High expression of CDKN2A in oral squamous cell carcinoma is associated with distant metastasis: Cell-free DNA as a new prognostic feature

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Abstract. Oral squamous cell carcinoma (OSCC) shows a high incidence and is associated with a higher rate of mortality, particularly in developing countries. Although the development of treatment strategies has been remarkable, the 5-year survival rate remains low (often <50%). Surgery and chemoradiotherapy are the standard treatments; however, there are limited factors that can predict metastasis. Liquid biopsy is promising in treating various cancers, including OSCC. In previous studies on OSCC, associations were identified among circulating tumour cell clusters, cell-free DNA (cfDNA) size/quantity and prognosis. The present study aimed to explore the role of CDKN2A and TP53 in OSCC using immunohistochemistry. Next-generation sequencing analysis of both cfDNA and tissues from two patients with poor prognoses was performed, and two common mutated genes, *TP53* and *CDKN2A*, were detected. The CDKN2A-positive group showed metastasis in 50% of cases and shorter overall survival in OSCC. Overall, CDKN2A protein expression is a potential prognostic marker for OSCC.

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Abbreviations: OSCC, oral squamous cell carcinoma; CTCs, circulating tumour cells; cfDNA, cell-free DNA; NGS, next-generation sequencing

Key words: oral squamous cell carcinoma, liquid biopsy, cfDNA, next-generation sequencing, TP53, CDKN2A, immunohistochemistry

Introduction

Oral cancer is a significant public health issue, with approximately 300,000 new cases reported annually worldwide (1). Approximately 90% of oral cancer pathologies are squamous cell carcinomas (SCCs). Oral squamous cell carcinoma (OSCC) shows high incidence and mortality rates, particularly in developing countries. Although the development of treatment strategies has been remarkable, the 5-year survival rate often remains below 50% (2). Despite the good local control of OSCC, distant metastases often result in poor outcomes. In the existing standard treatment regimen for OSCC, surgery is the first choice, and chemoradiotherapy is strongly recommended as postoperative adjuvant therapy in patients with a high risk of postoperative recurrence. High-risk factors for recurrence include T size, resection margin positivity, extranodal extension, and multiple lymph node metastases. However, only a few factors can be used to evaluate future metastasis.

Liquid biopsy has attracted attention for its usefulness in various types of cancers, including colorectal and breast cancers. Its utility in OSCC is anticipated. In a previous study, associations were reported between the number of circulating tumour cells (CTC) clusters and prognosis, as well as between the size and quantity of cfDNA and prognosis (3). In this study, next-generation sequencing (NGS) analysis of cfDNA was performed in two patients with inferior prognoses due to distant metastases. They were compared with mutation data from a clinical cancer panel test and two commonly mutated genes were detected, *TP53* and *CDKN2A*, in both cases. *CDKN2A* and *TP53* are frequently mutated genes in head and neck squamous cell carcinoma (HNSCC) (4) and OSCC (5). *CDKN2A*, or p16, is an essential tumour suppressor gene that regulates cell growth by preventing the progression from the G1 phase to the S phase of the cell cycle (6). *TP53* is a suppressor gene referred to as 'the guardian of the gene'. TP53 responds to various cellular stresses to control the expression of target genes, which subsequently trigger cell cycle arrest, apoptosis, senescence, or DNA repair. The IHC for CDKN2A and TP53 in patients with pathologically positive lymph node metastases were performed, thereby offering a new prognostic feature for metastasis.

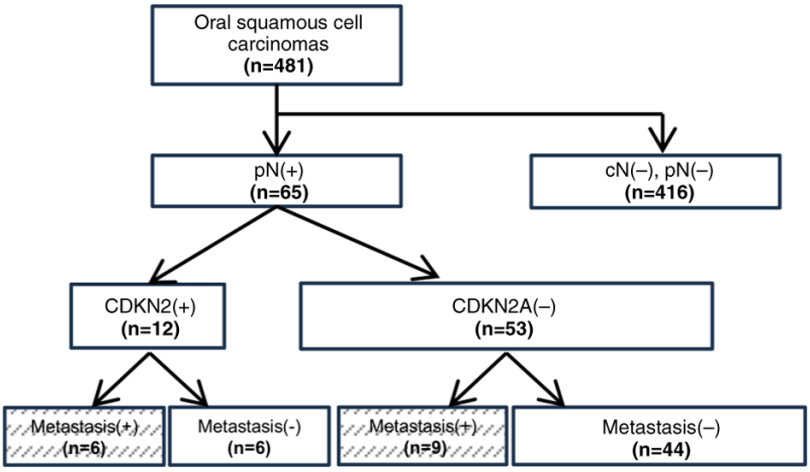

Figure 1. Flow chart of OSCC case selection with radical surgery. Of the 53 patients who visited the Department of Oral and Maxillofacial Surgery at Hiroshima University Hospital between January 2014 and December 2023, 481 underwent radical surgery. Among the 481 patients, 65 were pathologically confirmed as pN(+). Among the pN(+) patients, CDKN2A was positive in 18.5% (12/65) and negative in 81.5% (53/65). Among the CDKN2A-positive group, 50% (6/12) showed metastasis, whereas 17% (9/53) of patients in the CDKN2A-negative group showed metastasis. OSCC, oral squamous cell carcinoma; pN, pathological lymph node metastasis; cN, clinical lymph node metastasis.

