Inhibition of Endometrial Cancer by n-3 Polyunsaturated Fatty Acids in Preclinical Models

Hang Zheng1, Hongjun Tang1, Miao Liu1, Minhong He1, Pinglin Lai2, Heling Dong2, Jun Lin2, Chunhong Jia2, Mei Zhong3, Yifan Dai4, Xiaochun Bai2, and Liping Wang3

Abstract

Although preclinical and epidemiologic studies have shown the importance of n-3 polyunsaturated fatty acids (PUFA) in the prevention of hormone-responsive cancers such as breast cancer, evidence of the association between n-3 PUFAs and endometrial cancer risk is limited and no previous study has examined the effect of n-3 PUFAs on endometrial cancer in cellular and animal models. In this study, we demonstrated that docosahexenoic acid (DHA) dose- and time-dependently inhibited endometrial cancer cell proliferation, colony formation, and migration and promoted apoptosis. Dietary n-3 PUFAs efficiently prevented endometrial cancer cell growth in xenograft models. Moreover, ectopic expression of fat-1, a desaturase, catalyzed the conversion of n-6 to n-3 PUFAs and produced n-3 PUFAs endogenously, also suppressed endometrial tumor cell growth and migration, and potentiated apoptosis in endometrial cancer cell lines. Interestingly, implanted endometrial cancer cells were unable to grow in fat-1 transgenic SCID mice. Further study revealed that mTOR signaling, which plays an essential role in cell proliferation and endometrial tumorigenesis, is a target of n-3 PUFAs. Exogenous or endogenous n-3 PUFAs efficiently suppressed both mTOR complex 1 (mTORC1) and mTORC2 in vitro and in vivo. Moreover, both dietary n-3 PUFAs and transgenic expression of fat-1 in mice effectively repressed mTORC1/2 signaling and endometrial growth elicited by unopposed estrogen. Taken together, our findings provide comprehensive preclinical evidences that n-3 PUFAs efficiently prevent endometrial cancer and establish mTORC1/2 as a target of n-3 PUFAs. Cancer Prev Res; 7(8); 1–11. ©2014 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy and a major cause of morbidity and mortality in women worldwide, with nearly 200,000 cases diagnosed every year and a rising incidence in postmenopausal women (1–4). Although the precise cause of endometrial cancer is unknown, various associated risk factors for the disease have been identified. The main risk factors for the development of endometrial carcinoma are obesity and chronic unopposed estrogen stimulation of the endometrium (5–7). Previous publications demonstrated that parity, oral contraception, body mass index, physical activity, and diet may explain up to 80% of the risk of endometrial cancer, emphasizing the importance of lifestyle modification for prevention of this disease (8). On the other hand, although it is highly treatable by surgery when diagnosed at an early stage and grade, therapies for advanced and recurrent disease are rarely curative (1, 9, 10). Currently, the treatment of metastatic or recurrent disease is based on conventional chemotherapy combination regimens (11). Advances in the understanding of the molecular pathology of endometrial carcinoma have lead to the development and testing of targeted therapies (12, 13). Of the potential therapeutic targets identified to date, the mTOR signaling pathway is a major target for treatment of this disease (14).

mTOR is a highly conserved Ser/Thr kinase that integrates diverse signals, including nutrients, growth factors, energy, and stresses to control cell growth, proliferation, survival, and metabolism (15–18). mTOR elicits its pleiotropic functions in the context of two functionally distinct signaling complexes, termed mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 plays a key role in translation initiation by directly phosphorylating p70 S6 kinase 1 (S6K1) and 4E-BP1, and is sensitive to rapamycin. mTORC2 is not susceptible to acute rapamycin inhibition (17, 18). The function of mTORC2 is less clear, but it has been shown to phosphorylate Akt (S473) and to regulate cell survival and cell motility (15, 18). As a critical drug target, mTOR signaling is upregulated in endometrial cancer and plays key
roles in the carcinogenesis and progression of the disease (19). However, clinical trials have shown that responses to indirect (allosteric) mTORC1 inhibitors are modest, which, in part, relate to positive feedback from mTORC2 on the Akt pathway that can continue following inhibition of mTORC1 (20). Therefore, second-generation catalytic mTORC1/2 inhibitors that can act directly on mTOR are being developed and have entered clinical trials (21, 22).

