Polymorphism in heme oxygenase-1 (HO-1) promoter is related to the risk of oral squamous cell carcinoma occurring on male areca chewers

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Areca (betel) chewing is associated with the high incidence of oral squamous cell carcinoma (OSCC) and oral submucous fibrosis (OSF) in Asians. Heme oxygenase-1 (HO-1), encoding an oxidative response protein, plays protective roles in cells. A (GT)n microsatellite repeat in HO-1 promoter shows polymorphisms and modulates the level of gene transcription. We examined allelotypic frequencies of (GT)n repeats in 83 controls, 147 OSCC and 71 OSF. All subjects were male areca chewers. Logistic regression was used to adjust the age confounding for odds ratio (OR). (GT)n repeat polymorphism was classified into short (S), medium (M) and long (L) alleles. The adjusted OR in OSCC subjects carrying L allele relative to S allele was 1.75. Buccal squamous cell carcinoma (BSCC) is the most common OSCC subset in areca chewers. L allele implied the risk of BSCC with adjusted OR of 2.05, whereas M allele type appeared protective for non-BSCC with adjusted OR of 0.49. Our findings indicated that longer (GT)n repeat allele in HO-1 promoter is associated with the risks of areca-related OSCC, while the shorter (GT)n repeat allele may have protective effects for OSCC.

Keywords: gene polymorphism; HO-1; mouth; neoplasm; oral submucous fibrosis

Heme oxygenase (HO) is a rate-limiting enzyme that degrades heme to produce biliverdin, CO and free iron. It was also named as heat shock protein 32 (Maines, 1988). Three HO isoforms have been identified in human. Heme oxygenase-1 (HO-1) is an inducible isoform of HO in response to stresses, including heat shock, UV irradiation, hydrogen peroxide, heavy metals, glutathione deletion, hypoxia and NO (Maines, 1988; Shibahara, 1988; Keyse and Tyrrell, 1989; Oguro et al, 1996; Doi et al, 1999; Motterlini et al, 2000). The induction of HO-1 represents a cytoprotective defence mechanism against oxidative insults. Like other heat shock proteins, high expression of HO-1 was found in malignant tumours (Maines and Abrahamsson, 1996; Goodman et al, 1997). Administration of the HO inhibitor suppressed the growth of tumour cells, which suggests a vital role of HO-1 in tumour growth (Doi et al, 1999; Fang et al, 2003). In addition, HO inhibitor also induced the apoptosis of tumour cells, suggesting the roles of HO-1 in maintaining cancer cell survival (Fang et al, 2003). Reports have addressed that HO-1 plays important roles in angiogenesis of prostate cancers (Sunamura et al, 2003).

Oral squamous cell carcinoma (OSCC) is the third most common malignancy in developing countries and the sixth worldwide. It accounts for up to 50% of malignant tumours in some South Asia countries due to the popularity of areca (betel)-chewing habit. Around 200–600 million Asians chew areca (Liu et al, 1996; Jeng et al, 2001; Lin et al, 2002; Lo et al, 2003; Sharma, 2003). OSCC is also a prevalent disease in Taiwan as the fourth leading malignancy in male population (Lin et al, 2000, 2002; Jeng et al, 2001; Kao et al, 2002; Lo et al, 2003; Wong et al, 2003). Areca chewing is also exclusively associated with the occurrence of oral squamous fibrosis (OSF), which is a precancerous condition exhibiting disturbances in homeostasis of fibrous tissue and altered epithelial components (Chi et al, 2001, 2002; Yang et al, 2001; Ko et al, 2003; Liu et al, 2004; Shin et al, 2004). Oral submucous fibrosis was originally called idiopathic scleroderma of the mouth. This disease is characterised by mucosal rigidity due to the fibro-elastic transformation of the juxta-epithelial layer. A subepithelial inflammatory reaction and epithelial atrophy is frequently accompanied with the fibrous changes of the lamina propria (Shin et al, 2004). It is interesting that OSF seems to be a...
public health issue in many parts of the world, including the UK and South Africa, due to the spreading of areca chewing (Caniff et al, 1986; Yang et al, 2001; Shin et al, 2004). However, the genetic susceptibility or disease nature of OSF is still largely undefined. The (GT)n repeats were identified in the proximal region of HO-1 promoter. Reports indicated these microsatellites are highly polymorphic, and longer (GT)n repeat exhibits lower HO-1 transcriptional activity (Yamada et al, 2000; Chen et al, 2002, 2004; Kaneda et al, 2002). Subjects carrying longer (GT)n repeats are associated with the higher susceptibility of cardiovascular or chronic obstructive pulmonary diseases (Yamada et al, 2000; Chen et al, 2002, 2004; Kaneda et al, 2002). Oxidative stress is associated with the pathogenesis of cancers (Xie and Huang, 2003). However, the association between HO-1 promoter polymorphism and cancer risk has not been established. Although HO-1 expression might be related to the biological expression of OSCC (Yanagawa et al, 2004), the involvement of functional HO-1 polymorphisms in the risk of oral diseases has not been defined. We hypothesised that higher (GT)n repeats may be associated with the risk of OSCC and OSF. We showed that (GT)n repeats polymorphism in HO-1 was a risk factor for OSCC. In addition, polymorphic profile differences seemed to differentially predict BSCC and non-BSCC subsets.

