

International Journal of **Cancer Research**

ISSN 1811-9727



Academic
Journals Inc.

www.academicjournals.com



Research Article

Role of Free Fatty Acid Receptor (FFAR3) in Growth and Proliferation of Colorectal Cancer Cell Line

¹Saeed Al Mahri, ²Amal Al Ghamdi, ¹Sameer Mohammad and ²Mohammad Azhar Aziz

¹King Abdullah International Medical Research Center [KAIMRC], Department of Experimental Medicine, National Guard Health Affairs, King Saud Bin Abdulaziz University for Health Sciences, P.O. Box 22490, 11426 Riyadh, Saudi Arabia

²King Abdullah International Medical Research Center [KAIMRC], Colorectal Cancer Research Program, National Guard Health Affairs, King Saud Bin Abdulaziz University for Health Sciences, P.O. Box 22490, 11426 Riyadh, Saudi Arabia

Abstract

Background and Objective: Short chain free fatty acids (SCFAs) like butyrate, propionate and acetate are produced by microbiota in the gut. SCFAs have been shown to exert their metabolic effects through their cognate receptors (FFAR2 and FFAR3). These receptors are abundantly expressed in colonic epithelium and several studies have shown that these receptors play an important role in the metabolic homeostasis of colonic epithelial cells and are possibly involved in colorectal carcinogenesis. This study was initiated to understand the role of FFAR3 gene in colorectal cancer cell growth and proliferation. **Materials and Methods:** The HCT116 colorectal cancer cell line was engineered to develop stable FFAR3 knockdown using shRNA mediated gene silencing technology. Down regulation of FFAR3 mRNA was confirmed by RT-PCR and consequences of FFAR3 down regulation on cell growth and proliferation were analyzed. **Results:** The data revealed that FFAR3 down regulation had no impact on proliferation and growth rate of engineered HCT116 cells. Moreover, FFAR3 knockdown cells did not show any difference in the glucose uptake rate or cyclic adenosine monophosphate (cAMP) levels as compared to control cells. The most likely explanation of lack of FFAR3 effect on HCT116 cell metabolism was that FFAR2 compensated for the loss of FFAR3 and maintained the functionality of SCFAs. **Conclusion:** This study showed that FFAR3 gene alone does not impact growth and cell proliferation of colorectal cancers. Further studies are needed to fully understand the role of FFAR3 by using double knockdown cells (FFAR2/FFAR3).

Key words: Free fatty acid receptors, colorectal cancer, HCT116, glucose uptake, cell proliferation, cAMP

Citation: Saeed Al Mahri, Amal Al Ghamdi, Sameer Mohammad and Mohammad Azhar Aziz, 2019. Role of free fatty acid receptor (FFAR3) in growth and proliferation of colorectal cancer cell line. *Int. J. Cancer Res.*, 15: 17-22.

Corresponding Author: Mohammad Azhar Aziz, King Abdullah International Medical Research Center [KAIMRC], Colorectal Cancer Research Program, National Guard Health Affairs, King Saud Bin Abdulaziz University for Health Sciences, P.O. Box 22490, 11426 Riyadh, Saudi Arabia Tel: +966 11 4294582

