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# A hemagglutinin isolated from Northeast China black beans induced mitochondrial dysfunction and apoptosis in colorectal cancer cells

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ABSTRACT

Incidence of colorectal cancer is closely related with the lifestyle, especially the dietary habits of patients. Epidemiological researches have demonstrated a negative correlation between legume consumption and colorectal cancer incidence. Lectins/hemagglutinins are a type of carbohydrate binding proteins which are abundantly stored in legumes. Their eminent pH-stability allows them to survive digestion and remain active in the intestine where they may have direct contact with colorectal tumors. It is therefore interesting to explore the direct interaction between lectins/hemagglutinins and colorectal cancer. In the present research, we reported a detailed research on the interaction between a hemagglutinin isolated from an edible legume with two colorectal cancer cell lines. This hemagglutinin (NCBBH) was found to first bind to tumor cell membrane as early as 30 min post treatment and was gradually transported inside the cytoplasm within 3 h, with some of it localized in the Golgi apparatus and some in the lysosomes. After its entrance, the hemagglutinin induced aggregation of the Golgi apparatus, which in turn adversely affected the transportation of protein from endoplasmic reticulum (ER) to the Golgi apparatus, resulting in protein accumulation in ER and ER stress. The hemagglutinin-treated cells also manifested severe mitochondrial malformation and membrane depolarization, accompanied by obvious apoptosis characteristics, like chromatin condensation, phosphatidylserine exposure and caspase activation. Collectively, our results indicate that the hemaggltuinin could successfully enter the cytoplasm of colorectal cancer cells and adversely affect their growth, providing a mechanism in support of the application of edible legumes to the prevention and treatment of colorectal cancer.

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## 1. Introduction

Colorectal cancer (CRC) is listed as the third most common malignancy worldwide. In the United States, it is the second leading cause of cancer related deaths among adults [1]. The incidence of CRC varies markedly with countries and regions. According to the reports analyzing the data collected from 1998 to 2002, the registries from Europe, North America and Oceania had the highest incidence rates while those from Asia, Africa and South America ranked the lowest [2]. The same research has also pointed out a positive correlation between the incidence rates of CRC and the degree of westernization in lifestyle which have included diets containing high-calorie-dense foods, especially animal protein and fat [2]. Researchers also found the incidence rate of CRC among African Americans and Native Africans were respectively 52 per 100,000 and <5 per 100,000 and this significant difference could unlikely be explained by genetic susceptibility but environmental influences, especially lifestyle [1]. It has even been estimated that all cancer deaths are potentially avoidable by changing the dietary pattern and for colon cancer this percentage could be as high as 50% [3].

Consumption of food rich in dietary fiber is documented to have protective effects on CRC since dietary fiber is capable of capturing fats and biliary acids [4]. As one kind of the commonly consumed foods, legumes have been found to contain a variety of dietary proteins, vitamins, lignans and isoflavones [5]. Epidemiological studies indicated that consumption of legumes could help in reducing the incidence of CRC since the incidence of CRC in countries where people consumed an abundance of dry beans was comparatively low [6]. Experiments on rats also disclose a significant lower CRC tumor multiplicity induced by azoxymethane among bean-fed rats than casein-fed rats (5 vs. 22 tumors, 21 rats in each group) [6]. Though in this report the specific anticarcinogenic

*Abbreviations:* AAL, *Aleuria aurantia* lectin; ATF6, activating transcription factor 6; Apaf-1, apoptotic peptidase activating factor 1; BSA, bovine serum albumin; Con A, concanavalin A; CGN, *cis* Golgi network; CRC, colorectal cancer; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; FITC, fluorescein isothiocyanate; FLICA, fluorescent inhibitor of caspases; GalNAc, N-acetylgalactosamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRE1, inositol-requiring enzyme 1; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCBBH, purified hemagglutinin from Northeast China black beans; P21, cyclin-dependent kinase inhibitor 1; PAGE, polyacrylamide gel electrophoresis; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; PI, propidium iodide; PS, phosphatidylserine; TEM, transmission electron microscopy; TGN, *trans* Golgi network; UPR, unfolded protein response; WGA, wheat germ agglutinin; $\Delta\Psi m$, mitochondrial membrane potential.

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components were not identified, other research had indicated the importance of isoflavone genistein, protease inhibitor, saponins and phytate etc. isolated from soybeans in cancer prevention [7,8].

In the present study, we focused on the study of the anti-colorectal cancer function of a defense protein (a hemaggltuinin) isolated from an edible legume. Lectin/hemagglutinin is a type of protein which is found to be commonly and abundantly stored in the seeds of legumes. It is characterized with the unique property to initiate reversible binding with specific carbohydrate motifs in a non-catalytic way [9,10]. The majority of this type of protein is highly pH-stable and thermalstable. It could resist digestion and remain active in the colon [11].

Interestingly, when compared with normal colonic mucosa, there were more lectin/hemagglutinin receptors expressed in hyperplastic and neoplastic colonic tissues [12]. The interaction between dietary lectins and colonic mucosa was postulated to regulate the proliferation [12] and differentiation [11] of colorectal tumors. According to the report of Jordinson and his colleagues, *Vicia faba* agglutinin could stimulate the differentiation of an undifferentiated colon cancer cell line into gland-like structures, reversing the malignant phenotype of colon cancer cells [11]. This process was prospected to be completed through an interaction between dietary lectin and epithelial cell adhesion molecules [11].

Due to the difference of sugar specificity of lectins/hemagglutinins and diversity of molecule glycosylation, different lectins/hemagglutinins have been reported to initiate distinct reactions with different types of cells [11]. In our research, we have monitored the influence of the target hemagglutinin on proliferation of the colorectal cancer cell lines as well as the mechanisms involved. Its interaction with cell membranes and organelles was also the research focus of this paper.

