Clin Oral Invest (2015) 19:2273–2283
DOI 10.1007/s00784-015-1467-7

ORIGINAL ARTICLE

Downregulation of *CRNN* gene and genomic instability at 1q21.3 in oral squamous cell carcinoma

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Received: 4 October 2014 / Accepted: 23 March 2015 / Published online: 8 April 2015

Abstract
Objectives This study includes the direct sequencing of cornulin (CRNN) gene to elucidate the possible mechanism of *CRNN* downregulation and explore the genetic imbalances at 1q21.3 across oral squamous cell carcinoma (OSCC) samples.
Materials and methods In mutation screening of *CRNN* gene, gDNA from OSCC tissues were extracted, amplified, and followed by direct sequencing. OSCC samples were also subjected to fragment analysis on *CRNN* gene to investigate its microsatellite instability (MSI) and loss of heterozygosity (LOH). Immunohistochemistry was performed to validate CRNN downregulation in OSCC samples.
Results No pathogenic mutation was found in *CRNN* gene, while high frequency of allelic imbalances was found at 1q21.3 region. MSI was found more frequent (25.3 %) than LOH (9.3 %). Approximately 22.6 % of cases had high MSI which reflects higher probability of inactivation of DNA mismatch repair genes. MSI showed significant association with no betel quid chewing (p=0.003) and tongue subsite (p=0.026). LOH was associated with ethnicity (p=0.008) and advanced staging (p=0.039). The LOH at 1q21.3 was identified to be as an independent prognostic marker in OSCC (HRR=7.15 (95 % CI, 1.41–36.25), p=0.018). Downregulation of CRNN was found among MSI-positive OSCCs and was associated with poor prognosis (p=0.044).
Conclusion This study showed a significant correlation between LOH/MSI at 1q21.3 with clinical outcomes and that downregulation of *CRNN* gene could be considered as a prognostic marker of OSCC.
Clinical relevance Insights of the downregulation mode of *CRNN* gene lays the basis of drug development on this gene as well as revealing its prognostic value.

Keywords Oral squamous cell carcinoma · *CRNN* gene · Genetic instability · 1q23.1. LOH/MSI

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Introduction

Oral cancer is the 6th common cancer worldwide in which more than 90 % of oral cancers are squamous cell carcinoma [1–3]. Both genetic and environmental risk factors contribute to disease development. Oral cavity epithelium forms the first shield of protection during exposure to environmental risk factors. Hence, environmental risk factors might be able to induce some genetic alterations on these epithelial cells.

Genomic instability is one of the hallmarks of cancer that is associated with disease progression [4]. Chromosome 1q21.3 is one of the cancer-related regions that showed such instability across different types of cancers such as esophageal adenocarcinoma [5], gastrinomas [6], and sporadic insulinomas [7].

More recently, we detected genomic instability at the 1q21.3 chromosomal region in oral squamous cell carcinoma (OSCC) cases [8]. This region harbors the epidermal differentiation complex (EDC), a gene cluster that plays a critical role in late epithelial differentiation [9]. Downregulation of EDC genes is reported in the progression of normal esophageal epithelium to Barrett's esophagus [10] and esophageal adenocarcinoma [11]. Frequent loss of heterozygosity (LOH) of 1q21.3 in cases with esophageal adenocarcinoma [5] could be one of the possible explanations for downregulation of EDC genes.

Environmental stresses such as smoking, alcohol drinking, and betel-quid chewing play a critical role in causing oral lesions and reflux of acidic gastric fluid in causing oesophageal lesions [12–15]. Thus, heat shock proteins such as HSP70 and *CRNN* play a critical role in controlling these unusual environmental pressures placed on the squamous epithelial cells [12]. *CRNN* as a member of EDC genes on 1q21.3 is a squamous epithelial heat shock protein which is also known as *SEP53*. *CRNN* gene expression is confined to squamous cells, especially in esophageal squamous epithelial cells [16]. Upregulation of *CRNN* in response to squamous epithelial cell injury in porcine [12] and in the buccal mucosa of smokers reflects the stress respondent role of this gene [17]. High-level expression of *CRNN* induced by environmental pressures would arrest the cell cycle at G1/S checkpoint [12, 18, 19].

Thus, it seems that upregulation of *CRNN* plays a critical role to control environmental pressures and to prevent formation of lesions on the epithelial tissues. Significant downregulation of *CRNN* in lesional area of the skin compared with non-lesional area in patients with eczema provides evidence in agreement with this conclusion [20].

Oral epithelial cells are subject to damage from habitual risk factors with *CRNN* responding to DNA damages induced by environmental pressures [12]. *CRNN* gene expression has been shown to be significantly reduced or absent in cancer cell lines, esophageal tumors, and OSCC [11, 12, 16, 19]. Downregulation in OSCC tumors reflects that *CRNN* might not be able to respond to DNA damages that might be induced by habitual risk factors. Hence, we hypothesized that *CRNN* mutation or LOH at the relevant locus might be the possible mechanism of downregulation of *CRNN* gene expression in OSCC. Thus, we directly sequenced parts of the promoter region at 5′-UTR, coding regions, and 3′-UTR of *CRNN* and further explored the possibility of LOH at 1q21.3 using polymorphic microsatellites.

Materials and methods

Samples

Sixty-two tumor samples from patients with OSCC that consisted of 41 Indians, 3 Indigenous, 12 Malays, and 6 Chinese who had a history of risk habits were included for direct sequencing of *CRNN* gene. If any variation was detected in the tumor, the matched normal sample from peripheral blood of the same patient was used for excluding the germline variations. In addition, 75 OSCC samples comprised 35 from the initial set of 62 OSCC cases with the addition of 40 independent OSCCs with their matched DNA samples from peripheral blood were recruited for microsatellite instability (MSI) and LOH analysis. Samples were taken from OSCC of the gum, floor of mouth, lip, tongue, palate, and buccal mucosa.