Copyright: © 2019 Saeed Al Mahri *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Colorectal cancer (CRC) is a disease that is intricately associated with the dietary patterns, metabolism and inflammation. The site of this cancer is also the location for processing of food aided by the gut microbiota. The effect of diet on CRC has been studied with different perspectives of associated factors. Metabolism of nutrients, role of gut microbiota and familial factors are being studied to better understand the causal factors in diet related initiation and progression of CRC. Type of food intake, its digestion and metabolism is an upcoming area of research with potential to develop preventive and therapeutic strategies. Probiotic cultures are helping the nutrition based prevention of symptoms in diseases like antibiotic associated diarrhea, lactose intolerance and irritable bowel syndrome. Characterization of gut microbiota with newer technologies is allowing the possibility of customized probiotic treatment for the prevention of colorectal cancer^{1,2}. Digestion of food results into formation of building blocks which are assimilated in the small intestine. Short chain fatty acids (SCFAs) are produced in the distal gut by bacterial fermentation of macro-fibrous material that escapes digestion in the upper gastrointestinal tract and enters the colon^{3,4}. The SCFAs have been implicated in prevention against colon carcinogenesis⁵. Butyrate and Propionate SCFAs are known to have antitumor effect via induction of apoptosis^{6,7}. Evidence indicating successful use of free fatty acids such as eicosapentaenoic acid-free fatty acid (EPA-FFA) in chemoprevention strategy against certain types of CRC⁸ generates further interest in studying their role in CRC. In addition to being an important source of fuel, SCFAs play key role as signaling molecules. SCFAs have been shown to activate free fatty acid receptor 2 (FFAR2) also known as GPR43 and Free Fatty acid 3 (FFAR3) also known as GPR41⁹. FFAR2 and FFAR3 belong to the family of G-protein coupled receptors (GPCRs) and are expressed in human and rodent colon suggestive of possible involvement in normal development and functions of colon tissue¹⁰⁻¹². Some reports have suggested that FFAR2 functions as a tumor suppressor in CRC¹³. Not much is known about the role of FFAR3 in regulating cancer cell metabolism and growth. The connection between dietary fibers, short chain free fatty acid receptors and colorectal cancer is an important area of investigation that can explain the mechanism by which diet influences the initiation and progression. To address this problem this paper focused on receptors for short chain free fatty acids. In this report, an effort attempted at creating a cell line model that can help decipher the role of FFAR3 gene in colorectal cancer is described. Engineered colorectal cancer cell line (HCT116) was generated to down regulate FFAR3

gene, which was validated by qRT-PCR. Functional analysis of these cells revealed no significant difference in the growth and proliferation. Also, cellular glucose uptake was not altered in these cells. cAMP level in the engineered cell line was measured and was found to be unaltered. These results suggested that FFAR3 alone does not control cellular growth and proliferation of colorectal cancer cell line HCT116.

MATERIALS AND METHODS

This work was carried out at King Abdullah International Medical Research Center after obtaining approval from institutional review board. This project was started in October, 2017.

Generation of HCT116/FFAR3 knockdown cells: Colorectal cancer cell line (HCT116) was procured from American Type Culture Collection (ATCC) and maintained in advanced DMEM in 5% CO₂ and 37 °C culture conditions along with fetal bovine serum (FBs). FFAR3 knockdown in HCT 116 cells was achieved using lentiviral based shRNA (Origene) and following the manufacturer's instructions. Four different clones of FFAR3 were used to knockdown shRNA and the best knockdown was chosen for further analyses. Selection was done using blasticidin antibiotic. Cells transduced with scrambled control vector were used as control in all experiments.

RNA extraction and cDNA synthesis: The HCT116 cells were infected with lentiviral particles containing shRNA against FFAR3 and the cell colonies were selected in cell culture media supplemented with blasticidin (1.6 μ mL⁻¹). Cell pellets of 5-10 × 10⁶ cells were collected in 1.5 mL eppendorf tubes each time it had a confluent culture dish. Pellets were stored at -80 °C until required. Total RNA was isolated from each sample using the PureLink RNA Mini Kit (Ambion by Life Technologies, USA), following the manufacturer's instructions. Single-stranded cDNA was prepared from 2.5 μg of total RNA in a 50 μL reaction volume using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's instructions. The 50 μL reaction was diluted 1:3 with nuclease-free water (Ambion, USA) and then stored at -20 °C until the qPCR analysis was performed.

Quantitative RT-PCR: The level of expression was determined by qRT-PCR using ABI7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). FFAR3 down regulation was confirmed by real time PCR using primers specific to human FFAR3. To this end, 2.5 μg of total RNA procured from the frozen pellets transcribed into cDNA using standard reagents and then used as template for PCR reaction

using specific primers for the candidate gene. PowerUp SYBR Green (Applied Biosystems™) was used for detection and analysis. Melting curve analysis done to assess the specificity of each amplified gene. All reactions performed in triplicate and the data analyzed using Relative Quantification (RQ) method by SDS RQ manager software. GAPDH (house keeping gene) expression was used as the endogenous control and the target gene was FFAR3.