**MATERIALS AND METHODS**

**Samples**
A total of 147 primary OSCC without previous treatment and 71 OSF cases were obtained from the Oral and Maxillofacial Surgery Department of the Taipei Mackay Memorial Hospital. In all, 83 control subjects were selected from people who came for physical checkup, and had no neoplastic minor oral operations or maxillofacial trauma. Those with autoimmune disorders, blood diseases and previous malignancies were excluded from the control group. The site, stage and TNM classification of OSCC subjects are described in Table 1. This study was approved by an ethics reviewing committee. Blood was drawn from the subjects. A leukocyte cell pellet was obtained from the buffy coat by centrifugating the whole blood. DNA was isolated by Blood Mini Kit (Qiagen, Valencia, CA, USA).

**Table 1 Clinical parameters of OSCC subjects**

| Site          | Number |
|---------------|--------|
| Buccal mucosa | 90     |
| Tongue        | 35     |
| Gingiva       | 14     |
| Palate        | 5      |
| Floor of mouth| 3      |

**Stage and TNM**

| Stage | Number |
|-------|--------|
| Stage I | 15 |
| Stage II | 35 |
| Stage III | 20 |
| T1N1M0 | 2 |
| T2N1M0 | 5 |
| T3N0M0 | 11 |
| T3N1M0 | 2 |
| Stage IV | 77 |
| T2N2M0 | 3 |
| T3N2M0 | 3 |
| T4N0M0 | 32 |
| T4N1M0 | 24 |
| T4N2M0 | 8 |
| T4N3M0 | 7 |

*According to UICC classification system.*

**Heme oxygenase-1 genotyping**

(GT)n repeat polymorphism in the HO-1 promoter was determined by PCR-based genotyping. The primers used to generate HO-1 amplicons of 98 – 142 bp were sense: 5'-AGAGGCTCGAGCTTCTCAGA-3' and antisense: 5'-ACAAAGTCTGGCATAGGAC-3' (Kaneda et al, 2002). The 5' site of the sense primer was labelled with FAM fluorescence dye. The amplification reaction mixture (15 μl) contained 20 ng genomic DNA, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.5 U Prozyme DNA polymerase (Promega, Madison, WI, USA). The fluorescence was detected automatically by Genescan Biosystem (Foster City, CA, USA). The fluorescence was detected automatically by Genescan 672 software (Applied Biosystem). At least two independent experiments were performed on each sample to assure the reliability of the analyses.

**DNA sequencing**

Selected amplicons with various allelic sizes were cloned into pGEM-T vector (Promega, Madison, WI, USA). Five clones from each allele were sequenced using a 377-18 DNA sequencer (Applied Biosystem) and vector primers were used to confirm the number of GT repeats revealed by genotyping.

