RESEARCH ARTICLE

High Serum Level of Retinol and α-Tocopherol Affords Protection Against Oral Cancer in a Multiethnic Population

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Abstract

Background: A comparative cross-sectional study involving oral cancer patients and healthy individuals was designed to investigate associations between retinol, α-tocopherol and β-carotene with the risk of oral cancer. Materials and Methods: This study included a total of 240 matched cases and controls where subjects were selected from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). Retinol, α-tocopherol and β-carotene levels and intake were examined by high-performance liquid chromatography (HPLC) and food frequency questionnaire (FFQ) respectively. Results: It was found that results from the two methods applied did not correlate, so that further analysis was done using the HPLC method utilising blood serum. Serum levels of retinol and α-tocopherol among cases (0.177±0.081, 1.649±1.670μg/ml) were significantly lower than in controls (0.264±0.137, 3.225±2.054μg/ml) (p<0.005). Although serum level of β-carotene among cases (0.106±0.159 μg/ml) were lower compared to controls (0.134±0.131μg/ml), statistical significance was not observed. Logistic regression analysis showed that high serum level of retinol (OR=0.501, 95% CI=0.254-0.992, p<0.05) and α-tocopherol (OR=0.184, 95% CI=0.091-0.370, p<0.05) was significantly related to lower risk of oral cancer, whereas no relationship was observed between β-carotene and oral cancer risk. Conclusions: High serum levels of retinol and α-tocopherol confer protection against oral cancer risk.

Keywords: Oral cancer - micronutrients - retinol - α-tocopherol - β-carotene - Malaysia

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Introduction

Oral cancer remains a major public health challenge in many parts of the world with over 275,000 new cases reported annually (Warnakulasuriya, 2009). High incidence was reported in developing nations (Krishna Rao et al., 2013) where it accounts for up to 40% of all malignancies in Southeast Asia (Neville and Day, 2002). This disease is prevalent among the Indian ethnic group in Malaysia where it was ranked among the ten most common cancers (Lim et al., 2008). The overall mortality rate remains high and only approximately 60% of people diagnosed with oral cancer will survive up to 5 years (Priebe et al., 2008). Unfortunately, the survival rate for oral cancer patients had not changed much for the last three decades despite significant advances in management strategies (Lane et al., 2006).

It is widely known that the aetiology of oral cancer is multi-factorial with well established risk habits of tobacco smoking, alcohol consumption and betel quid chewing (Talamini et al., 2002; Gallus et al., 2003; Aruna et al., 2011). A recent study from South East Asia found that tobacco smoking and alcohol consumption increases the risk of getting oral cancer of up to 5 times while betel quid chewing contributes to a higher risk of up to 9 times (Loyha et al., 2012). Although it has been established globally, unfortunately in Malaysia awareness is only in tobacco smoking, where as, alcohol consumption and betel quid chewing are not recognised as risk factors for oral cancer (Ghani et al., 2013).

Interestingly, some researchers reported that these traditional risk factors are not associated with the development of oral cancer (Krishnamurthy and Ramshankar, 2013). Therefore, other factors such as dietary intake have been postulated to play a role in the prevention and promotion of oral cancer (Zain, 2001;

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Greenwald et al., 2002). Whilst the intake of meat has been shown to increase the risk of multiple cancers (Aune et al., 2009; Pan et al., 2012), a diet high in fruits and vegetables has been found to be inversely associated (Levi et al., 1998; Hung et al., 2004; Benetou et al., 2008). The inverse associations has been hypothesized to be accounted for by the micronutrients found abundantly in fruits and vegetables which plays a significant role in maintaining health and preventing diseases through a wide range of mechanism such as anti-oxidant, anti-proliferation and repair of DNA damage (Gupta et al., 2012).

