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Regulation of zinc transporters by dietary flaxseed lignan in human breast cancer xenografts

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Abstract Zinc is essential for cell growth. Previous studies have shown that zinc concentration in breast cancer tissues is higher than that in normal breast tissues. Zinc cannot passively diffuse across cell membranes and specific zinc transporter proteins are required. Two gene families have been identified involved in zinc homeostasis. ZnT transporters reduce intracellular zinc while ZIP transporters increase intracellular zinc. In this study, three human zinc transporter members: ZnT-1, ZIP2 and LIV-1 were chosen. We aimed to determine the effect of flaxseed lignan on the growth of ER-negative breast cancer cells in a nude mice model and observe the effect of flaxseed lignan on the regulation of the three zinc transporter in mRNA level. Nude mice were xenografted with human breast cancer cell line MDA-MB-231 and 6 weeks later were fed either the basal diet (BD) or BD supplemented with 10% FS and SDG for 5 weeks. The SDG levels were equivalent to the amounts in the 10% FS. RT-PCR was performed. Compared with the BD group, the tumor growth rate was significantly lower ($P < 0.05$) in the FS and SDG group. ZnT-1 mRNA level in mammary tumor was increased in SDG group and decreased in FS group, but no significant difference was found. Extremely low amplification of ZIP2 from mRNA was detected, with no difference between the treatment groups. LIV-1 mRNA expression of SDG group increases compared with BD group. In FS group, it significantly increases nearly 9 times than that in BD group ($P < 0.005$).

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Introduction

Zinc is essential for cell growth and is a co-factor for more than 300 enzymes, representing over 50 different enzyme classes. Zinc has been shown to be important to mammary tumor growth in animal model system [1]. Previous studies have shown that zinc concentration in breast cancer tissues is higher than that in normal breast tissues [2, 3]. The elevated zinc concentration in breast cancer tissues could be an indication of its involvement in breast cancer development. Zinc homeostatic regulation is achieved at the level of influx, efflux, and retention. Zinc influx and efflux depend on transporters. Therefore, studying the Zinc Transporters is meaningful in the research of breast cancer.

Diet rich in phytoestrogens have exhibited anticancer activity [4, 5]. Flaxseed is the richest source of the plant lignan Secoisolariciresinol diglycoside (SDG). The mammalian lignans enterolactone (EL) and enterodiol are formed by colonic bacterial action on SDG. Studies showed that diet supplement with flaxseed or its lignan SDG inhibits the initiation, promotion, and progression of mammary carcinogenesis in rats [6–8] and inhibits tumor growth and metastasis in nude mice [9, 10]. The mechanism by which flaxseed and its lignan inhibits breast cancer is not clear, but it includes the weak estrogenic and antiestrogenic properties. There is also evidence showing that the lignans exhibit nonestrogenic activities, such as antioxidant activity [11–13] and inhibition of cell membrane ATPase [14] and angiogenesis [15]. There is no study showing flaxseed and its lignan modulate Zinc metabolism to regulate growth in human breast cancer cells.

Recent studies identified several proteins and genes involved in cellular zinc transport, which belong to two gene families: the ZnT (Zinc transporter) proteins [solute-linked carrier 30 (SLC30)] and the Zip (Zrt- and Irt-like proteins) family [solute-linked carrier 39 (SLC39)]. ZnT transporters reduce intracellular zinc while ZIP transporters increase intracellular zinc. LIV-1 is breast cancer-associated protein which belongs to a new subfamily of ZIP transporters [16, 17]. In this study, three human zinc transporter members: ZnT-1, ZIP2 and LIV-1 were chosen. We aimed to determine the effect of flaxseed lignan on the growth of ER-negative breast cancer cells in a nude mice model and observe the effect of flaxseed lignan on the regulation of these three zinc transporters in mRNA level. Meanwhile, the mice serum zinc concentration was measured. Through which, we tried to explore the possible mechanisms associated with those effects.

Materials and methods

Animals

Fifteen female athymic nude mice (BABL/C) aged 3–4 weeks were purchased from Shanghai Slac Laboratory Animal CO. LTD and maintained in microisolator cages (5/cage) within a pathogen-free isolation facility with a 12:12-h light–dark cycle at 22–24°C and 50% humidity. Animals were given free access to the assigned diet (described below) and distilled water.

Diets

Three kinds of diets were prepared: basal diet (BD), flaxseed diet (FS) and SDG diet. BD diet was in accordance with AIN-93 purified diets for laboratory rodents. FS was the BD supplemented with 10% freshly ground flaxseed. In the SDG diet, the amount of SDG supplemented in BD was equivalent to the amount in the 10% FS diet. All diets were sterilized and stored at 4°C until use.