All samples were obtained from the Malaysian Oral Cancer Database and Tissues Bank System (MOCDTBS) coordinated by the Oral Cancer Research and Coordinating Centre, University of Malaya [21]. The American Joint Committee on cancer staging criteria was used for tumor staging [22]. Written informed consent has been taken from all patients and normal healthy controls before collecting the samples [21]. This study was approved by Medical Ethics Committee, Faculty of Dentistry, University of Malaya (MEC No. DFOP1108/0083(L)).

Direct sequencing of *CRNN* gene

Specific primers were designed using Primer3 program V0.4.0 [23], covering the coding regions, untranslated regions (5′- and 3′-UTR) and splicing sites (Table 1). The primers were designed approximately 100 bp beyond the splicing sites into the intronic region. PCR amplification was performed in a total volume of 25 μl, including 40 ng genomic DNA, 0.4 μm of each primer, and 12.5 μl GoTaq Mater Mix, 2× (Promega, USA). The PCR conditions were as follows: initial denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s and elongation at 72 °C for 30 s, and final elongation at 72 °C for 10 min.

Table 1 *CRNN* gene primer sequence on 1q21

Primer	Primer sequence	Tm	Size (bp)
5′-UTR	F: 5′-GACCAAGGCAGTCACACATCA-3′	62	484
	R: 5′-GCTGATCCAGGAAGGCACAT-3′	62	
Exon 1	F: 5′-AGCTGAACGTGCAAGGAAAG-3′	60	351
	R: 5′-TAGCTGCTGCCTCCTCAAAT-3′	60	
Exon 2a	F: 5′-AGCTGGCAGCAGATTGGAAG-3′	63	588
	R: 5′-TCCCTGGTCTGTGGCTGTCT-3′	63	
Exon2b	F: 5′-AAGCTGGAGAAGGCAAGAGGA-3′	63	557
	R: 5′-CGTCTGGGTCTGTCCCTGTT-3′	62	
Exon 2c	F: 5′-GGAGCAGGACAGAAGCCAAA-3′	63	516
	R: 5′-GAGCTGATGTCCAGGGATGC-3′	63	
3′-UTR	F: 5′-GAGAGTTTGGCTTGTCCTGCAT-3′	63	521
	R: 5′-TCAGGGCCATCATAAAGAAAGC-3′	63	

The purified PCR products were directly sequenced using the BigDye terminator V3.1 sequencing standard kit (Applied Biosystems), as recommended by the manufacturer, and run on an automated Applied Biosystems 3730XL Genetic Analyzer. DNA sequences were analyzed using the Bioedit sequence alignment editor software, V5.0.9 [24], and then were BLASTed against reference sequences for *CRNN*. DNA sequence variants were confirmed by the sequencing of both forward and reverse strands. Reference sequences for *CRNN* coding DNA and protein were NM_016190.2, NP_057274.1, and NG_007081.1, respectively, according to the human genome assembly GRCh37.p10/hg19.

The PolyPhen-2 program was further used to predict the functional effects of non-synonymous variants. PolyPhen score is ranged from 0 to 1, where 1 indicates probable damage and 0 indicates that mutation is benign.

LOH and MSI analysis

To elucidate the LOH and MSI events on the 1q21.3 region, we have selected four highly polymorphic CA dinucleotide short tandem repeat (STR) markers with a heterozygosity of ≥8, covering the 1q21.3 chromosomal region (Table 2). Very highly heterozygous markers increase the probability of LOH detection. However, *CRNN* is located at this region, but there was no documented polymorphic STR marker inside the gene.

Specific primers were designed using Primer3 program, and the forward primers were labeled with fluorescent dyes. Multiplex PCR amplification was performed in a total volume of 50 μl, including 100 ng genomic DNA, 0.4 μm of each primer and 25 μl GoTaq Mater Mix, 2× (Promega, USA). The PCR conditions were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 2 min and elongation at 72 °C for 30 s, and final elongation at 60 °C for 45 min. The PCR products were capillary electrophoresed using Applied Biosystems 3730XL Genetic Analyzer and analyzed by Peak Scanner V2.0 and GeneMapper V4.0 software (Applied Biosystems).

Total loss or significantly reduced signal intensity of one allele in tumor sample was considered as LOH. LOH was estimated based on the previously reported formula as below, ratios >50 % was considered as LOH [25]. Presence of a new fragment in the tumor samples compared with matched normal was considered as MSI. Detecting a new allele in more than one STR marker was considered as high MSI (MSI-high).

$$\mathrm{LOH} = 1 - \left(\frac{\left(\text{peak high of smaller allele in tumor} \Big/ \text{peak high of larger allel in tumor} \right)}{\left(\text{peak high of smaller allele in matched normal} \Big/ \text{peak high of larger allel in matched normal} \right)} \right) \times 100$$

Immunohistochemistry analysis of CRNN protein

Of the 75 OSCC samples that were used for MSI/LOH analysis, 43 samples were recruited for immunohistochemical (IHC) analysis. However, only 31 samples were deemed as interpretable for analysis in this study. Non-interpretable data was the consequence of damaged sections. Lack of fresh tissue impeded further sectioning and analysis. Frozen tissues

