Association of Alpha B-Crystallin Genotypes with Oral Cancer Susceptibility, Survival, and Recurrence in Taiwan

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Abstract

Background: Alpha B-crystallin (CRYAB) is a protein that functions as “molecular chaperone” in preserving intracellular architecture and cell membrane. Also, CRYAB is highly antiapoptotic. Abnormal CRYAB expression is a prognostic biomarker for oral cancer, while its genomic variations and the association with carcinogenesis have never been studied.

Methodology/Finding: Therefore, we hypothesized that CRYAB single nucleotide polymorphisms may be associated with oral cancer risk. In this hospital-based study, the association of CRYAB A-1215G (rs2228387), C-802G (rs14133) and intron2 (rs2070894) single nucleotide polymorphisms with oral cancer in a Taiwan population was investigated. In total, 496 oral cancer patients and 992 age- and gender-matched healthy controls were genotyped and analyzed. A significantly different frequency distribution was found in CRYAB C-802G genotypes, but not in A-1215G and intron2 genotypes, between the oral cancer and control groups. The CRYAB C-802G G allele conferred an increased risk of oral cancer ($P = 1.49 \times 10^{-5}$). Patients carrying CG/GG at CRYAB C-802G were of lower 5-year survival and higher recurrence rate than those of CC ($P < 0.05$).

Conclusion/Significance: Our results provide the first evidence that the G allele of CRYAB C-802G is correlated with oral cancer risk and this polymorphism may be a useful marker for oral cancer recurrence and survival prediction for clinical reference.

Introduction

Oral cancer, which is a leading cause of death and disfigurement around the world [1–4], has ranked on the 4th cancer in Taiwanese male population [5]. There is an urgent need to develop routine preoperative markers to spare patients with poor prognosis after surgery or other treatment and on the other hand, identify patients at risk of early recurrence and justify prophylactic neck dissection and adjuvant concurrent chemoradiotherapy as well as those who could benefit from various treatments regardless of their tumor size or staging. Those who are identified at higher risk of oral cancer recurrence and/or metastasis should be detected earlier and followed up more frequently to enjoy longer life with the development of useful markers for prognosis prediction.

Alpha B-crystallin (CRYAB) is a member of the small heat shock protein (sHSP) family and a molecular chaperone expressed in various tissues [6,7]. Recent evidence has established that CRYAB presents not only in eye, but also in heart, skin, brain, spinal cord, and lung tissues [6,8]. In mammals, there are three classes of crystallins: alpha, beta, and gamma, each contributing equally to the total mass of the lens. From the proteomics or protein level studies, it has recently been recognized that CRYAB may have a role in cancer development. In 2005, it is reported that CRYAB was down-regulated at mRNA level from oral cancer patients compared with normal oral mucosa [9]. However, in contrast to the highly expression in normal oral mucosa, patients with negatively or lower CRYAB detected in their tumor sites had better disease-free survival rates than those patients whose tumors stained strongly. On the contrary, it was reported that from a proteomics screening in Taiwan, CRYAB was significantly up-regulated in the primary tissue from oral cancer patients [10]. In 2010, similar results were reported in a mice oral cancer model via concomitantly 8-week treatment with 4-NQO (200 μg/mL) and arecoline (500 μg/mL) [11]. Despite of the disagreements raised among different ethics and populations investigated, the genomic status of CRYAB and the linkage between its genotype and clinical outcome are largely unknown.

In order to understand the genomic role of CRYAB in oral cancer, we have chosen three single nucleotide polymorphisms (SNPs) of CRYAB, A-1215G (rs2228387), C-802G (rs14133), intron2 (rs2070894), and investigated their genotypic distribution in a large Taiwanese oral cancer population. In addition, the two clinical outcomes contribute to the highest death rate of oral cancer, metastasis and recurrence, were analyzed of their associations with CRYAB genotypes.

