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Prognostic value of human papillomavirus and squamous cell carcinoma antigen in head and neck squamous cell carcinoma

Zeyi Deng,[1,2,3] Masahiro Hasegawa,[1] Yukashi Yamashita,[1] Sen Matayoshi,[1] Asanori Kiyuna,[1] Shinya Agena,[1] Takayuki Uehara,[1] Hiroyuki Maeda[1] and Mikio Suzuki[1]

[1]Department of Otorhinolaryngology, Head and Neck Surgery, University of the Ryukyus, Okinawa, Japan; [2]Department of Otorhinolaryngology, Head and Neck Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, China

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To clarify the synergistic influence of human papillomavirus (HPV) status and squamous cell carcinoma antigen (SCCA) mRNA expression on head and neck squamous cell carcinoma (HNSCC) prognosis, HPV DNA presence and SCCA1 and SCCA2 mRNA expression were determined by PCR and quantitative real-time RT-PCR, respectively, in 121 patients with primary HNSCC who were receiving curative treatment. HPV DNA was detected in 28.1% (34/121) of HNSCC cases, and only high-risk types (HPV-16, HPV-33, HPV-35 and HPV-58) were observed. Positive HPV status showed a significantly better prognosis than negative HPV status ($P = 0.022$). An elevated SCCA2/SCCA1 mRNA ratio was an independent predictor of disease recurrence ($P = 0.004$). In addition, HPV-negative patients with a high SCCA2/SCCA1 ratio (>0.27) had a significantly lower recurrence-free survival rate than HPV-negative patients with a low SCCA2/SCCA1 ratio ($P < 0.011$). Our findings revealed that both HPV status and the SCCA2/SCCA1 mRNA ratio are independently associated with prognosis in HNSCC. Patients with both a HPV-negative status and a high SCCA2/SCCA1 ratio might need intensified treatment and rigorous follow up after treatment because of the high risk of recurrence. (*Cancer Sci* 2012; 103: 2127–2134)

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer, accounting for more than 600 000 new cases annually.[1] The two most important risk factors for the development of HNSCC are heavy smoking and extensive alcohol consumption.[2] Recently, the strongest correlation between human papillomavirus (HPV) and HNSCC has been found in oropharyngeal squamous cell carcinoma (SCC), particularly tonsillar carcinoma, with HPV DNA present in up to 70% of studied patients.[3–8] Furthermore, many studies have demonstrated that patients with HPV-positive oropharyngeal carcinoma have a better prognosis than those with HPV-negative oropharyngeal carcinoma.[3,9]

Squamous cell carcinoma antigen (SCCA), originally isolated from SCC tissue of the uterine cervix,[10] is a member of the family of serine protease inhibitors that map to the serine protease inhibitor (serpin) cluster at chromosome 18q21.3.[11] Molecular studies demonstrate that SCCA is transcribed by two almost identical genes (SCCA1 and SCCA2). Using column isoelectric focusing, SCCA was shown to contain at least 14 subfractions that were divided arbitrarily into two groups: acidic fractions (pI < 6.25) corresponding to SCCA2 and neutral fractions (pI > 6.25) corresponding to SCCA1.[12] SCCA2 inhibits chymotrypsin-like proteinases cathepsin G and mast cell chymase,[13] whereas SCCA1 inhibits cysteine proteinases, such as cathepsins K, L and S.[14] The serum SCCA level usually decreases after tumor resection and increases with the recurrence of tumors arising from the uterine cervix and head and neck.[15,16] The increased SCCA2/SCCA1 mRNA ratio in a primary tumor is also associated with recurrence of uterine cervical cancer and HNSCC.[17,18] However, there is no report on the mutual interaction between HPV status and SCCA in relation to the prognosis of HNSCC.

To clarify the synergistic influence of HPV status and SCCA on HNSCC prognosis, the present prospective study uses PCR for HPV DNA detection and quantitative real-time PCR for SCCA1 and SCCA2 mRNA expression in patients receiving curative treatment.

Materials and Methods

Study design. Between October 2006 and July 2011, 172 patients with HNSCC diagnosed by biopsy at the Department of Otorhinolaryngology, Head and Neck Surgery, at University of the Ryukyus, Japan provided written informed consent before being enrolled into this prospective study. During the same period, non-malignant tonsillar samples were obtained from 29 controls (age $\geq$ 18 years) who underwent tonsillectomy for chronic tonsillitis. The research protocol was approved by the ethics committee of the University of the Ryukyus.

Eligibility criteria were as follows: the presence of untreated, pathologically confirmed primary HNSCC without distant metastases (M0); age of 18 years or older; receiving curative treatment, including surgery alone, surgery combined with radiation therapy (RT) or chemoradiotherapy (CRT), concurrent CRT or RT alone with more than 66 Gy of total dosage; and complete remission after primary treatment.

Demographic and clinicopathologic parameters for each patient were collected at scheduled intervals during the follow-up period.

DNA extraction. Tissue samples were snap-frozen in liquid nitrogen during biopsy or surgical excision and stored in liquid nitrogen until further analysis. A Gentra Purification Tissue kit (Qiagen, Germantown, MD, USA) was used to isolate DNA from the samples, according to the manufacturer's specifications.