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA) are essential fatty acids necessary for human health. Laboratory and animal studies have shown that the long-chain n-3 PUFAs, eicosapentenoic acid (EPA), and docosahexenoic acid (DHA), inhibit tumorigenesis at various cancer sites (23). n-6 PUFAs such as arachidonic acid, on the other hand, have been shown to promote tumor growth and progression (24). Total fat intake and the ratio of n-6 to n-3 PUFAs in the Western diet have increased significantly since the Industrial Revolution, which is thought to contribute to obesity, inflammation, and cancer (24). Studies in human populations have linked high consumption of fish or fish oil (n-3 PUFAs) to a reduced risk of colon, prostate, and breast cancer, and combined with the demonstrated effects of n-3 PUFAs on cancer from preclinical (animal and in vitro) models, has motivated the development of clinical interventions using n-3 PUFAs in the prevention and treatment of these cancers (25, 26).

Although the role of dietary PUFAs in various cancers has received significant attention in the literature, and the preventive effect of n-3 PUFAs on obesity has been established (27), evidence on the association between n-3 PUFAs and endometrial cancer is limited. A recent epidemiologic study suggests that higher dietary intake of EPA and DHA in food and supplements were associated with lower risk of endometrial cancer (28). No previous study has examined the effect of n-3 PUFAs on endometrial cancer cell and animal models. We previously developed a transgenic mouse model that expresses fat-1 cDNA into the RAPAd CMV Adenoviral Expression System (Cell Biolabs).

Cell culture

Endometrial cancer cell lines HEC-1-A, HEC-1-B, and RL95-2 were obtained from ATCC. Cell lines were frozen in bulk when received and maintained in McCoy’s 5A (HEC-1-A) or minimum essential medium (MEM) (HEC-1-B) or D-MEM/F-12 (RL95-2) supplemented with 10% FBS at 37°C, 5% CO2, and 95% humidity. They had been passed for less than 6 months in culture when the experiments were carried out. Cell lines were authenticated using single tandem repeat analysis.

Wound healing assay

HEC-1-A and HEC-1-B cells were seeded in 12-well plates and DHA was added or the cells were transfected with recombinant adenovirus and grown until 80% confluent. The cells were then pretreated with 1 μg/mL of mitomycin C for 24 hours in a minimum medium (containing 0.5% FBS). After making a straight scratch using a pipette tip, the cells were incubated in medium containing 0.5% FBS in a 37°C humidified incubator for 48 hours and the wound distances were measured under a microscope.

Transwell assay

The cell culture inserts were placed in a 24-well plate. Before use, the cell culture inserts were rehydrated with 200 μL warm McCoy’s 5A or MEM for 30 minutes. HEC-1-A and HEC-1-B cells were plated in the top chamber with 500 μL McCoy’s 5A or MEM containing various concentrations of DHA. The bottom chamber was filled with 800 μL McCoy’s 5A or MEM containing 10% FBS. The cells were then incubated at 37°C for 24 hours. After swabbing of non-invaded cells in the top chambers, cells that migrated to the bottom chambers were fixed with formaldehyde and stained with crystal violet. For quantification, the cells that had migrated to the bottom surface were counted under a light microscope.

Tumor xenograft models

Four-week-old female BALB/c nude mice were purchased from Guangdong Medical Experiment Animal Centre (Guangzhou, China). Each animal was injected with 1 × 10^6/0.1 mL of HEC-1-A or RL95-2 cell suspension into the flanks. The mice were randomized into four groups (n = 6) and administered a normal (low) or high n-3 PUFAs diet (Supplementary Table S1). For cisplatin (DDP) combination therapy, cisplatin (50 μg per mouse) in 0.25 mL of saline was injected intraperitoneally once weekly. Bidimensional tumor measurements were taken every 3 days. At the end of the experiment, the mice were killed and tumors were removed, weighed, and the proteins extracted for analysis. Tumor volume was measured along two major axes using calipers. Tumor volume (mm^3) was calculated as follows: V = 1/2LW^2 (L, length; W, width).
Inhibition of Endometrial Cancer by n-3 PUFAs