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Results

The clinical characteristics of the oral cancer patients and controls are shown in Table 1. There were no significant difference between both groups in their age and sex, while the patients are much higher exposed to the environmental risky factors for oral cancer in Taiwan, smoking, alcohol drinking and betel quid chewing habits (Table 1). The frequencies of the genotypes and alleles for the CRYAB A-1215G, C-802G, and intron 2 for the participants are shown in Table 2. Genotype distribution of various genetic polymorphisms of CRYAB C-802G is significantly different between oral cancer and control groups (P<0.05), while those for A-1215G or intron 2 were not significant (P>0.05) (Table 2). Also, the allele distributions of the CRYAB C-802G (P = 1.49\times10^{-5}, OR = 1.51, 95% CI = 1.25–1.83), not those of A-1215G (P = 0.8593, OR = 0.91, 95% CI = 0.31–2.62) or intron 2 (P = 0.1366, OR = 1.16, 95% CI = 0.95–1.41), is found to be associated with the susceptibility for oral cancer (Table 2). To sum up, the G allele and GG or CG genotype of CRYAB C-802G are associated with oral cancer risk and may be biomarkers for oral cancer detection. The representative PCR-based restriction analyses for the CRYAB C-802G polymorphisms are shown in Figure 1.

To evaluate the prognostic value of CRYAB genotypes, the relationships among disease-free survival, recurrence, metastasis and CRYAB C-802G genotypes were analyzed. First, the oral cancer patients carrying the CRYAB C-802G GG had a significant trend toward decreased disease-free survival, and the patients carrying CRYAB C-802G GG had the shortest disease-free survival period (Figure 2). The short disease-free may mainly reflect local recurrence. More than 80% (30 of 37) of the patients carrying CRYAB C-802G GG had nodal recurrence without an advanced N stage (Figure 2). Interestingly, the patients would have frequent recurrence and high second primary tumor rates within the following five years. Second, compared with those with CC genotype, the patients carrying CRYAB C-802G CG or GG genotype had a higher recurrence rate within the following five years (P = 0.028, OR = 2.08, 95% CI = 1.11–3.92), but not a higher metastasis rate (Table 3).

Discussion

The study aimed to investigate the association of CRYAB genotypes and clinicopathological variations in Taiwan oral cancer patients. It has recently been recognized that CRYAB protein may play a role in oral cancer development. In previous literature, it was reported that CRYAB was significantly over expressed in the primary tissue from oral cancer patients in Taiwan [10]. In 2010, in a tongue cancer mice model performed by concomitantly 8-week treatment with 4-NQO (200 µg/mL) and arecoline (500 µg/mL) and withdraw in the following 20 weeks, the cells of the tumor sites had higher expression of CRYAB than the counterpart cells of the sham-treated mice [11]. However, there were also some findings challenging this up-regulation association [9,12]. This may be due to that different populations of different ethics, genetic background, cultures, and environment exposure were investigated. From the viewpoints of cell-line based studies, it was demonstrated that overexpression of CRYAB in transformed immortalized human mammary epithelial cells demonstrated neoplastic features and luminal growth and these changes were inhibited when CRYAB expression was silenced using RNA interference [13]. Overexpression of CRYAB in human mammary epithelial cells also formed invasive mammary carcinomas in nude mice, induced epidermal growth factor and anchorage independent growth, increased cell migration and invasion, and activated the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) pathway, suggesting that CRYAB could be considered an oncoprotein [14]. However, there is not yet any study performed from the DNA level to investigate the important role of CRYAB in carcinogenesis.

Based on the previous differential expression evidence, we were strongly interested and chose the three SNPs of CRYAB two at the promoter region (A-1215G and C-802G) and one at the intron 2 (intron2), to investigate their associations with oral cancer risk and prognosis. We found that CRYAB C-802G polymorphism, not A-1215G or intron2, was associated with increased risk of oral cancer (Table 2), and the local recurrence rate (Table 3). Also, the oral cancer patients carrying GG or CG at the polymorphic site had lower 5-year survival rate than those carrying homologous CC (Figure 2). Interestingly, the patients carrying GG at CRYAB C-802G were recorded to have much more frequent recurrence and second primary rates. This may indicate that CRYAB C-802G could be a predictor for oral cancer progression direction. Possibly the genetic polymorphism directly affects the differential patterns of the CRYAB protein, at the expression and/or functional levels, and indirectly imbalance the normal functions of other CRYAB-related genes and proteins, which may result in the oral carcinogenesis. At the same time, the alteration of CRYAB protein expression in the extracellular matrix may cause the subtle changes of the microenvironment near the primary oral tumor, for the recurrence, but not for the metastasis. This can be justified by the role of CRYAB in the tyrosine kinase signaling, that could be easily altered in cancer cells. The reduced expression of CRYAB has been firstly reported to be associated with a negative prognosis in 2003 [15].

