

Association of NCCRP1 expression with T category and *in vitro* growth-related characteristics of head and neck squamous cell carcinoma FaDu cells

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Abstract: Nonspecific cytotoxic cell receptor protein 1 (NCCRP1), which is expressed in normal squamous epithelium, has been reported as a prognostic marker for esophageal squamous cell carcinoma and triple-negative breast cancer. Here, we evaluated expression of NCCRP1 in head and neck squamous cell carcinoma (HNSCC) and adjacent normal mucosa, its association with clinicopathological features, and *in vitro* growth-related characteristics following forced NCCRP1 expression. In the present study, we evaluated expression levels of NCCRP1 in formalin-fixed, paraffin-embedded samples of specimens collected by biopsy and resection from 43 patients with HNSCC and examined association of NCCRP1 expression with clinicopathological characteristics. Human HNSCC FaDu cells with forced NCCRP1 expression were assessed using the MTT assay and the colony formation assay. We found that NCCRP1 was expressed in noncancerous spinosum and superficial epithelial layers of the oral cavity, nasopharynx, mesopharynx, hypopharynx, and larynx. NCCRP1 expression in HNSCC tissues showed considerable variation, contrasting to relatively uniform expression in noncancerous surrounding mucosa. Based on NCCRP1 staining proportion in tumor, 31 of the 43 patients (72.1%) were classified as NCCRP1-low and the remaining 12 (27.9%) patients as NCCRP1-high. NCCRP1 expression in HNSCC tissue showed an exploratory association with T category ($p = 0.0215$). HNSCC FaDu cells with forced NCCRP1 expression exhibited enhanced *in vitro* growth-related characteristics measured by the MTT and colony formation assays. NCCRP1 expression was associated with T category in this selected HNSCC cohort, and forced NCCRP1 overexpression in FaDu cells was associated with increased *in vitro* growth-related measurements. Nevertheless, these exploratory findings require validation in independent clinical cohorts and experimental models.

Keywords: nonspecific cytotoxic cell receptor protein 1, head and neck squamous cell carcinoma, T category, growth-related characteristics

1. Introduction

Head and neck cancer is the sixth most common cancer worldwide, with approximately 600,000 newly diagnosed and more than 400,000 deaths per year (1). Head and neck squamous cell carcinoma (HNSCC), the most common histologic type, can arise from anywhere within the head and neck region, including oral cavity, nasopharynx, mesopharynx, hypopharynx, and larynx (2). Treatment strategy for HNSCC depends on tumor location, and surgery and/or radiotherapy are the main therapeutic approaches in patients with early-stage HNSCC. The 5-year overall survival rate for patients with early-stage HNSCC has increased to 70–90% with advances in surgery and due to the relatively high

sensitivity of HNSCC to radiotherapy. However, early-stage HNSCC accounts for 30–40% of all cases and the clinical outcomes and prognosis of patients with advanced HNSCC remain poor, with 5-year overall survival rate remaining between 20% and 40% (3). Furthermore, HNSCC tends to overlap with cancers at other sites, such as esophageal cancer, and requires periodic evaluation even after treatment for HNSCC.

Nonspecific cytotoxic cell receptor protein 1 (NCCRP1) was originally identified in catfish as an antigen receptor expressed in nonspecific cytotoxic cells, which are considered evolutionary precursors of natural killer cells in mammals (4). Subsequent studies in zebrafish have revealed receptor-related signaling properties of NCCRP1 including signal transducer

and activator of transcription (STAT) signaling (5). Unlike the fish homologs, mammalian NCCRP1 lacks a transmembrane domain and has predominantly been reported to localize in the cytoplasm, indicating species-related functional differences (6). In humans, *NCCRP1* mRNA is distributed in tissues containing squamous epithelium, such as esophagus, oral cavity, skin, and tongue, but not in immune tissues. Recent studies have reported reduced expression of NCCRP1 in esophageal squamous cell carcinoma (ESCC) (7), pancreatic cancer (8), and triple-negative breast cancer (9); however, its impact on prognosis in these cancers may depend on context. Indeed, reduced NCCRP1 expression was associated with poor prognosis in ESCC whereas patients with NCCRP1-positive pancreatic cancer and triple-negative breast cancer had poor prognosis. Therefore, predicting the role of NCCRP1 in HNSCC is difficult based on current evidence.

