

## Modulation of MGMT and CD133 Expression in HCT15 Colon Cancer Cells Exposed To 5-Fluorouracile versus HCT15 Cancer Stem Cells

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**Abstract:** Colorectal cancer (CRC) is one of the cancers with more prevalence, incidence and mortality. The chemo resistance promotes its reappearance which has been related to a small subpopulation designated cancer stem cells (CSCs) associated to CD133 biomarker. Previous studies have related this chemoresistance to DNA repairing systems such as MGMT. The aim of this study is to compare the modulation of MGMT and CD133 expression in CSCs and CRC cells exposed to 5-FU chemotherapy. HCT15 resistant CRC cell line was used in the assays. Methylation status of both MGMT and CD133 promoters in HCT15 CRC cells was carried out by methylation-specific PCRs (MSP). CD133 and MGMT expression before and after 5-FU exposure was determined by reverse transcription-PCR. In addition, HCT15 cells were submitted to CSC enrichment medium to obtain CSCs. A comparative study of the expression of CD133 and MGMT expression was carried out between HCT15 cells and CSCs. Our results showed a CD133 increase in HCT15 resistant cells when were treated with 4xIC50 5FU. This increase maintained over time. By contrast, CD133 expression in CSCs decreased after 4 days in culture. Interestingly, only HCT15 cells treated with 5-FU showed a significant modulation of MGMT expression. Thus, both MGMT and CD133 biomarkers modulated in HCT15 colon cancer resistant cells in a different way depending on the use of high 5-FU doses or CSCs enrichment.

**Keywords:** Colorectal cancer, MGMT, CD133, cancer stem cells, chemoresistance.

### INTRODUCTION

Colorectal cancer (CRC) is the third most frequent cancer worldwide [1]. Treatment of CRC is based in resection by surgery combined with adjuvant therapy which normally includes 5-fluorouracile (5-FU) and radiotherapy [2].

5-FU induces a thymidylate synthase inhibition enhancing the incorporation of UTP and FdUTP into DNA. This effect promoting mRNA and protein synthesis inhibition and, finally, induces DNA breaking [3]. However, part of CRC patients does not response to 5-FU treatment. This fact could be due to a small fraction of cells named cancer stem cell (CSC) which shares properties with stem cells such as capacity of self-renewal and presence of specific biomarkers [4]. Previous studies have isolated CSCs using different biomarkers although the most widely biomarker used for this cellular population is CD133, a transmembrane glycoprotein related to cell-cell interaction and signal transduction [5]. The CD133 gene consists in 37 exons located in the chromosome 4 and has 152 kb length with 9 different exons. The resulting translated

glycoprotein has 865 amino acids with a weight about 120 kDa [6]. However, the role of CD133 in CRC has not yet been elucidated. On the other hand, a DNA repair mechanism related to CSC is O6-methylguanine-methyltransferase (MGMT) which avoids DNA alkylation damage [7]. MGMT prevents cell death due to cytotoxic drugs by repairing DNA, but it can be silenced by epigenetic methylation [8]. Some studies suggest that MGMT methylation may also be involved in CRC carcinogenesis [9]. At the moment, the relation between the exposure to high 5-FU doses and the selection of CSCs from CRC is not clear. In addition, it is unknown if the CSCs from CRC show similar biomarkers modulations that cancer cells selected by the presence of a drug such as 5-FU. The aim of our work is to compare both MGMT and

CD133 expression in CRC cells exposed to a high 5-FU dose and in CSCs.

## MATERIALS AND METHODS

### Cell lines and drugs and reagents

Human HCT15 cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were maintained in culture with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Lonza, Walkersville, MD, USA) and 1% of antibiotics mixture of penicillin (10,000 U/mL) and streptomycin (10 mg/mL) in an humidified 5% CO<sub>2</sub> incubator at 37 °C of temperature. All chemicals used, including 5-FU, were of analytical high quality from Sigma-Aldrich Chemical Co. (Spain).