Materials and methods

cfDNA isolation and NGS. As reported previously (3), peripheral blood (PB) was obtained from patients 1 and 2 prior to surgery; patient 1 (48 years old, male, Tongue cancer, Stage IVA) and patient 2 (23 years old, male, Tongue cancer, Stage IVA) were recruited in March and January 2023, respectively, and the blood samples were collected at Hiroshima University Hospital. cfDNA was extracted using the MagMAX cfDNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. NGS was carried out by Macrogen Inc. (Seoul, Korea) using the Axen Cancer Panel 2 (Table SI). First, shared mutated genes were identified between cfDNA mutation data and tissue mutation data from a clinical cancer panel test within the same patient. Next, the commonalities between patients 1 and 2 were determined. The clinical relevance of the variants was identified using the ClinVar or OncoKB database (7).

Patients and specimens. Overall, 537 patients with oral cancer visited the Department of Oral Maxillofacial Surgery at Hiroshima University Hospital between January 2014 and December 2023. Of them, 481 patients underwent radical surgery. Among the 481 patients, 65 with pathologically positive lymphoid metastases (pN(+)) were selected (Fig. 1) (Table SII). The medical records and tissue samples of the patients included in this study were accessed specifically for this research starting in May 2023. Inclusion criteria included (1) absence of distant metastases at diagnosis, (2) receipt of surgical treatment, and (3) complete follow-up data. Exclusion criteria included (1) distant metastases at diagnosis, (2) non-surgical treatment, and (3) incomplete follow-up data. All medical records, including disease characteristics, diagnostic methods, and treatments, were retrospectively reviewed. All patients were diagnosed according to the TNM classification of oral cancer (8). The clinical endpoints of this study were based on the FDA's 'Clinical Trial Endpoints for the Approval of Cancer Drugs and Biologics' guidelines, which include a 5-year evaluation period. The primary endpoint was overall survival (OS). OS was defined as the time from the date of the initial diagnosis to death from any cause.

IHC for CDKN2A. IHC for CDKN2A was performed as previously described (9). Briefly, 4-μm formalin-fixed paraffin-embedded tissue sections on amino silane-coated glass slides (MATSUNAMI, Osaka, Japan) were deparaffinised in xylene (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and rehydrated using an alcohol gradient (FUJIFILM Wako). Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in an autoclave (HV-5; HIRAYAMA, Saitama, Japan). Sections were incubated overnight at 4˚C with a 1:100 dilution of mouse monoclonal anti-CDKN2SA/p16INK4A antibody (sc-56330, Santa Cruz Biotechnology, TX, USA) or a 1:100 dilution of mouse monoclonal anti-p53 antibody (DO-1, Santa Cruz Biotechnology). Detection was performed using EnVision+ System-HRP anti-mouse (Dako, Agilent Technologies, Santa Clara, CA, USA) for 30 min, followed by visualisation with 3,3'-diaminobenzidine (DAB) (Dako) and counterstaining with haematoxylin (FUJIFILM Wako). The slides were dehydrated, mounted, and covered with coverslips (NEO cover glass; MATSUNAMI). Images were captured using a Nikon DS-Ri2 camera (Nikon Corporation, Tokyo, Japan) and a Nikon Eclipse E800 microscope (Nikon Corporation).

Classification of IHC positivity. Immunohistochemical staining was evaluated based on the intensity and percentage of positively stained cells. Cases were considered 'positive' when the CDKN2A-positive region was expressed more than 70%, and the TP53-positive region was expressed more than 60%, according to previously reported thresholds (10-12).

Ethical consideration. This study was approved by the Ethics Committee of Hiroshima University (approval numbers: epidemiology 2016-9191 and epidemiology 2023-0025).

