RESEARCH ARTICLE

Vitamin D3 and Beta-carotene Deficiency is Associated with Risk of Esophageal Squamous Cell Carcinoma - Results of a Case-control Study in China

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Abstract

Objective: The aim was to evaluate roles of vitamin D3 (VD3) and beta-carotene (BC) in the development of esophageal squamous cell carcinoma (ESCC) in a high-risk area, Huai'an District, Huai'an City, China. Methods: 100 new ESCC diagnosed cases from 2007 to 2008 and 200 residency- age-, and sex-matched healthy controls were recruited. Data were collected from questionnaires, including a food frequency questionnaire (FFQ) to calculate the BC intake, and reversed phase high-performance liquid chromatography (RP-HPLC) was used to measure the serum concentrations of BC and VD3. Odds ratios (OR) and 95% confidence intervals (CI) were calculated in conditional logistic regression models. Results: The average dietary intake of BC was 3322.9 μg (2032.4- 5734.3) in the case group and 3626.8 μg (1961.9-5827.9) in control group per capita per day with no significant difference by Wilcoxon test ($p>0.05$). However, the levels of VD3 and BC in the case group were significantly lower than in the control group ($p<0.05$). The OR values of the highest quartile and the lowest quartile of VD3 and BC in serum samples were both 0.13. Conclusion: Our results add to the evidence that high circulating levels of VD3 and BC are associated with a reduced risk of ESCC in this Chinese population.

Keywords: Vitamin D3 - beta-carotene - esophageal squamous cell carcinoma - case-control study

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Introduction

Esophageal cancer is one of the most common upper gastrointestinal malignancies which give rise to significant morbidity and mortality worldwide which are respectively ranked eighth and sixth in the world. China alone has 250000 new diagnosed cases annually which contribute to more than half of the world's cases, and the incidence cases in northern Jiangsu area are still fairly high (Wiseman, 2008). In Huai'an District, Huai'an City, a high-risk area in China, the new cases of esophageal cancer are 10147 during 1998 and 2006, accounted for 42.74% of all new cases of malignant tumors, with an incidence rate of approximately 93 per 100000 population in the crowd.

Substantial reports have shown the efficacy of vitamin D3 (VD3) and VD3 analogues on inhibition of the proliferation of cells of breast, prostate, skin and colon cancer *in vitro* (Fleet, 2008). However, the results are inconsistent (Gilbert et al., 2011). Bruggemann (Bruggemann et al., 2010) reported that VD3 inhibited cell growth *in vitro*, but was not effective against tumor cell growth in the *in vivo* animal models.

Although VD receptor mRNA has been detected in the human esophagus (Trowbridge et al., 2012), and previous studies demonstrated that the inhibition of VD3 on proliferation was via induction of differentiation, apoptosis and cell cycle arrest in human EC9706 cell line (He et al., 2009), few results of VD3 have been reported in human epidemiologic studies of esophageal cancer.

Beta-carotene (BC) is widely found in green and yellow vegetables and it is the most biological active form of carotenoids. Peto et al. (1981) postulated that BC may affect cancer risk in the late 20th century. Since then, BC has received a tremendous amount of attention as potential anti-cancer compounds (Beilby et al., 2010; Druesne-Pecollo et al., 2010). BC may be a protective factor for esophageal cancer as indicated in some studies (Nomura et al., 1997; Lu et al., 1999). BC can also reduce the risk of both ESCC and esophageal adenocarcinoma in a population-based case-control study in Sweden (Terry et al., 2000). Nevertheless, more and more negative effects, even opposite effects were reported in past 20 years. Abnet (Abnet et al., 2003) found that there were no strong associations between BC and esophageal cancer.

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There were no reports about the roles of VD3 and BC concentrations in serum in epidemiologic studies of esophageal cancer. Serum vitamins concentration is related with the degree of absorption in human body which is the best indicator to the intracellular level of vitamins (Wiseman, 2008). To making up this gap, we assessed the associations of serum VD3 and BC with the risk of ESCC in a population-based case-control study.

