylated in SCC9 cells. The effect of 5-aza-CdR at concentration of 0,3μM and 2μM after 24h of treatment was not significant. However, 5-aza-CdR at concentration of 0,3μM and 2μM after 48h of treatment was able to promote significant demethylation (25%) of *MGMT* gene in SCC9 ($p<0.05$). (B) No effect of 5-aza-CdR was observed on hTERT methylation pattern.

Figure 3. Effect of 5-aza-CdR on gene expression. (A) Effect of 5-aza-CdR at concentrations of 0,3μM and 2μM on *MGMT, BRCA1, APC, cMYC* and *hTERT* expression after 24h and 48h of treatment. (B) Effect of 5-aza-CdR at concentrations of 0,3μM and 2μM on *MGMT, BRCA1, APC, c-MYC* and *hTERT* expression after 48h and 48h of treatment.

Supplementary Figure 1. MGMT MS-HRM Graphic. Standard curve is represented by different colors. Green is 100% methylated DNA (top of the chart) and is also 100% unmethylated DNA (bottom of the chart). Grey is 10% methylated DNA; Red is 25% methylated DNA; Blue is 50% methylated DNA; Pink is 75% methylated DNA. DNA from no-treatment SCC9 cells is together blue line. It means 50% of SCC9 cells showed methylation at the analyzed CpG sites. After 48h of 5-aza-CdR incubating with 0,3μM or 2μM, 25% of SCC9 cells showed methylation at the analyzed sites.

Supplementary Figure 2. The heatmap shows *MGMT, APC, BRCA1 are* downregulated while *c-MYC and hTERT* are upregulated in SCC9 lineage. Data are from Cancer Cell Line Encyclopedia.

Table 1. MS-HRM data

GENE	PRIMER 5'-3'	Genome Position	CpG	Amplicon Size	Anneling
MGMT	F-GCGTTTCGGATATGTTGGGATAGT R-CCTACAAAACCACTCGAAACTACCA	chr10:131,265,469-131,265,578	12	110	56°C
hTERT	F-CGCCAACCCTAAAACCCCAAAACCCCAAA R-GGGAAGTTTTGGTTTCGGTTATTTT	chr5: 1,253,147-1,295,069	29	139	60°C
APC	F-AAGTAGTTGTGTAATTCGTTGGAT R-CACCTCCATTCTATCTCCAATA	chr5:112,073,406-112,073,476	11	149	58°C
BRCA1	F-TTGTTGTTTAGCGGTAGTTTTTTGGTT R-CAATCGCAATTTTAATTTATCTATAATTCC	chr17:41,277,396-41,277,474	4	79	58°C
cMYC	F-TGAGGATTTCGAGTTGTGTTGT R-CTTCTCGAAACAAAAAAAAACCAAAA	chr8:127,736,069-127,741,432	7	100	58°C

Table 2. RT-PCR primers sequences

GENE	PRIMER 5' -3'	Anneling
MGMT	F-GCTGAATGCCTATTTCCACCA R-CACAACCTTCAGCAGCTTCCA	62°C
hTERT	F-CGTGGTTTCTGTGTGGTGTC R-CCTTGTCGCCTGAGGAGTAG	62°C

APC	F-AAAGTGAGCAGCTACCACGG R-CCTGGAGTGATCTGTTAGTCG	58°C
BRCA1	F-GGATTTTCGGGTTCACTCTG R-CCAAAAGGAGCCTACAAGAAAG	58°C
c-MYC	F-TGAGGAGACACCGCCCAC R-CAACATCGATTTCTTCCTCATCTTC	56°C
GAPDH	F- CCACTCCTCCACCTTTGAC R- ACCCTGTTGCTGTAGCCA	56°C

3.Conclusão

Observamos que a aplicação de 5-aza-2CdR desmetilou parcialmente o gene *MGMT*, além disso, aumentou a expressão de três importantes genes supressores de tumor e promoveu a regulação negativa do oncogene *c-Myc*.

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Anexo 1

Submission Confirmation

Thank you for your submission

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Title

5-Aza-2CdR Promoted MGMT Demethylation and Modified Expression of Different Genes in SCC9 Cells

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