In the present study, we aimed to retrospectively evaluate association of NCCRP1 expression with clinicopathologic features of 43 patients with HNSCC. Furthermore, we investigated influence on *in vitro* growth-related characteristics following forced NCCRP1 expression in HNSCC FaDu cells.

2. Materials and Methods

2.1. Patients

This retrospective study included 43 patients with histologically confirmed HNSCC who were diagnosed in the National Center for Global Health and Medicine (NCGM) between 2010 and 2022 and who met predefined eligibility criteria. In particular, the inclusion criteria were as follows: with histologically confirmed HNSCC, with formalin-fixed paraffin-embedded tumor tissue suitable for immunohistochemical evaluation, and with complete clinicopathological information. Patients whose tissue specimen was inadequate for immunohistochemical evaluation or who lacked essential clinical information were excluded. All patients who met these predefined inclusion criteria, including adequate tissue specimens and availability of required clinicopathological data during the study period, were included in the present analysis. No additional case selection was conducted according to NCCRP1 expression or study outcome. During the diagnostic biopsy procedure in some patients with small, early-stage HNSCC, the tumor was completely removed en bloc. Resected specimens provided sufficient pathological information to confirm the pathologic diagnosis of HNSCC. Of the 43 patients, 25 (58.1%) were evaluated using biopsy specimens and 18 (41.9%) using surgically or endoscopically resected specimens. Formalin-fixed, paraffin-embedded sections of these specimens were used for immunohistochemical analysis. Data on clinicopathologic parameters were retrieved from hospital

records. T categories were determined according to the TNM Classification of Malignant Tumors, 8th edition, published by the Union for International Cancer Control. The specimen used for NCCRP1 immunohistochemical assessment did not necessarily correspond to the source used for T-category determination. In patients who subsequently underwent surgical resection, pathological T category was determined from the definitive resection specimen. In patients who did not undergo surgical resection, including those treated with radiotherapy and/or chemotherapy, T category was determined clinically based on imaging and other clinical findings.

For this retrospective study, an opt-out procedure was approved by the Research Ethics Committee of the NCGM (approval no: 2417).

2.2. Immunohistochemical analysis

Paraffin-embedded sections containing formalin-fixed HNSCC specimens were deparaffinized and rehydrated. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in an autoclave at 120°C for 10 min. An anti-NCCRP1 antibody (HPA048141; Sigma-Aldrich, St. Louis, MO) was used to determine NCCRP1 immunoreactivity. Diaminobenzidine staining was performed using the ImmPACT™ DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA), and hematoxylin was used for counterstaining. Pancreatic tissue sections were used as controls for NCCRP1 staining because acinar cells have been reported to specifically express NCCRP1 (10). All slides were reviewed by two or more observers who were blinded to the clinical and pathologic data. Tumor areas were identified with reference to hematoxylin and eosin-stained sections according to squamous cell carcinoma's characteristic histopathological features, including nuclear enlargement, nuclear pleomorphism, hyperchromasia, nuclear-to-cytoplasmic ratio increase, cellular polarity loss, keratinization, intercellular bridges, and atypical squamous cells' invasive nests or cords. We evaluated NCCRP1 staining within intratumoral areas, avoiding peripheral tumor regions near normal tissue. Clearly non-neoplastic areas, including necrotic areas, inflammatory regions, normal mucosa, and tumor margins near normal tissue, were excluded from assessment. Twenty fields were assessed in resection specimens and five in biopsy specimens. Because stromal components were often closely intermingled with tumor cells, the exact proportion of tumor tissues within the evaluated area could not be quantified. Therefore, we classified positive rates of NCCRP1 staining for each field into the following five categories: 15% or less, 15–30%, 30–50%, 50–70%, and 70% or more, and the median value across evaluated fields was used as the representative value for each case. The area occupied by tumor cells, including stromal components, was used as the denominator for positive rates of NCCRP1

staining. Based on NCCRP1 positivity, patients were further classified as NCCRP1-low ($\leq 30\%$) or NCCRP1-high groups ($> 30\%$). The five-category staining scores were assigned independently by observers before any consensus discussion. Agreement on the five-category scores was observed in 41 of 43 cases (95.3%). These percentages represent observed agreement; chance-corrected agreement was not formally assessed. When cases were classified as NCCRP1-high or NCCRP1-low using the 30% cutoff, the classification was concordant between observers in all 43 cases. Therefore, no disagreement occurred in final classification.