Materials and Methods

This population based case-control study recruited 100 patients with ESCC and 200 healthy controls. All subjects were unrelated ethnic Han Chinese and residents in a high esophageal cancer incidence region of northern Jiangsu, 5 townships of Huai'an District, Huai'an City, Jiangsu Province, China. Eligibility criteria for both cases and controls included not being pregnant and taking VD3 and BC supplements at the time of recruitment, no prior history of cancer and/or esophageal surgery and chemotherapy or radiation therapy. A unified questionnaire, administered by trained personnel, was used to obtain study information with one-to-one and face-to-face survey. All cases were newly diagnosed by endoscopy, X-ray or clinical histopathology with primary ESCC in the local hospitals during 2007-2008, based on a database at the malignant tumor registry of Huai'an Center for Disease Control and Prevention (CDC). Controls were randomly selected healthy individuals confirmed by physical examination, clinical and biochemical analyses during the same period of case recruitment, and matched with cases on age (±2 years), gender and the same townships. In addition to epidemiological and food frequency questionnaires, 5 mL blood samples were collected and separated into serum and stored at -80 °C until it was analyzed. Informed consent was obtained from each subject. The study protocol was approved by the Institutional Review Boards of the Texas Tech University and the Southeast University for human subject protection.

BC intakes estimation

All questionnaires were filled out by trained personnel on 8 categories including 1) staple diet; 2) fried food; 3) pickled and salty food; 4) animal meat, egg and milk; 5) bean and bean product; 6) vegetables; 7) fresh fruits; and 8) nuts and dried fruits. A total of 120 food items were included in these 8 categories, which represented the usual diets of local residents. As the content of various kinds of carotene is not given respectively in Chinese Food Composition, therefore, USDA Nutrient Database for Standard Reference was used to calculate dietary BC intake (Mangels et al., 1993; Holden et al., 1999; USDA.). Some Chinese special foods were substituted and estimated by similar foods (Yang, 2004; Song and Wang, 2007).

Standard preparation of BC and vitamin D

VD3 and BC authenic standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solution of VD3 was prepared at 1 mg/mL in methanol; Stock standard solution of BC was prepared at 1 mg/mL in methylene chloride. The stock standard solutions were further diluted 50 times in the same solvent to prepare for a working standard solution of VD3 and BC at 20 μg/mL. The stock standard solutions were stored in amber bottles at -20 °C and were found to be stable for at least one month. The working standard solutions were freshly prepared every week and stored in amber bottles at -20 °C.

Vitamins extraction

200 μL serum was dissolved in 200 μL absolute alcohol, then vigorously shook it for 30 s and placed it for 10 min in order to precipitate protein. 400 μL n-hexane was added and the suspension was vigorously shook for 60 s and then centrifuged for 5 min at 6000 r/min. Supernatant fluid was sucked up and dried by nitrogen at 40 °C. At last it was dissolved in 100 μL mobile phase and vigorously shook for 30 s. The solution was filtered through a 0.22 μm membrane filter with Nylon 66 from Pall (Tianjin, China) before the high-performance liquid chromatography (HPLC) analysis.

RP-HPLC analysis

The BC and VD3 concentrations in serum were analyzed concurrently by the reversed phase high-performance liquid chromatography (RP-HPLC; LC-10 AT, Shimadzu Corporation) (Zhao et al., 2004; Murao et al., 2005; Zhu et al., 2005). Chromatographic separation uses a diamond C18 analytical column (4.0 mm×150.0 mm). Mobile phase was consisted of a mixture of 20% Methylene chloride in 80% methanol. The chromatographic separation was performed at a flow rate of 1 mL/min for 6 min, followed by a linear gradient to 1 mL/min-2 mL/min during 6-7 min. Finally the flow rate was kept in 2 mL/min and held for 13 min. The best compromise for two analytes was to reach using two different operative wavelengths (263 nm for VD3, and

Table 1. Demographic Characteristics of EC Cases and Healthy Controls

Variable	EC patients	Healthy controls	*P*
Total (n)	100	200	
Age (year, mean±SD)			
Female	60.9±7.8	60.6±7.7	0.82
Male	61.0±7.1	61.2±6.9	0.86
Total	61.0±7.4	60.9±7.3	0.96
Education (n, %)			0.13
Illiteracy	65(65.0)	114(57.0)	
Primary school	26(26.0)	51(25.5)	
Middle school	7(7.00)	33(16.5)	
Senior high school	2(2.00)	2(1.00)	
Marital status (n, %)			0.53
Married	83(83.0)	173(86.5)	
Divorce	0(0.00)	0(0.00)	
Widow	16(16.0)	23(11.5)	
Single	1(1.00)	1(0.50)	
Ever smoker[a], %	53(53.0)	114(57.0)	0.51
Passive smoking[b], %	44(44.0)	90(45.0)	0.84
Ever drinker[c], %	26(26.0)	66(33.0)	0.22