Statistical analysis. All statistical analyses were conducted using JMP Pro (version 16.2.0, SAS Institute Inc., Cary, NC,

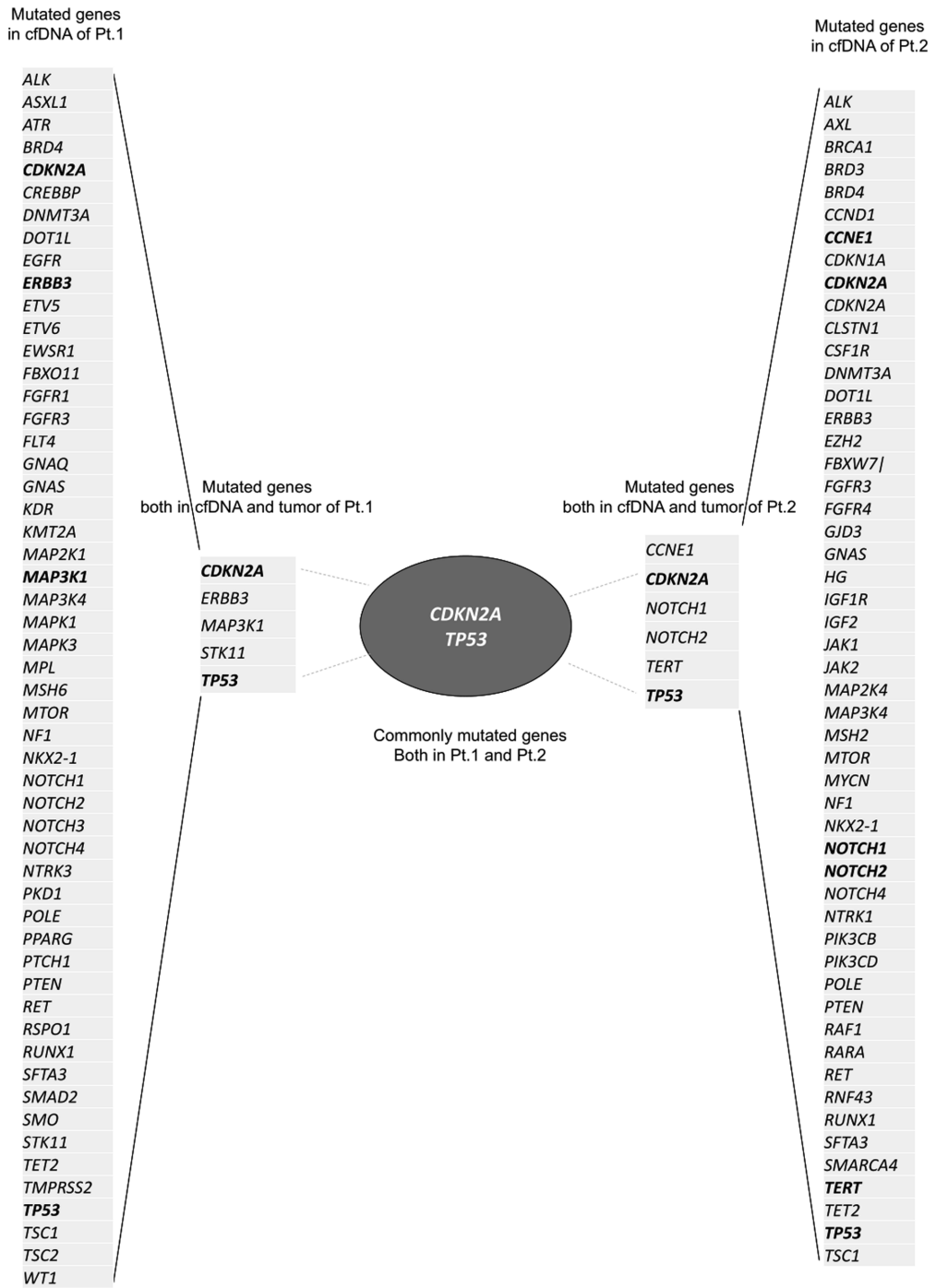

Figure 2. Detection of mutations in cfDNA and tumour. A total of 54 genetic mutations were identified in the cfDNA of patient 1, and 52 mutations were identified in patient 2. Some of these mutations overlapped with those previously detected in the tumour tissues of each patient. Notably, two genes, *CDKN2A* and *TP53*, were common in patient 1 and patient 2. cfDNA, cell-free DNA; Pt. 1, patient 1; Pt. 2, patient 2.

USA). The association between OS and factors and the IHC results of CDKN2A/TP53 expression and OS were examined using Kaplan-Meier survival curves and the log-rank test. To further assess the prognostic significance of CDKN2A positivity, a multivariate Cox proportional hazards regression analysis was performed.