Cell line

The estrogen-independent MDA-MB-231 human breast cancer cell line was maintained in RPMI-1640 medium containing 10% bovine serum, plus penicillin (100 U/ml) and streptomycin (1 μg/ml) at 37°C in the presence of 5% CO_2. The cells were grown to 70–90% confluence in T-75 flasks and fed fresh medium a day before cell harvest. For injection, the cells were trypsinized, then washed with stroke-physiological saline solution and resuspended at 5×10^7 cells/ml after cell counting.

Experimental design

The mice were acclimatized for one week, while being fed BD. A total volume of 200 μl of cell suspension containing 1×10^7 cells was injected subcutaneously into the right-sided axillary fossa. Tumors were palpated weekly with tumor surface area calculated using the formula (length/2 × width/2) × π. At Week 6, the mice were randomized into three groups ($n = 5$), such that the mean body weight and tumor size in each group were the similar, one continued BD and the others were changed to FS diet and SDG diet respectively. Body weight, food intake, and tumor were monitored weekly. Mice were sacrificed at Week 10. Serum and primary tumor tissues were collected and stored in liquid nitrogen expeditiously. At necropsy, body weight, primary tumor weight and volume were recorded. Primary tumor volume was calculated using the formula (length/2 × width/2 × thickness/2) × π [9, 10]. RT-PCR was performed. Serum zinc concentration was measured using the OLYMPUS AU2700 automatic biochemical analyzer.

RNA extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA was purified by a Trizol total RNA purification kit (BBI) from the tumor tissues. The cDNA was synthesized from 2 μg of total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas), following the manufacturer's instructions. Transcription products with 2 μl cDNA were then amplified by PCR using DNA polymerases [TaKaRa Biotechnology (Dalian) Co., Ltd)], Specific primers and cycling parameters are given below in Table 1 [7, 12]. PCR products were separated on a 1% agarose gel and stained with ethidium bromide and visualized by UV light. Relative band intensity was quantified using AlphaImager™ Imaging System. The mRNA expression was evaluated by examining the ratio of the band intensity of the specific PCR product to that of β-actin in each sample.

Results

Food intake and body weight gain

No significant difference in food intake or body weight change was recorded between treatment groups (Table 2).

Primary tumor growth

Palpable primary tumor growth rate was significantly reduced ($P < 0.05$) in the SDG and FS group after 1 week

Table 1 PCR primers used for amplification of cDNAs

MRNA	Sequence		PCR product size	No. of cycles
ZnT-1	Forward	5′-TCAGCCTGAATTTGCTAG-3′	264 bp	30
	Reverse	5′-GATTCAGGTTGTTTGTTTGGC-3′		
ZIP2	Forward	5′-GGCTTCTTTCTTGTCTTCTTT-3′	431 bp	35
	Reverse	5′-CCCCGCCCTCCTTCAGAGTCC-3′		
LIV-1	Forward	5′-AATCCTCAGGAGTGTTTC-3′	277 bp	35
	Reverse	5′-CCGATTCATGAGAGGCAC-3′		
β-actin	Forward	5′-GTGGGGCGCCCCAGGCACCA-3′	540 bp	30, 35
	Reverse	5′-CTCCTTAATGTCACGCACGATTT-3′		

Table 2 Change of nude mice body weight and food intake in each group before and after treatment (means ± SD)

Group	Body weight/g (n = 5)		Food Intake, g/day/mouse (n = 5)	
	Before treatment	After treatment	Before treatment	After treatment
BD	22.99 ± 0.91	24.59 ± 0.80	3.13 ± 0.31	3.16 ± 0.32
SDG	22.5 ± 1.12	24.49 ± 0.92*	2.85 ± 0.34	3.12 ± 0.33*
FS	23.12 ± 1.10	24.65 ± 1.12*	2.92 ± 0.31	2.97 ± 0.40*

* $P < 0.05$

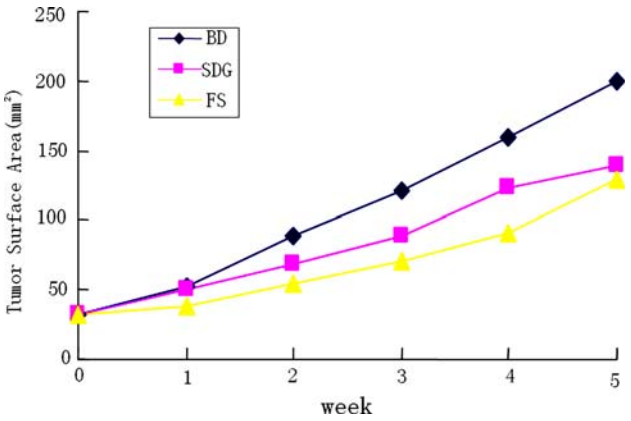

Fig. 1 Effect of flaxseed and SDG on growth of established human breast cancer, MDA-MB-231, in nude mice

of diet separation, and this reduction continued until Week 10 (Fig. 1). At necropsy at Week 10, the final tumor volume and weight were lowered significantly in SDG and FS group ($P < 0.05$) (Table 3).