Note: [a]Ever smoker: Smoked at least 1 cigarette every day for more than a year; [b]Passive smoking: Families were ever smoker; [c]Ever drinker: ≥1 times per week

Table 2. The Regression Equation, Linearity and Limit of Detection (LOD) of Serum Beta-carotene and Vitamin D3

Vitamin	Regression equation	Related coefficient	Linearity (μg/mL)	LOD (μg/mL)
Beta-carotene	$C=10^{-7}S+0.0348$	0.9993	0.02-4.00	0.02
Vitamin D3	$C=6^{-7}S-0.0018$	0.9967	0.03-4.00	0.03

Table 3. Serum Vitamin D3 and Beta-carotene Concentrations of Case and Control Groups (μg/dL)

Vitamin (μg/dL), *M* (25th–75th)	EC patients	Healthy control	*P*
Vitamin D3	2.43 (1.29-4.99)	4.24 (2.52-6.99)	0
Beta-carotene	4.20 (2.36-9.80)	3.85 (2.63-6.21)	0

452 nm for BC). Two analytes were completely eluted within 11 min and the whole chromatographic operating was completed in 20 min.

Statistical analysis

Data were entered into a database with EpiData software and analyzed by Statistical Package for the Social Sciences 13.0 software (SPSS). Medians and 25th–75th percentiles were calculated for VD3 and BC, and Wilcoxon test was used to compare the intakes of dietary BC, and *t* test was used to compare serum concentrations of VD3 and BC respectively among cases and controls after log transformation. Serum concentrations of VD3 and BC were categorized into quartiles based on the distribution of serum concentrations of these vitamins in the control participants. Conditional logistic regression models were used to estimate odds ratios (ORs) and corresponding 95% confidence intervals (CIs) and to assess whether serum concentrations of VD3 and BC are associated with the risk of esophageal cancer.

Results

Subject characteristics

Demographic characteristics of EC patients and healthy controls are presented in Table 1. A total of 100 EC cases and 200 age- and sex-matched healthy controls were included in the case-control study. Males accounted for 53% of the studied subjects. There are no significant difference on age, nationality, education, profession, marital status and tobacco and alcohol consumption status (ever vs. never) through balance test ($p>0.05$).

Dietary BC intake

Dietary BC intake was calculated by a program for calculating nutrition designed by ourselves. Data was showed with median (*M*) and the 25th and 75th percentiles (25th–75th) due to the skewness distribution. The dietary intake of BC is 3322.92 μg (25th-75th, 2032.43-5734.27) in the case group and 3626.83 μg (25th-75th, 1961.91-5827.96) in the control group per capita per day. There was no significant difference on BC intakes between case and control group by Wilcoxon test ($p>0.05$).

Serum VD3 and BC concentrations

The standard curves were obtained by plotting concentration (μg/mL) against peak area (Figure 1).

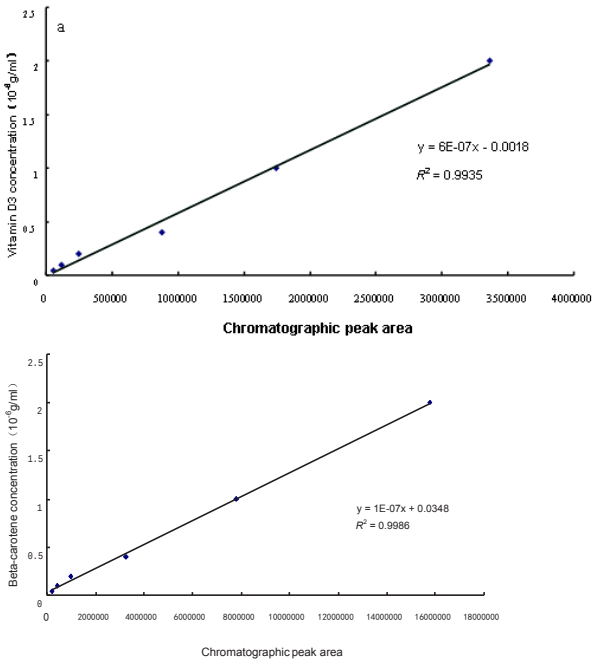

Figure 1. (a) Standard curve of serum vitamin D3. (b) Standard curve of serum beta-carotene