A. HEC-1-A

- Con
- 12.5 μmol/L
- 25 μmol/L
- 50 μmol/L

B. HEC-1-B

- Con
- 12.5 μmol/L
- 25 μmol/L
- 50 μmol/L

C. RL95-2

- Con
- 12.5 μmol/L
- 25 μmol/L
- 50 μmol/L

D. HEC-1-A

- Colony formation (% control)

E. HEC-1-B

- Colony formation (% control)

F. RL95-2

- Colony formation (% control)

G. HEC-1-A

- Tumor volume (mm³)

- Tumor weight (mg)

H. RL95-2

- Tumor volume (mm³)

- Tumor weight (mg)
Homozgyous SCID mice (Jax Number: 001131) (Balb/c background) were bred with fat-1 transgenic mice (originally on the C57BL/6 background) produced previously (29) to generate fat-1-SCID double-hybrid mice. These mice were backcrossed for 10 generation onto Balb/c. Female littermates lacking the fat-1 transgenic gene were used as controls. DNA extractions from the tail tips of offspring were subjected to PCR for genotyping in accordance with the protocol on the Jackson Laboratory webpage, using primers listed in Supplementary Table S2. Four-week-old female homozygous SCID mice (n = 6) with or without the fat-1 gene were injected with 100 μL (1 × 10^5) of RL95-2 cell suspension. Subsequent bidimensional tumor measurement and tumor sample analysis were performed as described above.

Tamoxifen-elicited endometrial growth

The mouse model of endometrial growth induced by unopposed estrogenic stimulation was established as described previously (32). Briefly, ten-week-old Balb/c mice underwent midline laparotomy and bilateral oophorectomy and were randomly assigned (n = 8) to a normal (low), high n-3 PUFAs, or high n-6 PUFAs (6 g/kg/d arachidonic acid) diet. One week after recovery, the mice received saline or 1 or 3 mg/kg/d tamoxifen citrate with the normal, high n-3 PUFAs diet. To investigate the potential protective role of n-3 PUFAs on endometrial cancer growth in vivo, we established endometrial tumor xenograft models by subcutaneously implanting HEC-1-A and RL95-2 cells into nude mice. We found that high n-3 PUFAs diet efficiently prevented tumor growth and reduced the average tumor weight and tumor volume (Fig. 1G and H). Fatty acid composition analysis identified the significantly increased ratio of n-3/n-6 PUFAs in the tumors of mice with high n-3 PUFAs diet compared with that of mice with normal diet (Supplementary Table S3). Interestingly, high n-3 PUFAs diet could also enhance the inhibitory effect of cisplatin, a cytotoxic antiendometrial cancer drug (Supplementary Fig. S3). These results indicate that dietary n-3 PUFAs suppress endometrial cancer cell growth in vivo.

Endogenously produced n-3 PUFAs inhibit endometrial cancer cell growth in vitro and in xenograft models

Diet or nutritional supplements contain many nutrients and other components that may interact, which adds a layer of complexity to their evaluation. Transgenic expression of fat-1 is capable of converting n-6 to n-3 PUFAs, leading to an increase in n-3 PUFAs and a decrease in the n-6/n-3 ratio, and allows well-controlled studies to be performed in the absence of restricted diets (33). We further established endometrial cancer cell culture and tumor xenograft models producing endogenous n-3 PUFAs. First, proliferation and
colony formation of HEC-1-A, HEC-1-B, and RL95-2 cells transfected with recombinant adenovirus with or without the fat-1 gene (Ad-fat-1 or Ad) were assessed. As expected, both proliferation rate (Fig. 2A–C and Supplementary Fig. S4) and colony formation (Fig. 2D–F and Supplementary Fig. S5) of fat-1–expressing cells were significantly decreased compared with the control cells in all tested endometrial cancer cell lines.