### DNA extraction and methylation-specific PCR

DNA was extracted from culture cells using QIAamp DNA Mini Kit (EpiTect Bisulfite kit, Qiagen, USA, Maryland) in accordance with the manufacturer's standard recommendations. DNA from each cell line (2 µg) was denatured, modified, and purified by EpiTect Bisulfite kit (Qiagen, USA, Maryland). MGMT and CD133 promoter CpG islands methylation status of different cell lines was based on the chemical modification by bisulfite of methylated cytosine to uracil. Methylation-specific PCRs (MSP) were performed with specific primers for either methylated or unmethylated DNA in MGMT and CD133 promoter. Primer sequences for MGMT were 5'-TTTGTGTTTGTATGTTTGTAGGTTTTTGT-3' (forward primer) and 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (reverse primer) for the unmethylated (UM) reaction and were 5'-TTTCGACGTTCTAGGTTTTTCGC-3' (forward primer) and 5'-GCACTCTTCCGAAAACGAAACG-3' (reverse primer) for the methylated (M) reaction. Primer sequences for CD133 were 5'-TTCGGGATAGAGGAAGTCGTAA-3' (forward primer) and 5'-CTCCC GCCCTAATCACC GCT-3' (reverse primer) for unmethylated reaction and 5'-TTTGGGATAGAGGAAGTTGTAA-3' (forward primer) and 5'-CTCCCACCCTAATCACC ACT-3' (reverse primer) for methylated reaction. Agarose electrophoresis, visualization by ethidium bromide and UV illumination for PCR amplified products were performed. For both biomarkers, samples were classified as hyper methylated (M) when only observed methylation amplification product, partially methylated (UM/M) when both unmethylated and methylated amplification products were seen, and unmethylated when it showed unmethylated amplification (UM) products alone.

### Exposure of HCT15 cells to 5-fluorouracil.

To determine 5-FU IC<sub>50</sub>, HCT15 cells were seeded in 96 well plates in 200 µL/well of DMEM and incubated overnight. Then, 5-FU treatment during 72

hours was administered at different concentrations. Untreated cells were considered as control. Cells were exposed to 20 µL/well of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (10 mg/ml) during 4 hours after 5-FU treatment. The supernatant were removed and crystals were dissolved by 200 µL of DMSO. The absorbance was measured at 570 nm using Ascen Software Multiskan\* EX Microplate Photometer (Thermo Scientific, Spain). The percentage of relative inhibition (RI, %) was obtained.

### Cancer stem cell enrichment and culture

Sub-confluent cultures in maintaining-standard conditions were detached through soft conditions by PBS-EDTA at 37°C for 10 min, washed twice in PBS and transferred to CSC enrichment conditions following Hu K *et al.* [10]. CSC enrichment was carried out using serum-free RPMI-1640 medium supplemented with 2 mM glutamine, 4 ng/mL bFGF, 10 ng/mL EGF and insulin (10 µg/mL)/transferrin (5.5 µg/mL)/sodium selenite (5 ng/mL) in an humidified 5% CO<sub>2</sub> incubator at 37 °C of temperature were performed.

### MRNA expression analysis

Total RNA was extracted using mechanical homogenization by syringe in TRIzol<sup>®</sup> solution and RNA purification system (RNeasy, Qiagen, USA, and Maryland). RNA integrity number (RIN) was determined using an Agilent BioAnalyzer 2100 with the RNA 6000 Nano Assay (Agilent Technologies, Las Rozas, and Spain). A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC) was used for RNA quantification. Reverse transcription-PCR process was performed with 1,5 µg of isolated total RNA using high Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. MGMT and CD133 primers for RT-PCR were 5'-TCACGGCCAGTCCTCCGGAG-3' (forward primer) and 5'-GTTCCCCGTGCCGGCTCTTC-3' (reverse primer) for MGMT (259 bp) and 5'-CTGGGGCTGCTGTTTATTATTCTG-3' (forward primer) and 5'-ACGCCTTGCTTGGTAGTGTG-3' (reverse primer) for CD133 (336 bp). Relative expression in RT-PCR assays were obtained by densitometry through Quantity One software.