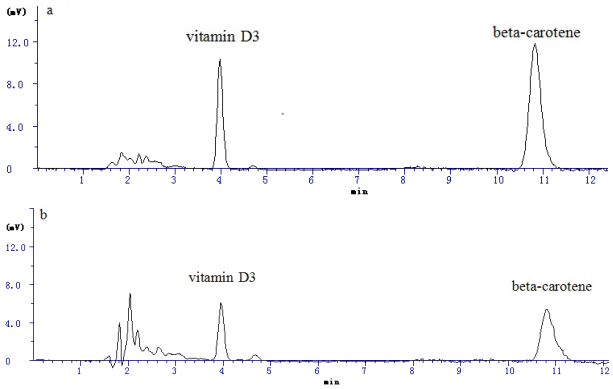

Figure 2. (a) Chromatogram of a standard mixture of vitamin D3 and beta-carotene, each at a concentration of 20 μg/mL. (b) Chromatogram of serum vitamin D3 and beta-carotene

RSD% (RSD, Relative standard deviation) of VD3 and BC were 32.34 and 2.14 respectively by analyzing the standard mixture during 7 days. BC was not stabile, so it had to be freshly prepared before determination.

RSD% of the intraday precision of VD3 and BC were 4.79 and 1.38 by analyzing the same serum sample 6 times in one day, and RSD% of inter-day precision of VD3 and BC was 3.78 and 6.03 by analyzing a sample for three days continuously. It indicated that serum BC and VD3 had good reproducibility by RP-HPLC.

The recovery rates of spiked standard of VD3 and BC were 88.50%-102.50% and 85.50%-96.90% respectively. Chromatogram of a standard mixture of VD3 and BC was given in Figure 2. Table 3 showed that the concentrations of serum VD3 and BC in case group was significant lower than control group by *t* test ($p<0.05$). Data was showed with median (*M*) and the 25th and 75th percentiles (25th–75th) as it was skewness distribution.

Levels of VD3 and BC in the case group were significantly lower than control group ($p<0.05$) (Table 3). Levels of VD3 and BC were further divided into four

Table 4. The Association Between the Levels of Vitamin Concentrations and the Risk of EC

Vitamin	Concentration	*P*	OR	95% CI
Vitamin D3 (μg/dL)	<2.00 (Q1)			
	2.00-3.70 (Q2)	0	0.14	0.05-0.35
	3.70-6.10 (Q3)	0	0.1	0.04-0.26
	>6.10 (Q4)	0	0.13	0.05-0.32
Beta-carotene (μg/dL)	<3.30 (Q1)			
	3.30-6.60 (Q2)	0	0.16	0.06-0.42
	6.60-11.1 (Q3)	0	0.08	0.03-0.22
	>11.1 (Q4)	0	0.13	0.05-0.33

quartiles: the first (highest) quartile, the second quartile, the third quartile, and the fourth (lowest) quartile, which named as Q1, Q2, Q3, and Q4 respectively. The OR and 95% CI were calculated using conditional logistic regression models to compare these quartiles, respectively (Table 4).

Discussion

Chromatographic conditions selection: VD3 was detected in 5 min and BC was detected in 36 min, and then the mobile phase was changed by adding methylene chloride to shorten the coming time of BC (80:20, v/v). It was difficult to separate VD3 from impurities in very short time, therefore, a gradient elution technique was applied to achieve a baseline separation of the vitamins and to shorten the total analysis time. The optimal gradient was as follow. At first, an initial isocratic step chromatographic separation was performed at a flow rate of 1 mL/min for 6 min, followed by a linear gradient to 1 mL/min - 2 mL/min during 6-7 min, then, the flow rate was kept in 2 mL/min and held for 13 min. Based on the UV spectra of each vitamin, VD3 and BC were detected at 263 nm and 452 nm respectively. So they were completely eluted within 11 min and the whole chromatographic operating was completed in 20 min. The limit of detection (LOD), precision and recovery rates of spiked standard was detected and it indicated that serum BC and VD3 had good sensitivity and reproducibility by RP-HPLC.