Direct and indirect relationships between micronutrients and cancer have been described in various epidemiological and clinical trial studies. However, the overall evidence for the relationship between micronutrients and the risk of oral cancer has been inconsistent and controversial. While micronutrients such as α-tocopherol and β-carotene have been found to confer protection against oral cancer (Negri et al., 2000; Pavia et al., 2006; Tavani et al., 2012), other researchers have found otherwise (De Stefani et al., 1999; Marchioni et al., 2002; Petridou et al., 2002). For the Malaysian population to date, no studies have been done to associate micronutrient intake with the risk of oral cancer. Malaysia is a diverse nation populated by various ethnic groups, thus the blend of food available from the different cultures provides a unique combination of nutrients sources. Furthermore, the dynamics of different ethnic groups co-existing in the same community serves as the best platform to compare nutrient level and risk for oral cancer between different ethnicities. Hence, this study aims to investigate the relationship between micronutrients (retinol, α-tocopherol and β-carotene) and risk of oral cancer in a multi-ethnic Malaysian population. This study would provide further understanding of this association which could result in the development of a national dietary plan targeted towards the prevention of oral cancer.

Materials and Methods

Study Population

Data and specimens for this study were extracted from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS) which collects socio-demographic, epidemiological and clinico-pathological data and specimens from 7 hospital-based centres nationwide. A total of 240 subjects were included in this study with 120 cases and 120 controls matched for age, gender and ethnicity respectively. An equal sampling of cases and controls were taken from the 4 ethnic groups (Malays, Chinese, Indians and Indigenous people of East Malaysia) that constitute the population of Malaysia. As dietary practices are largely influenced by culture, this type of sampling method is required to avoid bias. Cases were subjects who are histologically diagnosed with oral squamous cell carcinoma of the oral cavity and controls were selected among healthy individuals attending participating centres/hospitals for minor ailments without the disease of interest. For each respondent, data on socio-demographic background, risk habits and dietary intake were obtained via face-to-face interview by trained personnel at each participating centre/hospital using a set of validated questionnaire. Only respondents with complete information/data and sufficient amount of specimen were included in this study. The research protocol for this study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (Reference number: DFCO1302/0035(P).

Data collection from measurement tools

This study uses two measurement tools to measure micronutrient level/intake. The first measurement is obtained from high-performance liquid chromatography (HPLC) using blood serum, while the second is taken using data gathered via a specific food frequency questionnaire (FFQ). If data obtained from the two measurement tools are comparable, the FFQ can be used as a standard measurement tool, which is significantly less invasive than acquiring blood samples from individuals. This would encourage increased public participation in studies of this nature.

High-performance liquid chromatography (HPLC)

(a) Standard solution, quality control and calibration: Stock solutions of standards, all-trans-retinol (0.1mg/ml); α-tocopherol (1mg/ml); β-carotene (1mg/ml) and internal standards, retinol acetate (0.1mg/ml); α-tocopherol acetate (1mg/ml) was prepared individually in ethanol. Working solutions consisting of a mixture of analytes were prepared by diluting standard solutions in ethanol. The mixed calibration samples were prepared in ethanol in triplicates in the following concentration: 0.0390, 0.0781, 0.1560, 0.3125 and 0.6250μg/ml for all-trans-retinol, and 0.390, 0.781, 1.560, 3.125 and 6.250μg/ml for α-tocopherol and β-carotene. Internal standard concentration was maintained at 25μg/ml. Three levels quality control were used for reproducibility and validation purposes. Calibration was performed prior to injection on a weekly basis and the calibration curve was generated with application of the OpenLab CDS chemstation software.

(b) Sample pre-treatment: Blood samples obtained from the MOCDTBS were centrifuged at 3000g for 10 minutes to obtain serum. Isolated serum was stored at -80°C prior to use in the laboratory. Matched case-control serum samples were analysed for serum level of retinol, α-tocopherol and β-carotene using high performance liquid chromatography (HPLC) method. The serum samples prepared for analysis was a modified version of an earlier reported procedure (Siluk et al., 2007). Briefly, a mixture of 100μl serum, 50μl internal standards, 100μl distilled water was vortex-mixed and the resulting solution was added to a 200μl aliquot of ethanol. The components of interest were then extracted into 400 μl of n-hexane using liquid-liquid extraction technique. A 300μl of n-hexane aliquot was evaporated to dryness under stream of nitrogen and the resulting pellet was suspended in 200μl acetonitrile. 25μl of dissolved aliquot was injected into the HPLC system (Agilent 1260 Infinity Quaternary LC System).

