Ann Surg Oncol (2019) 26:2294–2303
https://doi.org/10.1245/s10434-019-07287-x

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

All-Exon *TP53* Sequencing and Protein Phenotype Analysis Accurately Predict Clinical Outcome after Surgical Treatment of Head and Neck Squamous Cell Carcinoma

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ABSTRACT

Background. This study elucidates the clinical impact of surgical treatment of head and neck squamous cell carcinoma (HNSCC) based on a detailed search of all exons of the *TP53* gene and p53 protein phenotypic analysis using formalin-fixed paraffin-embedded (FFPE) specimens.

Methods. Clinically well-annotated FFPE specimens from 317 patients with HNSCC treated by surgery were examined by all-exon *TP53* sequencing using a next-generation sequencer and p53 protein phenotype by immunohistochemistry. After excluding human papillomavirus-associated oropharyngeal carcinomas, two risk categories were classified as "p53 adverse function" and "p53 favorable function" based on *TP53* mutation status and p53 protein phenotype. Mutation in *PIK3CA*, *AKT*, and *HRAS* was also evaluated by target sequence. Cox proportional hazards regression models were used for statistical analysis of clinical outcomes. Receiver operating characteristic curve analysis was used to determine the optimal surgical margin cutoff for local recurrence. Local control rates were compared between the risk groups using Fisher's exact test.

Results. Multivariate analysis identified "p53 adverse function" as an independent poor predictor of overall survival, local control, and distant metastasis-free survival. In oral cavity cancer, the optimal surgical margin cutoff associated with local recurrence was 6 mm. In patients with surgical margin > 6 mm, the "p53 adverse function" group demonstrated significantly higher local recurrence rate than the "p53 favorable function" group. *PIK3CA*, *AKT*, or *HRAS* mutation did not correlate with improved overall survival.

Conclusions. All-exon *TP53* sequencing and p53 protein phenotype analysis using FFPE specimens can accurately predict clinical outcomes.

Somatic mutations in *TP53* represent the most frequent alteration in head and neck squamous cell carcinoma (HNSCC), occurring in 60–80% of patients with human papillomavirus (HPV)-negative HNSCC.[1–3] Mutation of this gene is closely related to prognosis, but testing for *TP53* alterations has not become routine in clinical practice due to the different clinical effects of various *TP53* missense mutations as well as discrepancies between the genomic mutation and protein phenotype.

First, *TP53* nonsense/frameshift/splice mutations result in unfavorable clinical outcomes because of the loss of wild-type p53 function. By contrast, the effects of *TP53* missense mutations vary depending on the nucleotide change. Thus, certain *TP53* missense mutations are

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-019-07287-x) contains supplementary material, which is available to authorized users.

First Received: 29 May 2018;
Published Online: 21 March 2019

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associated with similarly favorable clinical outcomes as wild-type *TP53*, whereas others are linked to similarly poor clinical outcomes as *TP53* nonsense/frameshift/splice mutations. Moreover, a recent study illustrated that some point mutations induced mutant p53 gain of function (GOF).[4] Some GOF phenotypes are associated with poorer outcomes in patients with HNSCC. Neskey et al. developed the Evolutionary Action p53 (EAp53) scoring system to predict the outcomes of *TP53* missense mutations.[5] However, it was not fully generalized.

Second, the information encoded by DNA is converted into the amino acid sequences of proteins via a multistep process. According to recent genomic and proteomic analyses, protein expression cannot be reliably predicted using DNA- or RNA-level measurements. Messenger RNA (mRNA) and protein levels are moderately correlated, but only one-third statistically significantly.[6,7]

In recent years, genomic information has become available using formalin-fixed paraffin-embedded (FFPE) biopsy or surgical specimens. The aim of this study is to determine whether protein phenotype analysis using preoperative biopsy specimens and mutation status analysis using surgical specimens could facilitate determination of surgical margin distance and prediction of clinical outcomes.

PATIENTS AND METHODS

Study Population

In the current retrospective cohort study, medical records and surgical specimens of 400 patients with HNSCC who were surgically treated as primary treatment at The National Cancer Center Hospital of Japan between January 2013 and December 2015 were reviewed. Forty-two patients were excluded based on the following criteria: secondary primary tumor, primary tumor of the nasopharynx, external ear canal, or salivary gland, and cervical metastasis of unknown primary tumor. As a result, the primary tumor location was limited to the oral cavity, pharynx, and larynx. Preoperative biopsy specimens were used for protein phenotype analysis, and postoperative surgical specimens were used for DNA sequencing. Overall, tumor specimens from 317 of 358 patients were available for next-generation sequencing (NGS) analyses. Human papillomavirus (HPV)-associated oropharyngeal carcinoma (OPC) manifests different biological characters compared with those of other HNSCCs. We excluded the 33 patients with HPV-associated OPC from the following analysis. The remaining 284 patients were considered eligible for this retrospective analysis. The detailed patient characteristics are presented in Table 1.

The treatment strategy was based on National Comprehensive Cancer Network (NCCN) guidelines. The detailed treatment policy is described in the supplementary information files.