Results

Result of NGS. cfDNA NGS was performed for patients 1 and 2 as preliminary exploratory observations. Forty-one and 40 genetic mutations were detected in cfDNAs of patients 1 and 2, respectively. Some common tumour tissue mutations were previously detected in each patient. *CDKN2A*, *ERBB3*, *MAP3K1*, *STK11*, and *TP53* were common mutations identified in patient 1. In patient 2, *CCNE1*, *CDKN2A*, *NOTCH1*, *NOTCH2*, *TERT*, and *TP53* were common between tissues and cfDNA. Among these, two genes, *CDKN2A* and *TP53*, were shared between patients 1 and 2 (Fig. 2). Specifically, TP53_c.524G>A_p.R175H/CDKN2A_ c.340C>T_p. P114 and TP53_c.569delC_p.P190fs*57, _c.733G>A _p.G245S/CDKN2A_c.247C>A_p. H83N were shared both in tumour

Table I. TP53 and CDKN2A mutations in cfDNA and tumour.

Patient no.	Origin	Gene	Protein change	Annotation	AF (%)
1	Tumour	*TP53*	R175H	Pathogenic	62.8
		CDKN2A	P114S	Pathogenic/VUS	58.1
	cfDNA	*TP53*	R175H	Pathogenic	1.6
			G245D	Pathogenic	0.3
		CDKN2A	P114S	Pathogenic/VUS	1.8
2	Tumour	*TP53*	P190fs*57	Pathogenic	28.5
			G245S	Pathogenic	12.3
		CDKN2A	H83N	Likely Pathogenic	56
	cfDNA	*TP53*	P190fs*57	Pathogenic	14.3
			G245S	Pathogenic	6.7
		CDKN2A	H83N	Likely pathogenic	24.3
			G89C	VUS	23.2

VUS, uncertain significance; cfDNA, cell-free DNA; AF, allele frequency.

Table II. Patient characteristics.

Characteristic	Number	%
Age (median), years	68 (21-89)	-
Sex		-
Male	43	66.2
Female	22	33.8
Site		
Tongue	21	32.3
Mandibular gingiva	18	27.7
Floor of mouth	11	16.9
Buccal mucosa	7	10.8
Maxillary gingiva	7	10.8
Other	1	1.5
Tumour size		
Tis/T1-2	17	26.2
T3-4	48	73.8
Metastasis		
No	49	75.4
Yes	16	24.6

Tis, Carcinoma *in situ*.

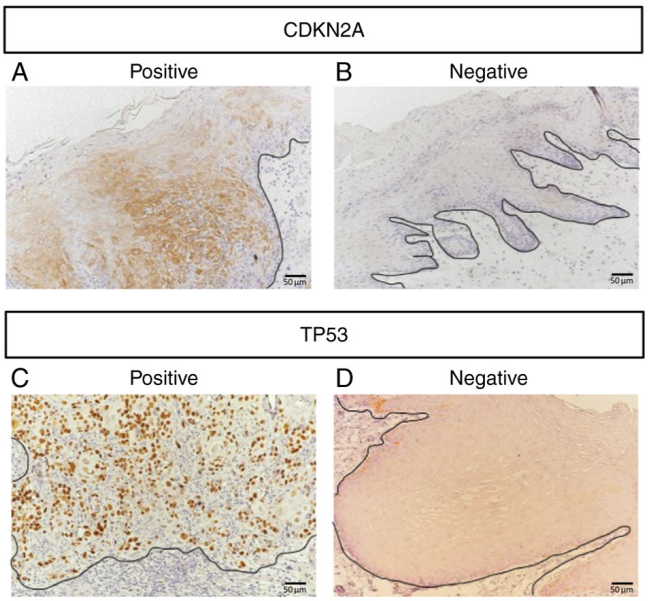

Figure 3. Images of IHC. IHC staining images of CDKN2 and TP53 in tumor and adjacent normal tissues. Tumor tissues are outlined, and the boundary between tumor and normal tissue is clearly marked with a demarcation line. Scale bars, 50 μm. (A) CDKN2A-positive (≥70%), (B) CDKN2A-negative (<70%), (C) TP53-positive (≥60%) and (D) TP53-negative (<60%). IHC, immunohistochemistry.

tissue and cfDNA, respectively, in patients 1 and 2 (Table I). Although the positions of the mutated base differed between patients 1 and 2, they were all pathological mutations. Therefore, the relationship between IHC staining for CDKN2A and TP53 and OSCC prognosis was investigated.

Patient characteristics. The characteristics of the 65 patients with SCC included in this study are summarised in Table II. Of the 65 patients, 43 (66.2%) were males and 22 (33.8%) were females; the average age was 68 years. The primary tumour sites included the tongue (32.3%), mandibular gingiva (27.7%), the floor of the mouth (16.9%), buccal mucosa (10.8%), maxillary gingiva (10.8%), and others (1.5%). Tumour size distribution showed that 17 patients (26.2%) had Tis/T1/T2 tumours, and 48 patients (73.8%) had T3/T4 tumours.

