doi: 10.13241/j.cnki.pmb.2017.13.004

Inositol Hexaphosphate (IP6) Regulates the mRNA Expression of E-cadherin, TGF-β and VEGF in DMH-induced Colorectal Cancer Rat Model*

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ABSTRACT Objective: To explore the effect of Inositol Hexaphosphate (IP6) on expression of E-cadherin, TGF-β and VEGF in DMH-induced Colorectal Cancer (CRC) rat model. **Methods:** 60 Wistar rats were assigned to 5 groups: Control group (CG), DMH group (DG), Low IP6 dose group (LG), Moderate IP6 dose group (MG) and High IP6 dose group (HG). The rats in LG, MG and HG were given sodium phytate with a dose of 0.25 g/kg, 0.5 g/kg, 1.0 g/kg for each group by gavage every day, respectively. Meanwhile, the rats in CG and DG were given the same volume of normal saline by gavage every day. The rats in DG, LG, MG, HG were subcutaneously injected with DMH (30 mg/kg) once a week for 18 weeks. The rats were fed for 16 weeks after the induced-tumor experiment. The mRNA expression levels of E-cadherin, TGF-β, VEGF were measured by real-time PCR. **Results:** The mRNA level expression of E-cadherin decreased obviously in DG ($P<0.05$). With the increase of IP6 concentration, the E-cadherin gradually increased. By contrast, the expression of TGF-β and VEGF was the highest in DG ($P<0.05$) and both of them gradually decreased with increase of IP6 concentration. **Conclusions:** IP6 could increase the mRNA level expression of E-cadherin in DMH-induced CRC rat model and decrease the expression of TGF-β and VEGF.

Key words: Inositol Hexaphosphate (IP6); DMH; E-cadherin; TGF-β; VEGF

Chinese Library Classification(CLC): R735.3 Document code: A

Article ID: 1673-6273(2017)13-2414-04

Introduction

Colorectal cancer, which includes colon cancer and rectal cancer, is the second common malignant neoplasm in females and the third in males all over the world [1]. Meanwhile, CRC is always easy to metastasize to other tissues and most of the patients with distant metastasis are usually not eligible for traditional therapy, with a poor prognosis and a five-year overall survival rate of less than 10%[2-4].

IP6, as a naturally-occurring compound, is highly existed in fiber content, especially in wheat bran and flaxseed (0.4%-6.4%) [5], has been confirmed to have significant antitumor properties in tumor growth of many types of tumor both in vivo and in vitro [6-8]. EMT is an initial step and a major phenotype of cancer metastasis and invasion. E-cadherin is a hallmark of Epithelial-mesenchymal transition (EMT). In the process of EMT, the molecules of TGF-β, β-catenin are risk factors and can be regulated by IP6.

Our previous study suggested that IP6 could inhibit the growth and metastasis of CRC rats induced by DMH [9]. In the following study, an experiment was conducted to investigate whether IP6 could inhibit CRC metastasis to liver, and the result indicated that IP6 played an important role in the extracellular matrix (ECM) components where the liver cancer cells existed [10]. Studies have investigated the association between IP6 and distant metastasis of cancer. However, the mechanism of IP6 affect the tumors in the primary site in vivo experiment was still unclear. Therefore, in the present study, we explored the effect of IP6 on EMT and VEGF, in order to explore the mechanism of the impact of IP6 on the metastasis of CRC.

1 Material and Methods

1.1 Reagents

Sodiym phytate was purchased from Huamaike Boitechology Co., Ltd. (Beijing, China). DMH (1, 2-dimethylhydrazine) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The experimental rats are fed with AIN 93G and AIN 93M (Trophic Animal Feed High-tech Co., Ltd, Nantong, China). Total RNA was isolated using TRIzol Reagent (Invitrogen). Real-Time-PCR was performed using a two-step RT-PCR FastQuantRT Kit (KR106) and SuperReal PreMix Plus (SYBR Green FP205) (Tiangen Biotech Company, Beijing, China).The primers were designed using Primer Premier 5.0 (Premier, Palo Alto, CA, USA) and Oligo 6 software (Molecular Biology Insights, Cascade, CO, USA) and were synthesized by a biological engineering company (Shanghai, China).