Approximately 10% of early-stage head and neck squamous cell carcinoma patients develop locoregional recurrence and 15% to
25% develop second primary tumors within 5 years of initial diagnosis [16,17]. As diagnostic and therapeutic approaches continue to develop, the ability to accurately predict second primary tumor/recurrence in early-stage oral cancer patients would facilitate intensive surveillance or targeted interventions for high-risk patients and thereby reduce mortality and morbidity. In this study, the patients carrying CRYAB C-802G CG or GG genotype were found to have a higher recurrence rate within the following five years, but not a higher metastasis rate (Table 3).

The occurrence of second primary tumors may be due to the subtle alterations of the microenvironment which have been accumulated to reach the threshold of tumorigenesis in the

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**Table 2.** Distribution of CRYAB genotypes and allelic frequencies among oral cancer patients and controls.

| Polymorphism         | Controls | %  | Patients | %  | P   | OR (95% CI) |
|----------------------|----------|----|----------|----|-----|-------------|
| A-1215G (rs2228387)  |          |    |          |    |     |             |
| Genotype             |          |    |          |    |     |             |
| GG                   | 981      | 98.9% | 491      | 99.0% | 1.00 (reference) |
| AG                   | 11       | 1.1%  | 5        | 1.0%  | 0.91 (0.31–2.63) |
| AA                   | 0        | 0%    | 0        | 0%    | 0.9999 |
| Allele               |          |      |          |      |     |             |
| G                    | 1973     | 99.4% | 987      | 99.5% |     |             |
| A                    | 11       | 0.6%  | 5        | 0.5%  | 0.8593 (0.31–2.62) |
| C-802G (rs14133)     |          |    |          |    |     |             |
| Genotype             |          |    |          |    |     |             |
| CC                   | 703      | 70.9% | 301      | 60.7% | 1.00 (reference) |
| CG                   | 245      | 24.7% | 158      | 31.9% | 1.51 (1.18–1.92) |
| GG                   | 44       | 4.4%  | 37       | 7.4%  | 0.0002 (1.24–3.10) |
| Allele               |          |      |          |      |     |             |
| C                    | 1651     | 83.2% | 760      | 76.6% | 1.00 (reference) |
| G                    | 333      | 16.8% | 232      | 23.4% | 1.49*10^2 (1.25–1.83) |
| Intron2 (rs2070894)  |          |    |          |    |     |             |
| Genotype             |          |    |          |    |     |             |
| CC                   | 688      | 69.4% | 325      | 65.5% | 1.00 (reference) |
| CT                   | 268      | 27.0% | 150      | 30.3% | 1.18 (0.93–1.51) |
| TT                   | 36       | 3.6%  | 21       | 4.2%  | 0.3241 (0.71–2.15) |
| Allele               |          |      |          |      |     |             |
| C                    | 1644     | 82.9% | 800      | 80.6% | 1.00 (reference) |
| T                    | 340      | 17.1% | 192      | 19.4% | 0.1366 (0.95–1.41) |

*P based on two-sided Chi-square test without Yate’s correction.

**Figure 1.** PCR-based restriction analysis of the CRYAB C-802G rs14133 polymorphism shown on 2.5% agarose electrophoresis. M: 100 bp DNA size marker, G/G: enzyme indigestible homozygote, C/G: heterozygote, and C/C: enzyme digestible homozygote.
patients of risky genotypes, such as GG at CRYAB C-802G. The functional study of this SNP and how the CRYAB protein interacts with proteins in extracellular matrix in oral carcinogenesis also need further investigations. In the future, collective evidence from larger and different cohorts using this SNP may help to oral cancer staging, outcome direction prediction, and more effective and integrative strategy.

