日本財団 The Nippon Foundation 助成事業

財団法人　日中医学協会

2011年度共同研究等助成金報告書－在留中国人研究者－

2012年　3　月　12　日

財団法人　日中医学協会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

中国人研究者氏名：　　鄧　澤義　㊞

指導責任者名:　鈴木幹男

所属部署名:　琉球大学大学院　　職名：教授

所　在　地:沖縄県中頭郡西原町上原207番地

電　　話:098-895-1183　　内線：1183

1．助成金額：　600,000　　円

2．研究テーマ

頭頸部癌におけるHPV感染の役割及びSCCAと予後に関する研究

3．成果の概要

頭頸部癌組織の生検と手術サンプルを用いて、PCR法及びリアルタイムPCR法を利用して検討を行い、頭頸部癌発症におけるHPV感染の役割及びSCCAと予後の関係を明らかにし、国際の学会及び英文論文を発表した。

4．研究業績

(1)学会における発表　　無・㊒(学会名・演題)

1) Detection and prognosis of HPV in head and neck cancer · 27th International Papillomavirus Conference and Clinical Workshop. Poster presentation.

2) Viral load, physical status and prognosis of human papillomavirus in head and neck squamous cell carcinoma11th · Japan-Taiwan Conference on Otolaryngology Head and Neck Surgery. Oral presentation

(2)発表した論文　　無・㊒(雑誌名・題名)

1) **Deng Z**, Hasegawa M, Kiyuna A, et al. Viral load, physical status, and E6/E7 mRNA expression of human papillomavirus in head and neck squamous cell carcinoma. Head Neck, 2012 (in Press)

2) Hasegawa M, **Deng Z**, Maeda H, et al. Human papillomavirus load and physical status in sinonasal inverted papilloma and squamous cell carcinoma. Rhinology. 2012 (in Press)

3) **Deng Z**, Hasegawa M, Matayoshi S, et al. Prevalence and clinical features of human papillomavirus in head and neck squamous cell carcinoma in Okinawa, southern Japan. Eur Arch Otolaryngol. 2011; 268:1625-1631.

5．指導責任者の意見(指導責任者がご記入・ご捺印ください)

300 以上の臨床検体を用いて，詳細な検討をおこなった。日本，アジア地域ではじめての prospective study であり，国際的にも高く評価される。

予後に関する論文は，現在投稿中である。

指導責任者署名 鈴木幹男 鈴木印

－日中医学協会助成事業－

頭頚部癌における HPV の役割及び SCCA と予後に関する研究

研 究 者氏名　鄧　澤義
中国所属機関　中国広西医科大学耳鼻咽喉科
日本所属機関　琉球大学大学院医学研究科耳鼻咽喉頭頚部外科
指 導 責任者　教授　　鈴木 幹男
共同研究者名　長谷川昌宏，又吉　宣，山下　懐，上原貴行
喜友名朝則，安慶名信也，真栄田裕行

要旨

To clarify the synergistic influence of HPV status and *SCCA* mRNA expression on HNSCC prognosis, HPV DNA presence and *SCCA*1 and *SCCA*2 mRNA expression were determined by polymerase chain reaction (PCR) and quantitative real-time reverse transcription-PCR, respectively, in 121 patients with primary HNSCC who were receiving curative treatment. 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 = 0.007). Although no significant correlation between HPV status and the *SCCA2/SCCA1* mRNA ratio was observed, HPV-negative patients with a high *SCCA2/SCCA1* mRNA ratio (>0.27) had a significantly lower survival rate compared with HPV-positive patients and those with a low *SCCA2/SCCA1* mRNA ratio (P = 0.001). 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 may need more aggressive treatment and rigorous follow-up after treatment because of the high risk of recurrence.

Key words　human papillomavirus; squamous cell carcinoma antigen; disease prognosis; head and neck squamous cell carcinoma

緒言

During the last decade, the strongest correlation between human papillomavirus (HPV) and head and neck squamous cell carcinoma (HNSCC) has been found in oropharyngeal squamous cell carcinoma, particularly tonsillar carcinoma, with HPV DNA present in up to 70% of studied patients.[1-3] Furthermore, many studies have demonstrated that patients with HPV-positive oropharyngeal carcinoma have a better prognosis than HPV-negative oropharyngeal carcinoma.[1] Nevertheless, there are few reports regarding the relationship

between HPV presence and other prognostic factors in patients with HNSCC. Squamous cell carcinoma antigen (SCCA) is a member of the family of serine protease inhibitors that map to the serine protease inhibitor (serpin) cluster at chromosome 18q21.3.[4] Molecular studies have demonstrated that SCCA is transcribed by two almost identical genes (*SCCA1* and *SCCA2*). In previous studies, the correlation between prognosis and *SCCA* mRNA expression in the uterine cervix and head and neck has also been investigated.[5-7] To clarify the synergistic influence of HPV status and SCCA on HNSCC prognosis, the present prospective study employed polymerase chain reaction (PCR) for HPV DNA detection and quantitative real-time PCR for *SCCA*1 and *SCCA*2 mRNA expression in patients receiving radical treatment.