1.2 Animals

60 rats of SPF clean grade, male, four-week old, 80～100 g of body weight, were purchased from Lukang Pharmaceutical Co., Ltd. (certificate of quality number: SCXK Lu 20130001) and were fed in the clean grade animal room of institute of nutrition of med-

*Foundation items: Natural Science Foundation of China (81373001)

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(Received: 2016-11-24 Accepted: 2016-12-21)

ical college, Qingdao University. The rats were kept in separate cages with 4 for each in an environment of 12-hour illumination time, room temperature of 25℃, humidity of 52%, freely access to drinking water.

1.3 Treatments

60 Wistar rats, after a week of adjustable feeding, were assigned to 5 groups (12/group), which were named Control group (CG), DMH group(DG), Low IP6 dose group(LG), Moderate IP6 dose group (MG) and High IP6 dose (HG), by randomized block design with weight as matching factor. All of the five groups were fed with AIN 93G (age below 20 weeks) and AIN 93M (age above 20 weeks). The rats in LG, MG and HG were given sodium phytate with a dose of 0.25 g/kg, 0.5 g/kg, 1.0 g/kg for each group by gavage every day, respectively. Meanwhile, the rats in CG and DG were given same volume of normal saline by gavage every day. After four-week gavage, the rats except those in CG were injected subcutaneously once a week for 18 weeks with a dose of 30 mg/kg DMH for each time. The rats were fed for 16 weeks after the induced-tumor experiment and then were executed to collect related tissue for detection and analysis.

1.4 Real-Time PCR

Total RNA was isolated using a TRIzol Reagent. Total RNA Kit II (Norcross, GA, USA).The IMPLEN Nano photometer P-330 nucleic acid detector (Munich, Germany) was performed to detect mRNA levels. A Primer Script RT Reagent Kit and a Bio-Rad My Cycler PCR instrument (Hercules, CA, USA) was performed to reverse transcribe the total RNA (2 μg).The Real-Time PCR primer sequences used and sizes of products obtained were shown in Table 1. According to the manufacturer's instructions, a 20 μL reaction volume with an Eppendorf Master cycler EP Gradient System (Eppendorf Hamburg, Germany) was strictly used to carry out the PCR. The relative gene expression levels was calculated by comparative Ct formula $2^{-\Delta\Delta Ct}$.

Table 1 Real-Time PCR primer sequences used and sizes of products obtained

Genes	Primer Sequences	Product Size(bp)
E-cadherin	F 5'-TCTCTTGTCCCTTCCACAGC-3' R 5'-CTCCAGACCCACACCAAAGT-3'	120
TGF-β	F 5'-ATTCCTGGCGTTACCTTGG -3' R 5'- AGCCCTGTATTCCGTCTCCT-3'	120
VEGF	F 5'-CGTCCTGTGTGCCCCTAAT-3' R 5'-TGGCTTTGGTGAGGTTTGAT-3'	121
B-Action	R 5'-TGTCACCAACTGGGACGATA -3' R 5'-GGGGTGTTGAAGGTCTCAAA -3'	165

1.5 Statistical Analysis

The mean ± standard deviation (SD) was used to express all of the values. All statistical analyses were conducted by SPSS software (version 17.0; IBM Corporation, NY, USA). A two tailed $P<0.05$ was considered statistically significant. All assays were performed in triplicate for an independent repetition of three times.

2 Results

2.1 Effect of IP6 on DMH-induced CRC and metastasis

After 18 weeks of DMH injection, continued 16 weeks feeding, all rats were euthanized in the 39-week. At the end of the study, upon necropsy, one sample of pulmonary metastasis was found in the DG; meanwhile, two samples and one sample of liver metastasis were also found in the DG and in LG, respectively. The incidence of lymphonodus metastasis was the highest in DG. Colons were separated from the bodies and analyzed. The formation of tumors was distinguished by histopathological examination. The incidence rates of tumorwere 0%, 100%, 100%, 88.89%, 66.67% in the CG, DG, LG, MG, HG respectively, most of tumors were colonic adenocarcinoma by treating with DMH alone.