(c) Calculations: Sample concentrations were calculated from the following formula (Semeraro et al., 2009):

$$C = \frac{\text{peak area of sample x CSTD}}{\text{peak area of standard}} \times \frac{\text{peak area of IS for STD}}{\text{peak area of IS for sample}} \times DF$$

Where, IS=internal standard; STD=standard; DF (dilution factor)=0.5; CSTD (working standard concentration) is 0.6250μg/ml for all-trans-retinol and 6.250μg/ml for α-tocopherol and β-carotene.

(d) Chromatographic conditions: Micronutrients were separated by isocratic elution and the operational conditions for HPLC method were as follows: stationary phase, Zorbax Eclipse XDB-C18 column (Agilent); mobile phase, 60% acetonitrile: 25% methanol: 15% dichloromethane; detector wavelength, 325nm for retinol, 292nm for α-tocopherol and 450nm for β-carotene (Thibeault et al., 2009); flow rate, 1.0ml/min.

Food frequency questionnaire (FFQ)

The FFQ which was developed and validated for the Malaysian population was used to collect information on dietary intake. This questionnaire consists of 99 food items and beverages where commonly used serving size were specified. The FFQ is an interview guided questionnaire where trained personnel from each participating centre will ask subjects to estimate the average frequency of intake for each food items in the questionnaire. Oral cancer patients were asked to estimate their usual dietary intake for 1 year prior to diagnosis while controls were asked to estimate dietary intake from the previous year.

Subjects were given nine options to indicate the frequency of each food items consumed where the options ranges from never to more than six times per day. For each food items consumed by the subjects, a score of daily equivalent will be tabulated to estimate nutrient intake. A nutrient composition database was then used whereby micronutrient intake was computed based on food intake input.

Statistical analysis

Data from FFQ was categorized according to the Recommended Daily Allowances (RDA) namely 'above RDA' and 'below RDA'. The RDA of each nutrient for the Malaysian population was obtained from nutrition book based on the guidelines provided by the National Coordinating Committee on Food and Nutrition (NCCFN). Association between micronutrient intake and oral cancer was analysed using Chi-square test while HPLC data was analysed using independent t-test to obtain mean differences in serum level of micronutrient. Pearson's correlation coefficient was employed to examine the relationship between the two methods used in this study. As both methods do not correlate, further analysis was only done using data from HPLC. In order to assess relative risk, the available continuous data from the HPLC method was categorized into 'low serum micronutrients' and 'high serum micronutrients'. The cut-off point to discriminate high serum micronutrients from low serum micronutrients was done using Receiver Operating Characteristics (ROC) curve analysis. Logistic regression analysis was then done to derive the relative risk (odds ratio) for oral cancer risk. All statistical analysis was carried out using the Statistical Programme for Social Science (SPSS) Version 21.0 software.

Results

Socio-demographic characteristics of the study sample which includes reference to the age and gender distribution of all subjects and their practice of risk habits are presented in Table 1. The population selected for this study was in the age range of 23-87 years with the mean age of 57.48±12.96. It was observed that the prevalence of oral cancer in the sample increases with increasing age group where more than 70% of oral cancer cases were seen in >50 years age group. There were slightly more females (n=134) than males (n=106) in this study population. Since cases and controls were matched, no difference was observed in age, gender and ethnicity between cases and controls.

In general, it was observed that a higher proportion of cases practiced risk habits compared to controls. It is also observed that the prevalence of risk habits is related to ethnicity where tobacco smoking habit is mostly seen among the Malays (46.7%), while alcohol drinking and betel quid chewing habits are most prevalent among the Chinese (38.3%) and Indians (71.7%) respectively.