## 2. Results

### 2.1. NCBBH suppressed proliferation of colorectal cancer cells

The proliferation of both HT29 and HCT116 cells was found to be suppressed by treatment of NCBBH in a dose- and time-dependent manner. As presented in Fig. 1A, though the anti-proliferative potency of NCBBH on HT29 and HCT116 cells appeared to be equivalent at 24 h, divergence was observed at 48 h that the proliferation of HT29 tended to be more potently suppressed than HCT116 cells that received the same dosage of protein. The $IC_{50}$ values for HT29 and HCT116 cells at 48 h were respectively around 0.625 μM and 10 μM.

The binding specificity of NCBBH has been discussed in our previous published paper [13] which introduced the purification and characterization of this protein. Briefly, we have tested on the inhibitory effect of 13 sugars, including GalNAc, D-galactose, L-fucose, L-arabinose, D-fructose, D-mannose, D-mannitol, D-xylose, D-glucose, maltose, α-lactose, raffinose, and L-rhamnose on the hemagglutinating activity of NCBBH and GalNAc was demonstrated to be the only one which could partially inhibit the hemagglutinating activity. The presence of the sugar GalNAc was also found to slightly attenuate the anti-proliferative effect of NCBBH on colorectal cancer cells. To eliminate the influence of GalNAc on cell proliferation, the percentage of inhibition (of different concentrations of NCBBH) was calculated by comparing treated cells with the corresponding control cells cultured in medium containing the same concentration of GalNAc but without NCBBH. As shown in Fig. 1B, there was a consistent trend observed in both cell lines that with the increase of concentrations of GalNAc, the anti-proliferative potency of NCBBH was crippled in a higher degree.

NCBBH reduced the efficiency of colony formation in both HT29 and HCT116 cells. The number and size of cell colonies were compared between cells cultured in the presence and absence of NCBBH. For HCT116 cells, no statistical significance was found in colony number between the control cells and treated cells. However, the size of HCT116 cell colonies after treatment with 1 and 2 μM NCBBH was respectively 56% and 65% smaller than the control. For HT29 cells, at the concentrations of 1 and 2 μM NCBBH, the number of cell colonies was respectively 8% and 66% less than the control while the size of colony was respectively 65% and 80% smaller than the control, as presented in Fig. 1C.

### 2.2. NCBBH induced cell cycle arrest in colorectal cancer cells

In the present study, the effect of NCBBH on the cell cycle was measured by two methods which included the application of flow cytometry to monitor the percentage of cells that contained DNA at a relative level and western blotting assay to detect two proteins which have been documented to be important in the regulation of cell cycle. As presented in Fig. 2A, after the treatment with NCBBH for 24 h, the percentage of cells in G1 phase was higher than that in the control. This trend was consistent in both cell lines that the percentages of HT29 and HCT116 cells in G1 phase respectively increased from 49% to 74% and 33% to 63% when treated with 5 μM NCBBH. The cell cycle arresting effect of NCBBH was further confirmed by the results presented in Fig. 2B. The expression of cyclin D1 which can promote cell proliferation and cell phase transition from G1 to S was found to be down-regulated in a time- and dose-dependent manner. Consistently, the expression of P21 is up-regulated to inhibit the activity of Cyclin-dependent kinases.

### 2.3. Transportation and colocalization of NCBBH with different organelles

Cells were cultured with 0.02 mg/ml FITC-labeled NCBBH for 30 min, 90 min, 120 min or 180 min and observed by using a confocal system. The 3D images were constructed by using Imaris software and the dynamic images were available in supplementary data Video clip 1a and b. In order to make it convenient for viewing, the static images were presented in Fig. 3A. At the time point of 30 min, the bulk of NCBBH was found to be localized on the membrane of HT29 cells and partially transported into the cytoplasm in HCT116 cells, which indicated divergence of this protein transportation efficiency in HT29 cells and HCT116 cells. At the time point of 90 min and 120 min, proteins on cell membrane of both HT29 and HCT116 cells were successively transported into cytoplasm. The majority of NCBBH was detected in the cytoplasm of cells and appeared to be distributed in an aggregated status in HCT116 cells at the time point of 180 min.

The localization of NCBBH in the cytoplasm was then further determined by co-localization analysis with different organelles inside the cells. As displayed in Fig. 3B and C, endoplasmic reticulum and mitochondria in HT29 and HCT116 cells were generally evenly distributed around the nucleus, which was significantly different from the pattern of NCBBH distribution. Co-localization analysis with Golgi apparatus (Fig. 3D) presented an interesting phenomenon that the Golgi apparatus appeared to be enwrapped and aggregated by the protein (as indicated by the arrow). The distribution of Golgi apparatus in cells receiving no treatment with NCBBH is presented in Fig. 3F. The results of co-localization analysis with lysosomes indicated that after cells were incubated with the protein for 3 h, parts of the protein was transported to the lysosome. The co-localization condition of NCBBH with lysosomes at 10 h was present in 3D image in supplementary data Video Clip 2 that at this time point NCBBH was still detectable in cell cytoplasm and only some of the protein were transported to the lysosomes.

### 2.4. Specific binding of NCBBH on the cell-cell interaction membrane

As it has been present in Fig. 3A, at the initial stage of interaction NCBBH was first bound on cell membranes and subsequently transported into the cytoplasm. At the time point of 3 h, the protein was barely observed on cell membranes except that at the interface between two cells (as displayed in Fig. 3E), which indicated that in a single cell the protein expression at the interface where it has contact with another cell is different from the contact-free membranes. In order to

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