Next, we established fat-1 transgenic SCID mice. Fatty acid composition analysis identified a significantly increased ratio of n-3/n-6 PUFAs in these mice compared with wild-type SCID mice lacking fat-1 expression (Supplementary Table S4). Interestingly, although xenograft tumors with an average volume of 223 mm$^3$ were observed within 3 weeks in control SCID mice, we failed to observe tumor growth in any of the fat-1 SCID animals (Fig. 2G and H). We suggest that endometrial tumor cells are unable to proliferate or survive in the presence of high levels of endogenously produced n-3 PUFAs in vivo. Taken together, these data unequivocally demonstrate that endogenously produced n-3 PUFAs suppress endometrial cancer cell growth in vitro and in vivo.

n-3 PUFAs prevent endometrial cancer cell migration

Metastasis is the major cause of mortality and morbidity in patients with endometrial cancer. Invasion of cancer cells into surrounding tissue and the vasculature is an initial step in tumor metastasis. This requires migration of cancer cells. To investigate the potential role of n-3 PUFAs in endometrial cancer cell migration, the effects of DHA on cell migration were examined using a wound-healing assay in serum-free medium. We found that 25 μmol/L of DHA-treated HEC-1-A and HEC-1-B cells filled the gap more slowly than vehicle control cells, suggesting that DHA prevented endometrial cancer cell migration (Fig. 3A and B and Supplementary Fig. S6). We further confirmed these results by the cell migration Transwell assay. Cells were
n-3 PUFAs promote endometrial cancer cell apoptosis in vivo and in vitro

n-3 PUFAs have been shown to promote apoptosis in a variety of cancer cells (26). We next examined whether n-3 PUFAs potentiate endometrial cancer cell apoptosis in vitro and in xenografts. It was found that DHA dose-dependently increased the number of dead cells in serum-starved HEC-1-A, HEC-1-B, and RL95-2 cell lines (Fig. 4A–C and Supplementary Fig. S7). Cleavage of PARP was enhanced by DHA in a dose- and time-dependent manner (Fig. 4D). DHA could also enhance the proapoptotic effect of cisplatin on HEC-1-A and RL95-2 cells (Supplementary Fig. S8). These results suggest that DHA promotes endometrial cancer cell apoptosis in vitro. The effect of n-3 PUFAs on apoptosis was further examined in xenograft models. The results revealed that administration of n-3 PUFAs promoted xenografted endometrial tumor cell apoptosis as manifested by the increased apoptotic cell numbers and enhanced cleavage of PARP in tumors in mice with high n-3 PUFAs diet (Fig. 4E). We conclude that n-3 PUFAs promote endometrial cancer cell apoptosis in vivo and in vitro.

n-3 PUFAs inhibit mTORC1/2 signaling in endometrial cancer cell lines and xenograft models

Previous studies have shown that activation of mTOR and phosphorylation of 4E-BP1 occur frequently in advanced stage and high-grade endometrial tumors, respectively, and
are associated with cancer progression and reduced survival (34). mTOR signaling plays important roles in tumorigenesis and progression of endometrial cancer and have revealed a clinical advantage in targeting this pathway (12, 14). We recently reported a critical role for n-6 PUFA-activated mTORC1/2 signaling in mammary tumorigenesis and angiogenesis (30). We then determined whether n-6 PUFAs stimulate mTORC1/2 activity in endometrial cancer cells. As expected, arachidonic acid (n-6 PUFA) acutely stimulated mTORC1-directed phosphorylation of S6 (S235/235) and mTORC2-directed phosphorylation of Akt at position Ser 473 (Fig. 5A). We next examined whether DHA inhibits mTORC1/2 in endometrial cancer cell lines. In HEC-1-A cells, DHA rapidly and dose-dependently suppressed arachidonic acid-stimulated phosphorylation of S6 (S235/235) and Akt (S473) (Fig. 5B). We further examined the role of endogenously produced n-3 PUFAs on mTORC1/2 signaling. In HEC-1-A cells transfected with fat-1 cDNA, the phosphorylation of S6 (S235/235) and Akt (S473) were significantly reduced compared with cells transfected with the control vector (Fig. 5C). These results were further repeated in HEC-1-B cells (Supplementary Fig. S9). It is suggested that both mTORC1 and mTORC2 signaling pathways are targets of exogenous and endogenous n-3 PUFAs in endometrial cancer cells.