Table 2 shows the serum level of micronutrients

Table 1. Sociodemographic Characteristics of the Study Population

		Ethnicity														Total (n)
		Malay				Chinese				Indian				Indigenous		
		Case		Control		Case		Control		Case		Control		Case		Control		
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
Subjects		30	25	30	25	30	25	30	25	30	25	30	25	30	25	30	25	240
Age	<35	4	13.3	5	16.7	3	10	3	10	0	0	0	0	2	6.7	2	6.7	19
	35-49	8	26.7	10	33.3	2	6.7	3	10	4	13.3	3	10	2	6.7	2	6.7	34
	≥50	18	60	15	50	25	83.3	24	80	26	86.7	27	90	26	86.7	26	86.7	187
Gender	Male	17	56.7	17	56.7	20	66.7	20	66.7	8	26.7	8	26.7	8	26.7	8	26.7	106
	Female	13	43.3	13	43.3	10	33.3	10	33.3	22	73.3	22	73.3	22	73.3	22	73.3	134
Risk habits	Smoking																	
	No	16	53.3	16	53.3	11	36.7	23	76.7	28	93.3	27	90	17	56.7	22	73.3	160
	Yes	14	46.7	14	46.7	19	63.3	7	23.3	2	6.7	3	10	13	43.3	8	26.7	80
	Drinking																	
	No	30	100	30	100	14	46.7	23	76.7	25	83.3	26	86.7	17	56.7	21	70	186
	Yes	0	0	0	0	16	53.3	7	23.3	5	16.7	4	13.3	13	43.3	9	30	54
	Chewing																	
	No	29	96.7	26	86.7	30	100	30	100	7	23.3	10	33.3	11	36.7	23	76.7	166
	Yes	1	3.3	4	13.3	0	0	0	0	23	76.7	20	66.7	19	63.3	7	23.3	74

obtained from HPLC. Mean of serum retinol and α-tocopherol levels among cases (0.177±0.081, 1.649±1.670μg/ml) were significantly lower than in controls (0.264±0.137, 3.225±2.054μg/ml) (p<0.005). β-carotene level among cases (0.106±0.159μg/ml) were also lower compared to controls (0.134±0.131μg/ml), however, the difference was not statistically significant. Analysis of micronutrient intake from FFQ shows that significantly more controls consume retinol (21.7%) above recommended daily allowances (RDA) as compared to cases (10.8%), where as, differences in the intake of α-tocopherol and β-carotene among cases and controls was found to be insignificant (Table 3).

Pearson's correlation coefficient found that there was no correlation between the two methods used for all the three micronutrients (Table 4). Therefore, further analysis on risk assessment was done only on results obtained from the HPLC analysis. ROC curve defined 'high' level of serum retinol as >0.20μg/ml (AUC=0.699, p=0.000) while for α-tocopherol and β-carotene 'high' is categorized as >2.90μg/ml (AUC=0.734, p=0.000) and >0.05μg/ml (AUC=0.639, p=0.000) respectively. Logistic regression analysis (Table 5) showed that high serum level of retinol (OR=0.501, 95% CI=0.254-0.992, p<0.05) and α-tocopherol (OR=0.184, 95% CI=0.091-0.370, p<0.05) was significantly related to lower risk of oral cancer and the significant effect remains unchanged even after adjusting for known risk habits. No relationship was observed between serum level of β-carotene and oral cancer risk. When sub-group analysis (Table 5) was done for the different ethnic groups in Malaysia slight differences were seen in the effects of micronutrient level on oral cancer risk. The protective effect of retinol seen earlier is only significant among the Indians after adjusting for confounding factors. As for the Malays and Indigenous people, only α-tocopherol appeared to confer protective effect, while, none of the micronutrients studied is associated with oral cancer risk reduction among the Chinese.