2.3. Cell lines and culture

FaDu, a human HNSCC cell line obtained from the American Type Culture Collection, was cultured in Minimum Essential Medium supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. Cells were treated with MC-210 (KAC Co Ltd., Hyogo, Japan) before use. A separate mycoplasma test was not performed. Cell growth was assessed using the MTT assay following the manufacturer's protocol (Nacalai Tesque, Kyoto, Japan). For the MTT assay, different wells were used at different time points. In the colony formation assay, FaDu transfectants (400 cells/well) were seeded into 24-well plates. Following a 2-week incubation, colonies were stained with crystal violet. Staining image was obtained using a fluorescence microscope BZ-X710 (KEYENCE). Semi-quantification from images was performed using a BZ-X Analyzer (KEYENCE). All *in vitro* experiments were performed independently three times, with three technical wells per condition in each experiment. For each of the three independent experiments, the three technical wells were first summarized into a single experiment-level value, and the independent biological experiment was used as the unit of analysis. FaDu cells, which were authenticated by the supplier using short tandem repeat testing, were passaged in our laboratory for fewer than 6 months after resuscitation.

2.4. Transfection of *NCCRP1*

To construct the expression vector containing *NCCRP1*, a DNA fragment encoding the full-length *NCCRP1* open reading frame (OriGene Technologies, Rockville, MD) was used as a template. Briefly, *NCCRP1* gene was amplified by polymerase chain reaction using Primestar (Takara Bio, Shiga, Japan) and ligated into the pIRES2-EGFP vector (Clontech, Palo Alto, CA) using the In-Fusion HD cloning kit (Clontech). The resulting pIRES2-EGFP-*NCCRP1* vector and empty pIRES2-EGFP vector (for mock-transfection) were transfected into FaDu cells using Lipofectamine LTX reagent (Life Technologies, Carlsbad, CA). Stable transfectants were isolated using a MoFlo™ XDP cell sorter (Beckman Coulter, Brea, CA).

2.5. RT-qPCR

Total RNA was isolated from cultured cells using the RNA easy Mini Kit (QIAGEN, Hilden, Germany). After treating RNA with DNase I, double-stranded complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed using ABI TaqMan. Threshold cycle numbers (Ct) were determined using Sequence Detector software and relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ formula (11) as described by the manufacturer, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as calibrator gene according to the manufacturer's instructions. The TaqMan Gene expression assay IDs for the genes used in this study were *NCCRP1*, Hs01583969_m1 and *GAPDH*, Hs00266705_g1 (Applied Biosystems).

2.6. Western blotting

Protein extraction was prepared from cultured cells using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). After centrifugation at $12,000 \times g$ for 10 min at 4°C , supernatant was collected. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis for cell lysates (10 $\mu\text{g}/\text{lane}$) was performed using 5–20% gradient gels under reducing conditions. After electroblotting, the membrane was stained with anti-NCCRP1 (1:500, Sigma-Aldrich #HPA052812) or anti-tubulin (1:20,000, MBL #PM054) antibodies, followed by horseradish peroxidase-conjugated goat antirabbit antibodies (1:5,000, Santa Cruz #sc-2030) and Amersham ECL Prime Western Blotting Detection Reagent (Cytiva). Staining image was obtained using ImageQuant LAS4000 (Cytiva).

2.7. Statistical analysis

Continuous variables with approximately symmetric distributions were expressed as means $\pm$ standard deviation. Welch's *t* test was used for age comparison and mean difference with a 95% confidence interval (CI) was reported. For categorical variables, we used a two-sided Fisher's exact test for 2×2 tables and analyzed tables with more than two categories using a Fisher–Freeman–Halton test. In clinicopathological comparisons, exact unadjusted *p* values were reported. Missing values were not imputed, and analyses were conducted using all available data for each variable. For RT-qPCR, statistical testing was performed using ΔCt values obtained from three independent biological experiments using a paired-sample approach. Two-way ANOVA was used to analyze overall group, time, and group-by-time pattern for MTT data, and Sidák's multiple-comparisons test was used to compare mock and NCCRP1 transfectants on each day. For the colony formation assay, experiment-level values

were compared by paired *t*-test. All statistical analyses were performed using GraphPad Prism 8 statistical program (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. NCCRP1 expression in HNSCC and normal adjacent mucosa