CDKN2A-positive group shows metastasis in 50% of OSCC cases. IHC images were assessed, as shown in Fig. 3. CDKN2A expression was positive in 12 patients (18.5%) and negative in 53 (81.5%). Of the CDKN2A-positive and CDKN2A-negative patients, six (6/12, 50%) and nine (9/53, 16.9%) showed metastasis (Fig. 1). In the Fisher's exact test for distant metastasis,

Table III. Fisher's exact test for distant metastasis and other factors.

Characteristic	No metastasis, n (%)	Metastasis, n (%)	Total, n (%)	P-value
Sex				
Male	32 (49.2)	10 (15.4)	42 (62.6)	1.0
Female	18 (27.7)	5 (7.7)	23 (35.4)	
Age				
≤65	23 (35.4)	3 (4.6)	26 (40.0)	0.06
>65	27 (41.5)	12 (18.5)	39 (60.0)	
Tumor size				
Tis/T1-T2	15 (23.1)	2 (3.1)	17 (26.2)	0.2
T3-T4	35 (53.9)	13 (20.0)	48 (73.9)	
IHC (TP53)				
Negative	29 (44.6)	7 (10.8)	36 (55.4)	0.46
Positive	21 (32.3)	8 (12.3)	29 (44.6)	
IHC (CDKN2A)				
Negative	44 (67.7)	9 (13.9)	53 (81.5)	0.046
Positive	6 (9.2)	6 (9.2)	12 (18.5)	

Tis, Carcinoma *in situ*; IHC, immunohistochemistry.

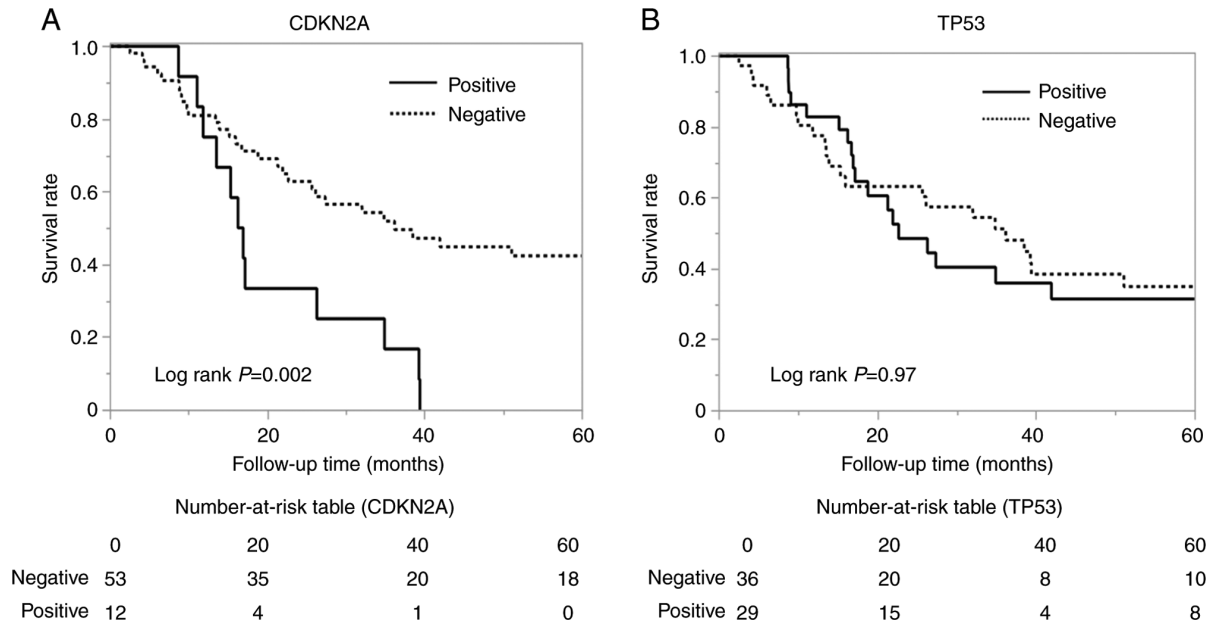

Figure 4. Relationship between IHC result for CDKN2A/TP53 and the 5-year survival rate. Kaplan-Meier survival curves for OS based on IHC results for CDKN2A/TP53 in OSCC tissues. Each graph shows the OS of patients with OSCC, with positive cases (blue line) and negative cases (red line) in the (A) CDKN2A and (B) TP53 groups. Statistical significance was assessed using the log-rank test. Statistical significance was set at P<0.05 (statically significant). (A) Median OS was 16.6 months (95% CI, 11.1-34.9) in the CDKN2A-positive group and 21.0 months (95% CI, 16.0-63.5) in the P16-negative group (P=0.002 log-rank test). (B) Median overall survival was 34.9 months in the TP53-negative group and 21.3 months in the TP53-positive group. The survival difference was not statistically significant (P=0.97 log-rank test). OS, overall survival; IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma; CI, confidence intervals.

the IHC CDKN2A-positive group showed a significantly higher metastasis rate (Table III).