PCR for detection of high-risk and low-risk human papillomavirus DNA. The presence and integrity of the DNA in all samples was verified by PCR β-globin gene amplification using the primers PC04 and GH20.[19] Water (negative control) and the DNA of HPV-16-positive CaSki cells (positive control) were included in each amplification series. The presence of high-risk and low-risk HPV DNA was analyzed by PCR using the general consensus primer sets GP5+/GP6+ and MY09/11.[20,21]

[3]To whom correspondence should be addressed.
E-mail: jordandzy@hotmail.com

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DNA samples that were negative in the GP5+/GP6+ or MY09/11 PCR were re-amplified in an (auto-) nested PCR using the GP5+/GP6+ primer pair, as previously described.[8,22]

RNA extraction and reverse-transcription. Total RNA was isolated from 5–10 mg frozen HNSCC and non-malignant tonsil samples using the ToTALLY RNA kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Before cDNA synthesis, any residual DNA was removed through incubation with 1 U DNase I (Ambion) at room temperature for 25 min. cDNA was then synthesized from DNA-free total RNA using the RETROscript Kit (Ambion), according to the manufacturer's instructions. To examine the presence of contaminating DNA in RNA samples, all the assays were performed both with and without reverse transcriptase.

Detection of squamous cell carcinoma antigen 1 and squamous cell carcinoma antigen 2 gene expression by quantitative real-time PCR. To estimate SCCA1 and SCCA2 genes expression, quantitative real-time PCR was performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) and TaqMan PCR Master Mix II (Roche Molecular Systems, Foster City, CA, USA). Primers and TaqMan probes were used as previously described.[18] Both SCCA1 and SCCA2 probes were labeled with FAM at the 5′ end and with TAMRA at the 3′ end (Applied Biosystems Japan, Tokyo, Japan). Amplification conditions were: 2 min at 50°C, 10 min at 95°C, and a two-step cycle of 95°C for 15 s and 60°C for 60 s for a total of 40 cycles. Two standard curves for the SCCA1 and SCCA2 genes were generated by amplification of serial 10-fold dilutions (3×10^1, 3×10^2, 3×10^3, 3×10^4, 3×10^5 and 3×10^6 copies) of a plasmid pDNR-LIB carrying SCCA1 and SCCA2 cDNA, respectively (Open Biosystems, Huntsville, AL, USA). A linear relationship was found between the threshold cycle values plotted against the log of the copy number over the entire range of dilutions. All experiments were performed in duplicate using similar ratios.

For precise quantification, the SCCA1 and SCCA2 mRNA expression level of each sample was normalized using the expression of the β-actin gene. Primers and TaqMan probes that target the β-actin mRNA, were used, as previously described.[23] An external standard curve was generated using known 10-fold serial-diluted plasmid pCAG-mGFP-Actin clones as templates (Addgene, Cambridge, MA, USA). The quantitative value of SCCA1 or SCCA2 mRNA was described as each value relative to β-actin mRNA, and relative signal intensity (RSI; value of 100 000 × SCCA1/β-actin and 100 000 × SCCA2/β-actin) was measured for quantification.[24,25]

Squamous cell carcinoma antigen 1 and 2 immunohistochemistry. Serial 4-μm-thick sections of formalin-fixed paraffin-embedded samples were deparaffinized and dehydrated in a graded series of alcohol. Epitope retrieval was performed by heating at 95–99°C for 10 min in Tris/EDTA buffer (pH 9.0). Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide containing 15 mmol/L sodium azide for 5 min. The sections were subsequently incubated overnight at 4°C with primary monoclonal mouse anti-SCCA1 antibody (clone 8H11, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-SCCA2 antibody (clone 10H12, 1:1000, Santa Cruz Biotechnology). After extensive washing in PBS, the slides were incubated for 30 min at room temperature with a secondary HRP-conjugated goat anti-mouse antibody (MTM Laboratories AG, Heidelberg, Germany). The sections were then color-developed in 3-3′-diaminobenzidine (DAB) for 3 min and counterstained with hematoxylin.

Statistical analysis. Descriptive statistics were used to summarize baseline characteristics and clinicopathological variables of the patients. The Mann–Whitney *U*-test or the Kruskal–Wallis test, and Pearson's χ^2-test or Fisher's exact test, were used for continuous variables and categorical variables, respectively.

Recurrence-free survival was defined as the time from the end of treatment to cancer recurrence or final follow up. Disease-specific survival was defined as the time from the end of treatment to subsidence of disease or final follow up. Survival curves were estimated by the Kaplan–Meier method, and survival distributions were compared using the log-rank test. We assessed the univariate prognostic significance of tumor variables using proportional hazards models, and multivariate analyses were performed using Cox's proportional hazards model. Estimates for adjusted and unadjusted hazard ratios (HR) were reported with 95% confidence intervals (CI). All tests were two-sided, and *P*-values less than 0.05 were considered to indicate statistical significance. Analyses were performed using the SPSS statistical package (SPSS for Windows version 12.0; SPSS, Chicago, IL, USA).

Results

Exclusion of patients. Of the 172 registered patients, 38 were excluded from the study because they did not meet the eligibility criteria. An additional 13 were excluded because of insufficient specimens for analysis of both HPV DNA presence and SCCA mRNA expression. The remaining 121 patients were eligible for investigation. Both included and excluded patients had similar distributions of data with regard to age ($P = 0.202$), sex ($P = 0.384$), T stage ($P = 0.179$), nodal stage ($P = 0.287$), TNM stage ($P = 0.726$) and histological differentiation ($P = 0.695$).

Characteristics of eligible patients and follow up. Primary tumor location was the nasopharynx in 8 patients (6.6%), the oropharynx in 38 (31.4%), the hypopharynx in 29 (24.0%), the larynx in 22 (18.2%) and the oral cavity in 24 (19.8%). The follow-up period ranged from 6 to 54 months, with a median of 22 months for patients whose data were censored. Treatment details were as follows: 52 were treated with concurrent CRT, 37 with surgery and postoperative RT or CRT (radiation dosage 50–54 Gy), 21 with surgery alone and 11 with RT alone. Demographic and clinical characteristics are summarized in Table 1.

