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

Vimmitra Athirajan¹, Ishak Abdul Razak¹,², Nalina Thurairajah³, Wan Maria Nabillah Ghani¹, Helen-Ng Lee Ching¹, Yi-Hsin Yang⁴, Karen-Ng Lee Peng¹, Zainal Ariff Abdul Rahman⁵, Wan Mahadzir Wan Mustafa⁶, Mannil Thomas Abraham⁶, Tay Keng Kiong⁶, Yuen Kar Mun⁶, Norma Jalil⁶, Rosnah Binti Zain¹,⁵ *

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
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 have 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 (Negrí 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 20μl 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):
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 2. Comparison of Micronutrient Level Between Cases and Controls Using HPLC

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>Cases Mean (µg/ml)</th>
<th>Cases S.D</th>
<th>Control Mean (µg/ml)</th>
<th>Control S.D</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.177 ± 0.081</td>
<td>0.264</td>
<td>0.137 ± 0.131</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td>1.649 ± 1.670</td>
<td>3.225</td>
<td>2.054 ± 2.054</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>0.106 ± 0.159</td>
<td>0.134</td>
<td>0.141 ± 0.141</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>Case n (%)</th>
<th>Control n (%)</th>
<th>Chi-square test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>13 (10.8)</td>
<td>26 (21.7)</td>
<td>0.023</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>8 (6.7)</td>
<td>6 (5.0)</td>
<td>0.582</td>
</tr>
<tr>
<td>β-carotene</td>
<td>72 (60.0)</td>
<td>84 (70.0)</td>
<td>0.104</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>Pearson’s Correlation</th>
<th>Sample size</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>-0.29</td>
<td>240</td>
<td>0.658</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>-0.5</td>
<td>240</td>
<td>0.437</td>
</tr>
<tr>
<td>β-carotene</td>
<td>-0.23</td>
<td>240</td>
<td>0.723</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Micronutrients</th>
<th>high vs low serum level (n)</th>
<th>Crude OR (95%CI)</th>
<th>p-value</th>
<th>Adjusted OR* (95%CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>93/147</td>
<td>0.495 (0.255-0.958)</td>
<td>0.037</td>
<td>0.501 (0.254-0.992)</td>
<td>0.047</td>
</tr>
<tr>
<td>Retinol</td>
<td>87/153</td>
<td>0.184 (0.093-0.364)</td>
<td>&lt;0.001</td>
<td>0.184 (0.091-0.370)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>158/81</td>
<td>0.529 (0.280-1.002)</td>
<td>0.051</td>
<td>0.569 (0.293-1.103)</td>
<td>0.095</td>
</tr>
<tr>
<td>β-carotene</td>
<td>27/33</td>
<td>0.618 (0.131-3.523)</td>
<td>0.646</td>
<td>0.604 (0.104-3.466)</td>
<td>0.569</td>
</tr>
<tr>
<td>Malay</td>
<td>27/33</td>
<td>0.039 (0.008-0.197)</td>
<td>&lt;0.001</td>
<td>0.029 (0.005-0.172)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chinese</td>
<td>25/35</td>
<td>0.259 (0.069-0.980)</td>
<td>0.047</td>
<td>0.253 (0.062-1.034)</td>
<td>0.056</td>
</tr>
<tr>
<td>Indian</td>
<td>36/243</td>
<td>0.556 (0.148-2.087)</td>
<td>0.384</td>
<td>0.699 (0.169-2.884)</td>
<td>0.62</td>
</tr>
<tr>
<td>Indigenous</td>
<td>17/843</td>
<td>0.208 (0.040-1.074)</td>
<td>0.061</td>
<td>0.184 (0.035-0.960)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

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 α-tocopherol, however, this item is not specified in the FFQ, thus, α-tocopherol intake was only measured by
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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