Cell proliferation assay using xCELLigence: This instrument (RTCA-DP) is available from ACEA biosciences (San Diego, USA). E-16 plates were used for monitoring cell adhesion and growth while CIM plates were used to monitor cell invasion and migration patterns. This system works on the principle of electrical impedance. As given in the systems manual, a unitless parameter termed Cell Index (CI) is used to measure the relative change in electrical impedance to represent cell status. CI is a relative and dimensionless value since it represents the impedance change divided by a background value. When there were no cells present in the medium, the sensor's electronic property will not be affected and the impedance will be small. When there are more cells on the electrodes, the impedance will be larger. CI calculation is based on the following formula: $CI = (Z_i - Z_0) / 15 \zeta$, where Z_i is the impedance at an individual point of time during the experiment and Z_0 is the impedance at the start of the experiment. CI is a self-calibrated value derived from the ratio of measured impedances.

Glucose uptake assay: HCT 116 cells (control and FFAR3 knockdown cell lines) were plated on a 96 well plates (~30,000 per well). Next day, cells were washed twice with PBS and incubated in glucose free DMEM. Glucose uptake was determined using glucose uptake assay kit (Cayman Chemicals, MI, USA). Briefly, cells were incubated with $100 \mu\text{g mL}^{-1}$ fluorescent 2-NBDG in glucose free medium for 1 h. Cells were washed with assay buffer three times and analyzed immediately. 2-NBDG taken up by cells was detected on a Tecan Infinite 200 pro fluorimeter (Tecan Group Ltd. Männedorf, Switzerland) with fluorescent filters usually designed to detect fluorescein (excitation/emission = 485/535 nm). Cells without any 2-NBDG incubation were used as blank control. Statistical significance was determined by using paired students t-test.

cAMP assay: HCT 116 cells (control and FFAR 3 knockdown) were plated on a 48 well plates (~30,000 per well). Next day cells were incubated with 500 μM IBMX (3-isobutyl-1-methylxanthine) for 1 h. cAMP in each well was measured using cAMP Immunoassay System (Thermo-fisher scientific) by following the manufactures instructions. Statistical

significance was determined by using paired students t-test. Each bar represents an average of two independent experiments with 8 technical replicates in each experiment.

RESULTS

Loss of FFAR3 did not impact growth and proliferation of HCT 116 cells: shRNA mediated silencing of FFAR3 resulted in more than 70% down regulation of FFAR3 gene (Fig. 1a). The cell proliferation rate was determined using xCELLigence assay. There was no significant difference between the proliferation rates of controls HCT116 cells and FFFAR3 knockdown cells (Fig. 1b).