Table 2 STR marker features and the relevant primers for LOH analysis of 1q21.3

STR	Physical position	Het.	Primer sequence	Tm
D1S2346	153169533–153169697	0.83	F: 5′HEX-CCTGCCAAGTCCACTCCATC-3′	63
			R: 5′-TCCAGGATACAGCCGCCATC-3′	65
D1S498	151301496–151301856	0.81	F: 5′HEX-GCCCATTACAGGGTTTTGTTAGG-3′	63
			R: 5′- GCTTTCAGAATGCTCGCTTGT-3′	62
D1S2345	151533267–151533616	0.8	F: 5′6FAM-GGTGATTTACTCTGCCTTGTTAGCC-3′	63
			R: 5′-AAGACTCCGTCTCAAACACTCACAC-3′	63
D1S305	154281903–154282282	0.83	F: 5′6FAM-GAAAATGGACTAACACAGACGCTG-3′	62
			R: 5′-TGAGTGAAGGAGCAGCTCAAGTG-3′	64

Physical position of STR markers based on human hg19 assembly

Het heterozygosity, *F* forward, *R* reverse

were sectioned at 5 μm thickness and placed onto SuperfrostExcell™ microscope slides (Fisher Scientific, Pittsburgh, PA) for IHC study. Prior to performing IHC using the Envision technique, DAKO REAL EnVision Detection System and Peroxidase/DAB+ (Dako, USA), all sections were immersed in precooled acetone (−20 °C) for 10 min at room temperature for tissue fixation. The sections were immersed in blocking solution (Dako Corporation, Carpinteria, CA, USA) for 10 min at room temperature followed by incubation with primary antibody (1:200, anti-CRNN rabbit polyclonal antibody, 11799-1-AP, Proteintech Group, Chicago, IL, USA) for 1 h at room temperature. Visualization was achieved by incubation with the peroxidase-labeled secondary antibody from the Envision kit (Dako Corporation, Carpinteria, CA, USA) for one hour at room temperature followed by staining with 3'3 diaminobenzidine substrate chromogen (Dako Corporation) and counterstaining with Mayer's hematoxylin, dehydration, and mounting. Normal oral mucosal tissues were used as positive controls because CRNN is highly expressed in oral mucosa. For negative control, the primary antibody was replaced with phosphate-buffered saline. The scoring assessment was done by two oral pathologists independently based on the semi-quantitative scoring system. The intensity scores were quantified using the following scores: negative = 0, weak = 1, moderate = 2, and strong = 3. The proportion of immune-positive cells was quantified as follows: 0 = negative, 1=0–5 % 2=6–50 %, and 3=51–100 % of positive cells. The final immunoreactive score (IRS) was determined by multiplying the positive intensity and the positive proportion scores to obtain an immunoreactive score ranging from 0 to 9. The consolidated immunoreactive scores for each case were recorded. Protein expression of CRNN was classified into two groups; high and low with a cutoff value based on the median of the respective immunoreactive score, by which IRS scores of ≥6 and <6 were used as high and low levels of CRNN expression, respectively.

Statistical analysis

The association between the LOH, MSI, CRNN protein expression, and the clinico-pathological parameters were analyzed by Chi-square test (or Fisher's exact test where appropriate). Survival curves were plotted using the Kaplan–Meier analysis and compared using the log rank tests. Of 75 OSCC

