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Effect of cyasterone on intestinal flora in a *BRAF*^{V600E}-mutant mouse model of colorectal cancer

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BRAF^{V600E}-mutated colorectal cancer (CRC) is very aggressive and responds poorly to standard treatment. In this study, *BRAF*^{V600E}-mutant mice with CRC were treated with intragastric cyasterone, a compound commonly used in traditional Chinese herbal medicine, for 21 days. Microbial DNA was extracted from mouse intestinal contents for 16S ribosomal RNA gene amplicon sequencing and analyzed. Our results indicated that cyasterone enhanced the diversity of the gut microbiota. The abundance of beneficial bacteria, such as Prevotellaceae, Muribaculaceae, and Ruminococcaceae was significantly higher in cyasterone-treated mice than controls. The abundance of Erysipelotrichaceae, a family of bacteria that promotes inflammation in the gut, was significantly positively correlated with tumor weight. Cyasterone is a potential inhibitor of *BRAF*^{V600E}-mutant CRC *via* its effects on intestinal flora.

1. Introduction

Worldwide, colorectal cancer (CRC) ranks third in terms of incidence but second in terms of mortality (Sawicki et al. 2021; Chen et al. 2021). The incidence of CRC has been increasing in East Asian countries, including China (Bray et al. 2018). Among all cases of CRC-related morbidity and mortality worldwide, China accounts for 18.6% and 20.1% of cases, respectively, which ranks first in the world for both (Loupakis et al. 2015; Davies et al. 2002). CRC is associated with *KRAS/BRAF* gene mutations (Li et al. 2020; Bellio et al. 2021). Aberrations of the RAS-RAF-MAPK signaling pathway in *KRAS* gene mutation are well studied. RAS genes include *HRAS, KRAS*, and *NRAS*. *KRAS* mutations have been related to the human epidermal growth factor receptor (EGFR) cascade and development of CRC and non-small cell lung cancer (Román et al. 2018; Huang et al. 2021). In CRC, *KRAS* mutations are more common in adenocarcinoma and mucinous adenocarcinoma and mostly located in codons 12 and 13 (Krajnović et al. 2016). *RAF* is downstream of *RAS* and its activation has an important effect on the development of many cancers, including CRC. The RAF family includes serine/threonine kinases (ARAF, BRAF, and CRAF). *RAF* mutations are common in *BRAF* and widely detected at the V600E site (Tjensvoll et al. 2016; Lou et al. 2017). *KRAS/BRAF* mutations result in abnormal conduction of the RAS-RAF-MAPK pathway that leads to excessive cell proliferation and differentiation. This causes tumor induction and promotes tumor proliferation, invasion and metastasis. Therefore, *KRAS/BRAF* mutations affect CRC treatment response (Guan et al. 2013). Currently, vemurafenib and dabrafenib are used to treat more than 80% of *BRAF*^{V600E}-mutant melanomas (Patel et al. 2020). However, the inhibition rate for *BRAF*^{V600E}-mutant CRC is less than 5%. *BRAF*^{V600E}-mutant CRC is difficult to treat and has a poor prognosis (Ducreux et al. 2019).

Human intestinal microbial flora has been associated with CRC development and treatment response (Iida et al. 2013; Feng et al. 2015; Dai et al. 2018; Tilg et al. 2018; Han et al. 2020). A recent study has shown that the intestinal flora significantly differs between patients with *BRAF*^{V600E}-mutant CRC and those with other CRC types (Trivieri et al. 2020). Drugs that improve the intestinal flora of patients with *BRAF*^{V600E}-mutant CRC may provide clinical benefit. Cyasterone is a compound isolated from *Ajuga decumbens* Thunb (Labiatae) or *Cyathula officinalis* Kuan that is used in traditional Chinese herbal medicine (Zhou et al. 2015; Zeng et al. 2000). Cyasterone has several pharmacological activities, including anticancer and anti-osteoporosis effects (Takasaki et al. 1999; Mamadalieva et al. 2013; Lu et al. 2016; Pu et al. 2019). This study aimed to examine the effects of cyasterone on intestinal flora and *in vivo* tumor suppressor activity in a *BRAF*^{V600E}-mutant mouse model of CRC.