It is firstly found that the SNP at the promoter region of CRYAB, C-802G, is associated with oral cancer susceptibility, recurrence, and 5-year disease-free survival, but not metastasis. Since poor local-regional control and easy recurrence are the main causes of treatment failures in oral cancer therapy, the results of this study may provide more predictive guidance information for not only the prevention, but the care, therapy and follow-up of those patients at higher risk of cancer recurrence and lower 5-year survival rate.

Materials and Methods

Study population and sample collection

Four hundred and ninety six cancer patients diagnosed with oral cancer were recruited at the outpatient clinics of general surgery between 2005–2008 at the China Medical University Hospital, Taichung, Taiwan, Republic of China. The clinical characteristics of patients include histological details were all graded and defined by expert surgeons. All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. Double number of non-cancer healthy volunteers as controls were selected by matching for age, gender and some indulgences after initial random sampling from the Health Examination Cohort of the hospital. The exclusion criteria of control group included previous malignancy, metastasized cancer from other or unknown origin, and any familial or

Table 3. Association of CRYAB C-802G genotype with oral cancer recurrence and metastasis.

| Patient Status      | CRYAB C-802G rs14133 | P     | OR (95% CI)  |
|---------------------|----------------------|-------|--------------|
|                      | CC       | CG+GG |       |              |
| Recurrence Status   |          |       |       |              |
| No recurrence >5 years | 282    | 171  | 1.00  |              |
| Recurrence ≤5 years | 19       | 24   | 0.0228 | 2.08 (1.11–3.92) |
| Metastasis Status   |          |       |       |              |
| No metastasis >5 years | 279    | 178  | 1.00  |              |
| Metastasis ≤5 years | 22       | 17   | 0.6101| 1.21 (0.63–2.34) |

*P based on two-sided Chi-square test without Yate’s correction.

The ORs were estimated with multivariate logistic regression analysis.

Statistically identified as significant.

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genetic diseases. Both groups finished a short questionnaire which included some inducences and they were recorded. Our study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all participants.

Genotyping assays

Genomic DNA from oral cancer and health control subjects were prepared from peripheral blood leukocytes using a QiAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to previous studies [10–24]. Briefly, the following primers were used for CRYAB intron 9 (rs14133): 5'-TTGCACTACCTTGCACTCTC-3'; for CRYAB intron 145 (rs12225537): 5'-ACCTGGAGGTGCTT-GATCTT-3' and 5'-ATGCACCTCAATCACATCCT-3'; for CRYAB C-802G (rs14133): 5'-TTGACCATGAGTCACAACTCAAGT-3' and 5'-TTGGACATGACAGCATAACC-3'; for CRYAB intron 2 (rs2070894): 5'-GTCCTAGAGACTAAGTTAAGG-3' and 5'-AGAGAGTCCACACTCAAGT-3'. The following cycling conditions were performed: one cycle at 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were studied after digestion with Fnu I, Fai I, and Dpn I, restriction enzymes for A-1215G (cut from 212 bp A type into 67 bp G type), C-802G (cut from 363 bp G type into 85+278 bp C type), and intron 2 (cut from 363 bp T type into 74+339 bp C type), respectively.

Statistical analyses

In our study, only those matches with all SNPs data (case/control = 496/992) were selected into final analyzing. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of CRYAB SNPs in the control subjects from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson’s two-sided Chi-square test or Fisher’s exact test (when the expected number in any cell was less than five) was used to compare the distribution of the CRYAB genotypes between cases and controls.

The primary outcome was disease-free survival. The endpoints included local cancer recurrence and metastasis. Follow-up information was available for all patients at the 3-year time point. Disease-free survival time was calculated from the date of treatment until the time of recurrence, defined as disease recurrence at the same site or the detection of metastases, including recurrence in the neck lymph nodes. The genotypes were coded assuming an allele dose-effect (CC wild-type = 0, CG heterozygous carrier of the mutated allele = 1, GG homozygous carrier of the mutated allele = 2). Disease-free survival curves were generated by the Kaplan-Meier method and verified by the log-rank test. The significance level was set at P<0.05.

Author Contributions

Conceived and designed the experiments: D-TB M-HT. Performed the experiments: D-TB. Analyzed the data: C-WT C-CL. Contributed reagents/materials/analysis tools: C-CL M-HT D-TB C-WT. Wrote the paper: D-TB R-YT.

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