2.2 IP6 regulated the mRNA expressions of E-cadherin, TGF-β and VEGF in the colons of CRC rats

The mRNA expressions of E-cadherin, TGF-β, VEGF in the colons of CRC rats were measured by real-time PCR to further investigate the mechanism of IP6 on the CRC metastasis. Figure1 showed the effects of different concentrations of IP6 on the mRNA expressions of the E-cadherin, TGF-β, VEGF. It was found that the expression of E-cadherin was obviously decreased in DG ($P<0.05$, compared to other groups). Along with the increase of IP6 concentration, the mRNA E-cadherin expression was gradually increased. By contrast, the mRNA expression of TGF-β was the highest in DG ($P<0.05$, compared to other groups) was gradually decreased along with increase of IP6 concentration. Meanwhile, the mRNA expression of VEGF was also the highest in DG and gradually decreased along with increase of IP6 concentration($P>0.05$, compared to LG).

3 Discussion

Colorectal cancer is always easy to metastasize to other tissues. The mechanism, pathogenic process and metastasis of colorectal cancer are very complicated, with a number of complex signal pathways. Previous study on CRC metastasis was mainly focused on distant metastasis to liver [10], and the corresponding molecular mechanisms about the primary sites of CRC generation

were seldom explored. The relevant parameters of the primary sites of tumor metastasis and early metastasis could be researched based on the establishment of this model.

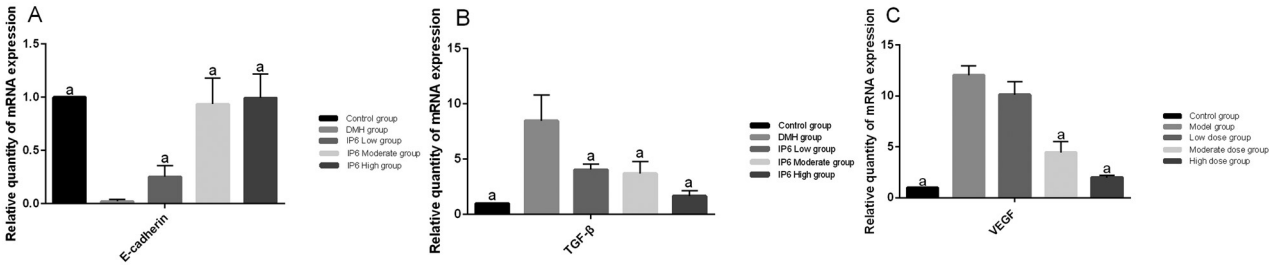

Fig. 1 Real-time PCR analysis of the mRNA expression of E-cadherin, TGF-β, VEGF. RT-PCR experiments were repeated in triplicate. (A) E-cadherin expression and (B) TGF-β expression and (C) VEGF expression in five groups; The results are showed as the mean ± standard deviation from three independent experiments. a P<0.05, compared to DG.

Our study showed that, the distant metastasis to liver or pulmonary appeared in DG and LG, and other groups did not appear tumor metastasis. This result implied that tumor metastasis could be inhibited by IP6. Through experiments, we further examined the expression of E-cadherin, TGF-β and VEGF in CRC tissue. Tumor metastasis is closely related to both tumor itself and the microenvironment of the tumor. Metastatic cancer can be characterized by the ability of tumour cell overcoming cell-cell adhesion and the ability of generating new blood vessels and new lymphatic vessel.