CDKN2A-positive group shows shorter OS in OSCC. Five-year survival rates were examined based on the positive and negative immunohistochemical results for CDKN2A and TP53. The CDKN2A-positive group showed a significantly decreased survival rate (hazard ratio=3.63, 95% CI: 1.36-9.68, P=0.01) (Fig. 4A), but there were no significant differences for TP53 (Fig. 4B). Furthermore, the results of the multivariate cox proportional hazards model indicated that CDKN2A positivity remained a significant independent predictor of poor

Table IV. Multivariable cox proportional hazards model for overall survival.

Variable	HR	95% CI	P-value
P16 positivity ≥70% (yes vs. no)	1.75	0.17-1.60	0.0179
TP53 positivity ≥60% (yes vs. no)	0.01	-0.64-0.64	0.9787
Age >60 years (yes vs. no)	0.95	-0.12-1.16	0.1126
Smoking history (never vs. ever)	0.27	-0.56-1.22	0.5321
Alcohol dose (never vs. ever)	0.05	-0.95-1.01	0.8980
T stage (T1-2 vs. T3-4)	0.13	-0.72-0.55	0.7415

HR, hazard ratio; CI, confidence interval; T, tumor.

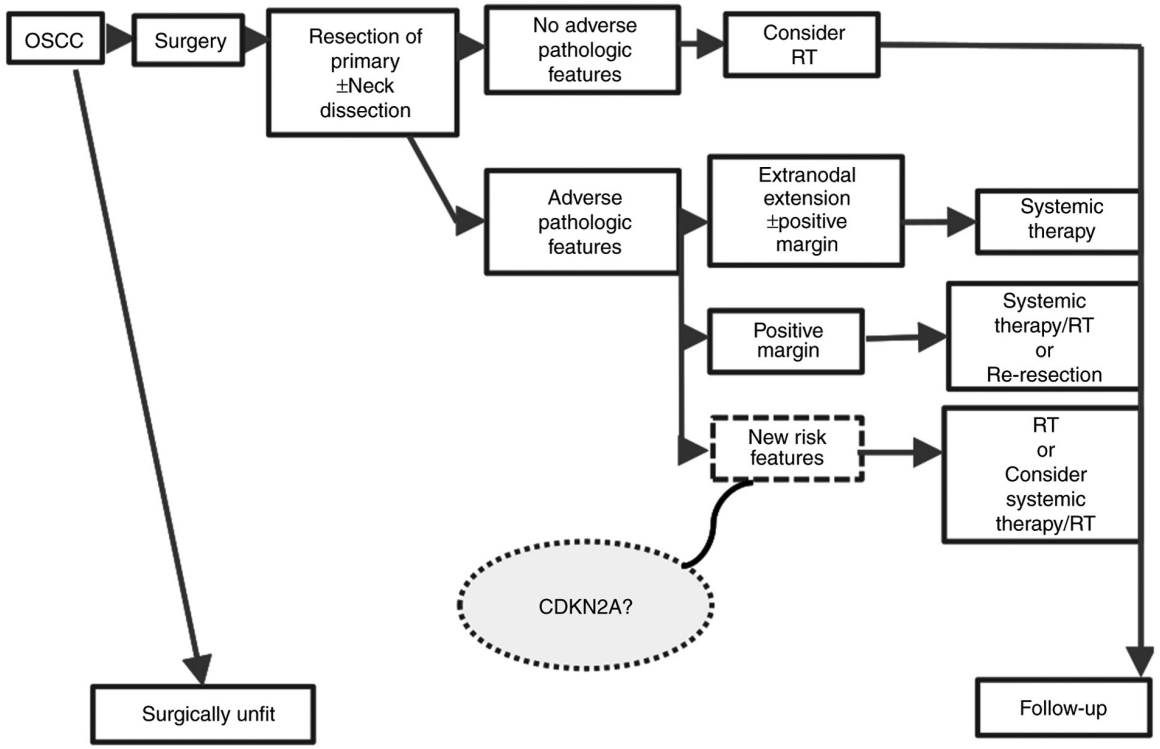

Figure 5. A new risk factor for OSCC. This figure was drawn by editing the Clinical Practice Guidelines for Oral Cancer 2023 (24). CDKN2A may be a new risk factor for OSCC treatment or management. OSCC, oral squamous cell carcinoma; RT, radiotherapy.

prognosis after adjusting IHC results, age, smoking history, alcohol dose, and tumour size (hazard ratio=1.75, 95% CI: 017-1.60, P-value=0.0179) (Table IV).