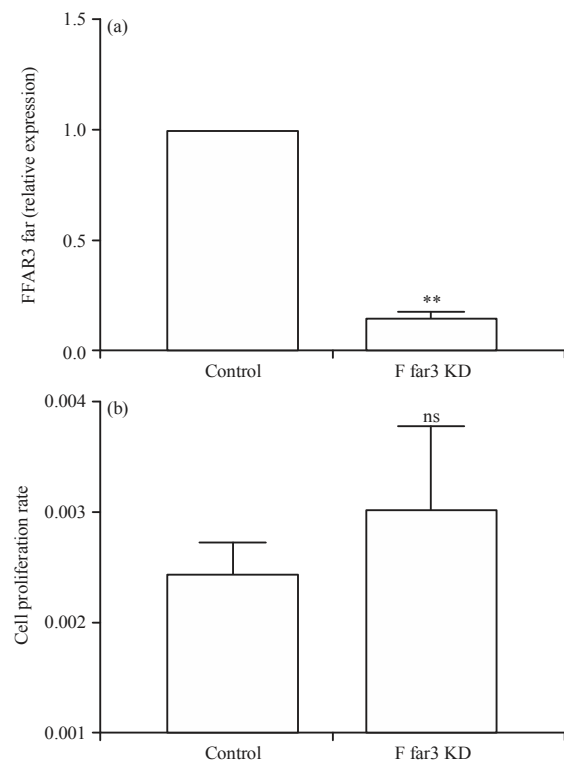


Fig. 1(a-b):FFAR3 down regulation does not change proliferation of HCT116 cells, (a) Real time PCR showing FFAR3 knockdown in HCT116 cells. FFAR3 knockdown cells were generated using lentiviral expression system as detailed in the Method section. The knockdown was confirmed by determining FFAR3 mRNA levels using quantitative RT-PCR. Each bar represents an average of at least three independent experiments. ** $p < 0.001$ and (b) Cell proliferation rates of control and FFAR3 knockdown HCT 116 cells. Proliferation rates of control and FFAR3 knockdown cells were determined by using xCELLigence system ns: Not significant

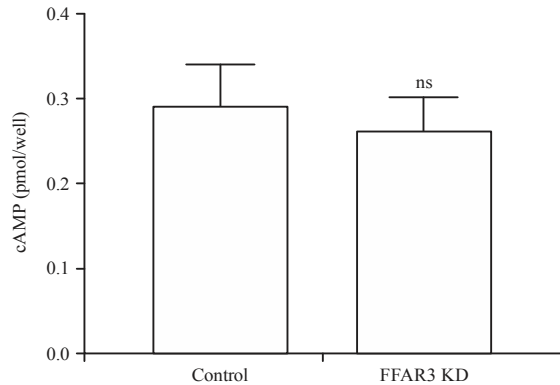


Fig. 2: Cellular cAMP levels in control and FFAR3 knockdown HCT116 cells

cAMP in control and FFAR3 knockdown cells was measured using cAMP Immunoassay System (Thermo-fisher scientific) by following the manufactures instructions, ns: Not significant

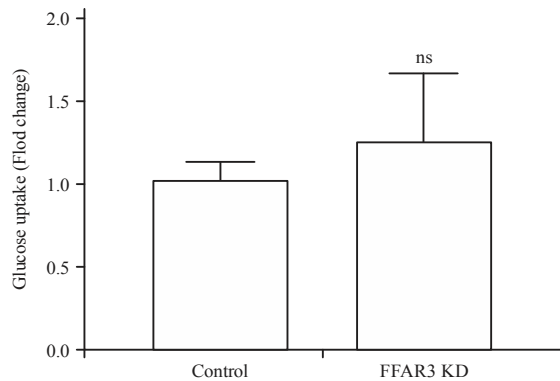


Fig. 3: Glucose uptake rates of control and FFAR3 knockdown HCT116 cells

Glucose uptake in control and FFAR3 knockdown cells was determined using Glucose uptake assay kit (Cayman Chemicals, MI, USA) as described in the method section, ns: Not significant

The FFAR3 is known to signal through G-Protein complex (Gi) by inhibiting adenylate cyclase and reducing levels of cAMP in the cell. Loss of FFAR3 would affect cellular cAMP levels. Surprisingly, there was no change in the cellular cAMP levels of control and FFAR3 knockdown cells (Fig. 2).

Loss of FFAR3 has no impact on glucose uptake rate of HCT116 cells: Cancer cells showed a high rate of glucose uptake that is necessary to support their rapid growth rate. Therefore, glucose uptake rates were measured in control and FFAR3 knockdown HCT116 cells and no significant difference was found (Fig. 3).

DISCUSSION

In this manuscript there was an attempt to study the role of FFAR3 in colorectal cancer cell line. Results suggest that FFAR3 alone did not affect the growth and proliferation of colorectal cell line HCT116. It is likely, that FFAR2, which shares considerable structural and functional homology compensated for the loss of FFAR3 and maintained the metabolism of HCT116 cells. Both FFAR2 and FFAR3 are activated by short chain free fatty acids (acetate, propionate and butyrate) and the functional redundancy is on expected lines¹⁴. These results suggested generating cell lines that will have down regulation of both FFAR2 and FFAR3 (double KD) and assess the impact of these receptors on the growth and proliferation of colorectal cancer cell lines. It has been recently reported that heterodimerization of FFAR2 and FFAR3 are required for short chain fatty acid sensing¹⁵.