Table 2. Comparison of Micronutrient Level Between Cases and Controls Using HPLC

Micronutrients	Cases		Control		t-test
	Mean (μg/ml)	S.D	Mean (μg/ml)	S.D	p-value
Total					
Retinol	0.177	0.081	0.264	0.137	<0.001
α-tocopherol	1.649	1.67	3.225	2.054	<0.001
β-carotene	0.106	0.159	0.134	0.131	0.141

Table 3. Comparison of Micronutrient Intake Between Cases and Controls Using FFQ

Micronutrients		Case n (%)	Control n (%)	Chi-square test p-value
Retinol	> RDA	13 (10.8)	26 (21.7)	0.023
	< RDA	107 (89.2)	94 (78.3)	
α-tocopherol	> RDA	8 (6.7)	6 (5.0)	0.582
	< RDA	112 (93.3)	114 (95.0)	
β-carotene	>RDA	72 (60.0)	84 (70.0)	0.104
	< RDA	48 (40.0)	36 (30.0)	

Table 4. Correlation Between Two Measurements Tools (HPLC and FFQ)

Micronutrients	Pearson's Correlation	Sample size	p-value
Retinol	-0.29	240	0.658
α-tocopherol	-0.5	240	0.437
β-carotene	-0.23	240	0.723

Discussion

Studies with regards to micronutrients and its association with risk for oral cancer originating from the South East Asian region where food and nutrient intake is distinctly different from the Western world are limited. In this study, subjects were included from four ethnic groups in Malaysia and from various parts of the country to better elucidate the relationship between micronutrients and occurrence of oral cancer. In this study two dietary assessment methods were used. The HPLC was used to assess serum level of micronutrients while FFQ was used to assess micronutrient intake. It is interesting to note that results from both methods are consistent for retinol and β-carotene but not for α-tocopherol. This may be attributed to lack of food sources of α-tocopherol in the FFQ. For example, plant oil is an important source of a-tocopherol, however, this item is not specified in the FFQ, thus, α-tocopherol intake was only measured by

Table 5. Logistic Regression Analysis of Micronutrients and Oral Cancer Risk

Micronutrients		high vs low serum level (n)	Crude OR (95%CI)	p-value	Adjusted OR* (95%CI)	p-value
Total	Retinol	93*vs*147	0.495 (0.255-0.958)	0.037	0.501 (0.254-0.992)	0.047
	α-tocopherol	87*vs*153	0.184 (0.093-0.364)	<0.001	0.184 (0.091-0.370)	<0.001
	β-carotene	159*vs*81	0.529 (0.280-1.002)	0.051	0.569 (0.293-1.103)	0.095
Malay	Retinol	27*vs*33	0.618 (0.131-3.523)	0.646	0.602 (0.104-3.466)	0.569
	α-tocopherol	27*vs*33	0.039 (0.008-0.197)	<0.001	0.029 (0.005-0.172)	<0.001
	β-carotene	40*vs*20	0.446 (0.086-2.320)	0.337	0.708 (0.102-4.916)	0.727
Chinese	Retinol	31*vs*29	0.452 (0.127-1.610)	0.22	0.565 (0.146-2.183)	0.408
	α-tocopherol	25*vs*35	0.259 (0.069-0.980)	0.047	0.253 (0.062-1.034)	0.056
	β-carotene	36*vs*24	0.556 (0.148-2.087)	0.384	0.699 (0.169-2.884)	0.62
Indian	Retinol	18*vs*42	0.208 (0.040-1.074)	0.061	0.184 (0.035-0.960)	0.045
	α-tocopherol	18*vs*42	0.34 (0.066-1.752)	0.197	0.33 (0.061-1.778)	0.197
	β-carotene	52*vs*8	0.104 (0.009-1.178)	0.068	0.079 (0.005-1.171)	0.065
Indigenous	Retinol	17*vs*43	0.62 (0.171-2.245)	0.467	0.338 (0.030-1.898)	0.218
	α-tocopherol	17*vs*43	0.238 (0.063-0.907)	0.035	0.216 (0.047-0.994)	0.049
	β-carotene	31*vs*29	0.82 (0.259-2.599)	0.736	0.762 (0.206-2.820)	0.684

*Adjusted for risk habits of smoking, alcohol consumption and betel quid chewing

other items like green leafy vegetables, margarine and wheat germ (Wardlaw and Smith, 2011) which may not represent the actual intake. The underreporting of this nutrient suggested that results from the HPLC method may be more suitable to achieve the objective of this study. Furthermore, analysis indicated that the two methods do not correlate, thus, further analysis was carried out using data obtained from HPLC.

