

## RESEARCH COMMUNICATION

# Effect of Riboflavin-fortified Salt Nutrition Intervention on Esophageal Squamous Cell Carcinoma in a High Incidence Area, China

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### Abstract

**Background:** Riboflavin-fortified salt is now supplied in the diet for residents who live in high incidence areas for esophageal squamous cell carcinoma in China. **Patients and Methods:** All residents from 21 townships in Cixian, Hebei province were divided into an intervention group (9 townships, 11,382 people) who took up riboflavin-fortified salt and a control group (12 townships, 10,711 people) who were free from riboflavin-fortified salt. Some 1,300 of the control group and 950 of the intervention group were randomly selected to undergo endoscopy examination using iodine dye with multi-point biopsy and histopathology examination. Among them 155 of the intervention group and 120 of the control group were tested for the blood riboflavin level with reference to the erythrocyte glutathione reductase activity coefficient (EGRAC). Esophageal squamous cell carcinoma incidence data were further obtained from the Cixian Cancer Registry. **Results:** The results of endoscopy suggested the mucosal status of the intervention group to be better than that of the control group. It showed 82.1% of the mucosal status of control group to be normal, 14.8% to have dysplasia, and 3.1% pre-cancer or cancer, respectively, as compared to 84.8%, 13.6% and 1.6%, respectively, for the intervention group. The mean EGRAC values for the intervention and control groups were 1.452 and 1.606, respectively ( $P < 0.01$ ); compared with normal mucous membrane of esophagus, the lack of riboflavin increased the risk of esophageal squamous cell carcinoma ( $OR = 3.921$ , 95%  $CI = 1.853 \sim 11.936$ ), but the risk of dysplasia did not increase ( $OR = 3.421$ , 95%  $CI = 0.912 \sim 10.159$ ); after intervention, the six years average esophageal squamous cell carcinoma incidence of the intervention group (112.46/100,000) was lower than in the control group (142.11/100,000), although there was no statistical significance ( $u = 1.858$ ,  $P > 0.05$ ). **Conclusion:** It proved practical and effective to improve the status of riboflavin and esophageal mucosa by taking up riboflavin-fortified salt.

**Key Words:** Esophageal SCC - riboflavin-fortified salt - intervention - high incidence area

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### Introduction

Esophageal cancer is the sixth most common cancer and about four hundreds of thousands of people died of it on the global in 2002. China has the highest incidence and mortality rate of esophageal cancer all over the world, which accounts above half of people who died of esophageal cancer in the world. Esophageal squamous cell carcinoma (SCC) is the predominant type of esophageal malignancy in China. Cixian county of Hebei province neighboring to Linxian county of Henan province is the highest incidence of esophageal cancer in China, even in the world. The incidence rate is 160.1/100,000 in males, and 82.6/100,000 in females (He et al., 2008). Some studies suggested that the nutritional imbalance played an important role in the tumor occurrence and development. The joint U.S.-China nutritional intervention study in Linxian county found the odds ratio for subjects in the treatment group (versus those in the placebo group) having esophageal or gastric dysplasia or cancer was 0.84

and riboflavin was a protective factor for occurrence of the esophageal cancer (Van Rensburg, 1981; Taylor et al., 1995). The 1990's dietary investigation and biochemical analysis showed that the riboflavin levels of Cixian residents only accounted for 53.3 percent of the standard level (Cui and Luo, 2006). Adding riboflavin to daily salt will be more acceptable than taking drugs. To explore the potential protection role of the riboflavin in the pathogenesis of esophageal SCC, we investigated the effect of the riboflavin-fortified salt nutritional intervention in esophageal SCC in Cixian County in Hebei province China from May 2000 to May 2007.