Prevalence of human papillomavirus in head and neck squamous cell carcinoma. The prevalence of HPV DNA in HNSCC was 28.1% (34/121). HPV DNA was most frequently observed in the oropharynx (18 of 38 cases, 47.4%). The palatine tonsil was the most common site in the oropharynx infected by HPV (15 of 22 cases, 68.2%). The presence of HPV DNA varied among other sites as follows: 25.0% (2/8) in the nasopharynx, 33.3% (8/24) in the oral cavity, 10.3% (3/29) in the hypopharynx and 13.6% (3/22) in the larynx (Table 1).

Among HPV-positive HNSCC samples, 29 (85.3%) were infected with HPV-16 and the others were infected with non-16 high-risk types (2 with HPV-33, 1 with HPV-35 and 2 with HPV-58).

Quantitative analysis of squamous cell carcinoma antigen 1 and 2 mRNA expression in head and neck squamous cell carcinoma. The frozen HNSCC samples used for quantitative real-time PCR consisted of more than 70% malignant cells (Fig. 1A).

In the 29 non-malignant tonsillar specimens, the RSI of SCCA1 and SCCA2 mRNA expression ranged from 1.72×10^{-1} to 7.92×10^2 (median 1.24×10^1) and from 2.90×10^{-2} to 4.92×10^1 (median1.55×10^0), respectively, whereas the RSI of SCCA1 and SCCA2 in HNSCC ranged from 6.22×10^{-1} to 1.77×10^4 (median 4.08×10^2) and from 1.00×10^0 to 3.73×10^2 (median 1.44×10^2), respectively. Each expression of SCCA1 and SCCA2 mRNA in HNSCC was significantly higher than that in non-malignant

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Table 1. Demographic and clinical characteristics

Characteristics	Total number	HPV+	HPV−	*P*-value	SCCA2/SCCA1 ratio		*P*-value
		n = 34	*n* = 87		Low; *n* = 50	High; *n* = 71	
Sex, number (%)							
Male	106	29 (27.4)	77 (72.6)	0.630	45 (42.5)	61 (57.5)	0.502
Female	15	5 (33.3)	10 (66.7)		5 (33.3)	10 (66.7)	
Age							
Mean, years	64.1	62.7	64.6	0.415	63.3	64.6	0.533
Range, years	28–89	39–89	28–83		28–82	39–89	
≤50, number (%)	15	6 (40.0)	9 (60.0)	0.273	6 (40.0)	9 (60.0)	0.912
>50, number (%)	106	28 (26.4)	78 (73.6)		44 (41.5)	62 (58.5)	
Tobacco smoking, number (%)†							
Never	23	9 (39.1)	14 (60.9)	0.409	5 (21.7)	18 (78.3)	0.069
≤400	14	4 (28.6)	10 (71.4)		8 (57.1)	6 (42.9)	
>400	84	21 (25.0)	63 (75.0)		37 (44.0)	47 (56.0)	
Alcohol use, number (%)‡							
Never	19	6 (31.6)	13 (68.4)	0.926	7 (36.8)	12 (63.2)	0.896
≤50	39	11 (28.2)	28 (71.8)		16 (41.0)	23 (59.0)	
>50	63	17 (27.0)	46 (73.0)		27 (42.9)	36 (57.1)	
T classification, number (%)							
T1	13	1 (7.7)	12 (92.3)	0.167	5 (38.5)	8 (61.5)	0.453
T2	48	14 (29.2)	34 (70.8)		20 (41.7)	28 (58.3)	
T3	36	9 (25.0)	27 (75.0)		18 (50.0)	18 (50.0)	
T4	24	10 (41.7)	14 (58.3)		7 (29.2)	17 (70.8)	
Node status, number (%)							
N0 or N1	70	20 (28.6)	50 (71.4)	0.892	28 (40.0)	42 (60.0)	0.729
N2 or N3	51	14 (27.5)	37 (72.5)		22 (43.1)	29 (56.9)	
TNM Stage, number (%)							
Early (I and II)	34	6 (17.6)	28 (82.4)	0.110	13 (38.2)	21 (61.8)	0.666
Advanced (III and IV)	87	28 (32.2)	59 (67.8)		37 (42.5)	50 (57.5)	
Differentiation, number (%)							
Well	54	8 (14.8)	46 (85.2)	<0.001	16 (29.6)	38 (70.4)	0.056
Moderately	51	15 (29.4)	36 (70.6)		25 (49.0)	26 (51.0)	
Poorly	16	11 (68.8)	5 (31.2)		9 (56.3)	7 (43.8)	
Tumor location, number (%)							
Hypopharynx	29	3 (10.3)	26 (89.7)	0.007	9 (31.0)	20 (69.0)	0.058
Oropharynx	38	18 (47.4)	20 (52.6)		19 (50.0)	19 (50.0)	
Oral cavity	24	8 (33.3)	16 (66.7)		8 (44.4)	10 (55.6)	
Larynx	22	3 (13.6)	19 (86.4)		13 (59.1)	9 (40.9)	
Nasopharynx	8	2 (25.0)	6 (75.0)		1 (12.5)	7 (87.5)	

†Brinkman index: daily cigarettes × years. ‡Light drinker ≤ 50 g alcohol per day; heavy drinker > 50 g alcohol per day. HPV, human papillomavirus; SCCA, squamous cell carcinoma antigen.

tissue ($P < 0.001$ and $P < 0.001$, respectively, Mann–Whitney *U*-test), shown in Figure 2A. There were no significant differences in the RSI of SCCA1 and SCCA2 mRNA expression among the sites ($P = 0.484$ and $P = 0.829$, respectively, Kruskal–Wallis test, Table 2).