対象と方法

One hundred and seventy-two patients with HNSCC provided written informed consent before being enrolled into this prospective study. Demographic and clinicopathologic parameters for each patient were collected at scheduled intervals during the follow-up period. After isolating DNA from the clinical fresh-frozen samples, the presence of HPV DNA was analyzed by PCR using the general consensus primer sets GP5+/GP6+ and MY09/11. Positive PCR products were purified and directly sequenced. Obtained sequences were aligned and compared with those of known HPV types in the GenBank database using the BLAST program.

cDNA was synthesized from DNA-free total RNA after total RNA was isolated from frozen samples of HNSCCs. To estimate *SCCA1* and *SCCA2* genes expression, quantitative real time-PCR was performed with the ABI Prism 7300 Sequence Detection System and TaqMan PCR Master Mix II. Primers and TaqMan probes were used as previously described.[7] Two standard curves for the *SCCA1* and *SCCA2* genes were generated by amplification of serial 10-fold dilutions of a plasmid pDNR-LIB carrying SCCA1 and SCCA2 cDNA, respectively. A linear relationship was found between the threshold cycle values plotted against the log of the copy number over the entire range of dilutions. For precise quantification, the *SCCA1* and *SCCA2* mRNA expression level of each sample was normalized using the expression of the *β-actin* gene. The quantitative value of *SCCA1* or *SCCA2* mRNA was described as each value relative to *β-actin* mRNA (relative signal intensity, e.g. RSI: value of 100,000 × *SCCA*/*β-actin*).

A Mann-Whitney U-test or Kruskal-Wallis test for continuous variables and Pearson's Chi-square test or Fisher's exact test for dichotomous variables were used to compare patients with and without mutations at baseline. Survival curves were estimated according to the Kaplan-Meier method, and survival distributions were compared using the log-rank test. Multivariate analysis for recurrence-free survival and disease- specific survival were performed using the Cox proportional hazards model. Analyses were performed using the SPSS statistical package.

結果

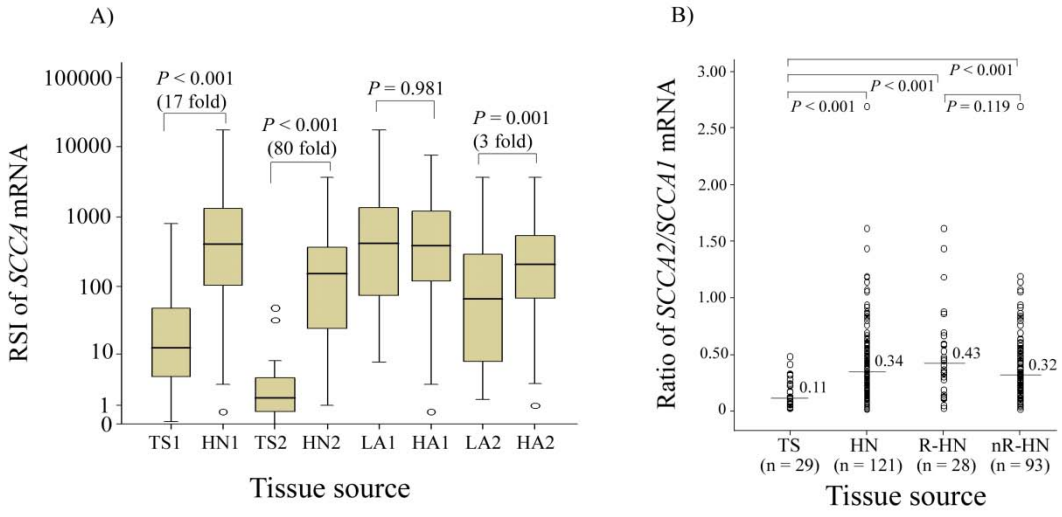
Prevalence of HPV in HNSCC

Of the 172 registered patients, 38 were excluded from the study, since they did not meet eligibility criteria. The remaining 121 patients were eligible for investigation. 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%). Among HPV-positive HNSCC samples, 29 (85.3 %) were infected with HPV-16 and the others were infected with non-16 high-risk types, in particular, 2 with HPV-33, 1 with HPV-35, and 2 with HPV-58. There were no cases of HPV-18, and no multiple HPV infections were detected.