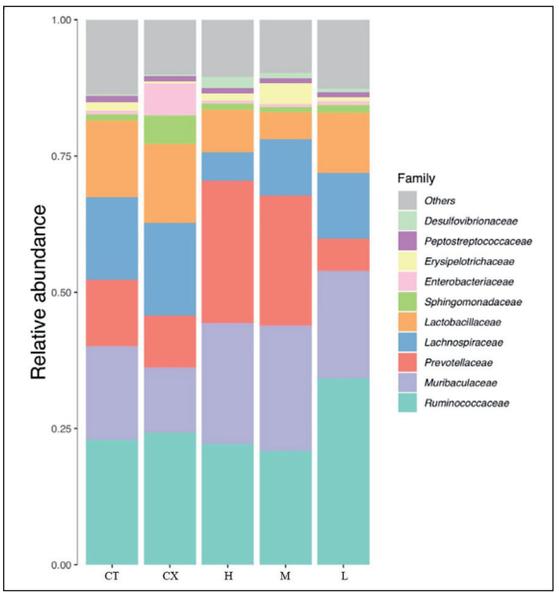

Fig. 1: Effect of cyasterone on bacterial distribution at the level of family,

2. Investigations, results and discussion

A total of 574,077 sequences were obtained through 16S ribosomal RNA high-throughput sequencing analysis. After quality control, 512,198 high-quality sequences were available and an average of 34,146 sequences were available per sample analysis. At the phylum level, the dominant microbiota were Bacteroidetes and Firmicutes; the proportions of Proteobacteria and Teneribacteria significantly differed between the experimental groups. As shown in Fig. 1, at the family level, the abundance of Prevotellaceae was significantly higher in the high-dose and medium-dose cyasterone groups. The abundance of Lachnospiraceae and cyasterone dose were negatively correlated. The abundance of Muribaculaceae was significantly higher in the cyasterone treatment groups.

Bacterial community diversity was measured using the Simpson and Shannon index. As shown in Fig. 2, the α-diversity of the cyasterone treatment group was significantly different from the control group and the positive group. In the treatment groups, cyasterone concentration and α-diversity were significantly negatively correlated.

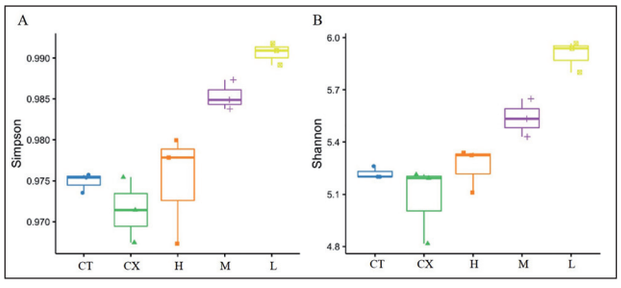

Fig. 2: Effect of cyasterone on alpha-diversity analysis of the gut microbiota (A) simpson, (B) Shannon.

β-Diversity analysis results were obtained after weighting for species abundance data. As shown in Fig. 3, β-diversity was similar between the control group and cetuximab group, and slightly different between the high- and medium-dose groups. However, β-diversity was significantly different between the low-dose group and all other groups.

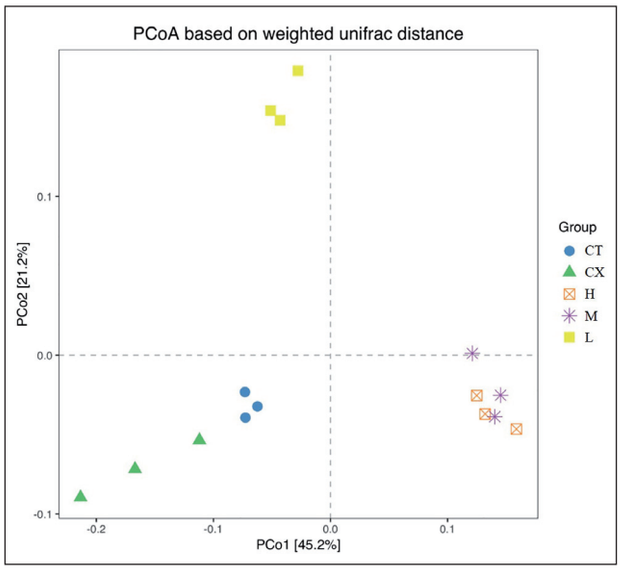

Fig. 3: Effect of cyasterone on beta-diversity analysis of the gut microbiota.