The median of the SCCA2/SCCA1 ratio was 0.11 with a range of 0.03–0.49 for non-malignant tonsillar tissue and 0.32 with a range of 0.02–1.62 for HNSCC. HNSCC had a significantly higher value than non-malignant tissue for the SCCA2/SCCA1 ratio ($P < 0.001$, Mann–Whitney *U*-test, Fig. 2B). Based on the finding of the upper 99% confidence interval of the median in non-malignant tonsillar tissue, which was 0.27, patients were divided into a high SCCA2/SCCA1 ratio group (>0.27, $n = 71$) and a low SCCA2/SCCA1 ratio group (≤0.27, $n = 50$). SCCA2 expression in samples with a high SCCA2/SCCA1 ratio was significantly increased and approximately threefold higher than samples with a low SCCA2/SCCA1 ratio (median 1.93×10^2 *vs* 7.06×10, $P = 0.002$, Mann–Whitney *U*-test). A significant difference in the SCCA2/SCCA1 ratio was found between five primary sites ($P = 0.017$, Kruskal–Wallis test), and the nasopharynx had the highest ratio of SCCA2/SCCA1 (Table 2). The SCCA2/SCCA1 ratio in recurrent HNSCC was significantly higher than that in non-recurrent HNSCC ($P = 0.037$, Mann–Whitney *U*-test, Fig. 2B). In addition, there was no significant difference in the SCCA2/SCCA1 ratio between sex, age, T stage, node status, TNM stage, HPV status or death due to disease, as shown in Table 2. No significant correlation was found between HPV status and the SCCA2/SCCA1 ratio ($P = 0.400$, Pearson's χ^2-test, Table 3).

Immunohistochemistry. SCCA1 and SCCA2 were distributed diffusely in tumor tissues and in the cytoplasm of cancer cells (Fig. 1). The intensity of SCCA1 and SCCA2 in tumor cells was moderate to strong, compared with the weak intensity seen in normal squamous epithelium of non-malignant tonsillar tissues (Fig. 1H and I).

Prognosis in relation to human papillomavirus DNA presence and the squamous cell carcinoma antigen 1/squamous cell carcinoma antigen 2 mRNA ratio. During the observation period, 28 (23.1%) of the 121 patients developed recurrent lesions: 6

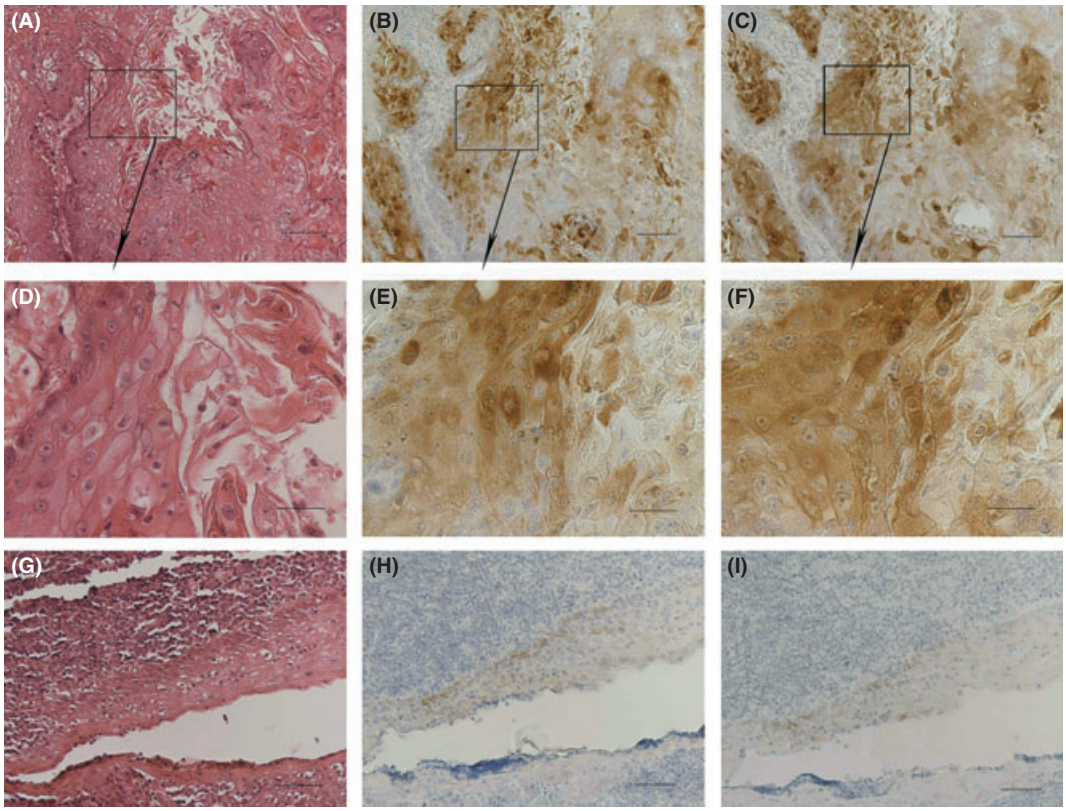

Fig. 1. Immunohistochemistry of squamous cell carcinoma antigen (SCCA) 1 and 2 in head and neck squamous cell carcinoma (A–F, oropharyngeal carcinoma) and non-malignant tonsil (G–I). (A), (D) and (G) show specimens stained with H&E (A, ×100, bar = 100 μm; D, ×400, bar = 30 μm; and G, ×200, bar = 50 μm). (B) and (E), and (C) and (F) exhibit strong cytoplasmic accumulation of SCCA1 and SCCA2, respectively (B and C, ×100, bar = 100 μm; E and F, ×400, bar = 30 μm). (H) and (I) show weak immunoreactivity of SCCA1 and SCCA2, respectively (H and I, ×200, bar = 50 μm).