Tobacco smokers were defined as individuals with heavy tobacco use (20 pack-years or more, i.e., 1 pack per day for 20 or more years), and alcohol drinkers were defined as individuals with heavy alcohol use (15 drinks or more per week for at least 15 or more years). The 8th edition of the tumor–node–metastasis (TNM) classification from the International Union Against Cancer and the American Joint Committee on Cancer was adopted for clinical staging. Each of vascular, lymphatic, and perineural invasion was classified as either positive or negative.

Ethical approval for the retrospective biomarker analysis was obtained from our ethics committee (approved number 2010-77).

Assessment of p16 and p53 Immunohistochemistry (IHC)

Immunohistochemical analysis of all patients was performed using FFPE preoperative biopsy specimens as described previously.[8,9] HPV status and p53 protein phenotype were determined by p16 and p53 immunohistochemistry, respectively. p53 expression was classified as +/−, +, 2+, and lost (Supplementary Fig. 1a–e). The detailed protocols are provided in the supplementary information files. All IHC data were independently assessed by two histopathologists (T.M. and N.H.), who were blinded to the clinical outcome data because of the preoperative evaluation. There was no discordance between the assessors.

Sequencing Analysis

Mutations of *TP53*, *PIK3CA*, *AKT*, and *HRAS* were assessed using NGS. The detailed protocols are provided in the supplementary information files and Supplementary Table 1.

Definition of "p53 Functional Classification"

We classified p53 function via combined gene sequencing and immunostaining analysis, termed "p53 functional classification." We categorized *TP53* mutation with p53 protein phenotype 2+ or lost as "p53 adverse function" and *TP53* wild-type or mutation with p53 protein phenotype + or +/− as "p53 favorable function."

Statistical Analysis

Differences in mutation status between any two groups were analyzed using Fisher's exact test. Clinical outcomes were evaluated using overall survival (OS), local failure-

TABLE 1 Patient characteristics

Characteristic	All patients (N = 284) No. of patients	p53 favorable function (N = 144) No. of patients (%)	p53 adverse function (N = 140) No. of patients (%)	p value
Sex				
Male	220	106 (48%)	114 (52%)	0.076
Female	64	38 (64%)	26 (41%)	
Age at study entry				
> 75	219	108 (49%)	111 (51%)	0.24
< 75	65	36 (55%)	29 (45%)	
Primary tumor site				
Oral cavity	153	85 (56%)	68 (44%)	0.093
Hypopharynx	74	36 (49%)	38 (51%)	
Oropharynx	32	10 (31%)	22 (69%)	
Larynx	25	13 (52%)	12 (48%)	
p16 status				
Positive	20	14 (70%)	6 (30%)	0.059
Negative	264	130 (49%)	134 (51%)	
Pathological tumor stage				
T1	79	36 (46%)	43 (54%)	0.42
T2	100	57 (57%)	43 (43%)	
T3	66	31 (47%)	35 (53%)	
T4	39	20 (51%)	19 (49%)	
Pathological nodal stage				
N0	182	93 (51%)	89 (49%)	0.40
N1	23	10 (43%)	13 (57%)	
N2	32	20 (63%)	12 (37%)	
N3	47	21 (45%)	26 (55%)	
Clinical TNM stage				
I	74	34 (46%)	40 (54%)	0.82
II	62	33 (53%)	29 (47%)	
III	50	26 (52%)	24 (48%)	
IV	98	51 (52%)	47 (48%)	
Treatment				
Surgery	242	124 (51%)	118 (49%)	0.40
Surgery + postoperative therapy	42	20 (48%)	22 (52%)	
Reconstruction				
Free Flap	174	90 (52%)	84 (48%)	0.38
Primary suture	110	54 (49%)	56 (51%)	
Smoking status				
Smokers	112	63 (56%)	49 (44%)	0.083
Nonsmokers	172	81 (47%)	91 (53%)	
Drinking status				
Drinkers	172	88 (51%)	84 (49%)	0.47
Nondrinkers	112	56 (50%)	56 (50%)	

free survival (LFFS), and distant metastasis-free survival (DMFS). Failure was defined as death due to any cause (OS), local recurrence (LFFS), or distant progression (DMFS). Survival curves were estimated using the Kaplan–Meier method and compared between risk groups using the log-rank test. Hazard ratios and interactions between risk parameters were estimated using Cox regression models. Baseline variables with $p < 0.05$ on

univariate analysis were included in the multivariable models.

All surgical margin distances were remeasured in two dimensions, namely the mucosal margin and deep margin, by a pathologist and a surgeon (M.T. and K.K. in all cases). The closest margin distance of the two lengths was used to determine the surgical margin distance. We used receiver operating characteristic (ROC) curve analysis in patients without microscopically positive surgical margin to determine the optimal surgical margin cutoff for predicting local recurrence. Local control rates between the risk groups were analyzed using Fisher's exact test.

Statistical analyses were performed using SPSS Statistics software (version 22.0.0, IBM, Armonk, NY, USA). The level of significance was set at $p < 0.05$.