**Statistical analysis**

Associations between the HO-1 polymorphisms and risk of disease genesis were estimated by odds ratio (OR) and associated 95% confidence interval (CI), which were calculated by logistic regression models using SPSS version 8.0 (SPSS Inc., Chicago, IL, USA). Differences between the variants were considered significant when P<0.05.

**RESULTS**

All subjects were male areca chewers. The ages (mean ± s.d.) of OSCC, OSF and control subjects were 51.3 ± 9.8, 39.0 ± 10.8 and 47.1 ± 10.0, respectively. In all, 61% (90 cases) of the OSCC subjects were BSCC, 37% (54 cases) of the OSCC patients presented with lymph node metastasis (LNM) and 63% (97 cases) of patients had late stage lesions (Table 1).

The genotyping of (GT)n microsatellite polymorphism in HO-1 promoter region was carried out by Genescan system. It distinguished (GT)n repeats on the basis of differential mobility of amplicons with different sizes. The repeat numbers were derived from the amplicon size and the sequencing reading in cloned alleles. The allelotypic distribution of HO-1 polymorphism of control, OSCC and OSF is shown in Figure 1. In controls, the GT repeat numbers ranged from 19 to 37, and the most common alleles were (GT)26 and (GT)30, which were consistent with previous Taiwanese studies (Chen et al, 2002, 2004). The alleles were classified into three subgroups: the shorter component (≤ 25 repeats) was designated class 'S', the medium component (26 – 30 repeats) was designated class 'M' and the longer component (≥ 31 repeats) was designated class 'L'. Table 2 describes the genotypes from Genescan analysis. Table 3 describes that the frequency of L allelotype was significantly increased in OSCC subjects in relation to control subjects, with an age-adjusted OR of 1.75. The frequency of L allelotype was also significantly increased in OSF subjects relative to control subjects before adjusting. However, after adjusting for age, OR remarkably declined to an insignificant level (Table 3).
is critical for converting heme to bilirubin, CO and iron, we have examined the correlation between HO-1 polymorphisms and serum bilirubin to support the notion that subjects with longer GT repeat might have higher serum bilirubin. Although we observed a trend that subjects carrying L allelotype have a higher serum bilirubin level, the increase did not reach a statistical significance (detailed analysis not shown). It is plausible that the induction of HO-1 only occurs in local tissue and may not necessarily stand out from measuring circulating bilirubin level, since serum bilirubin level is affected by multiple systemic factors including liver function. Male areca chewers with L allelotype have 1.75 or 2.05 times higher risk for OSCC or BSCC, respectively, compared to those carrying S allelotype. To our knowledge, this is the first study investigating the roles of HO-1 promoter polymorphism on OSCC risks.

Oral submucous fibrosis is a unique disease characterised by the unbalance between synthesis and degradation of extracellular matrix (Chiu et al, 2002), and occurred exclusively in areca chewers (Chiu et al, 2001, 2002; Ko et al, 2003; Liu et al, 2004). It is considered as an inflammatory reaction in response to areca ingredients or physical irritation, while the covering epithelium may exhibit precancerous changes undertaking malignant transformation (Ko et al, 2003). The fact that areca disrupts cytokine production and molecules for organising the extracellular matrix seems to play important roles in OSF pathogenesis. Studies have demonstrated that the functional polymorphism of genes on immune reaction, such as MICA (Liu et al, 2004), CTLA4 (Shin et al, 2004), collagen-related genes (Chiu et al, 2002) and cytokines (Chiu et al, 2001), were associated with the risk of OSF. The OSF patients usually came for medical helps for resolution of oral symptoms early in their lives. Thereby, the mean age of OSF subjects is around 10 years younger than control or OSCC subjects in our study cohort. In this study, the risk of L allelotype in HO-1 promoter for OSF became not significant after adjusting for age. It was speculated that the protection driven by HO-1 is not sufficient for counteracting the oxidative stress elicited by areca, which might cause the pathogenesis of OSF (Liu et al, 1996). Age-related confounding factors or genetic events contributive to OSF genesis deserve further dissection.