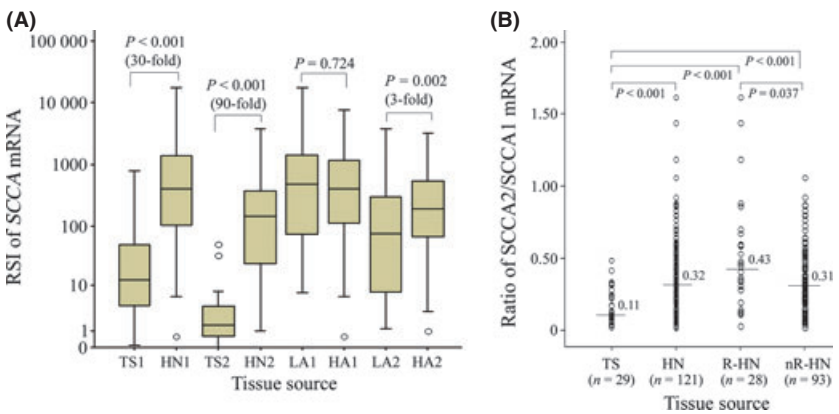

Fig. 2. Relative signal intensity (RSI) of squamous cell carcinoma antigen (SCCA) mRNA expression in non-malignant tonsillar tissue and head and neck squamous cell carcinoma (HNSCC). (A) SCCA1 and SCCA2 mRNA expression. TS1, SCCA1 of non-malignant tonsils; HN1, SCCA1 of HNSCC; TS2, SCCA2 of non-malignant tonsils; HN2, SCCA2 of HNSCC; LA1, SCCA1 of HNSCC with a low SCCA2/SCCA1 ratio; HA1, SCCA1 of HNSCC with a high SCCA2/SCCA1 ratio; LA2, SCCA2 of HNSCC with a low SCCA2/SCCA1 ratio; HA2, SCCA2 of HNSCC with a high SCCA2/SCCA1 ratio. (B) Comparison of SCCA2/SCCA1 mRNA ratios. TS, SCCA2/SCCA1 mRNA ratio in non-malignant tonsillar tissue; HN, SCCA2/SCCA1 mRNA ratio in HNSCC; R-HN, SCCA2/SCCA1 mRNA ratio in recurrent HNSCC; nR-HN, SCCA2/SCCA1 mRNA ratio in non-recurrent HNSCC.

(17.6%) of 34 early stage HNSCC and 22 of 87 (25.3%) advanced stage HNSCC. A total of 13 patients died of disease and 4 died of unrelated causes, and all had advanced stage HNSCC.

Impact of human papillomavirus DNA presence on prognosis. Kaplan–Meier analysis revealed that patients with HPV-positive HNSCC had better recurrence-free survival than patients with HPV-negative HNSCC (P = 0.022, Fig. 3A). The 3-year rate of recurrence-free survival was 91.2% (95% CI = 81.7–100%) in the HPV-positive group and 66.6% (95% CI = 54.2–78.9%) in the HPV-negative group. However, no significant difference was observed in disease-specific survival between HPV-positive and HPV-negative patients (P = 0.084; Kaplan–Meier curves not shown).

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Table 2. RSI† of SCCA1 and SCCA2 and SCCA2/SCCA1 ratio by clinicopathological status

	RSI of SCCA1	RSI of SCCA2	SCCA2/SCCA1 ratio
	Median (minimum–maximum)	Median (minimum–maximum)	Median (minimum–maximum)
Sex			
Female	1.54×10^3 (6.84×10^1–8.73×10^3)	5.40×10^2 (1.34×10^1–2.33×10^3)	0.35 (0.12–0.86)
Male	3.63×10^2 (6.20×10^{-1}–1.77×10^4)	1.30×10^2 (1.00×10^0–3.73×10^3)	0.32 (0.02–1.62)
P-value	0.017	0.014	0.598
Age, years			
≤ 50	7.02×10^2 (7.34×10^0–2.30×10^3)	2.98×10^2 (1.44×10^0–8.70×10^2)	0.33 (0.09–1.19)
>50	3.90×10^2 (6.20×10^{-1}–1.77×10^4)	1.38×10^2 (1.00×10^0–3.73×10^3)	0.32 (0.02–1.62)
P-value	0.540	0.374	0.729
T stage			
T1 or T2	4.19×10^2 (6.20×10^{-1}–1.77×10^4)	1.52×10^2 (1.00×10^0–3.73×10^3)	0.34 (0.02–2.69)
T3 or T4	3.36×10^2 (6.34×10^0–7.64×10^3)	1.38×10^2 (1.19×10^0–2.58×10^3)	0.32 (0.03–1.44)
P-value	0.832	0.850	0.913
Node status			
N0 or N1	5.09×10^2 (7.34×10^0–1.77×10^4)	1.57×10^2 (1.44×10^0–3.73×10^3)	0.33 (0.02–1.44)
N2 or N3	2.99×10^2 (6.20×10^{-1}–3.71×10^3)	9.06×10^1 (1.00×10^0–2.58×10^3)	0.31 (0.03–1.62)
P-value	0.044	0.037	0.472
TMN stage			
Early	5.31×10^2 (1.49×10^1–1.77×10^4)	1.56×10^2 (1.98×10^0–3.73×10^3)	0.34 (0.02–1.19)
Advanced	3.70×10^2 (6.20×10^{-1}–7.64×10^3)	1.23×10^2 (1.00×10^0–2.58×10^3)	0.32 (0.03–1.62)
P-value	0.145	0.125	0.649
Subsite of tumor			
Nasopharynx	2.69×10^2 (1.01×10^1–5.72×10^2)	1.06×10^2 (9.32×10^0–6.05×10^2)	0.48 (0.19–1.06)
Oropharynx	3.44×10^2 (7.34×10^0–8.73×10^3)	1.03×10^2 (1.44×10^0–2.58×10^3)	0.29 (0.04–0.87)
Hypopharynx	4.25×10^2 (6.20×10^{-1}–7.64×10^3)	2.04×10^2 (1.00×10^0–2.43×10^3)	0.37 (0.02–1.62)
Larynx	5.70×10^2 (6.24×10^0–1.77×10^4)	1.54×10^2 (2.80×10^0–3.73×10^3)	0.22 (0.03–0.69)
Oral cavity	3.88×10^2 (9.91×10^0–5.02×10^3)	1.80×10^2 (1.19×10^0–2.33×10^3)	0.41 (0.10–0.86)
P-value	0.484	0.829	0.017
HPV status			
Positive	4.16×10^2 (6.34×10^0–5.15×10^3)	1.56×10^2 (1.19×10^0–1.75×10^3)	0.33 (0.04–0.80)
Negative	3.74×10^2 (6.2×10^{-1}–1.77×10^4)	1.36×10^2 (1.00×10^0–3.73×10^3)	0.32 (0.02–1.62)
P-value	0.804	0.984	0.867
Recurrent cancer			
Yes	4.36×10^2 (6.2×10^{-1}–5.02×10^3)	1.67×10^2 (1.00×10^0–2.33×10^3)	0.43 (0.03–1.62)
Not	4.08×10^2 (6.24×10^0–1.77×10^4)	1.07×10^2 (1.19×10^0–3.73×10^3)	0.31 (0.02–1.06)
P-value	0.685	0.341	0.037
Death of disease			
Yes	1.76×10^2 (6.2×10^{-1}–3.33×10^3)	9.06×10^1 (1.00×10^0–2.33×10^3)	0.46 (0.03–1.62)
Not	4.22×10^2 (6.24×10^0–1.77×10^4)	1.53×10^2 (1.19×10^0–3.73×10^3)	0.32 (0.02–1.19)
P-value	0.228	0.422	0.116