VD3 and esophageal carcinoma: It is well known that VD3 not only plays a crucial role in maintenance of calcium absorption and control of bone mineralization, but also acts an effective regulator of cell cycle arrest, differentiation, and apoptosis in normal and transformed cells (Baek et al., 2011). 1,25(OH)2D3, the active form of VD3, contributes to defend cells against risk for carcinogenic conversion (van den Bemd et al., 2000; Beer and Myrthue, 2004). Substantial evidence supports a possible anticancer role for VD3 against breast, prostate, skin and colon cancers *in vitro* and *in vivo* experimental model (Wu et al., 2007; Gonzalez Pardo et al., 2012). However, recently published epidemiologic studies show conflicting results (Li et al., 2007; Schwartz, 2012). Studies have also indicated that VD3 metabolites suppress the growth and stimulate the differentiation of human esophageal cancer cells *in vitro*, but no enough evidence demonstrates the clear relationship between VD3 and risk of esophageal cancer.

In this study, the serum concentrations of VD3 were detected by HPLC and the results showed that VD3 concentrations in the case group were significantly lower than that in the control group. According to quartiles, the ORs of Q4, Q3, and Q2 compared with Q1 were 0.13, 0.10 and 0.14 respectively. It means VD3 may be a protective factor for esophageal cancer in all range.

BC and esophageal carcinoma: BC is probably the most well known of the carotenoids, a phytonutrients family that exists in the widespread plants and fruits. It is a precursor to vitamin A in the body and can be found in green and yellow vegetables primarily. In the late 20th century, BC has received a tremendous amount of attention as potential anti-cancer compounds. But Druesne-Pecollo's review (Druesne-Pecollo et al., 2010) and Schaumberg' study (Schaumberg et al., 2004) indicated that BC supplementation has not any beneficial effect on cancer prevention such as lung, gastric, pancreatic, colorectal, prostate, breast and skin cancers.

There is even some concern that high doses of BC supplements can cause a slight increase in the risk of cancer. Synthetic BC supplements are found to increase the risk of both colorectal and lung cancer in smokers, especially those who also drink alcohol (Shiels et al., 2011). However, getting nutrients from real food instead of supplements can avoid worrying about these types of issues. Observational studies suggested that a diet high in fruits and vegetables, both of which are rich in antioxidants, may prevent cancer developing. Unlike supplements, foods rich in BC don't result in cancer risk. A study (Neuhouser et al., 2003) indicated that BC consumed as part of whole foods has no such negative effects.

This study indicated that BC was inversely associated with risk of EC. Our study used USDA Nutrient Database for Standard Reference to calculate dietary BC intake. The dietary intake of BC had no statistically significant difference between the two groups. The reason may be the common sense that eating vegetables and fruits is good for health and people are willing to improve intakes of them.

In this investigation the serum concentrations of BC is detected by HPLC. Results showed that the BC of case group was lower than the control group significantly. It suggested that BC may be inversely associated with esophageal cancer risk which is consistent with Nomura's research (Nomura et al., 1997). The value of BC was divided into four ranks according to quartiles, and we analyzed the association of the different levels of concentrations and the risk of esophageal cancer. The ORs of Q4, Q3, and Q2 compared with Q1 were 0.13, 0.08 and 0.16 respectively. It means BC may be a protective factor for esophageal cancer in all range.

One potential limitation of the current study is the relatively smaller sample size, which may reduce the credibility of the study results by introducing imprecision into the measurements and limit our ability to estimate the association more precisely. Hence, further studies with larger sample size and prospective cohort are mandated to confirm the present findings and the dietary intakes of BC and the risk of esophageal cancer. However, despite the limitation due to the sample size, we observed a statistical significance with serum VD3 and BC and the risk of esophageal cancer.

In conclusions, our finding provides important evidences that a reduced risk of esophageal cancer may be associated with increased circulating concentrations of VD3 and BC. But it is not significantly associated with the dietary BC intake. This study suggests that BC and VD3 are the protective factors for esophageal cancer.

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