The assays for measuring the impact of reduced levels of FFAR3 were well supported by previous reports where increased cAMP levels were shown to correlate with cancer cell growth and proliferation¹⁶⁻¹⁸. Real time measuring of cell growth and proliferation using xCELLigence has been successfully used earlier in colorectal cancer cells. Glucose uptake had been known to be increased in cancer cells and down-regulating an important mediator of increased cell proliferation was expected to show significantly higher glucose uptake which was not the case with FFAR3 alone¹⁹⁻²¹.

Taken together, previous studies on colorectal cancer patients²²⁻²⁴ also did not show any significant down regulation in FFAR3 gene, which supported our observations. But since it is an important mediator of short chain free fatty acid metabolism, it should be further studied along with FFAR2 to establish a crucial link between gut microbiota and colorectal cancer.

CONCLUSION

Short chain free fatty acid receptors could be crucial link between microbiota, dietary fibers and colorectal cancer. This report provide evidence that suggest change in expression level of FFAR3 is not alone in mediating increased proliferation of colorectal cancer cells.

SIGNIFICANCE STATEMENT

This study is significant in terms of addressing an important connection between short chain free fatty acids and

colorectal cancer. These results provide evidence that suggests the role of other receptor (FFAR2) either alone or in combination with FFAR3. Future work in this direction will help unravel the connection between dietary fibers, colorectal cancer and gut microbiota.

ACKNOWLEDGMENTS

This work was supported by a research grant awarded to MAA [Grant number [M-S-20-36] by King Abdul Aziz City for Science and Technology [KACST] in Riyadh, Saudi Arabia.