Random Forest analysis and difference testing were used to examine microbial biomarkers according to group. The results were judged by decline in Gini index. As shown in Fig. 4, Prevotella-9 had a greater impact, and its abundance was significant among groups. Its abundance was higher in the high- and medium-dose cyasterone groups than the others.

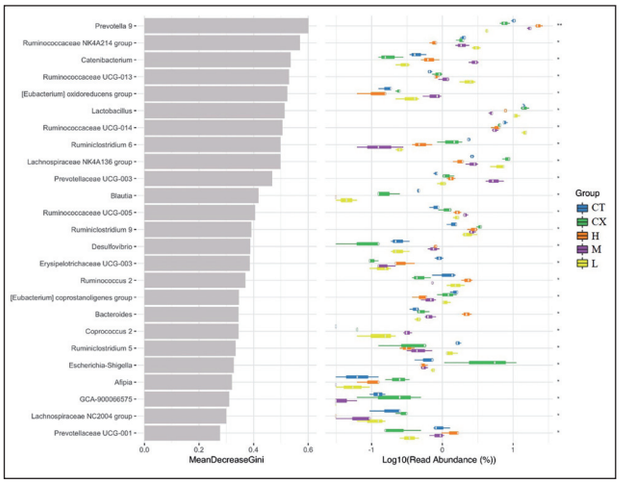

Fig. 4: Random Forest differential species analysis after treated with cyasterone.

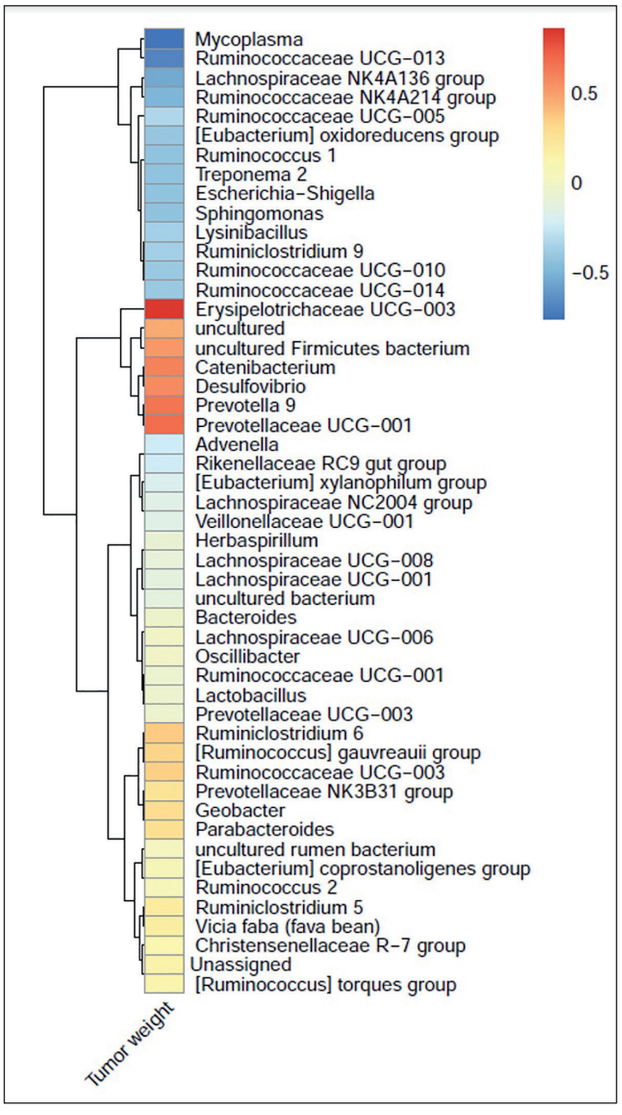

Fig. 5: Correlation analysis between tumor weight and microbiota after treated with cyasterone.

Fig. 5 shows the correlation analysis between tumor weight and bacterial genus. Erysipelotrichaceae and tumor weight were significantly positively correlated, which is consistent with the differential sample analysis results.