†RSI (relative signal intensity): value of 100 000 × SCCA/β-actin. HPV, human papillomavirus; SCCA, squamous cell carcinoma antigen.

Table 3. Correlation between HPV status and SCCA2/SCCA mRNA ratio

HPV status	SCCA2/SCCA1 ratio		*P*-value	OR (95% CI)†
	High‡	Low§		
Positive	22	12	0.400	1.422 (0.625–3.232)
Negative	49	38		

†OR (95% CI), odds ratio (95% confidence interval). ‡High SCCA2/SCCA1 ratio: >0.27. §Low viral load: ≤ 0.27. HPV, human papillomavirus; SCCA, squamous cell carcinoma antigen.

Of the various primary lesions, HPV-positive patients with oropharyngeal carcinoma had better recurrence-free survival than HPV-negative patients with oropharyngeal cancer ($P = 0.037$, Fig. 4A), but no significant difference between HPV-positive and HPV-negative cohorts with oropharyngeal carcinoma was found for disease-specific survival ($P = 0.174$; Kaplan–Meier curves not shown). In contrast, no significant differences in recurrence-free survival and disease-specific survival existed between HPV-positive and HPV-negative patients with non-oropharynx cancer ($P = 0.370$ and $P = 0.385$, respectively; Kaplan–Meier curves not shown).

Impact of squamous cell carcinoma antigen 2/squamous cell carcinoma antigen 1 mRNA expression ratio on prognosis. Kaplan–Meier survival analysis showed that patients with a low SCCA2/SCCA1 ratio had better recurrence-free survival than patients with a high SCCA2/SCCA1 ratio ($P = 0.011$, Fig. 3B). However, the Kaplan–Meier survival curve revealed no statistically significant difference of disease-specific survival between the high and the low SCCA2/SCCA1 ratio cohorts ($P = 0.338$; Kaplan–Meier curves not shown).

Synergistic relationship between human papillomavirus presence and squamous cell carcinoma antigen 2/squamous

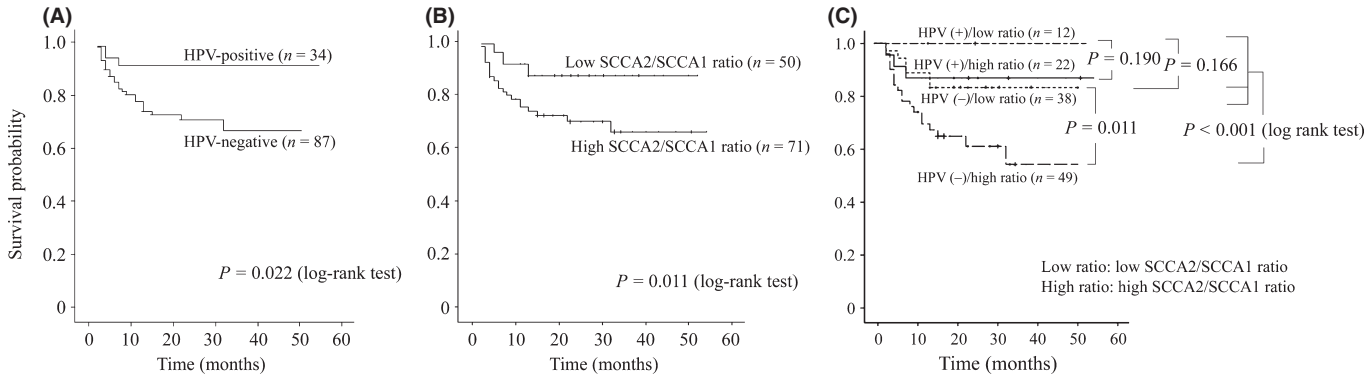

Fig. 3. Kaplan–Meier curves of recurrence-free survival in head and neck squamous cell carcinoma (HNSCC) patients. (A) Recurrence-free survival between human papillomavirus (HPV)-positive and HPV-negative HNSCC. (B) Recurrence-free survival between the low and high SCCA2/SCCA1 ratios of HNSCC. (C) Recurrence-free survival in the four groups defined by HPV presence and the SCCA2/SCCA1 ratio for HNSCC. High SCCA2/SCCA1 ratio, >0.27; low SCCA2/SCCA1 ratio, $\leq$0.27. SCCA, squamous cell carcinoma antigen.