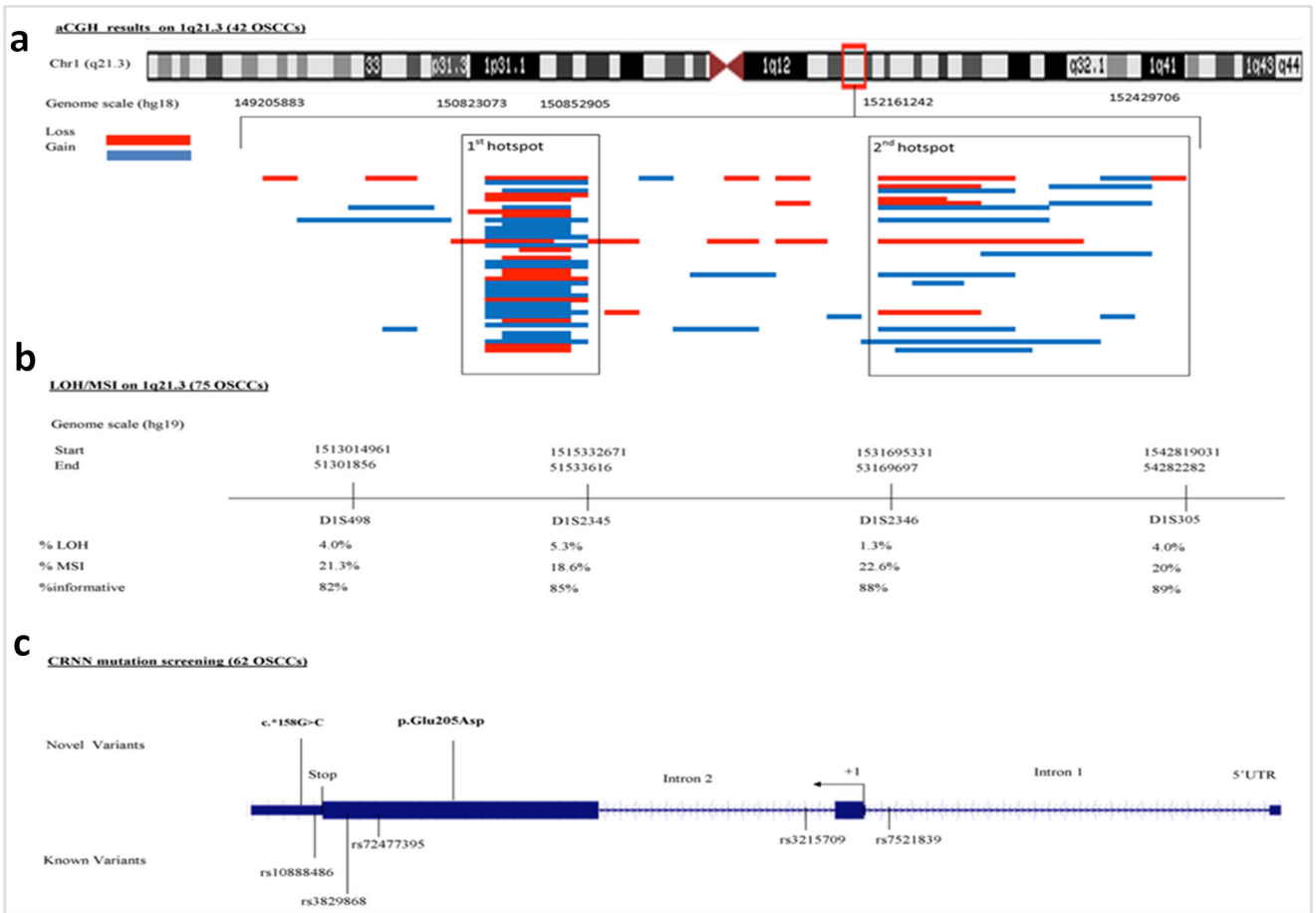

Fig. 1 Heat map of gains and losses at 1q21.3 among 42 OSCCs using aCGH (**a**). Frequency of MSI/LOH for each STR marker at 1q21.3 and the marker among 75 OSCCs (**b**). Novel and known variants in *CRNN* gene among 62 OSCC cases (**c**)

samples that have been used for MSI and LOH analysis, 10 samples with no survival data and 7 samples that were diagnosed after 2012 were excluded for survival analysis. Multivariate Cox regression analysis was conducted to evaluate the LOH and MSI at 1q21.3 as an independent prognostic factor. The protein expression of CRNN was compared between tumor and normal tissues using the Mann–Whitney *U* test. All statistical analyses were performed using the SPSS statistical package (SPSS version 12.0, Chicago, IL, USA), and the *p*-values <0.05 was considered significant.

Results

LOH/MSI at 1q21.3 chromosomal region

Revisiting aCGH data revealed that 64.3 % of OSCC samples had a chromosomal gain in the 1q21.3 region in contrast to 35.7 % that had losses [8]. Two hotspots for alteration in copy numbers were detected in 1q21.3 region, where the *CRNN* gene is located in the 2nd hotspot (Fig. 1a). MSI/LOH analysis was performed on 75 OSCC samples using highly polymorphic STR markers. Most of the cases were informative (≥82 %) and remained into analysis (Fig. 1b). LOH at single STR marker was ranged from 1.3 to 5.3 %, while MSI was ranged from 18.6 to 22.6 %. LOH plus MSI were detected in 29.3 % of all cases. When all loci were considered, the overall MSI was more frequent (25.3 %) than LOH (9.3 %). Most of the cases had instability in more than one marker (22.6 %) than single marker. LOH at D1S2345 and MSI at D1S2346 markers, closest markers to the *CRNN*, were more frequent.

Association of LOH and MSI at 1q21.3 with socio-demographic and clinico-pathologic parameters

A significant statistical association was found with LOH at 1q21.3 among OSCC cases (Table 3). As, LOH was significantly associated with Malay ethnicity (Malay, 71.4 % vs. non-Malay, 28.6 %; *p* value=0.008) and advanced staging (advanced staging, 100 % vs. early stage, 0 %, *p* value=0.039). Of 18 Malay patients, 15 patients were diagnosed as having advanced stage disease. MSI was found to be significantly associated with non-betel quid chewing (non-chewer, 78.9 % vs. chewer, 21.1 %, *p* value=0.003) and tongue SCC (tongue, 52.6 % vs. other subsites, 47.4 %, *p* value=0.026) (Table 3).

Table 3 Association analysis between LOH and MSI at 1q21.3 with socio-demographic and clinico-pathologic parameters

Variables	Category	No. of patients (%)	LOH (*n*, %)		*p* value	MSI (*n*, %)		*p* value
			No	Yes		No	Yes	
Total			68	7		56	19	
Gender	Male	27	25 (36.8)	2 (28.6)	NS	17 (30.4)	10 (52.6)	NS
	Female	48	43 (63.2)	5 (71.4)		39 (69.6)	9 (47.4)	
Age (years)	<45	8	7 (10.3)	1 (14.3)	NS	5 (8.9)	3 (15.8)	NS
	≥45	67	61 (89.7)	6 (85.7)		51 (91.1)	16 (84.2)	
Ethnicity	Indian	41	40 (58.8)	1 (14.3)	**0.008**	35 (62.5)	6 (31.6)	NS
	Malay	18	13 (19.1)	5 (71.4)		11 (19.6)	7 (36.8)	
	Chinese	12	12 (17.6)	0 (0.0)		7 (12.5)	5 (26.3)	
	Others	4	3 (4.5)	1 (14.3)		3 (5.4)	1 (5.3)	
Smoking	No	50	47 (69.1)	3 (42.9)	NS	40 (71.4)	10 (52.6)	NS
	Yes	25	21 (30.9)	4 (57.1)		16 (29.6)	9 (47.4)	
Drinking	No	63	56 (82.4)	7 (100.0)	NS	49 (87.5)	14 (73.7)	NS
	Yes	12	12 (17.6)	0 (0.0)		7 (12.5)	5 (26.3)	
Betel quid chewing	No	37	31 (45.6)	6 (85.7)	NS	22 (39.3)	15 (78.9)	**0.003**
	Yes	38	37 (54.4)	1 (14.3)		34 (60.7)	4 (21.1)	
Tumor site	Non-tongue*	51	46 (67.6)	5 (71.4)	NS	42 (75.0)	9 (47.4)	**0.026**
	Tongue	24	22 (32.4)	2 (28.6)		14 (25.0)	10 (52.6)	
Lymph node metastasis	Negative	35	34 (50)	1 (14.3)	NS	27 (48.2)	8 (42.1)	NS
	Positive	40	34 (50)	6 (85.7)		29 (51.8)	11 (57.9)	
pTNM staging	Early stage	29	29 (42.6)	0 (0.0)	**0.039**	21 (37.5)	8 (42.1)	NS
	Advanced stage	46	39 (57.4)	7 (100.0)		35 (62.5)	11 (57.9)	