First, we performed immunohistochemical analysis to investigate protein expression and localization of NCCRP1 in HNSCC and normal adjacent mucosa specimens. Of 43 cases, NCCRP1 expression in noncancerous mucosa was evaluable in 40 samples. Among these 40 cases, NCCRP1 expression was detected in 97.5% (39/40), including oral cavity, nasopharynx, mesopharynx, hypopharynx, and larynx. NCCRP1 expression was observed in spinous and superficial epithelial layers of stratified squamous epithelium, where epithelial cells showed NCCRP1 staining in cytoplasm and nuclei (Figure 1A). In contrast, NCCRP1 expression in HNSCC tissues showed considerable variation. Therefore, we categorized the HNSCC cases based on proportion of NCCRP1-positive area in tumor tissues into the following five categories: 15% or less, 15–30%, 30–50%, 50–70%, and 70% or more (Figure 1D). Based on the bimodal distribution of NCCRP1 positivity,

we classified patients into NCCRP1-low ($\leq 30\%$) and NCCRP1-high ($> 30\%$) groups. Of the 43 tumor specimens, 31 (72.1%) HNSCC cases were classified as NCCRP1-low (Figure 1B) and the remaining 12 (27.9%) HNSCC cases as NCCRP1-high (Figure 1C).

3.2. Association of NCCRP1 expression with clinicopathologic features of HNSCC

We next investigated association of NCCRP1 expression with the clinicopathologic characteristics of HNSCC. Of the 31 NCCRP1-low cases, 26 (84%) showed 0–15% positivity, and 5 (16%) showed 15–30% positivity. Of the 12 NCCRP1-high cases, 9 (75%) showed 30–50% positivity, 2 (17%) showed 50–70% positivity, and 1 (8%) showed $\geq 70\%$ positivity (Figure 1D). In the NCCRP1-low group, 17 of 31 patients (54.8%) had biopsy specimens and 14 (45.2%) had resection specimens, whereas in the NCCRP1-high group, 8 of 12 patients (66.7%) had biopsy specimens and 4 (33.3%) had resection specimens. Distribution of specimen type across T categories is presented in Supplementary Table S1 (<https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=132>).

In the overall cohort, mean patient age was 65.2 ± 9.23 years, and the majority of patients were male (95.3%). Mean age was 61.4 ± 12.67 years in the