Discussion

This study was designed based on the commonalities of tumour tissue and cfDNA sequencing data to identify new prognostic features of metastasis. Most of the mutations detected in cfDNA sequencing are not pathogenic mutations. However, a few pathogenic mutated genes, *TP53* and *CDKN2A*, were common in both tumour tissues and cfDNA. TP53_p.R175H/_p.G245S mutation, previously reported pathogenic variant, is located in the DNA binding domain, resulting in loss of function. TP53_p.R175H is found in acute myeloid leukaemia (13). TP53_p.G245S has been frequently found in hepatocellular carcinoma (14). TP53_p.P190fs*57 is a truncated mutation, resulting in the loss of function. Those truncated mutations show a relatively strong association with poor prognosis in HNSCC (15). CDKN2A_p. P114S/_p. H83N mutation is located in the ankyrin repeats of the p16/INK4A protein, resulting in loss of function. CDKN2A_p. P114S, previously reported pathogenic variant, was identified as a germline variant in families affected by melanoma (16). Though TP53_c.569delC_p.P190fs*57 and CDKN2A_c.247C>A_p. H83N are not reported mutations, they are predicted as pathogenic mutation in silico analysis using mutation taster (https://www.mutationtaster.org/index.html).

cfDNA was previously assessed at two points, pre- and post-operation, and reported that the group with distant metastases showed significantly higher amounts of cfDNA

than those without distant metastases both preoperatively and postoperatively (3). In reference 3, we showed that though only three genes (TP53, HRAS, and MLH1) were classified as pathogenic in 13 patients, two patients, who commonly possessed TP53_c.215C>G_p.Pro72Arg, showed short-term distant metastasis. Despite the limited overlap in mutations among patients, these findings underscore the clinical relevance of cfDNA mutation profiling (3). If the same genetic mutations in cfDNA can be detected both preoperatively and postoperatively or the comparison of those genetic mutations can be performed, it would further support the clinical relevance of such findings.

Specifically, the relationship between CDKN2A/TP53 IHC expression and prognosis was investigated in 65 patients with pN(+) OSCC. pN(+) was selected to evaluate the high-risk group for metastasis because, as commonly reported, the pN(+) group had a higher distant metastasis rate of 22% (15/65), than those of the pN(-) group, 5% (2/40) (Table SII). The following were key findings: in 65 patients with pN(+) OSCC, CDKN2A expression was positive in 18.5% (12/65) of the patients and negative in 81.5% (53/65), whereas no such difference was found for TP53. The CDKN2A-positive group resulted in metastasis in 50% of cases (6/12). This result contradicts our expectations. Regarding the oropharynx, Fakhry *et al* (17) assessed whether HPV infection was present or absent and concluded that the HPV-positive group (CDKN2A-positive group) in the oropharynx had a better prognosis, showing better radiosensitivity. This present case may have been different because it consisted of 86.2% (56/65) cases of surgical alone and 13.8% (9/65) surgical plus adjuvant radiation therapy with or without chemotherapy. Hong *et al* (18) reported that, in oropharyngeal cancer, while surgery alone cases were deemed insufficient in number for their multivariable analysis, HPV status predicts better outcomes treated with surgery plus adjuvant radiotherapy as well as with definitive radiation therapy with or without chemotherapy. In Fakhry *et al* (17) and Hong *et al* (18), the assessment was not based on IHC-CHKN2A negativity or positivity but also considered the presence of HPV16 DNA using in situ hybridisation or PCR. Ni *et al* (19) reported that CDKN2A overexpression decoupled from HPV infection was not a prognostic marker for patients with OSCC.

CDKN2A is widely recognised as a tumour suppressor gene that controls cell proliferation by preventing the progression from the G1 phase to the S phase of the cell cycle (20). In normal cells with proliferative potential, the expression of CDKN2A is very low, and CDKN2A has little function. However, when normal cells reach the mitotic lifespan or undergo oncogenic stress, CDKN2A gene expression is markedly elevated, and cellular senescence occurs (21). Beyond phenomena related to invasion and metastasis, Shi *et al* (22), reported that in the colorectal cancer cell line, HT-29, CDKN2A could induce the epithelial-mesenchymal transition, showing that knock downed CDKN2A expression was followed by enhanced E-cadherin expression and suppression of N-cadherin and vimentin expression. Cheng *et al* (23), reported that CDKN2A mediates cuproptosis, a type of cell death characterised by excessive copper-lipid reactions in the tricarboxylic acid cycle, resistance through regulating glycolysis and copper homeostasis, accompanied by a malignant phenotype and pro-tumour niche, also using colorectal cancer cell line. They concluded that radiation and chemotherapy are expected to potentially serve as therapeutic approaches for cuproptosis-resistant colorectal cancer with high CDKN2A expression (23). Given the limited literature investigating these phenomena in OSCC, future studies should aim to 1) elucidate the molecular pathways by which CDKN2A influences cell motility, epithelial-mesenchymal transition, and invasion using *in vitro* OSCC models 2) evaluate metastatic potential *in vivo* between CDKN2A-over expressing and CDKN2A-deficient OSCC xenografts 3) use single-cell transcriptomics or proteomics analyses to gain deeper insight into how CDKN2A expression modulates cellular behavior at the invasive front of OSCC lesions.