Significant *p* values were highlighted in bold

*Non-tongue: buccal mucosa, gingiva, lip, floor of mouth, palate

Association of LOH and MSI of at 1q21.3 with survival outcome as prognostic indicators

In the present study, the follow-up time for patients ranged from 1 to 88 months (mean, 23.78 months; median, 20.0 months). Two-year survival rates for negative and positive LOH at 1q21.3 chromosomal region were 61.43 and 25.0 %, respectively. Results of Kaplan–Meier analysis revealed a significant association between LOH at 1q21.3 and poor prognosis (p=0.029) (Fig. 2a). In multivariate Cox regression analysis, LOH at 1q21.3 remained as a significant prognostic factor for survival after adjustment for age, gender, habitual risk factors, and lymph node metastasis (LNM) which are the common confounding factors in OSCC (HRR=7.15 (95 % CI, 1.41–36.25), p=0.018).

Three-year survival rates for negative and positive MSI at 1q21.3 chromosome region were 59.1 and 37.3 %, respectively. Results of Kaplan–Meier analysis showed no significant association between MSI at 1q21.3 and poor prognosis (p= 0.177) (Fig. 2b).

Mutation screening of *CRNN* gene

In direct sequencing of *CRNN* gene in 62 OSCC samples, five known and two novel variants were found (Fig. 1c). The novel variant of c.*158G>C was found as heterozygous at 3′-UTR in an Indian patient with a history of betel-quid chewing. Sample was taken from the gum of a patient who was at the advanced stage. The p.Glu205Asp was found as heterozygous non-synonymous variant in which glutamic acid in the position site of 205 was substituted with an aspartic acid. This missense variation was detected in a Malay OSCC patient who was at advanced stage and with lymph node metastasis. Sample was taken from tongue.

With the exception of three Indian patients who were heterozygous for rs3215709 variant, an intronic insertion in intron 2, almost all other samples were homozygous for rs3215709 variant (Table 4). However, the wild-type allele G of rs72477395 is reported ≥98 % in the HAPMAP population, but in contrast, the mutant allele was more frequent in all four ethnicities in the present study (Table 4).

CRNN protein expression among OSCCs

Immunohistochemical analyses showed high (IRS >6) CRNN expression in 28/31 (90.3 %) cases. Strong and extensive staining in the nuclei and the cytoplasm of the tumor cells was observed in these cases. Weak (IRS <6) staining of the tumor cells was seen in 3/31 (9.7 %) cases (Fig. 3). The socio-demographical and clinico-pathological parameters of the 31 OSCC patients recruited for IHC study is shown in Table 5. In all normal oral mucosal tissues (positive control), the epithelial cells showed strong (IRS=9) cytoplasmic and nuclear staining with anti-CRNN antibody in the spinous and keratinized layer of the epithelium (Fig. 3).

Correlation of CRNN protein expression with LOH, MSI, and clinico-pathological parameters

Expression of CRNN protein was significantly higher in normal oral mucosa tissues compared with OSCC samples (p<0.05), but it showed no significant association with clinico-pathologic factors. In contrast, low expression of CRNN protein was significantly correlated with MSI (MSI yes, 100 % vs. MSI no, 0 %, p value=0.037). We were able

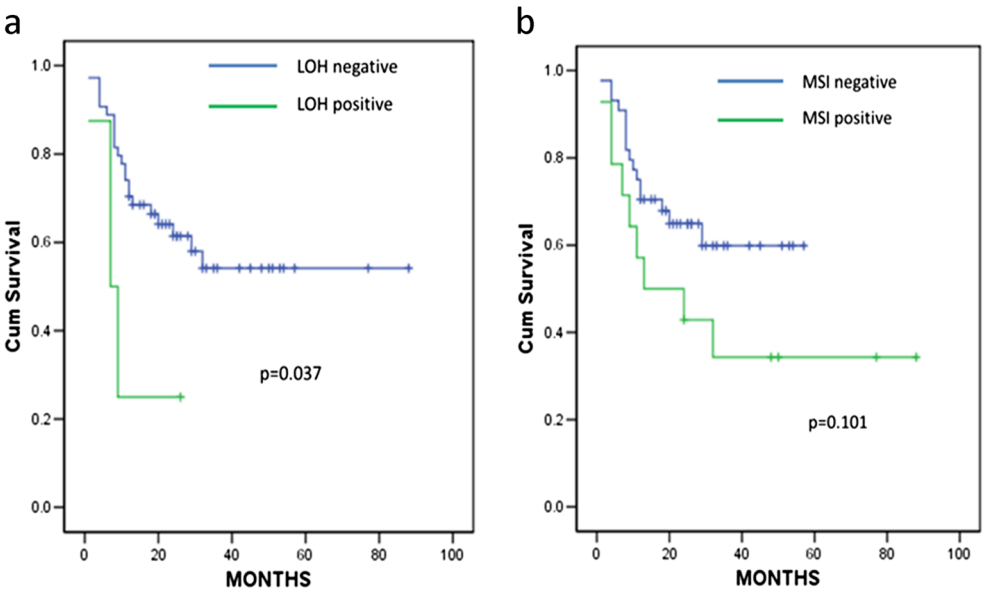

Fig. 2 Overall survival curves were analyzed according to LOH (**a**) and MSI (**b**) using Kaplan–Meier estimate with log rank test