Zinc status assessment

To detect the effect of dietary lignan supplement on the organism, we measured the mice serum zinc concentration. However, no significant difference was recorded between the treatment groups (Table 4). We did not detect the zinc excretion in gastrointestinal tract and zinc status in the primary mammary gland tumor. The discrepancy may be caused by versatile regulations, perhaps small sample size as well, and is to be further researched.

ZnT-1, ZIP2 and LIV-1 expression in primary tumor

To elucidate the possible effect of dietary flaxseed lignan on zinc transporters, the expression of ZnT-1, ZIP2 and LIV-1 in primary tumor was assessed. Compared with BD group, ZnT-1 mRNA level in mammary tumor was increased in SDG group and decreased in FS group. But there was no significant difference between the three groups (Figs. 2, 4). Extremely low amplification of ZIP2 from mRNA was detected, with no difference between the treatment groups (data not shown). LIV-1 mRNA expression in SDG group increases more than 4 times than that in BD group. In FS group, it was increased nearly 9 times than BD group ($P < 0.005$) (Figs. 3, 4).

Discussion

Diet rich in phytoestrogens have exhibited anticancer activity [4, 5]. Flaxseed is the richest source of the plant lignan Secoisolariciresinol diglycoside (SDG). Previous studies showed that flaxseed may exhibit an antiangiogenesis potential and down regulate expression of insulin-like growth factor and epidermal growth factor receptor [9], which is consistent with our result.

Table 3 The mean volume and weight of xenografted tumor in each group

Group	Tumor volume/mm^3	Tumor weight/g
BD	1489.7 ± 256.33	2.19 ± 0.18
SDG	639.35 ± 110.52*	1.01 ± 0.15*
FS	459.28 ± 110.04*	0.83 ± 0.05*

* $P < 0.05$

Table 4 Serum zinc concentration (means ± SD)

Group	Serum zinc concentration (μg/ml, $n = 5$)
BD	2.69 ± 0.56
SDG	1.99 ± 1.02*
FS	3.06 ± 0.36*

* $P < 0.05$

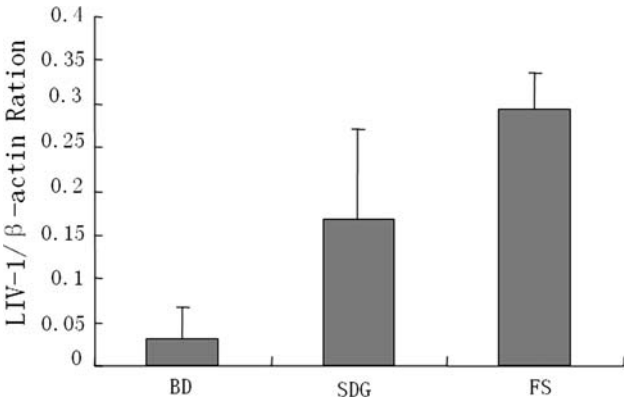

Fig. 3 Expression of LIV-1 mRNA, determined using RT-PCR, in MDA-MB-231 mammary tumor cells in nude mice exposed to diets containing 10% flaxseed and SDG (equivalent to the amount in the 10% FS diet). mRNA expression was evaluated by examining the ratio of the band intensity of the specific PCR product to that of β-actin in each sample. Means + SD of 5 tumors per group are shown. $P < 0.005$

Zinc is essential for cell growth and is involved in protein, nucleic acid, carbohydrate and lipid metabolism, as well as in the control of gene transcription, growth, development and differentiation [18, 19]. Zinc cannot passively diffuse across cell membranes; therefore, specific zinc transporter proteins are required to transport zinc into and out of cells. Two gene family had been characterized, ZnT and ZIP. There are at least 9 ZnT and 15 Zip transporters in human cells [16–17]. As zinc is essential for cell growth, membrane proteins capable of transporting zinc into and out of cells will have a crucial role in maintaining the cellular balance between apoptosis and cell growth, aberrations of which could lead to cancer.