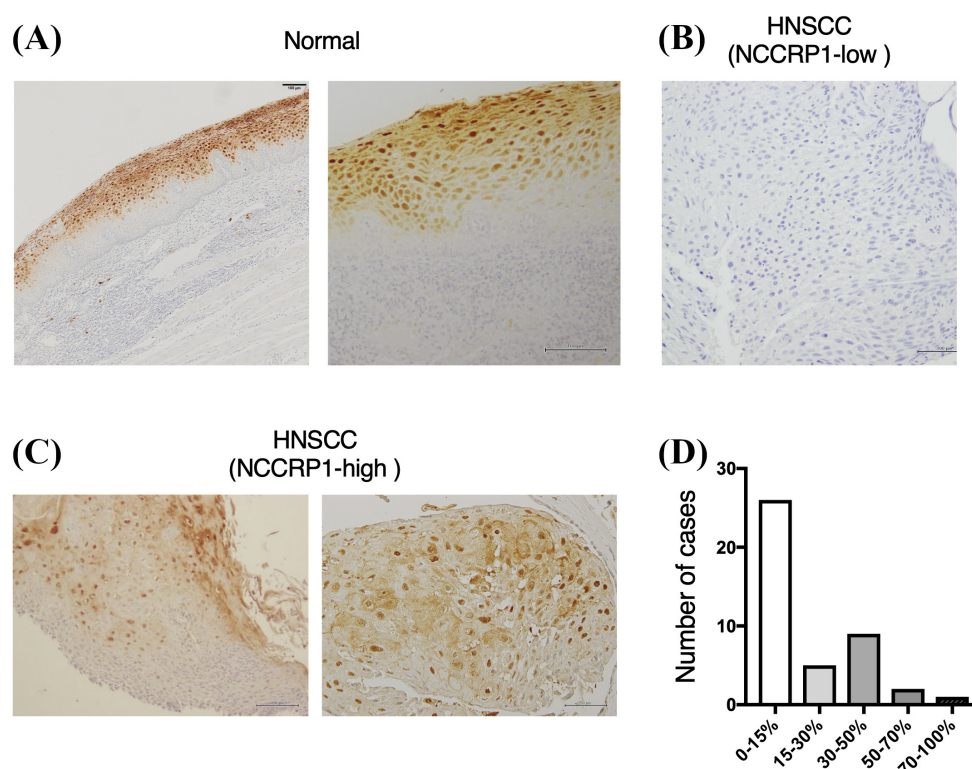


Figure 1. Nonspecific cytotoxic cell receptor protein 1 (NCCRP1) expression in head and neck squamous cell carcinoma (HNSCC) and adjacent noncancerous mucosa. (A) Representative images of formalin-fixed, paraffin-embedded samples of noncancerous head and neck mucosa stained with anti-NCCRP1 antibody. **(B)** Representative image of NCCRP1-low HNSCC. **(C)** Representative images of NCCRP1-high HNSCC. **(D)** Bimodal distribution of NCCRP1 positivity in HNSCC tissue.

NCCRP1-high group and 66.6 ± 7.25 years in the NCCRP1-low group; mean difference between groups was -5.23 years (95% CI, -13.56 to 3.11 , $p = 0.200$). A two-sided Fisher–Freeman–Halton exact test revealed a statistically significant difference of T category between the NCCRP1-high and NCCRP1-low groups ($p = 0.0215$, Table 1). Given sparse distribution of patients across T categories, we additionally summarized the T categories as T3–T4 in comparison with Tis–T2 as a secondary descriptive analysis to provide an interpretable effect estimate without fitting an overparameterized regression model. We found that T3–T4 tumors were observed in 5 of 12 patients (41.7%) in the NCCRP1-high group and 2 of 29 patients (6.9%) in the NCCRP1-low group, reflecting a conditional odds ratio of 8.97 (exact 95% CI, 1.18–113.19). The very wide CI further emphasizes substantial statistical uncertainty arising from the small and sparse dataset. No significant differences were identified for sex, tumor location, history of alcohol consumption, smoking, and pN stage between the NCCRP1-high and NCCRP1-low groups.

3.3. Association of forced NCCRP1 expression and *in*

vitro growth-related characteristics in HNSCC cells

To elucidate significance of NCCRP1 expression in HNSCC, we examined effect of NCCRP1 expression on *in vitro* growth-related characteristics by comparing FaDu cells expressing NCCRP1 to those that were mock-transfected. Basal *NCCRP1* mRNA expression in FaDu cells was extremely low, but the mock-transfected FaDu cells yielded measurable Ct values. As shown in Figure 2A, the NCCRP1-transfected FaDu cells exhibited $> 1,000$ fold higher *NCCRP1* mRNA levels than the mock-transfected FaDu cells. Western blotting further confirmed NCCRP1 overexpression at the protein level (Figure 2B). Supplementary Figure S1 (<https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=132>) presents original Western blot image for Figure 2B. For MTT data, two-way ANOVA showed significant effects of group ($p = 0.002$), time ($p < 0.001$), and the group-by-time interaction ($p < 0.001$). In Sidák's multiple comparisons test, no significant differences between NCCRP1- and mock-transfected FaDu cells on days 1–3, whereas MTT signals of NCCRP1-transfected

Table 1. Comparison of clinicopathologic features of patients with NCCRP1-high and NCCRP1-low HNSCC

Characteristic	Total (%)	Number of patients (%)		<i>p</i> value
		NCCRP1-high	NCCRP1-low	
Number of patients	43	12	31	
Sex				0.0731
Male	41 (95.3)	10 (83.3)	31 (100)	
Female	2 (4.7)	2 (16.7)	0 (0.0)	
Mean age $\pm$ standard deviation (years)	65.2 ± 9.23	61.4 ± 12.67	66.6 ± 7.25	0.200
Alcohol ¹ ($n = 39$)				0.573
No	4 (10.3)	2 (16.7)	2 (7.4)	
Yes	35 (89.7)	10 (83.3)	25 (92.6)	
Smoking ² ($n = 39$)				0.4026
No	7 (17.9)	1 (8.3)	6 (22.2)	
Yes	32 (82.1)	11 (91.7)	21 (77.8)	
Tumor location				0.289
Oral cavity	5 (11.6)	2 (16.7)	3 (9.7)	
Oropharynx	3 (7.0)	2 (16.7)	1 (3.2)	
Hypopharynx	17 (39.5)	3 (25.0)	14 (45.2)	
Larynx	18 (41.9)	5 (41.7)	13 (41.9)	
T category ³ ($n = 41$)				0.0215*
Tis	6 (14.6)	1 (8.3)	5 (17.2)	
T1	19 (46.3)	5 (41.7)	14 (48.3)	
T2	9 (22.0)	1 (8.3)	8 (27.6)	
T3	3 (7.3)	1 (8.3)	2 (6.9)	
T4	4 (9.8)	4 (33.3)	0 (0.0)	
pN status ($n = 40$)				0.0865
N0	35 (87.5)	9 (81.8)	26 (89.7)	
N1	3 (7.5)	0 (0.0)	3 (10.3)	
N2	2 (5.0)	2 (18.2)	0 (0.0)	
Complicated by ESCC				0.723
No	15 (34.9)	5 (41.7)	10 (32.3)	
Yes	28 (65.1)	7 (58.3)	21 (67.7)	