We next determined whether n-3 PUFAs suppress mTORC1/2 in vivo. Interestingly, high dietary n-3 PUFAs repressed both mTORC1 and mTORC2 activities in the endometrial tumor xenograft model, as manifested by decreased phosphorylation levels of S6 (S235/235) and Akt (S473) in mice with a high n-3/n-6 PUFAs diet (Fig. 5D). Moreover, levels of phosphorylated S6 (S235/235) and Akt (S473) were lower in the livers of fat-1 SCID mice compared with wild-type mice (Fig. 5E). Importantly, PP242, an mTORC1/2 inhibitor, did not enhance the inhibitory effects of n-3 PUFAs on endometrial cancer cells (Fig. 5F and G). We suggest that mTORC1 and mTORC2 signaling are targets of n-3 PUFAs in vivo and the suppression of mTORC1/2 signaling by n-3 PUFAs may contribute to their inhibitory effects on endometrial tumor growth.
n-3 PUFAs repress the endometrial growth elicited by unopposed estrogenic stimulation in mouse model

Many known endometrial cancer risk factors are associated with unopposed estrogenic stimulation of the endometrium (35, 36). To elucidate the effect of PUFAs on the growth of endometrium in response to estrogen exposure, a mouse model of unopposed tamoxifen-elicited uterotrophy was established. Mice treated with tamoxifen had a significantly increase in uterine wet weights, luminal epithelial cell heights ($P < 0.05$), and phosphorylation of S6 (S235/236) and Akt (S473) in the endometrium compared with the vehicle-only group (Fig. 6A–D and Supplementary Fig. S10A). The high n-3 PUFAs diet decreased the uterine wet weights, epithelial cell heights, and the levels of P-S6 (S235/236) and P-Akt (S473; Fig. 6A–D and Supplementary Fig. S10A; Supplementary Table S5), whereas the high n-6 PUFAs (arachidonic acid) diet significantly increased the uterine growth in response to tamoxifen (Supplementary Fig. S10B and S10C). Furthermore, the prevention of endometrial growth and inhibition of mTORC1/2 by n-3 PUFAs was reproducible in fat-1 transgenic mice (Fig. 6E–G and Supplementary Fig. S10D). These data indicate that both dietary and endogenous n-3 PUFAs inhibit mTORC1/2 and prevent unopposed estrogen-elicited endometrial growth.

Discussion

Although n-3 PUFAs have been shown to prevent carcinogenesis and progression of hormone-responsive cancers such as breast cancer in vitro and in animal experiments, and epidemiologic studies suggest that higher dietary intake of n-3 PUFAs in food and supplements lowers the risk of these
cancers (23–26), evidence of the association between n-3 PUFAs and endometrial cancer risk remains sparse (28). Multiple case–control studies and cohort studies reported no association between total fish or polyunsaturated fats intake and endometrial cancer risk, but did not separately analyze n-3 and n-6 fatty acids (37–40). A nationwide case-control study in Sweden showed that the major dietary source of long-chain n-3 PUFAs, fatty fish consumption, lowers the risk of endometrial cancer (41). Another recent case-control study suggested that total fish intake is not associated with risk, but higher intakes of EPA and DHA or fish oil supplement use are significantly associated with reduced risk of endometrial cancer (29, 33). Using fat-1 transgenic mouse models, combined with a conventional dietary approach, our results unequivocally demonstrate that n-3 PUFAs efficiently inhibit endometrial carcinogenesis and tumor progression. Because the fat-1 mouse produces a mixture of different n-3 PUFAs, the in vivo function of individual n-3 PUFA, as suggested by animal and human studies. Since transgenic expression of fat-1 enables the host to produce n-3 PUFAs endogenously while concomitantly reducing the levels of n-6 PUFAs, the fat-1 transgenic mouse is capable of increasing n-3 content with a balanced n-6/n-3 PUFAs ratio in all tissues and allows carefully controlled studies to be performed in the absence of restricted diets (29, 33). Using fat-1 mouse and fat-1 transgenic SCID mouse models, combined with a conventional dietary approach, our results unequivocally demonstrate that n-3 PUFAs efficiently inhibit endometrial carcinogenesis and tumor progression. Because the fat-1 mouse produces a mixture of different n-3 PUFAs, the in vivo function of individual n-3 PUFA in suppression of endometrial cancer need to be further defined. Previous study has shown that DHA is a more potent inhibitor of breast cancer than EPA (44). The high ratio of DHA in total PUFAs of fat-1 mice (Supplementary Table S4 and S5) and our in vivo data that DHA effectively represses endometrial cancer cell growth, proliferation, and migration.