REFERENCES

1. Azcarate-Peril, M.A., M. Sikes and J.M. Bruno-Barcena, 2011. The intestinal microbiota, gastrointestinal environment and colorectal cancer: A putative role for probiotics in prevention of colorectal cancer? *Am. J. Physiol.-Gastrointest. Liver Physiol.*, 301: G401-G424.
2. Aziz, M.A., Z. Yousef, A.M. Saleh, S. Mohammad and B. Al Knawy, 2017. Towards personalized medicine of colorectal cancer. *Crit. Rev. Oncol./Hematol.*, 118: 70-78.
3. Cummings, J.H. and G.T. Macfarlane, 1997. Role of intestinal bacteria in nutrient metabolism. *JPENJ Parenter Enteral Nutr.*, 21: 357-365.
4. Russell, W.R., L. Hoyles, H.J. Flint and M.E. Dumas, 2013. Colonic bacterial metabolites and human health. *Curr. Opin. Microbiol.*, 16: 246-254.
5. Young, G.P., 1996. Prevention of colon cancer: Role of short chain fatty acids produced by intestinal flora. *Asia Pac. J. Clin. Nutr.*, 5: 44-47.
6. Matthews, G.M., G.S. Howarth and R.N. Butler, 2007. Short-chain fatty acid modulation of apoptosis in the Kato III human gastric carcinoma cell line. *Cancer Biol. Ther.*, 6: 1051-1057.
7. Gui, H. and Z. Shen, 2016. Concentrate diet modulation of ruminal genes involved in cell proliferation and apoptosis is related to combined effects of short-chain fatty acid and pH in rumen of goats. *J. Dairy Sci.*, 99: 6627-6638.
8. Piazza, G., G. D'argenio, A. Prossomariti, V. Lembo and G. Mazzone *et al.*, 2014. Eicosapentaenoic acid free fatty acid prevents and suppresses colonic neoplasia in colitis associated colorectal cancer acting on Notch signaling and gut microbiota. *Int. J. Cancer*, 135: 2004-2013.
9. Le Poul, E., C. Loison, S. Struyf, J.Y. Springael and V. Lannoy *et al.*, 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. *J. Biol. Chem.*, 278: 25481-25489.
10. Karaki, S.I., H. Tazoe, H. Hayashi, H. Kashiwabara, K. Tooyama, Y. Suzuki and A. Kuwahara, 2008. Expression of the short-chain fatty acid receptor, GPR43, in the human colon. *J. Mol. Histol.*, 39: 135-142.
11. Tazoe, H., Y. Otomo, I. Kaji, R. Tanaka, S.I. Karaki and A. Kuwahara, 2008. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. *J. Physiol. Pharmacol.*, 59: 251-262.
12. Kim, M.H., S.G. Kang, J.H. Park, M. Yanagisawa and C.H. Kim, 2013. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology*, 145: 396-406.
13. Pan, P., K. Oshima, Y.W. Huang, K.A. Agle and W.R. Drobyski *et al.*, 2018. Loss of FFAR2 promotes colon cancer by epigenetic dysregulation of inflammation suppressors. *Int. J. Cancer*, 143: 886-896.
14. Nohr, M.K., M.H. Pedersen, A. Gille, K.L. Egerod and M.S. Engelstoft *et al.*, 2013. GPR41/FFAR3 and GPR43/FFAR2 as cosensors for short-chain fatty acids in enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes. *Endocrinology*, 154: 3552-3564.
15. Ang, Z., D. Xiong, M. Wu and J.L. Ding, 2017. FFAR2-FFAR3 receptor heteromerization modulates short-chain fatty acid sensing. *FASEB J.*, 32: 289-303.
16. Iguchi, H., T. Mitsui, M. Ishida, S. Kanba and J. Arita, 2011. cAMP response element-binding protein (CREB) is required for Epidermal Growth Factor (EGF)-induced cell proliferation and serum response element activation in neural stem cells isolated from the forebrain subventricular zone of adult mice. *Endocr. J.*, 58: 747-759.
17. Vitali, E., V. Cambiaghi, A. Spada, A. Tresoldi and A. Zerbi *et al.*, 2015. cAMP effects in neuroendocrine tumors: The role of Epac and PKA in cell proliferation and adhesion. *Exp. Cell Res.*, 339: 241-251.
18. Sirotkin, A.V., A. Benco, A. Tandmajerova, M. Laukova and D. Vasicek *et al.*, 2018. cAMP response element-binding protein 1 controls porcine ovarian cell proliferation, apoptosis and FSH and insulin-like growth factor 1 response. *Reprod. Fertil. Dev.*, 30: 1145-1153.
19. Maddalena, F., G. Lettini, R. Gallicchio, L. Sisinni and V. Simeon *et al.*, 2015. Evaluation of glucose uptake in normal and cancer cell lines by positron emission tomography. *Mol. Imaging*, 14: 490-498.
20. Moon, S.H., S.J. Lee, K.H. Jung, C.H.T. Quach and J.W. Park *et al.*, 2016. Troglitazone stimulates cancer cell uptake of ¹⁸F-FDG by suppressing mitochondrial respiration and augments sensitivity to glucose restriction. *J. Nucl. Med.*, 57: 129-135.

21. Zhang, R., J. Li, X. Yan, K. Jin and W. Li *et al*, 2017. SODD promotes glucose uptake of colorectal cancer cells via AKT pathway. *Cell Biol. Int.* 10.1002/cbin.10907
22. Eldai, H., S. Periyasamy, S. Al Qarni, M. Al Rodayyan and S.M. Mustafa *et al*, 2013. Novel genes associated with colorectal cancer are revealed by high resolution cytogenetic analysis in a patient specific manner. *PLoS One*, Vol. 8. 10.1371/journal.pone.0076251.
23. Aziz, M.A., S. Periyasamy, Z. Al Yousef, I. AlAbdulkarim, M. Al Otaibi, A. Alfahed and G. Alasiri, 2014. Integrated exon level expression analysis of driver genes explain their role in colorectal cancer. *PLoS One*, Vol. 9. 10.1371/journal.pone.01110134.
24. Aziz, M.A., S. Periyasamy, Z. Yousef, A. Deeb and M. AlOtaibi, 2014. Colorectal cancer driver genes identified by patient specific comparison of cytogenetic microarray. *Genomics Data*, 2: 29-31.