Intestinal flora is an important part of the microbiota ecosystem in the human gut. The gut is colonized by 10^{14} microbes, which is ten times the number of cells in the human body. The intestinal flora provides essential nutrients, synthesizes vitamin K, digests cellulose, and promotes angiogenesis and enteric nerve function. When the intestinal microbial composition changes because of antibiotic use, disease, stress, aging, poor diet, or lifestyle change, harmful effects on health may occur (Zhang et al. 2015). Dysbiosis of the intestinal flora community may cause chronic diseases such as inflammatory bowel disease, obesity, cancer, and autism (Guarner et al. 2003; Mueller et al. 2006).

Naturally occurring cyasterone has many biological activities. This study examined its role in regulating the intestinal flora of *BRAF*^{V600E}-mutant mice with CRC by analyzing microbial DNA extracted from the mouse intestinal contents using 16S ribosomal RNA gene amplicon sequencing. Our results showed that the abundance of Prevotellaceae and Muribaculaceae family bacteria, which are beneficial, was significantly higher in the cyasterone-treated mice. The α-diversity and β-diversity analyses indicated that cyasterone had an impact on bacterial community structure. In previous studies, the gut abundance of Erysipelotrichaceae was higher in human CRC patients than healthy controls (Chen et al. 2012) and significantly higher in the tumor group of a rat model of 1,2-dimethylhydrazine-induced colon cancer (Zhu et al. 2014). In our study, Erysipelotrichaceae was significantly positively correlated with tumor weight. Cyasterone may regulate the composition of intestinal flora by inhibiting *BRAF*^{V600E}.

In conclusion, our findings suggest that cyasterone may significantly affect the intestinal flora composition of *BRAF*^{V600E}-mutant mice, which then affects their physiological state.

3. Experimental

3.1. Animals and reagents

BRAF^{V600E} spontaneous tumor model mice were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China). The mice were aged 4 to 6 weeks and weighed 18 to 20 g. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Sichuan University. Cyasterone was obtained from Dalian Meilun Biotech Co., Ltd (Dalian, China). Cetuximab was purchased from MCE (NJ, USA). ZymoBIOMICS DNA microprep kit was supplied from Zymo Research (Irvine, CA, USA).

3.2. Effect of cyasterone on intestinal flora in BRAF^{V600E} spontaneous tumor model mice

One week after adaptation, 15 mice were randomly assigned to five groups, named the control group (CT), cetuximab group (CX), high-dose cyasterone group (H), medium-dose cyasterone group (M) and low-dose cyasterone group (L). The control group received intragastric administration 400 µl of sterilized water daily. Cetuximab (1 mg/kg) was administered by intraperitoneal injection in a volume of 200 µl three times a week. The high-, medium- and low-dose of cyasterone groups were administered 15 mg/kg, 10 mg/kg and 5 mg/kg cyasterone by oral gavage every day, respectively. After 3 weeks, the mice were sacrificed and the tumors were removed and weighed. Total DNA extraction was performed on the colon contents. Polymerase chain reaction was then performed using16S ribosomal RNA V4 region primers (ABI 9800 Fast Thermal Cycler; ThermoFisher, Waltham, MA, USA) to obtain amplicon fragments. Then, the NEB DNA fragment construction kit (New England Biolabs, Inc., Ipswich, MA, USA) was used to construct a 350bp sequencing library. Sequencing was performed in PE250 mode on a 2500 high-throughput sequencer (Illumina, San Diego, CA, USA). The data were subjected to quality control and microbial operational taxonomic unit (OTU) clustering using open-source Usearch (www.drive5.com/usearch) and QIIME (www.qiime.org) software. Based on the existing OTU representative sequences, the on-line SILVA database was used to classify species classification information and reconstruct the evolutionary tree. Finally, abundance information, community composition, α-diversity, β-diversity, correlation, and differential species analysis at each classification level were calculated.

Author contributions: Yongling Gong and Yi Luo contributed to the study conception and design. Ying Xiong, Xinyue Zheng, and Yongmei Xie participated in data collection and interpretation. Ying Xiong, Xinyue Zheng, Yongling Gong, and Yi Luo analyzed data and drafted the manuscript. All authors reviewed and approved the final manuscript.

Ethical considerations: The study was approved by the Institutional Animal Care and Treatment Committee of Sichuan University.

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Conflicts of interest: None declared.

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