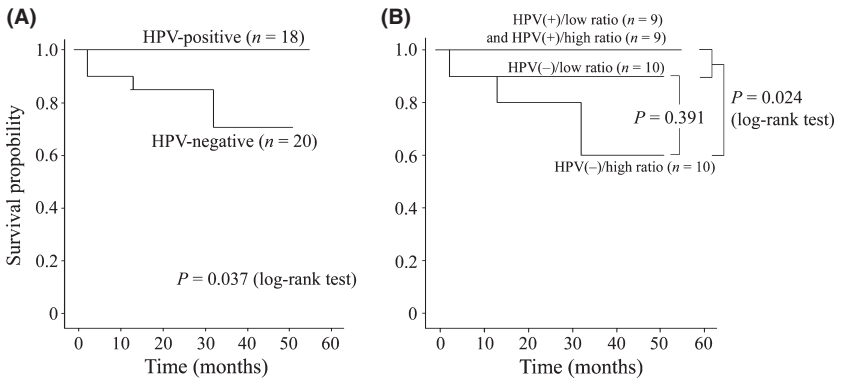

Fig. 4. Kaplan–Meier curves of recurrence-free survival in patients with oropharyngeal cancer. (A) Recurrence-free survival between human papillomavirus (HPV)-positive and HPV-negative oropharyngeal cancer. (B) Recurrence-free survival in the four groups defined by HPV presence and SCCA2/SCCA1 ratio for oropharyngeal cancer. High SCCA2/SCCA1 ratio, 0.27; low SCCA2/SCCA1 ratio, $\leq$0.27. Because HPV-positive patients with a low SCCA2/SCCA1 ratio had an identical recurrence-free survival to HPV-positive patients with a high SCCA2/SCCA1 ratio, the two Kaplan–Meier curves overlapped. SCCA, squamous cell carcinoma antigen.

cell carcinoma antigen 1 mRNA ratio in recurrence-free survival. The synergistic relationship between HPV DNA presence and the SCCA2/SCCA1 ratio with respect to disease prognosis was then investigated. Patients were classified into four groups as follows: an HPV-positive/low SCCA2/SCCA1 ratio group (n = 12); an HPV-positive/high SCCA2/SCCA1 ratio group (n = 22); HPV-negative/low SCCA2/SCCA1 ratio group (n = 38); and an HPV-negative/high SCCA2/SCCA1 ratio group (n = 49). HPV-negative patients with a high SCCA2/SCCA1 ratio had significantly lower recurrence-free survival compared with the other three groups ($P < 0.001$, Fig. 3C). The 3-year recurrence-free survival was 87.3% (95% CI = 79.6–95.1%) in HPV-positive or HPV-negative patients with a low SCCA2/SCCA1 ratio, and 50.72% (95% CI = 30.5–70.9%) in HPV-negative patients with a high SCCA2/SCCA1 ratio. In addition, among the HPV-negative patients, a low SCCA2/SCCA1 ratio revealed better prognosis than a high SCCA2/SCCA1 ratio (P = 0.011, Fig. 3C). In oropharyngeal carcinoma, HPV-negative patients with a high SCCA2/SCCA1 ratio also had significantly decreased recurrence-free survival compared with HPV-positive or HPV-negative patients with a low SCCA2/SCCA1 ratio (P = 0.024, Fig. 4B).

The final model of multivariate analysis using a Cox proportional hazards model for identification of independent risk factors of recurrence-free survival of HNSCC showed that advanced T stage (P = 0.027; adjusted HR = 2.43; 95% CI = 1.11–5.35), HPV-negative status (P = 0.005; adjusted HR = 5.95; 95% CI = 1.71–20.73) and a high SCCA2/SCCA1 ratio (P = 0.004; adjusted HR = 3.97; 95% CI = 1.57–10.04) were associated with a high risk of HNSCC recurrence (Table 4).

Discussion

Recent studies reveal that HPV is responsible for a subgroup of HNSCC[3–8] and that patients with HPV-positive HNSCC have a better prognosis than those with HPV-negative HNSCC.[3,9,26–28] A case-controlled study of HNSCC shows that HPV-16-positive HNSCC has different risk factor profiles from HPV-16-negative HNSCC (e.g. relating to sexual behavior and exposure to marijuana), indicating that they should be considered distinct cancers.[29] In the present study, HPV DNA, mainly HPV-16, was detected in 28.1% of HNSCC cases. The recurrence-free survival in HPV-positive patients with oropharyngeal SCC was significantly better than in HPV-negative patients, which is consistent with previous reports.[3,9,26–28]

A study on head and neck cancer cell lines reveals that SCC cells actively synthesize SCCA1 and SCCA2 but that the majority of these proteins are retained in the cytosol;[30] the authors consequently speculate that SCCA in the circulation of cancer patients might represent the size of SCCA-overproduc-

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Table 4. Results of univariate and multivariate analysis for recurrence-free survival