Note: * $p < 0.05$. ¹Alcohol consumption was categorized based on the presence of habitual alcohol consumption. ²Smoking was categorized as "No" for patients who have never smoked and "Yes" for those who ever smoked. ³T category was based on pathological assessment of definitive resection specimens when available and on clinical assessment, including imaging and other clinical findings, in patients who did not undergo surgical resection. Abbreviations: HNSCC, head and neck squamous cell carcinoma; NCCRP1, nonspecific cytotoxic cell receptor protein 1.

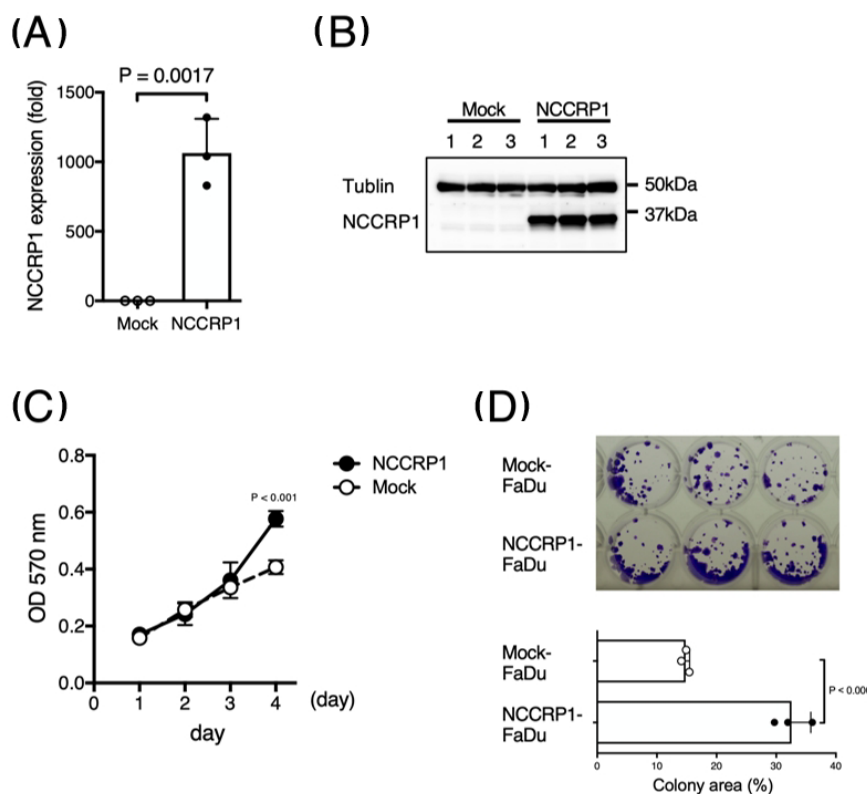


Figure 2. Effect of forced NCCRP1 expression on MTT signal and colony formation in FaDu cells. Transfection experiments were conducted three times independently and same results were obtained each time. There were three technical wells within each experiment. (A) NCCRP1 mRNA levels in mock-transfected and NCCRP1-transfected FaDu cells. Each point represents mean of three technical wells from one independent biological experiment; data are shown as mean $\pm$ standard deviation (SD) across three independent experiments. **(B)** Western blots of mock-transfected and NCCRP1-transfected FaDu cells for detection of NCCRP1 and tubulin. Lanes 1–3 illustrates independent biological repeats. **(C)** Growth curves of NCCRP1-transfected and mock-transfected FaDu cells determined by MTT assay. Cultures were started at a cell density of 2×10^3 /well. Different wells were used at different time points. Data are presented as mean $\pm$ SD from three independent biological experiments after averaging three technical wells within each experiment. **(D)** NCCRP1-transfected and mock-transfected FaDu cells were cultured for two weeks. Representative images of colonies visualized using crystal violet staining (upper panel). Areas with colonies were determined in three separate wells within each experiment. Each point represents mean of three technical wells from one independent biological experiment; data are shown as mean $\pm$ SD across three independent experiments (lower panel).