Although increasing evidence from animal and in vitro studies indicate that n-3 PUFAs present in fatty fish and fish oils inhibit the carcinogenesis of many tumors, inconsistencies remain (42, 43). Several factors may account for these inconsistent results, namely (i) wide variations in the amount and source (the type and even the bioavailability) of n-3 PUFAs consumed in each study; (ii) the ratio of n-6 to n-3 may be more important than the absolute amount of n-3 PUFA, as suggested by animal and human studies. Since transgenic expression of fat-1 enables the host to produce n-3 PUFAs endogenously while concomitantly reducing the levels of n-6 PUFAs, the fat-1 transgenic mouse is capable of increasing n-3 content with a balanced n-6/n-3 PUFAs ratio in all tissues and allows carefully controlled studies to be performed in the absence of restricted diets (29, 33). Using fat-1 mouse and fat-1 transgenic SCID mouse models, combined with a conventional dietary approach, our results unequivocally demonstrate that n-3 PUFAs efficiently inhibit endometrial carcinogenesis and tumor progression. Because the fat-1 mouse produces a mixture of different n-3 PUFAs, the in vivo function of individual n-3 PUFA, as suggested by animal and human studies.
and promotes apoptosis also implicate that DHA is the more bioactive component.

Endometrial cancer is a heterogeneous disease with distinct molecular characteristics. The most frequent aberration is activation of the PI3-K/mTOR pathway; however, the first generation mTOR inhibitors have been tested in clinical trials as single agents with only modest results (12, 14, 45). A significant problem in targeting mTOR with rapalogs is that these agents show partial inhibitory activity, which only block mTORC1 and have little effect on mTORC2, thereby increasing Akt activity. The development of mTORC1/2 kinase inhibitors that block mTORC1 and mTORC2 and consequent upregulation of Akt activity may obviate this issue. Our findings that n-3 PUFAs target to both mTORC1 and mTORC2 pathways may explain their strong inhibitory effects on endometrial cancer in vitro and in vivo. n-3 PUFAs, such as DHA, are able to target multiple intracellular signaling pathways (26) and numerous upstream signaling regulators and mechanisms involved in the regulation of mTOR (16), leading to the complexity of the regulation of mTOR by n-3 PUFAs. The mechanisms by which n-3 PUFAs inhibit mTORC1/2 remain to be identified.

Because unopposed estrogen exposure significantly increases endometrial hyperplasia and cancer risk (35), we examined the effect of n-3 PUFAs on unopposed estrogenic stimulation of endometrial growth. To our knowledge, no research studies have evaluated the efficacy of dietary changes and n-3 PUFAs in reversing the estrogen-elicited uterine growth. We found that both dietary and endogenous n-3 PUFAs effectively repressed the estrogen-stimulated endometrial growth in a mouse model. Investigators have found that treatment with rapalogs slowed the progression of endometrial hyperplasia and reduced tumor burden in animal models (46). Our results showing that mTOR is a potential target of n-3 PUFAs in this model further support the above finding. Future study of clinical interventions using n-3 PUFAs is therefore warranted.

In conclusion, this study provides comprehensive preclinical evidence that n-3 PUFAs efficiently inhibit endometrial cancer and implicates that n-3 PUFAs are anticarcinogenic nutrients of potential benefit in endometrial cancer. n-3 PUFAs may work through inhibition of mTORC1/2 signaling to decrease tumor cell proliferation, migration, and enhance tumor cell apoptosis. These evidences could have public health implications with regard to prevention of endometrial through dietary and lifestyle interventions such as fish oil supplementation and encourage public health authorities to design primary prevention campaigns promoting long chain n-3 PUFA consumption in populations at risk. However, studies are needed to investigate the potential negative effects before making any recommendations regarding the use of n-3 PUFAs or their dosage in endometrial cancer prevention. Future research will also have to answer the question of individual n-3 PUFA efficacy in endometrial cancer prevention and the exact mechanisms through which n-3 PUFAs inhibit endometrial cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Zheng, M. Zhong, Y. Dai, X. Bai, L. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Zheng, H. Tang, M. Liu, M. He, P. Lai
Writing, review, and/or revision of the manuscript: H. Tang, Y. Dai, X. Bai, L. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Zheng, H. Tang, M. Liu, J. Lin, C. Jia, Y. Dai
Study supervision: H. Tang, M. Zhong, Y. Dai

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