EMT is a pivotal component for tumor invasion and metastasis. The primary tumor cells can be detached from other cells or basement membrane and invaded into blood vessel or lymphatic vessel with the help of EMT. E-cadherin is a hallmark of EMT and one of the cell-adhesion molecules. The low-expression of E-cadherin, which is a marker of tumor invasion, can result in the loss of intercellular adhesion [11]. The result of Xiuli Bi [12]showed that the expression of E-cadherin declined in colorectal cancer, which was in accordance with ours. The expression of E-cadherin, was lowest in DG and there were no significant differences between MG and HG. TGF-β is an essential factor in the process of EMT. High expression of TGF-β could increased E-cadherin and indirectly induced EMT, thus promoting metastasis. E-cadherin binds to β-catenin as a compound [13], which will be essential for formation of cell- cell adhesion, thus leading to tumor invasion prevention[14]. Zinc finger proteins, which are parts of the Slug/Snail family, are repressors of E-cadherin gene transcription[15-17], and the TGF-β can active the expression of these proteins [18]. Meanwhile the TGF/β-catenin complex can bind to E-cadherin promoter and inhibit its function. The cell-cell adhesion and the concomitant accumulation of signaling β-catenin can be caused through the inhibition of cadherin expression by Slug/Snail or TGF/β-catenin complex. Our study showed IP6 decreased the expression of TGF-β, which implied IP6 may influence E-cadherin and TGF/β-catenin complex in this way.

Angiogenesis is the process of blood vessel formation that occurs under physiological and pathological conditions. In particular, angiogenesis is associated with progressive growth and metastasis of solid tumors, which depend on recruitment of new blood vessels [19-22]. VEGF is secreted by tumor cells, and promotes the proliferation of endothelial cells and remodeling of neo-vessels. In our study, we evaluated the anti-angiogenic activity of IP6 through the mRNA level of VEGF. The expression was the highest in DG and gradually decreased with increasing of IP6 concentration. Our findings are in accordance with those reported by Ivana Vucenik et al. They have investigated the effects of IP6 on secretion of VEGF from HepG2 cells, human liver cancer cells and found that IP6 reduces the expression of VEGF mRNA and protein[23].

In the future, we will be on the E-cadherin, TGF-β and VEGF protein were detected, expect to be able to explain the effect of IP6 on the protein level from them.

4 Conclusion

IP6 regulates the mRNA expressions of E-cadherin, TGF-β and VEGF in DMH-induced CRC rat model, which implied that the metastasis of CRC could be inhibited by IP6.

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肌醇六磷酸对二甲肼诱导的结直肠癌大鼠 E-cadherin，TGF-β 和 VEGF 表达的影响 *

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摘要 目的：探索肌醇六磷酸(IP6)对结直肠癌大鼠 E- 钙黏蛋白(E-cadherin)、转化生长因子 β(TGF-β)和血管生成因子(VEGF)表达的影响。**方法：**将 60 只 Wistar 大鼠分成五组分别为：空白组(CG)、DMH 组(DG)、低剂量 IP6 组(LG)、中剂量 IP6 组(MG)、高剂量 IP6 组(HG)。LG、MG 和 HG 大鼠每天分别以 0.25 g/kg、0.5 g/kg、1.0 g/kg 剂量的植酸钠灌胃，CG 和 DG 大鼠以同体积的生理盐水灌胃。除去空白组的大鼠，其他四组大鼠每周进行一次颈背部皮下注射 30 mg/kg 的 DMH 诱导结直肠癌肿瘤，一共注射 18 周。注射 18 周后继续喂养 16 周到实验结束。运用实时定量 PCR 的方法检测 E-cadherin、TGF-β 和 VEGF 的表达。**结果：**DG 组 E-cadherin mRNA 的表达明显低于其他各组(P＜0.05)，并且随着 IP6 浓度的增加 E-cadherin 的表达逐渐增加。相反，DG 里 TGF-β 和 VEGF 的表达高于其他各组(P＜0.05)，且随着 IP6 浓度的增加 TGF-β 和 VEGF 的表达在减少。**结论：**IP6 可以上调 DMH 诱导的结肠癌大鼠体内 E-cadherin 的表达和下调 TGF-β、VEGF 的表达。

关键词：肌醇六磷酸；二甲肼；E- 钙黏蛋白；转化生长因子 β；血管内皮生成因子

中图分类号：R735.3 **文献标识码：**A **文章编号：**1673-6273(2017)13-2414-04

* 基金项目：国家自然科学基金项目(81373001)

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（收稿日期：2016-11-24 接受日期：2016-12-21）