### Patients and Methods

#### Patients

**Basic information:** Cixian is located at latitude  $36^{\circ}30'$  north and longitude  $114^{\circ}40'$  east. It is situated on the east side of the Taihang Mountain, along the Zhanghe River and it lies to the south of Handan City. Across the Zhanghe

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River to the south is Anyang City of Henan Province. Cixian county occupies an area about 1,014 square kilometers, composed of 19 districts, and its population is 625,147, consisting of 316,876 males and 308,271 females according to 2000 census. There is a remarkable variation in the earth stratum of the county, with mountainous, hilly, and plain areas each constituting of about one-third of its total area. Cixian, an underdeveloped county, the incidence of esophageal SCC is 127.14/10,000 and the diet of the residents is monotonous, high in starch but low in fresh fruits and vegetables.

**Study subjects:** All residents from 21 townships in Cixian, Hebei province were divided into intervention group (9 townships, 11,382 people) who took up riboflavin-fortified salt and control group (12 townships, 10,711 people) who were free from riboflavin-fortified salt. Riboflavin-fortified salt was produced by the Hebei Franchise Salt Production Limited Company in accordance with national standards (riboflavin salt content of 100 ~ 150 mg / kg). Local residents used to store the salt in sealed cans in the dark, so the loss of riboflavin induced by photochemistry can be ignored. The residents of intervention group including 9 hamlets only use riboflavin-fortified salt from May 2000 through May 2007. All participants provided written informed consent. The investigators performed a sample survey every half year to observe the status of consumption. The usage rate is above 98 percent.

#### Methods

**Endoscopy examination:** 1300 of control group and 950 of intervention group from 40 to 69 years were randomly selected to undergo examination of electronics fiber endoscopy using iodine dye with multi-point biopsy and histopathology examination in 2007. At the beginning of the survey, the subjects were asked to fill out an epidemiological questionnaire, followed by physical examination performed by the physicians to exclude persons with serious contraindications to endoscopy. Endoscopic examinations were then performed by specialists following the procedures described by Wang et al (1995). The results were recorded and the biopsy specimens obtained were fixed in 80% alcohol and stained by hematoxylin-eosin (HE) for subsequent pathological diagnosis by pathologists. The result was recorded as normal (including esophagitis), dysplasia (including mild dysplasia, moderate dysplasia), pre-cancer and cancer (including severe dysplasia, carcinoma in situ and advanced carcinoma).

**Riboflavin status variables:** Among the residents (155 of intervention group and 120 of control group) who undergone the endoscopy examination were tested for the blood riboflavin level by the measure of erythrocyte glutathione reductase activity coefficient (EGRAC). 0.5ml venous blood samples were collected from the subjects under fasting conditions between 8:00 AM and 10:00 AM with ethylenediaminetetraacetate (EDTA) used as an anticoagulant. The blood were centrifuged and separated into plasma and red blood cell. 0.5ml RBC were put into a cuvette and added with 0.5ml distilled water and

**Table 1. Endoscopy Examination Results for the Intervention and Control Groups**

Group	Normal		Atypical hyperplasia		Cancer		Total
	N	%	N	%	N	%	
Control	1,067	(82.1)	193	(14.8)	40	(3.1)	1,300
Intervention	806	(84.8)	129	(13.6)	15	(1.6)	950
Total	1,873	(83.2)	322	(14.3)	55	(2.4)	2,250

$\chi^2=6.419$  P=0.040

preserved at -80°C then taken back to Hebei Cancer Institute for the following test which referred to Sauberlich et al (1972). For each sample to be assayed two cuvettes were used, both adding oxidized glutathione (GSSG) to the RBC demolysate. One cuvette, 0.1 ml of the lavin adenine dinucleotide (FAD) solution was added and the other, 0.1 ml of distilled water. Both cuvettes were then allowed an equilibration incubation period of 10min in 37°C. And then, 0.1 ml of reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) solution was added to each cuvette and the change in optical absorption lasted for 10 min, a reference blood sample was used routinely in the assays. Erythrocyte glutathione reductase assays were expressed in terms of "activity coefficients" (AC), representing the degree of stimulation resulting from the in vitro addition of FAD. The EGRAC, a biochemical indicator to value the riboflavin status, could reflect the GSSG level saturated by FAD in the blood. To some extent, the higher the EGRAC is, the worse the riboflavin status, vice versa. A threshold of 1.50 was used to indicate biochemical riboflavin deficiency (Landommenic et al., 1997).