Variable	Univariate analysis		Multivariate analysis	
	P-value	HR (95% CI)	*P*-value	HR (95% CI)
Age ($\leq$50 *vs* > 50)	0.747	1.24 (0.36–4.26)		
Sex (male *vs* female)	0.110	0.39 (0.13–1.22)	0.060	0.38 (0.14–1.04)
T stage (T3 or T4 *vs* T1 or T2)	0.179	1.80 (0.76–4.25)	0.027	2.43 (1.11–5.35)
Nodal stage (N0 or N1 *vs* N2 or N3)	0.931	1.04 (0.44–2.44)	0.975	1.01 (0.42–2.44)
Smoking (yes *vs* never)	0.357	0.62 (0.23–1.71)		
Alcohol use (yes *vs* never)	0.042	0.34 (0.12–0.94)	0.057	0.42 (0.17–1.03)
Differentiation				
Well		Reference		
Moderately	0.150	0.51 (0.20–1.29)		
Poorly	0.529	0.55 (0.14–2.19)		
Tumor location				
Oropharynx		Reference		
Nasopharynx	0.089	5.10 (0.87–29.85)	0.747	1.34 (0.23–7.92)
Hypopharynx	0.071	3.24 (0.87–12.09)	0.423	1.68 (0.47–5.98)
Larynx	0.267	2.50 (0.59–10.53)	0.107	3.43 (0.77–15.34)
Oral cavity	0.046	4.25 (1.11–16.22)	0.232	2.23 (0.60–8.29)
HPV status (negative *vs* positive)	0.020	4.17 (1.17–14.88)	0.005	5.97 (1.71–20.87)
SCCA2/SCCA1 ratio (high *vs* low)	0.015	3.29 (1.22–8.86)	0.004	3.97 (1.57–10.04)

CI, confidence interval; HPV, human papillomavirus; HR, hazard ratio; SCCA, squamous cell carcinoma antigen.

ing tumor cells and their turnover. Although SCCA has been recognized as a tumor marker for head and neck SCC as well as cervical carcinoma, SCCA1 expression is downregulated in head and neck SCC cells compared with normal keratinocytes.[31] In another study, SCCA1 suppressed migration of NK cells and this inhibitory effect was lost by mutation of the reactive site loop of SCCA1.[32] In contrast, previous studies have also shown that the mRNA copy number of SCCA2 is higher in malignant cervical tissues than in normal tissues.[18] Stenman *et al.* investigate the effect of variations in relation to the proportion of tumor tissue to normal epithelium with high versus low SCCA2/SCCA1 ratios.[17] Their results clearly show that the presence of normal tissue in the HNSCC samples has only a marginal effect on the measured SCCA2/SCCA1 ratio. In our study, HNSCC samples consisted of at least 70% cancer cells, which concurs with the results in the previous report.[17] We believe that our data accurately reflects SCCA1 and SCCA2 mRNA expression in the tumor tissue.

Previous *in vitro* studies have demonstrated that SCCA2 protects tumor cells from apoptosis.[13,33,34] A significantly reduced invasive potential is noted with SCCA1-transfected clones of the HNSCC cell line in *in vitro* and *in vivo* experiments.[35] In the present study, SCCA1 and SCCA2 mRNA expression in HNSCC was 30-fold and 90-fold higher than in non-malignant tissues, respectively. Although the high SCCA2/SCCA1 ratio in HNSCC correlated with worse recurrence-free survival, the mean absolute SCCA1 and SCCA2 mRNA expression in the recurrent cases was not significantly different from that in non-recurrent cases. These findings suggest that SCCA2 expression, compared with SCCA1 rather than the absolute SCCA2 expression, plays a more important role in the progression of cancer and in the protection of malignant cells from various HNSCC therapies than previously envisaged.

Stenman *et al.* first reported that HNSCC with an elevated SCCA2/SCCA1 ratio had an aggressive characteristic and frequently recurred independent of clinical stage in patients who responded to initial therapy.[17] The present study indicates that patients with a high SCCA2/SCCA1 ratio have a poor prognosis and that a high SCCA2/SCCA1 ratio is associated with disease recurrence. These results suggest that the SCCA2/SCCA1 ratio has potential for predicting disease severity and response to treatment. To the best of our knowledge, this is the first study to perform absolute quantification of SCCA1 and SCCA2 from malignant and non-malignant tissue of the head and neck. Further studies are also needed to clarify the detailed mechanism and the biological significance of a high SCCA2/SCCA1 ratio in HNSCC.

Multivariate analysis on recurrence-free survival in the present study clearly indicated that in addition to tumor stage, HPV status and the SCCA2/SCCA1 ratio are independent prognostic factors for HNSCC recurrence. In addition, a HPV-negative status and a high SCCA2/SCCA1 ratio indicated a markedly increased risk of recurrence after initial curative therapy. Moreover, HPV-negative patients with a low SCCA2/SCCA1 ratio showed significantly better prognosis than those with a high SCCA2/SCCA1 ratio. This finding suggests that the SCCA2/SCCA1 ratio becomes a prognostic predictor of HPV-negative HNSCC. A similar tendency of the SCCA2/SCCA1 ratio was also observed in patients with HPV-negative oropharyngeal carcinoma (Fig. 3B). However, no significant correlation was noted between HPV status and SCCA2/SCCA1 ratio, as shown in Table 3. Furthermore, no significant difference in SCCA1, SCCA2 or SCCA2/SCCA1 rate was observed between HPV-positive and HPV-negative cohorts. Thus, our data provides no evidence that HPV infection mediates the expression of SCCA in malignant cells.

In conclusion, our findings provide evidence that both HPV status and the SCCA2/SCCA1 ratio are independently associated with HNSCC prognosis. The results suggest that HPV-negative patients with a high SCCA2/SCCA1 ratio need intensified therapy and rigorous follow up after treatment.

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Disclosure statement

The authors have no conflicts of interest to declare.

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