### STATISTICAL ANALYSIS

Statistical evaluations were carried out using SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA). For all tests, the level of statistical significance was set at  $p < 0.05$ . The experimental data were expressed as mean ± standard deviation (SD). The results were compared using the student's t-test.

## RESULTS AND DISCUSSION

### Methylation status basal-pattern of MGMT and CD133 promoter

We analyzed the methylation status of both MGMT and CD133 promoters in HCT15 colon cancer

cells using RT-PCR and MSP. HCT15 cells showed a partially methylated MGMT and Cd133 promoter (UM/M) (Fig. 1A).

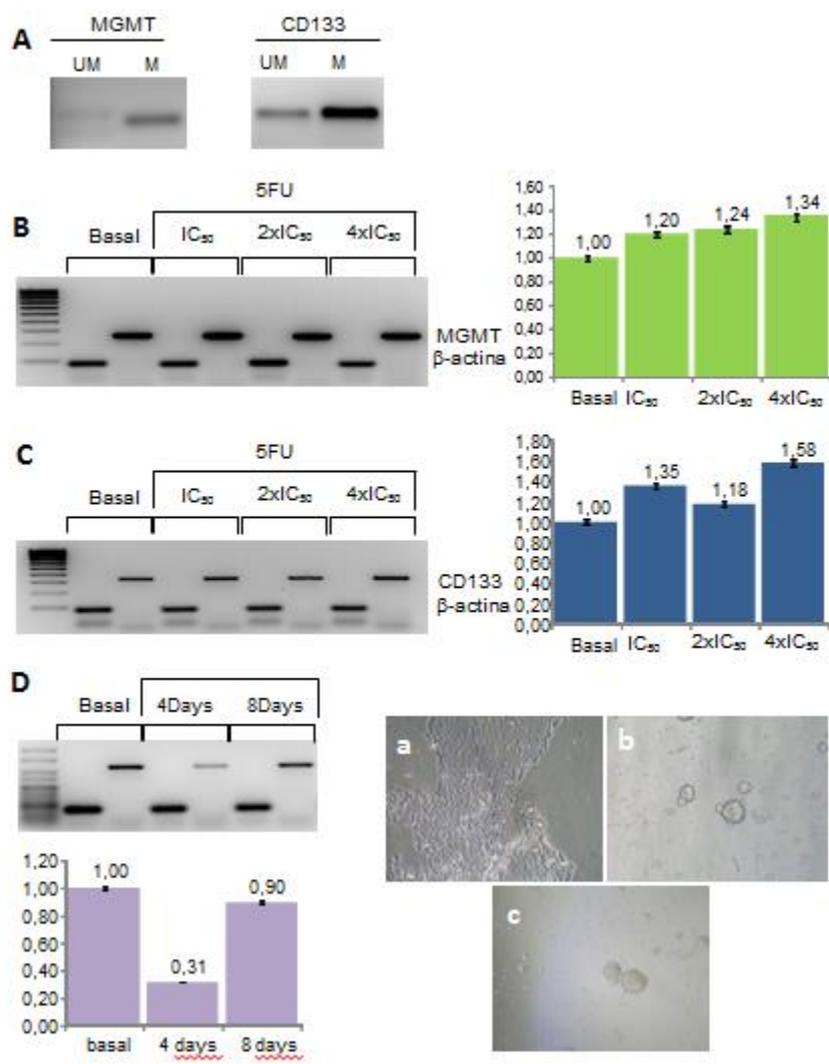
### MGMT and CD133 modulation by 5-fluorouracil treatment

To determine the 5-FU effect in the expression of MGMT and CD133 we detected the 5-FU IC<sub>50</sub> of HCT15 cells which was 8 μM. Next, we analyzed the modulation of mRNA expression after exposure to three 5-FU concentrations corresponding to IC<sub>50</sub>, IC<sub>50</sub> 2x and IC<sub>50</sub> 4x by PCR. Densitometry analysis of the relative MGMT expression showed a positive modulation of the MGMT expression after

exposure to 5-FU (Fig. 1B). In addition, a significant increase of CD133 expression was detected in HCT15 cells lines after 5-FU exposure (Fig. 1C).