**Esophageal SCC incidence data:** The esophageal SCC incidence data of the two groups were obtained from Cixian Cancer Registry. The Cixian Cancer Registry is a population-based registry established in 1974. The register was conducted by the three-level prevention web. Each clinic doctor in every township (prevention web I) was required to report each new case of cancer occurring in the township by a standard card, then the cards were sent to the clinic of the rural administration unit (prevention web II). They were sorted and sent to the Cixian Cancer Registry (prevention web III) once a month. These cards were checked, analyzed, coded and stored there. At the end of each year, a sample survey was conducted, to check the quality of the registration. For all the cases known from a death certificate only (DCO), we followed trace-back procedures before registering the case as a DCO notification. The percentage of Death Certificate Notification (DCN) cases was less than 10% in the period.

**Statistical Analysis:** The data were expressed as the mean±S.D. and percent. The  $\chi^2$ -test was used for the difference in different groups. p<0.05 was considered significant. Analyses were done in SPSS for Windows (version 13.0, SPSS Inc, Chicago, IL, USA).

## Results

**Endoscopy examination:** It showed 82.1% of the

**Table 2. Erythrocyte Glutathione Reductase Activity Coefficient (EGARC) Measurement Results**

Group	40~49		50~59		60~		Total							
	Male (78)	Female (76)	Male (42)	Female (42)	Male (20)	Female (17)								
Control	1.588	0.301	1.657	0.251	1.562	0.271	1.627	0.249	1.559	0.224	1.662	0.349	1.606	0.273
Intervention	1.465	0.331	1.477	0.324	1.505	0.297	1.312	0.218	1.544	0.201	1.401	0.157	1.452	0.297
Total	1.528	0.320	1.534	0.313	1.529	0.285	1.447	0.278	1.553	0.210	1.524	0.289	1.519	0.296

mucosal status of control group was normal, 14.8% had dysplasia, and 3.1% had pre-cancer or cancer, respectively, while the intervention group figures were 84.8%, 13.6% and 1.6% respectively. The esophageal mucosal status of intervention group was better than control group ( $\chi^2=6.419$   $P=0.040$ ) (Table 1).

**EGRAC:** The average value of 275 people was 1.519, which indicated the average level of riboflavin was still low in this area. The intervention group contained 155 persons, eating riboflavin-fortified salt. 65.8% of their level of riboflavin was sufficient, 23.2% was not enough and 11.0% was deficient. And the control group, 120 persons who had ate the common salt and 31.7% of their level of riboflavin was sufficient, 52.5% was not enough and 15.8% was deficient. The EGRAC of intervention group was 1.452 ( $\pm 0.297$ ), lower than 1.606 ( $\pm 0.273$ ) of control group, the difference is significant ( $t=4.412$ ,  $P<0.001$ ). There is not significant difference between intervention group and control group on sex and age (Table 2), the results showing that eating Riboflavin-fortified salt could increase the blood level of riboflavin.

**AC values reflecting activity of glutathione reductase were associated with esophageal cancer:** AC value  $>1.5$ , as reflected in the low activity of glutathione reductase, was the risk factor of atypical hyperplasia or cancer. Compared with normal mucous membrane of esophagus, the lack of riboflavin increased the risk of esophageal squamous cell carcinoma (OR=3.921, 95% CI=1.853~11.936), however, the occurrence of dysplasia did not show significant difference (OR=3.421, 95% CI=0.912-10.159) between the intervention group and the control group.

**Esophageal SCC data:** After intervention, the six years average esophageal cancer incidence of intervention group was 112.46/100,000, control group was 142.11/100,000. Intervention group was lower than control group, although there was no statistical difference between them ( $u=1.858$ ,  $P>0.05$ ).