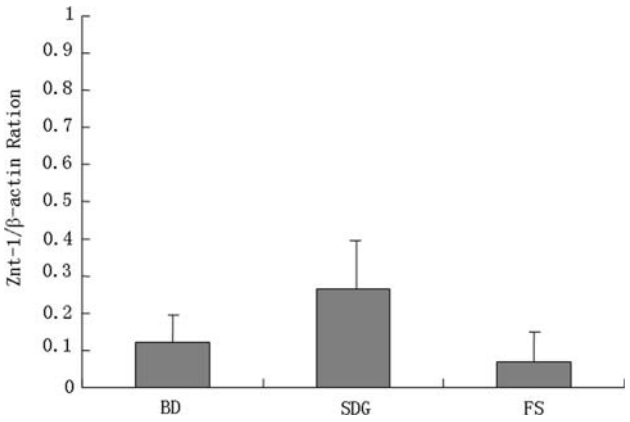

Fig. 2 Expression of ZnT-1 mRNA, determined using RT-PCR, in MDA-MB-231 mammary tumor cells in nude mice exposed to diets containing 10% flaxseed and SDG (equivalent to the amount in the 10% FS diet). mRNA expression was evaluated by examining the ratio of the band intensity of the specific PCR product to that of β-actin in each sample. Means + SD of 5 tumors per group are shown

Human mammary gland is a tissue that can accumulate and secrete zinc. Some results had shown that zinc concentration in breast cancer tissues is higher than that in normal breast tissues [2, 3]. A family of zinc-dependent endopeptidases, matrix metalloproteinases had been identified. They are apparently involved in breast cancer initiation, invasion and metastasis [20]. This clue which implies zinc transporter may also have play an important role in breast cancer.

ZnT-1 is a cellular zinc exporter and its mRNA expression has a wide tissue distribution. It is able to confer resistance to high levels of extracellular zinc in the zinc-sensitive cell and reduce the intracellular zinc level with a localization to cell plasma membrane [21]. In mammary gland tumor cells, low levels of ZnT-1 mRNA had been observed in rats [3]. In our study, mRNA level of ZnT-1 was about 12.4% of β-actin and was up-regulated by dietary SDG supplement. Although

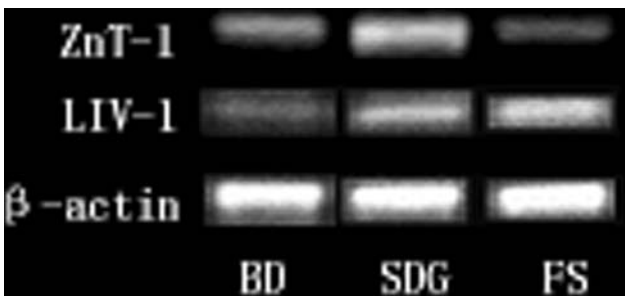

Fig. 4 Expression of ZnT-1 and LIV-1 mRNA, determined using RT-PCR, in MDA-MB-231 mammary tumor cells in nude mice exposed to diets containing 10% flaxseed and SDG (equivalent to the amount in the 10% FS diet)

the difference is not significant, we could produce a proposal that this transporter protect cells from damage related to toxic levels of zinc and lack of which would result in an assistance involved in the growth of human breast cancer. SDG may exhibit anticancer activity by up-regulating ZnT-1, and then mediately influence intracellular zinc status which is essential for tumor cell growth. The effect of FS in our study is not clear and need further study.

Previous report showed expression of hZIP2 was undetectable in human prostate malignant cell lines LNCaP and PC-3 [22], and in breast cancer, was low. In this nude mice model, extremely low amplification of ZIP2 from mRNA was detected, with no difference between the treatment groups which is similar to the previous results.

LIV-1 has been identified as a gene whose expression is stimulated by estrogen in MCF-7 and ZR-75 breast cancer cells [23]. In this study, we found LIV-1 mRNA expression was increased in SDG and FS group, which may result in the increasing intracellular zinc level. In Fong's study [24], Dietary zinc deficiency in mice, in cooperation with cyclin D1 overexpression, can deconstrols cell proliferation and ensure cell expansion, a prerequisite for cancer development. Accordingly, new directions is to show up in the association of zinc and tumor growth. SDG may be the main factor in flaxseed. The effect of flaxseed is significant may be due to its other component. In the estrogen-negative human breast cancer model, we presume that other regulation mechanism may exist by which SDG and flaxseed regulate LIV-1 expression.

In summary, as zinc is essential for cell growth, membrane proteins capable of transporting zinc into/out of cells will have a crucial role in maintaining the cellular balance between apoptosis and cell growth. Such a function would probably require a family of proteins under tight regulation of expression because faults in the system could easily lead to premature death or cancer.

In this study, we found dietary flaxseed lignan supplement inhibits the tumor growth as in other previous studies. Meanwhile, zinc transporters expression were regulated by dietary flaxseed lignan in human breast cancer xenografts. This is the first study involves in the relationship of flaxseed lignan and zinc metabolism which may explain a new mechanism about the anticancer effect of flaxseed and its lignan.

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