Quantitative analysis of *SCCA1* and *SCCA2* mRNA expression in HNSCC

Each expression of *SCCA1* and *SCCA2* mRNA in HNSCC was significantly higher than that in non-malignant tissue ($P < 0.001$ and $P < 0.001$, respectively), shown in Figure 1-A. HNSCC had a significantly higher value than non-malignant tissue for the *SCCA2/SCCA1* mRNA ratio ($P < 0.001$, Figure 1-B). *SCCA2* expression in samples with a high *SCCA2/SCCA1* mRNA ratio was significantly increased and 3-fold higher than samples with a low *SCCA2/SCCA1* mRNA ratio (median 2.10×10^2 vs 6.59×10, $P = 0.001$).

(Figure 1)



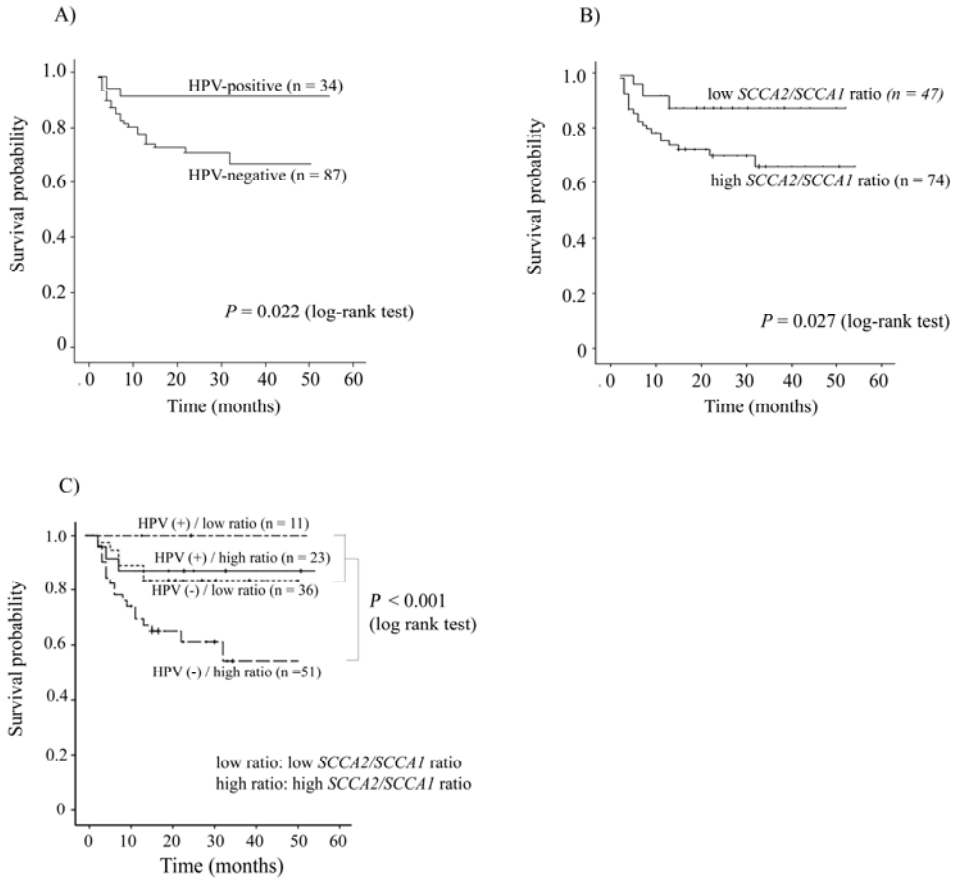
Prognosis in relation to HPV DNA presence and the *SCCA2/SCCA1* mRNA ratio

1) Impact of HPV DNA presence and *SCCA2/SCCA1* mRNA expression ratio, respectively, on prognosis

Kaplan-Meier analysis revealed that patients with HPV-positive HNSCC had better recurrence-free survival than patients with HPV-negative HNSCC ($P = 0.022$, Figure 2-A).

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, Figure 3-A). Patients with a low *SCCA2/SCCA1* mRNA ratio (≤0.27, n = 47) had better recurrence-free survival than patients with a high *SCCA2/SCCA1* mRNA ratio (>0.27, n = 74) (P = 0.027, Figure 2-B).