## Discussion

As a kind of B vitamin, vitamin B2 (riboflavin) deficiency can increase the activity of the nitrosamine metabolism activation enzyme, thereby promoting the metabolic activation of the nitrosamines. Formation of single strand breaks in nuclear DNA induced by N-nitrosodimethylamine and hepatocarcinogens aflatoxin B1 was observed to be more pronounced in rats maintained on a riboflavin-deficient diet compared with that on a normal diet. This increased damage was reversed on

riboflavin supplementation. The induction of repair enzymes poly (ADP-ribose) polymerase, DNA polymerase beta and DNA ligase was significantly higher in riboflavin-deficient rats following DNA damage caused by the administration of carcinogens. Riboflavin supplementation brought down the induction to the levels found in rats maintained on normal diet. Since damage to DNA and its altered repair may lead to carcinogenesis, modulation of these parameters by riboflavin suggests a potential chemo preventive role of this vitamin (Pangrekar et al., 1993; Webster et al., 1996). Furthermore, riboflavin can reduce glutathione in the liver. Glutathione is an important intracellular antioxidant; indirectly undermining the inhibition of glutathione metabolism detoxification nitrosamines. Some scholars think that one of the mechanisms of vitamin B2 evoked cancer is that it does not participate in the bio-oxidation reaction and energy metabolism in vivo, which may indirectly undermine the antioxidant capacity of the body (Mattson et al., 2002). Animal experiments show that low-dose nitrosamines present carcinogen obviously under the conditions of lack of riboflavin (Foy and Kondi, 1984). In 600~800 days low-dose nitrosamine induced rat's esophageal cancer experiments, 73.4% rats suffered from esophageal neoplasm in the riboflavin deficient feedstuff group, while no cancer occurrence detected in the control group.

Epidemiologic investigation suggested that dietary staples particular lack of riboflavin associated with a high risk for esophageal cancer, and vice versa (Van Rensburg, 1981; Chen et al., 2002). The genesis of esophageal cancer is a complex process as follows: normal esophageal epithelium, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ and advanced carcinoma. Esophagistis is also a risk factor for esophageal cancer. An epidemiologic survey among 538 young persons in a high-risk area for esophageal cancer in China revealed a high prevalence of esophagitis. Histologically, very mild, mild and moderate esophagitis was observed in 31.6%, 10.7%, and 1.1% of males and 30.4%, 4.3%, and 1.1% of females (Chang-Claude et al., 1992). An endoscopic survey in Cixian found that mild, moderate, and severe esophageal dysplasia were 8.6% (172/2,013), 7.8% (157/2,013) and 2.6% (53/2,013) respectively in the selected population (Lu et al., 2004). Riboflavin can prevent esophageal cancer by protecting the integrity of esophageal mucosa epithelial.

As a water-soluble vitamin, riboflavin need renewed to make up for the consumption of metabolism. It's an economic, convenient and suitable "effective" strategy Riboflavin supplement is a long-term work, so adding riboflavin to daily salt will be conducive to maintain its effect and popularize it. Our study indicated that supplement of riboflavin-fortified salt worked well as a

nutritional intervention measure on esophageal cancer. Long-term regular supplement would improve the nutritional level of riboflavin in vivo. It suggested that riboflavin salt might not only improve the nutritional status, but also could ameliorate the pathological state of esophageal mucosa, which was consistent with research previous findings (Siassi and Ghadirian, 2005). After six years intervention, the average esophageal cancer incidence of intervention group was lower than control group, although there was no statistical difference between them. Our study was accorded with the report of Mark at the 2001 American Association for Cancer Research 92nd Annual Meeting, who found that a quartile rise of riboflavin content could reduce the incidence of esophageal cancer by 6% based on his nutrition intervention experiment on esophageal cancer in Linxian.

In this study, we used EGRAC value measure and endoscopy to investigate riboflavin-fortified salt, which could thought to be more effective. To evaluate the status of riboflavin, the AC of glutathione reductase is more accurate and convenient. The red blood cell content of riboflavin is a reasonably sensitive and practical index for evaluating nutritive status and the convenient and available micro chemical methods are well adapted for its detection (Bessey et al., 1956). Compared with other methods, endoscope iodine staining indicative multi-point biopsy was more direct and exact. Pathological diagnosis was applied to those who had been tested as abnormal by endoscope, which could truly reflect the status of esophagus.

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