Serum level of retinol and α-tocopherol was found to be significantly higher in controls as compared to cases and was shown to confer protection of up to 50% and 80% respectively. Zheng et al. (1993) also reported significant association between retinol and α-tocopherol with oral cancer risk. This finding is further corroborated by an earlier study where serum level of vitamin A (a group of organic compound including retinol) and E (a group of organic compound including tocopherol) was also found to be lower in cases as compared to controls where low serum level of these vitamins was associated with 10.9 times and 5.6 times increased risk for oral cancer (Lawal et al., 2012). Inverse association of retinol and α-tocopherol have also been reported in other cancers such as lung (Klarod et al., 2011), breast (Shim et al., 2012) and gastric cancer (Jenab et al., 2006). The protective effect exerted by retinol and α-tocopherol could be explained by their role as potent regulators of cellular activities, thus having significant impact on oral carcinogenesis (Mukherjee et al., 2011). These micronutrients are also strong antioxidants that have been shown to suppress the development of malignancy in cell culture experiments and animal studies (Zou et al., 2001). Although the mechanism of action is rather complex and unclear, it has been hypothesized that antioxidant activity of these micronutrients prevents tissue damage by deactivating excited oxygen molecules and neutralizing free radicals (Poljsak, 2007) which then translates into the protective effects seen.

In contrast, this study found that serum β-carotene level was not statistically associated with lower risk of oral cancer although it has been shown to also exhibit antioxidant properties (Ross et al., 2007). This lack of protective effect is consistent with several findings on other cancers such as prostate and bladder cancer (Nomura et al., 1997; Zeegers et al., 2001; Wang et al., 2009). However, studies on oral cancer reports otherwise. Zheng et al. (1993) found that serum level of β-carotene was lower in subjects that subsequently developed oral and pharyngeal cancer while a study among the Japanese found that serum β-carotene was significantly lower in males with oral leukoplakia as compared to controls (Nagao et al., 2000). In this study, serum level of β-carotene was similar between cases and controls, thus, obviating the detection of any effect (Persson et al., 2008).

This study also attempted to elucidate ethnic differences (if any) in the level of serum micronutrients and its effects on oral cancer risk as ethnic differences in consumption of nutrients had been previously described. It was reported that non-Hispanic Blacks have lower level of serum vitamin E and D compared to non-Hispanic Whites (Kant and Graubard, 2007). Another study found that β-carotene level among Chinese women is higher than Japanese, Caucasian and Hispanic women (Huang et al., 2002). In this study, all the three micronutrients were found to exert protective effect in all the ethnic groups but only a few were found to be statistically significant. Retinol was found to reduce risk of oral cancer by almost 5 folds for the Indian ethnic group after confounding for risk habits. This suggests that protective mechanism of retinol may be masked by the effect of betel quid chewing as it is heavily practiced among the Indians in this population. α-tocopherol confers significant protection of more than 90% in the Malay and approximately 80% in the Indigenous ethnic group. This result is consistent with findings from a preliminary study done in Malaysia where α-tocopherol was found to be inversely associated with the risk of oral cancer among Indigenous people (Zain et al., 1999a). β-carotene does not show any significant association with oral cancer risk in all ethnic groups and this is especially true for the Indian ethnic group as another preliminary study found no association between serum micronutrient and risk of oral cancer among the Indians (Zain et al., 1999b). This finding is further strengthened by a study on dietary pattern of Malaysians where no significant relationship was found for a diet characterized by heavy consumption of fruits and vegetables (which is the primary source of β-carotene) with oral cancer risk (Helen-Ng et al., 2012).

In conclusion, high levels of retinol and α-tocopherol confers protection against oral cancer risk. Ethnic differences can be seen in the protective effect of selected micronutrients. This study, along with previous epidemiological and experimental studies provides further evidence that retinol and α-tocopherol are promising chemoprevention agents for cancer of the oral cavity.

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