Table 4 Frequency of genotypes for each variant among all subpopulations

Ethnicity (n=62)	rs3215709 c.138+73_138+74insA intron2	rs7521839 c.-13-37A>G intron 1	rs72477395 c.1065G>A p.Thr355=exon 3	rs10888486 c.*8C>T 3′UTR	rs3829868 c.1438C>T p.Gly480Ser exon 3	Novel variants
Indian (n=41)	A/− (3); AA (38)	AA (27), AG (12), GG (2)	AA (41)	CC (18), CT (17), TT (6)	CC (18), CT (17), TT (6)	g.152381912G>C, c.*158G>C
Indigenous (n=3)	AA (3)	AA (2), AG (1)	AG (1), AA (2)	CT (2), TT (1)	CT (2), CC (1)	–
Malay (n=12)	AA (12)	AA (9), AG (2), GG (1)	AG (3), AA (8), GG (1)	CC (6), CT (5), TT (1)	CC (6), CT (5), TT (1)	g.152382943:G>C, c.615G>C, p.Glu205Asp exon 3
Chinese (n=6)	AA (6)	AA (4), AG (1), GG (1)	AG (1), AA (5)	CC (2), CT (2), TT (2)	CC (2), CT (3), TT (1)	–

to recruit only two samples with LOH for IHC analysis. Therefore, lack of correlation between LOH and CRNN protein expression could be attributed to the small sample size of cases with LOH.

Significance of CRNN protein expression as a prognostic indicator in OSCC

The follow-up time of patients analyzed for CRNN expression ranged from 1 to 88 months (mean, 26.27 months; median, 22.5 months). Two-year survival rates for low and high CRNN expressions were 33.3 and 68.4 %, respectively. The low expression of CRNN protein was significantly correlated with poor prognosis (p=0.044) in Kaplan–Meier analysis (Fig. 4).

Discussion

Genomic instability as the hallmark of cancer refers to increased tendency of alteration such as copy number changes, MSI/LOH, single gene mutations, and epigenetic changes in the genome [26]. EDC genes are clustered at 1q21, and the mechanisms that regulate the normal balanced expression of these genes are still unknown [27]. CpG methylation appeared to play a role in this event but chromatin remodeling and other epigenetic modifications most likely are contributing predominantly [28]. In addition, pool of transcription factors that are acting in a gene- and cell-specific manner to regulate the normal balance of gene expression play pivotal role [27, 29].

It is however controversial that genomic instability is the consequence of cancer progression or the event that drives

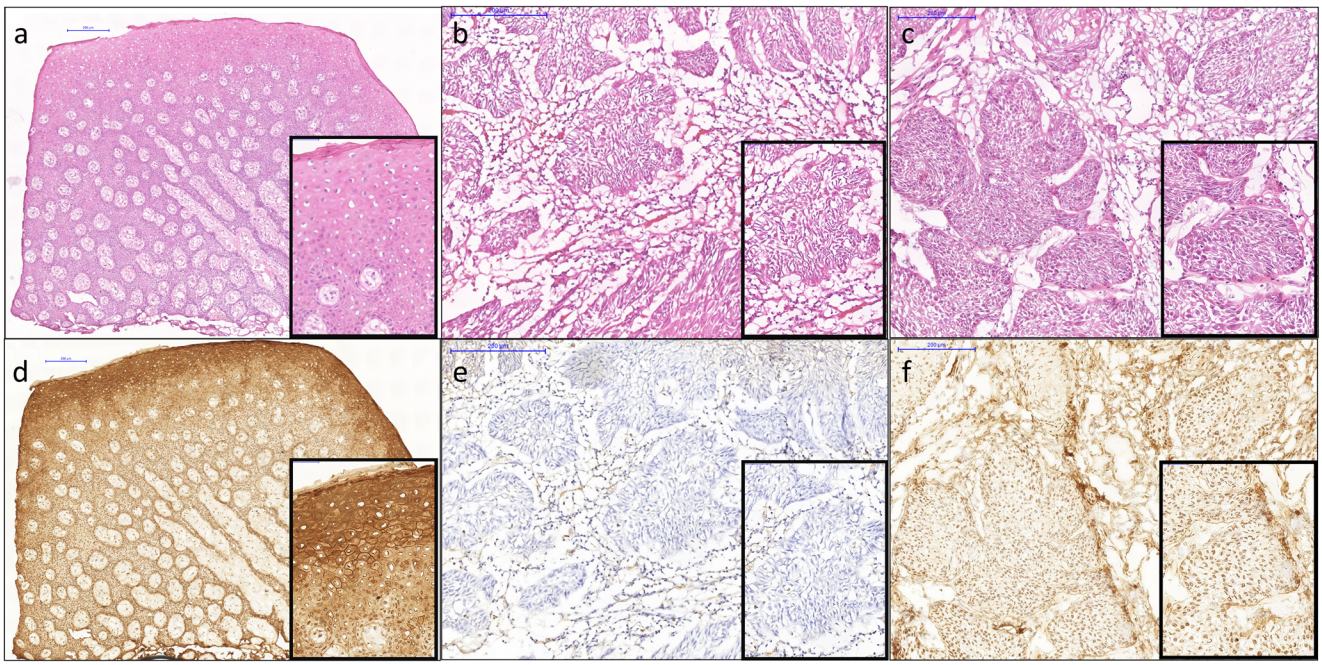

Fig. 3 Immunohistochemistry of CRNN. Normal oral mucosal tissue. **a** H&E stain (magnification×400 and×1600); **d** anti-CRNN antibody immunostain was strongly positive in normal oral mucosa (magnification×400 and×1600); OSCC (**b**, **c**) H&E stain (magnification×400 and×1600); **e** anti-CRNN antibody immunostaining showed low expression and **f** high expression in the cytoplasm and nuclei of the epithelial tumor cells (magnification×400 and×1600)