(Figure 2)

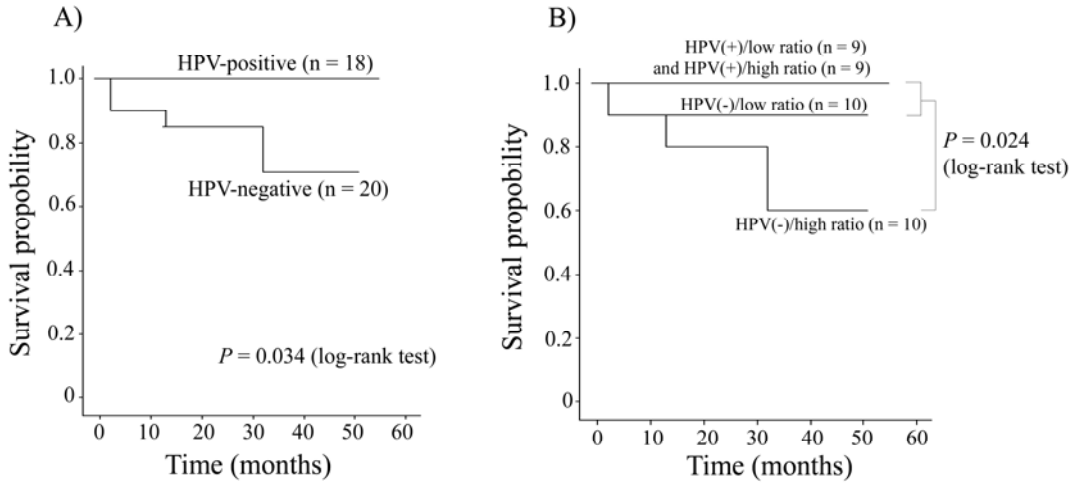


2) Synergistic relationship between HPV presence and *SCCA2/SCCA1 mRNA ratio* in recurrence-free survival

HPV-negative patients with a high *SCCA2/SCCA1* mRNA ratio had significantly lower recurrence-free survival compared with both HPV-positive patients and HPV-negative/low *SCC2/SCCA1* ratio patients (P < 0.001, Figure 2-C). In oropharyngeal carcinoma, HPV-negative patients with a high *SCCA2/SCCA1* mRNA ratio also had a significantly decreased recurrence-free survival compared with HPV-positive patients or with those with a low *SCCA2/SCCA1* mRNA ratio (P = 0.024, Figure 3-B) 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 female gender (P = 0.044; adjusted HR = 2.83; 95% CI = 1.03-7.78), advanced T stage (P = 0.020; adjusted HR = 2.56; 95% CI = 1.16-5.66),

HPV-negative status (P = 0.005; adjusted HR = 5.97; 95% CI = 1.71-20.87), and a high *SCCA2/SCCA1* ratio (P = 0.007; adjusted HR = 3.64; 95% CI = 1.42-9.30) were associated with a high risk of HNSCC recurrence.

(Figure 3)



考察

In the present study, HPV DNA, mainly HPV-16, was detected in 28.1% of HNSCC cases. The recurrent-free survival in HPV-positive patients with HNSCC, including the oropharynx, was significantly better than in HPV-negative patients with HNSCC, which was consistent with previous study.[1] *SCCA1* and *SCCA2* mRNA expression in HNSCC was 17-fold and 80-fold higher than in non-malignant tissues, respectively, suggesting that the high *SCCA2/SCCA1* mRNA ratio in HNSCC is due to elevation of SCCA2 mRNA expression. It seems that elevated *SCCA2* expression might play a more important role in the progression of cancer and in protecting malignant cells from various therapies for HNSCC than previously envisaged.

The present study indicated that patients with a high *SCCA2/SCCA1* mRNA ratio had a poor prognosis and that a high *SCCA2/SCCA1* mRNA ratio is associated with disease recurrence. These results suggest that the *SCCA2/SCCA1* mRNA 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.

Multivariate analysis on recurrence-free survival in the present study clearly indicated that in addition to tumor stage and gender, both HPV status and the *SCCA2/SCCA1* mRNA ratio are independent prognostic factors for recurrence in HNSCCs. In addition, a HPV-negative status and/or a high *SCCA2/SCCA1* mRNA ratio indicated a markedly increased risk of recurrence after initial radical therapy in patients with HNSCC, and a similar tendency was observed in patients with oropharyngeal carcinoma.

In conclusion, our findings provide evidence that both HPV status and the *SCCA2/SCCA1* mRNA ratio are independently associated with HNSCC prognosis. Positive HPV status and a low *SCCA2/SCCA* ratio are two independent factors for predicting good prognosis. On the other hand, patients with both high *SCCA2/SCCA1* mRNA ratio and negative HPV status had HNSCC recurrence after radical treatment. The present results suggest that HPV-negative patients with a high *SCCA2/SCCA1* mRNA ratio need more aggressive therapy and rigorous follow-up after treatment.

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注：本研究は、2011 年 9 月『第 27 回国際乳頭腫ウイルス学会』にてポスター発表、2011 年 12 月『第 11 回日台耳鼻咽喉科学学会』にて口演発表。

作成日：2012 年 3 月 9 日