Further analysis on different clinical parameters, including site, LNM and stage, was performed to elucidate the relationship between HO-1 polymorphism and OSCC. The allelotypic distributions of HO-1 polymorphism in control, BSCC and non-BSCC subjects are shown in Figure 2. A significantly higher difference in frequencies of L allelotype was noted in BSCC subjects compared to those carrying S allelotype. To our knowledge, this is the first study investigating the roles of HO-1 promoter polymorphism in OSCC risks.

DISCUSSION

In this case–control study, we have identified a significant association between GT repeat length in HO-1 promoter and the risks of OSCC occurring in male areca chewers, particular for the BSCC subset. Increases in risk were observed for subjects with a GT repeat ≥31, which suggested that shorter GT repeat alleles could have a protective effect on OSCC. The results also supported previous findings suggesting better protection against disease formations in subjects carrying shorter HO-1 GT repeats (Yamada et al, 2000; Chen et al, 2002, 2004; Kaneda et al, 2002). Since HO-1

![Figure 1 Allelotypic distribution of HO-1 (GT)_n repeat polymorphisms in control, OSCC and OSF subjects.](image1)

Table 2 HO-1 genotype in subjects

| Genotype | Control (n = 83) | OSCC (n = 147) | OSF (n = 71) | BSCC (n = 90) | Non-BSCC (n = 57) |
|----------|-----------------|----------------|--------------|---------------|------------------|
| SS       | 17              | 29             | 7            | 14            | 15               |
| SM       | 29              | 30             | 23           | 17            | 14               |
| SL       | 11              | 34             | 20           | 19            | 15               |
| MM       | 13              | 20             | 8            | 16            | 4                |
| ML       | 12              | 27             | 8            | 20            | 6                |
| LL       | 1               | 7              | 5            | 4             | 3                |

Table 3 Association between HO-1 allelotype and risks of OSCC and OSF subjects

|          | OSCC P | OR 95% CI | OSF P | OR 95% CI |
|----------|--------|-----------|-------|-----------|
| S        | 1.00   |           | 1.00  |           |
| M        | 0.88   | 0.57–1.34 | 0.91  | 0.55–1.51 |
| L        | 1.81   | 1.06–3.10 | 1.95  | 1.06–3.59 |
| *        | 1.00   |           | 1.00  |           |

*Adjusted for age.
promoter polymorphisms between BSCC and non-BSCC. Evidences accumulated might suggest that BSCC, which is quite prevalent in Asians, exhibits distinctive pathways for tumorigenesis.

Heme oxygenase-1 expression has been reported to enhance growth against apoptosis and induce angiogenesis through increase in angiogenic factor and VEGF in endothelial cells and cancer cells (Deramaudt et al, 1998; Doi et al, 1999; Kushida et al, 2002; Malaguarnera et al, 2002, 2003; Fang et al, 2003; Sunamura et al, 2003). Such phenotypes are advantageous for tumour progression and survival. However, a recent paper, denoting the lower HO-1 expression in head and neck carcinomas with LNM, has argued that the increase of HO-1 expression as a cause or a consequence of carcinogenesis (Yanagawa et al, 2004). Preliminary evidences from us indicated that HO-1 promoter polymorphism had no impact on tumour progression, reflecting by metastasis or advanced clinical stage. It might exclude the role of HO-1 polymorphism as a risk marker of tumour dissemination, although such polymorphism could affect transcription. This could be partially interpreted by the multiple factors involving in the tumour progression, which mask the crucial role of HO-1 genotype. Advanced tumours exhibited tremendous potentials to survive in a stress microenvironment, with hypoxia and free radical overproduction (Xie and Huang, 2003). Thereby, the extensive stress on advanced tumours may have secondary or selective effects on HO-1 expression. Additional genotypic surveys using more samples and confounders, together with HO-1 expression profile, are required to further insight the functional importance of HO-1 in cancer progression.

Overall, our clues indicated that longer GT repeat allele in HO-1 promoter is associated with the risks of areca-associated oral carcinogenesis. The findings also suggest that shorter GT repeat allele in HO-1 promoter may have protective effects for OSCC in our study cohort.

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