Table 5 Socio-demographic and clinic-pathologic parameters of 31 OSCC patients

Variables	Category	No. of patients (%)	CRNN expression (*n*, %)		*p* value
			Low level of expression	High level of expression	
Total		31	3	28	
Gender	Male	13	1 (33.3)	12 (42.9)	NS
	Female	18	2 (66.7)	16 (57.1)	
Age (years)	<45	4	1 (33.3)	3 (10.7)	NS
	≥45	27	2 (66.7)	25 (89.3)	
Ethnicity	Indian	15	1 (33.3)	14 (50.0)	NS
	Malay	4	1 (33.3)	3 (10.7)	
	Chinese	10	1 (33.3)	9 (32.1)	
	Others	2	0 (0.0)	2 (7.2)	
Smoking	No	20	1 (33.3)	19 (67.9)	NS
	Yes	11	2 (66.7)	9 (32.1)	
Drinking	No	24	1 (33.3)	23 (82.1)	NS
	Yes	7	2 (66.7)	5 (17.9)	
Betel quid chewing	No	17	2 (66.7)	15 (53.6)	NS
	Yes	14	1 (33.3)	13 (46.4)	
Tumor site	Non-tongue*	22	1 (33.3)	21 (75.0)	NS
	Tongue	9	2 (66.7)	7 (25.0)	
Lymph node metastasis	Negative	13	1 (33.3)	12 (42.9)	NS
	Positive	18	2 (66.7)	16 (57.1)	
pTNM staging	Early stage	10	1 (33.3)	9 (32.1)	NS
	Advanced stage	21	2 (66.7)	19 (67.9)	
LOH	No	29	3 (100.0)	26 (92.9)	NS
	Yes	2	0 (0.0)	19 (7.1)	
MSI	No	20	0 (0.0)	20 (71.4)	**0.037**
	Yes	11	3 (100.0)	8 (28.6)	

Significant *p* values were highlighted in bold

*Non-tongue: buccal mucosa, gingiva, lip, floor of mouth, palate

tumorigenesis, but these alterations can be indicative of a cancer-related gene. Detection of genetic instabilities from clinically benign appearing lesions to potentially malignant disorders (OPMD) to invasive oral tumors provide evidence that genomic instabilities play a role in both genesis and progression of cancer [30–34]. We more recently detected high frequency of chromosomal instabilities such as gains or losses among OSCC cases [8]. Revisiting our aCGH data revealed that 64.3 and 35.7 % of OSCC samples had gain and loss at 1q21.3, respectively. In addition, revisiting our unpublished gene expression microarray data showed that *CRNN* is downregulated among OSCCs. However, genes in a region with copy number changes might be amplified partially which are either non-functional or act as a gene expression silencer [35]. Here, we hypothesized that downregulation of *CRNN* gene might be the consequence of mutation or LOH at 1q21.3. Hence, we further explored this region using polymorphic STR markers and the *CRNN* gene was directly sequenced.

In the present study, MSI was found more frequent than LOH, which was consistent with the results of aCGH by which gains were more frequent than losses at 1q21.3. In addition, MSI showed a significant association with no betel-quid chewing and tongue SCC. In a study on Japanese cases with OSCC, a high frequency of MSI was detected among tongue SCC which is in agreement with our finding [36]. Lack of association between MSI and habitual risk factors and identifying MSI-H with a high frequency reflects the higher probability of inactivation in DNA mismatch repair (MMR) genes. MSI phenotype is strongly associated with mutation in genome, especially in MMR genes [37]. Inactivation of MMR genes either by mutation or methylation has been previously detected in oral cancer [38, 39]. MSI in various loci is reported in a range of 7 % up to 60 % among oral cancers with a higher frequency among Asian countries [33, 34, 40, 41]. In this study, LOH showed significant association with Malay ethnicity than other ethnicities in Malaysia. However, the sample size of Malays was not that much large in comparison with Indians and Indians had more habitual risk factors than Malays. Thus, these observations reflect that LOH/MSI at 1q21.3 might not be affected by habitual risk

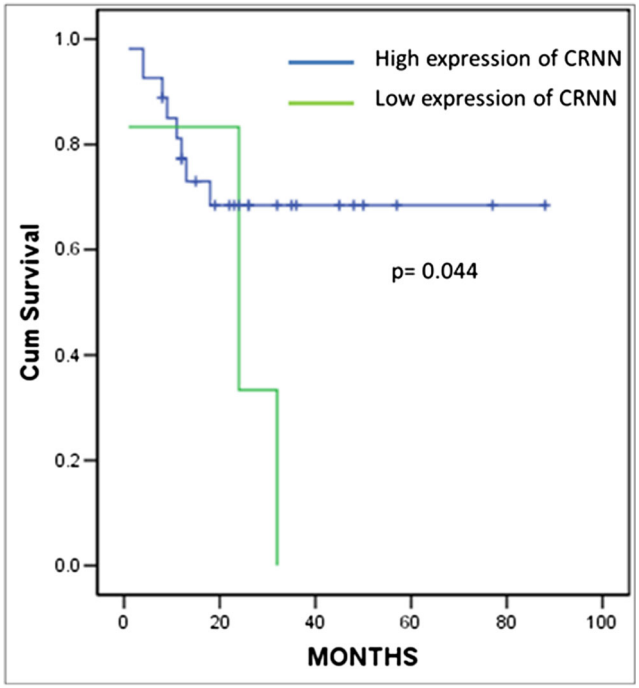

Fig. 4 Overall survival curves were analyzed according to CRNN protein expression using Kaplan–Meier estimate with log rank test