Despite these promising findings, the limitations of this study warrant further consideration. First, the retrospective nature of this study might have induced selection bias, as only patients who underwent surgery and had available pathology reports were included. Second is the lack of HPV status assessment. Since CDKN2A (p16) overexpression is often considered a surrogate marker for HPV-related oncogenesis, especially in head and neck cancers, the inability to account for HPV infection status may confound the interpretation of CDKN2A expression in this cohort. Although the prognostic significance of CDKN2A expression has been well established in oropharyngeal cancers, particularly in HPV-positive cases, less attention has been given to CDKN2A's role in OSCC. Most studies focus on the strong association between p16 expression and HPV status in oropharyngeal cancer, where p16 overexpression is often considered a surrogate marker for HPV infection. However, OSCC, which is primarily HPV-negative, presents a different biological context. Future studies should incorporate HPV testing to better clarify this relationship. This study addresses this gap by investigating the independent role of CDKN2A expression in OSCC, irrespective of HPV infection. Evidence that CDKN2A dysregulation in OSCC may be associated with tumour progression, metastasis, and poorer prognosis was provided, independent of HPV status. This finding is particularly important as it expands the understanding of CDKN2A as a prognostic marker in head and neck cancers beyond HPV-positive oropharyngeal cancers. Results suggest that CDKN2A could serve as a valuable biomarker in OSCC, offering insights into its potential role in cancer progression and therapeutic targeting. Third is the sample size. Because the sample size is very small, two samples for NGS and twelve samples for IHC CDKN2A-positive patients, these results should be seen as preliminary and interpreted with caution. Although further studies involving a larger patient cohort are necessary to confirm these preliminary observations, these findings suggest a potential prognostic role for CDKN2A expression. Assessing CDKN2A expression could help stratify patients based on their risks and tailor treatment strategies accordingly. For example, the CDKN2A-positive group, which we assumed to be a high-metastasis group, could be managed with more aggressive treatment regimens or closer follow-up. Further research will contribute to the development of personalised cancer therapies and improve OSCC prognosis (Fig. 5).

This study addresses this gap by investigating the independent role of CDKN2A expression in OSCC, irrespective of HPV infection. We provide evidence that CDKN2A dysregulation in OSCC may be associated with tumour progression, metastasis, and poorer prognosis, independent of HPV status. This finding is particularly important as it expands the understanding of CDKN2A as a prognostic marker in head and neck cancers beyond HPV-positive oropharyngeal cancers. The results suggest that CDKN2A could serve as a valuable biomarker in OSCC, offering insights into its potential role in cancer progression and therapeutic targeting. In conclusion, this study demonstrates that the CDKN2A-positive group had a high metastasis rate, resulting in a poorer prognosis. CDKN2A expression is a potential prognostic marker for OSCC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The following three mutations, NM_000546.6(TP53):c.524G>A (p.Arg175His), NM_000546.6(TP53):c.733G>A (p.Gly245Ser) and NM_000077.5(CDKN2A):c.340C>T (p.Pro114Ser), are reported mutations and their URLs are as follows, https://www.ncbi.nlm.nih.gov/clinvar/RCV000013173.22/, https://www.ncbi.nlm.nih.gov/clinvar/variation/12365/, and https://www.ncbi.nlm.nih.gov/clinvar/RCV001915587/, respectively. The NGS data have been deposited with links to Biosample accession number SAMD01605716 (patient 1) and SAMD01611905 (patient 2) in the DDBJ Biosample database, https://ddbj.nig.ac.jp/search/entry/biosample/SAMD01605716 and https://ddbj.nig.ac.jp/search/entry/biosample/SAMD01611905.

Authors' contributions

SoY and AH designed the experiments. NE, AH, AT, MH, FO, NI, SaY, RT, TS, KK and SoY performed the experiments. NE, AH and SoY confirmed the authenticity of all the raw data, and wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Research Ethics Board of Hiroshima University approved this study. The studies were conducted in accordance with the local legislation and institutional requirements. Patients 1 and 2 provided written informed consent to participate in the study, which was approved by the Ethics Committee of Hiroshima University (approval no. epidemiology 2016-9191). For IHC, written informed consent was not obtained from patients for publication because this was a retrospective study. This retrospective observational study was approved by the Research Ethics Board of Hiroshima University (approval no. epidemiology 2023-0025).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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