### Modulation of MGMT and CD133 in CSCs

We obtained CSCc from HCT15 cells (Fig. 1D) to analyze the MGMT and CD133 modulation and to compare with 5-FU exposure (see Material and Methods). HCT15 sphere showed a low proliferation rate in relation to standard conditions (monolayer). As shown in Fig. 1C, HCT15 CSCs showed an increase of CD133 at four days of culture in suspension which recuperated at 8 days. By contrast, MGMT did not show significant changes (data no shown).



**Fig-1: Analysis of the HCT15 colon resistant cells. A, MGMT and CD133 promoter methylation status. Representative image of electrophoresis bands of methylation-specific PCR and reverse transcription PCR of both MGMT and CD133 biomarkers. Methylated samples showed amplification of both unmethylated (UM) and methylated (M) lanes. B, Representative electrophoresis of the MGMT mRNA expression after 5-FU exposure (IC<sub>50</sub>, 2xIC<sub>50</sub>, 4xIC<sub>50</sub>). Densitometric analysis (right). C, Representative electrophoresis of CD133 mRNA expression by 5-FU exposure (IC<sub>50</sub>, 2xIC<sub>50</sub>, 4xIC<sub>50</sub>). Densitometric analysis (right). D, Representative electrophoresis of the CD133 mRNA in CSCs from HCT15. Densitometric analysis (below). Representative images (right) of cells in monolayer (a) and CSCs enrichment and sphere cell formation at 4 (b) and 8 (c) days. Magnification x10.**

The 5-FU treatment of the CRC shows important lacks allowing its reappearance and progression probably because this treatment not considers the heterogeneity of tumor. The studies of the CSCs in this type of tumors show numerous evidences that these subpopulations, which characterized to have similar properties those stem cells [4], are related with the therapy failure [11]. The interaction between CSC-CD133+ and 5-FU treatment have been analyzed previously. Various studies have observed that the CD133+ population proliferate faster than CD133-under 5-FU treatment enriching CSC in CRC (DLD1) [12], hepatoma (BEL-7402) [13] and lung adenocarcinoma [14]. The effect of 5-FU in the repairing DNA systems such as MGMT have been analyzed. Previous studies in CRC cell lines (LoVo and RPMI-4788) showed that MGMT mRNA levels were slightly reduced after 5-FU treatment although the ineteracion between 5-FU and MGMT is not clear [15]. However, Sideris M *et al.* [16] recently showed no association between the status of MGMT expression and response to neo-adjuvant therapy in patients Thus, the association between the modulation of the MGMT protein expression and 5-FU treatment remains unknown. On the other hand, Pistollato F *et al.* [17] showed that glioblastoma multiform (GBM) cancer stem cell (CD133+) express high levels of MGMT in tumor mass. Moreover, He J *et al.* [18] showed that SHG-44 CSCs glioma cells exhibited a characteristic radio resistance associated to high MGMT mRNA levels. Further, Liu G *et al.* [19] showed that primary culture of CSC (CD133 +) displayed strong capability of chemo resistance relating this to higher expression of MGMT and BRCP1. However, some clinical studies did not appreciate relation between MGMT and CRC patients [20]. However, there is not a comparative study between the modulation that a drug such as 5-FU a high concentration may induce in the MGMT and CD133 expression in colon cancer cells and the expression of both markes in CSCs obtained from the same cell line. We use de resistant HCT15 colon cancer cells to develop an assay with high 5-FU concentration and to obtain a CSCs population. Our results showed that HCT15 colon cancer cells treated with 4xIC50 5FU induced an increase of CD133 which maintained over time whereas CD133 expression in CSCs decreased after 4 days in culture. In addition, the MGMT modulation only was observed when cells were exposed to 5-FU.

## CONCLUSION

The CSC enrichment by the 5-FU exposure may be indicative of a CSC proliferation facilitating reappearance and progression of disease. These results support the necessity of new-drugs which are able to eliminate CSC from patients being necessary more researches focusing to the heterogeneity of tumor.

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