factors such as betel-quid chewing. This statement was in line with that from the significant association between no betel-quid chewing and MSI in the current study. In addition, detecting higher frequency of LOH among Malays could be attributed to similar predisposing hereditary and dietary factors that are different in other ethnicities in Malaysia. However, we were unable to exclude the possible role of these habitual risk factors in mutagenesis of MMR genes by which subsequently will cause genetic instability. In agreement with the current study, LOH has been detected in OSCC cases with advanced stage [42], as all cases with LOH had LNM as well. In this study, a significant difference was found among those with and without LOH in survival analysis. Poor prognosis in OSCC cases with LOH at 1q21.3 could be explained by the fact that over-expression of *CRNN* gene suppresses cell proliferation by arresting the cell cycle progression at the G_1/S phase and downregulation of cyclin D1 in oral cancer [19].

Downregulation of *CRNN* gene has been well documented in tumoregenesis of esophageal squamous cell carcinoma (ESCC) [18, 43, 44]. They found that significant loss of *CRNN* expression to be associated with advanced stage, invasive behavior of tumor, LNM, and poor survival [18, 43, 44]. This gene has shown downregulation among OSCCs as well [19], which is consistent with our findings. CRNN expression was downregulated in OSCCs compared with normal oral mucosal samples, and it was significantly correlated with poor prognosis. Loss of *CRNN* expression has been detected consistently across these studies with no further investigation on the possible mechanisms. Consistent with the current study, evidence has shown that single nucleotide changes might not have a remarkable impact on *CRNN* downregulation among ESCCs [45]. While LOH/MSI was frequent in this study, hence we suggested that genetic instabilities could be one of the possible mechanisms that diminish the expression of the genes that are located at 1q21.3.

To the best of our knowledge, mutation screening of *CRNN* in patients with ESCC is the only documented report, so that except for several known polymorphic variants, no novel variation was detected [45]. In direct sequencing of *CRNN* among OSCC patients with a history of habitual risk factors, five known and two novel germline variations were detected in the current research. Nucleotide substitutions were more frequently observed in exon three. All known variants have been previously reported in the National Center for Biotechnology Information (NCBI) SNP database. The novel variant of c.*158G>C was found at 3′-UTR of the gene in an Indian patient who had a history of betel-quid chewing. However, this position site is conserved among primates, but it is not conserved among placental mammals. The 3′-UTR of protein coding genes harbors the binding site of microRNAs (miRNAs) [46]. Disruption of these binding sites tends to be damaging and might be involved in disease development via regulation of gene expression [47, 48]. Somatic mutations that created or disrupted the miRNA binding sites have been reported in many cancer-related genes [49]. Thus, the potential impact of variants at 3′-UTR should be kept in mind.

The p.Glu205Asp was found in a Malay patient who was smoker. The p.Glu205Asp was predicted to be benign (PolyPhen score=0.001), and this position site is not conserved among different species. Both novel variants were detected in matched normal tissues as well, excluding somatic mutation. Hence, we did not further explore these variants among normal healthy controls. However, patients with novel variants were at advanced stage, but they had no LOH/MSI at 1q21.3. In addition, the p.Gly480Ser which is a synonymous variant was predicted as benign as well (PolyPhen score= 0.18), and this position site is not conserved.

With the exception of several known and two benign novel germline variants, no pathogenic mutation was found in mutation screening of *CRNN* gene among OSCC cases. Thus, it appears that habitual risk factors might not be associated with increased risk of mutation in *CRNN* and mutation in the coding regions might not be the mechanism of downregulation in gene expression. However, promoter plays a central role in regulation of gene expression, but no statistical difference in the expression of *CRNN* gene has been detected among patients with variations in the promoter region [45].

Identifying LOH in 9.3 % of our samples reflects that small part of CRNN downregulation could result from LOH, which is consistent with results of aCGH. Despite significant difference in CRNN expression between

normal oral mucosal samples and OSCCs, we were unable to draw a clear pattern between LOH and CRNN expression due to insufficient sample size. Evidence has shown that MSI would be able to alter the transcription factor binding and gene expression [50]. Consistent with this evidence, our MSI-positive OSCC samples showed low expression of CRNN reflecting the impact of MSI on CRNN expression. Thus, the MSI-H phenotype that was found in the current research could be associated with a higher mutation rate in the genome which in turn could alter the CRNN expression profile in OSCC. Downregulation of *CRNN* is detected to be significantly associated with lymph node metastases, advanced clinical stage, and overall survival rate, and it thus remained as an independent prognostic marker for ESCC [18]. Taken together, a part of downregulation of *CRNN* among OSCC cases could be resulted from LOH/MSI at 1q21.3 than mutation in the *CRNN* gene. However, epigenetic changes should be kept in mind as another possibility of downregulation for this gene.

In addition, this study showed a significant correlation between LOH/MSI at 1q21.3 with clinical outcomes. The LOH at this region was identified to be as an independent prognostic marker in OSCC. These findings provide further evidences on the roles of *CRNN* in tumor progression of OSCC and as a prognostic marker to predict the disease outcome. However, further investigation on the interaction between *CRNN* and other potential genes or environmental risk factors would shed light on the pathogenesis of OSCC.

Acknowledgments This study was supported by the High Impact Research MoE Grant UM.C/625/1/HIR/MoE/DENT/08. Special thanks to Lee Peng Karen-Ng for providing expression data for *CRNN* gene and to the Oral Cancer Research and Co-ordinating Centre (OCRCC), University of Malaya (UM) for providing tissue and data from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). The authors also thank the clinicians and pathologists from the Ministry of Health Malaysia for their clinical expertise required to successfully complete this study.

Conflict of interests The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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