cells were significantly higher than that of mock-transfected cells on day 4 ($p < 0.001$; Figure 2C). Furthermore, NCCRP1-transfected FaDu cells formed colonies more efficiently than mock-transfected FaDu cells in the colony formation assay ($p < 0.0001$; Figure 2D). Although these results do not directly establish a proliferative function of NCCRP1 but demonstrated the association of NCCRP1 expression and *in vitro* growth-related characteristics in HNSCC.

4. Discussion

In the present study, we used immunohistochemical analysis to demonstrate that NCCRP1 is expressed in normal mucosa across all areas of the pharynx and larynx, while its expression in HNSCC tissues showed considerable variation and NCCRP1-high ($> 30\%$) was associated with T category. *In vitro* overexpression of NCCRP1 in FaDu cells was associated with enhanced growth-related characteristics measured by MTT assay and colony formation. A previous study has

demonstrated that suppression of NCCRP1 with siRNA leads to a significant decrease in growth of HeLa cells (6). These findings support our observations, although these assays do not distinguish proliferation from altered metabolism, survival, or oncogenic clonogenicity. Contrasting to previous reports demonstrating predominant localization of mammalian NCCRP1 in the cytoplasm (6), we observed nuclear staining of NCCRP1 in some normal and tumor specimens (Figure 1, A and C). Such nuclear localization of NCCRP1 should be interpreted cautiously and further validated using complementary approaches.

In this study, NCCRP1 expression showed an exploratory association with T category. This association should be interpreted cautiously because the present cohort was a small sample size and was highly selected; 95.3% of patients were male, hypopharyngeal and laryngeal tumors predominated, and 28 of 43 patients with HNSCC (65.1%; Table 1) had concomitant ESCC. The proportion of patients with both HNSCC and ESCC concomitantly in this study is substantially higher than

that generally reported. In a systematic review and meta-analysis of 15 studies, approximately 15% of 3,386 patients with HNSCC had esophageal second primary neoplasms (12). The difference in percentages of patients with concurrent HNSCC and ESCC is likely explained by the fact that our institution's referral pathway differs from that of other institutions. Numerous patients with HNSCC treated at our institution are initially identified during screening or diagnostic work-up for ESCC and are subsequently referred for head and neck lesion management. This referral pathway may also explain the predominance of male patients and hypopharyngeal/laryngeal tumors in our cohort. Furthermore, sampling bias related to specimen type should be considered. Although no significant difference was observed in proportion of biopsy/resection specimens, nonsignificant comparison does not demonstrate equivalence between these specimen types. Therefore, our findings are exploratory and future validation in larger, independent cohorts with a more representative distribution of HNSCC subsites, sex, and other patient characteristics are warranted.

Several additional limitations should be acknowledged. First, sample size was small, limiting the power of our results and preventing prognostic analysis and overparameterized multivariable analysis. Residual confounding is possible. Second, association of NCCRP1 expression with histological differentiation or HPV/p16 status were not examined because this information was not systematically available. Third, the 30% cutoff value used to define NCCRP1-low and NCCRP1-high expression was derived from the present cohort and has not been externally validated. Multiple clinicopathological comparisons were performed without adjustment for multiplicity; therefore, the possibility of false-positive findings cannot be excluded. In addition, the robustness of the observed association with T category to the cohort-derived 30% cutoff value remains uncertain because sensitivity analyses using alternative cutoffs were not performed. Therefore, the observed association should not be interpreted as being independent of potential confounding factors and requires further validation in an independent cohort.

5. Conclusion

NCCRP1 expression varied among HNSCC tissues and showed an exploratory association with T category in this selected cohort. In FaDu cells, forced NCCRP1 expression was associated with increased MTT signals and colony formation, suggesting a potential relationship between NCCRP1 expression and *in vitro* growth-related characteristics. These findings should be interpreted cautiously given the small, selected clinical cohort, the cohort-derived expression cutoff, and use of a single cell model. Validation in larger, independent clinical cohorts and additional experimental models,

together with mechanistic studies, is needed to clarify the biological and clinical significance of NCCRP1 in HNSCC.

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