RESULTS

Somatic Mutation and p53 Protein Phenotype Landscape of All Patients

The landscape of genetic alterations across the 284 patients is shown in Fig. 1. The most frequently mutated gene was *TP53* (67%), followed by *PIK3CA* (8%), *AKT* (4%), and *HRAS* (3%). Regarding the types of *TP53* mutation, missense, nonsense, frameshift, and splice mutations comprised 37, 20, 13, and 8%, respectively. Conversely, the p53 protein phenotype was 2+, +, lost, and +/− in 34, 12, 30, and 11% of patients, respectively. The association between protein phenotype and mutation showed high sensitivity (84%) but low specificity (56%) in the 2+, +, and missense mutation/in-frame deletion/intron variant groups, but low sensitivity (67%) and high specificity (84%) in the lost and nonsense/frameshift/splice mutation groups.

AKT mutations were found in 26% of patients with *PIK3CA* mutations, versus 3% of those with wild-type *PIK3CA* ($p = 0.00017$). *HRAS* mutations were observed at high rates (87%) in patients with oral cancer.

Mutation Status, p53 Protein Phenotype, and Prognosis

TP53 nonsense/frameshift/splice mutations were associated with significantly worse 2- and 5-year OS rates than observed for wild-type *TP53* (2-year OS: 74 vs 85%; 5-year OS: 55 vs 72%, $p = 0.013$). However, there was no statistically significant difference in the rates of 2 and 5-year OS between the *TP53* missense mutation/in-frame deletion/intron variant and wild-type *TP53* (2-year OS: 84 vs 85%; 5-year OS: 62% vs 72%, $p = 0.15$) (Fig. 2a). Subsequently, we compared survival based on the p53 protein phenotype. *TP53* missense mutation/in-frame deletion/intron variant with p53 protein phenotype 2+ or lost had a significantly poorer 2-year OS rate than that for those with p53 protein phenotype +/− or + (2-year OS: 81 vs 90%; 5-year OS: 48 vs 85%, $p = 0.0051$) (Fig. 2b).

PIK3CA, *AKT*, or *HRAS* mutation was not significantly correlated with OS (Supplementary Fig. 2a–c).

p53 Functional Classification and Prognosis

"p53 adverse function" was associated with significantly worse 2- and 5-year OS rates than "p53 favorable function" (2-year OS: 78 vs 87%; 5-year OS: 53 vs 77%, $p = 0.00048$) (Fig. 2c).

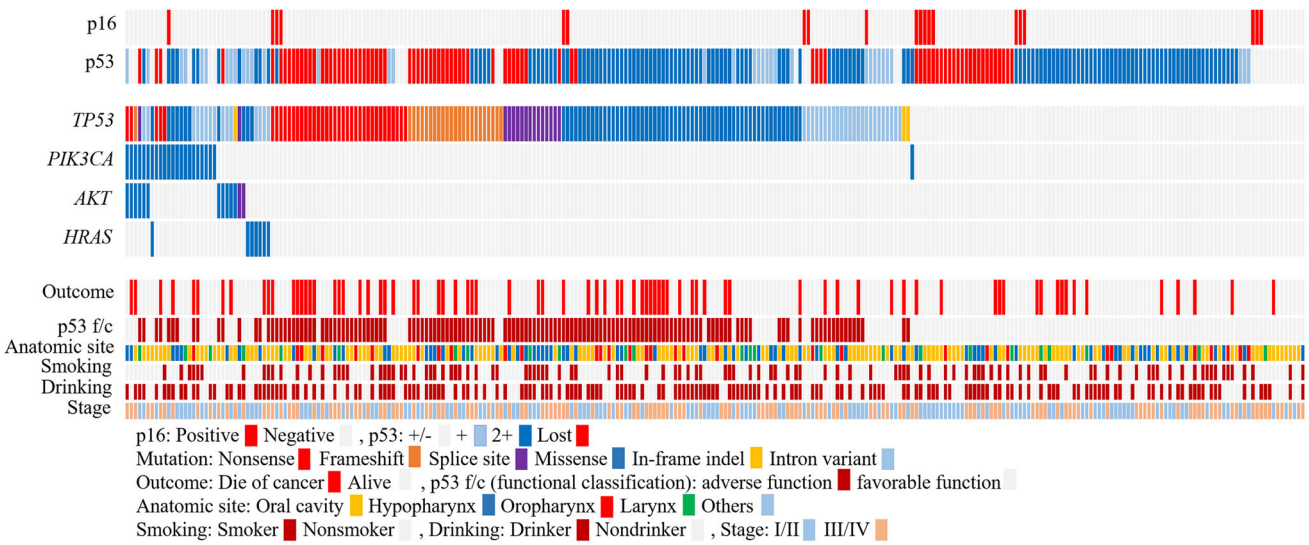


FIG. 1 Mutation status and clinical outcome: the landscape of genetic alterations and immunohistochemical status across the 284 head and neck squamous carcinoma samples sorted by p53 status and *TP53*/*PI3CK*/*AKT*/*HRAS* mutation status. Mutation types indicated by color

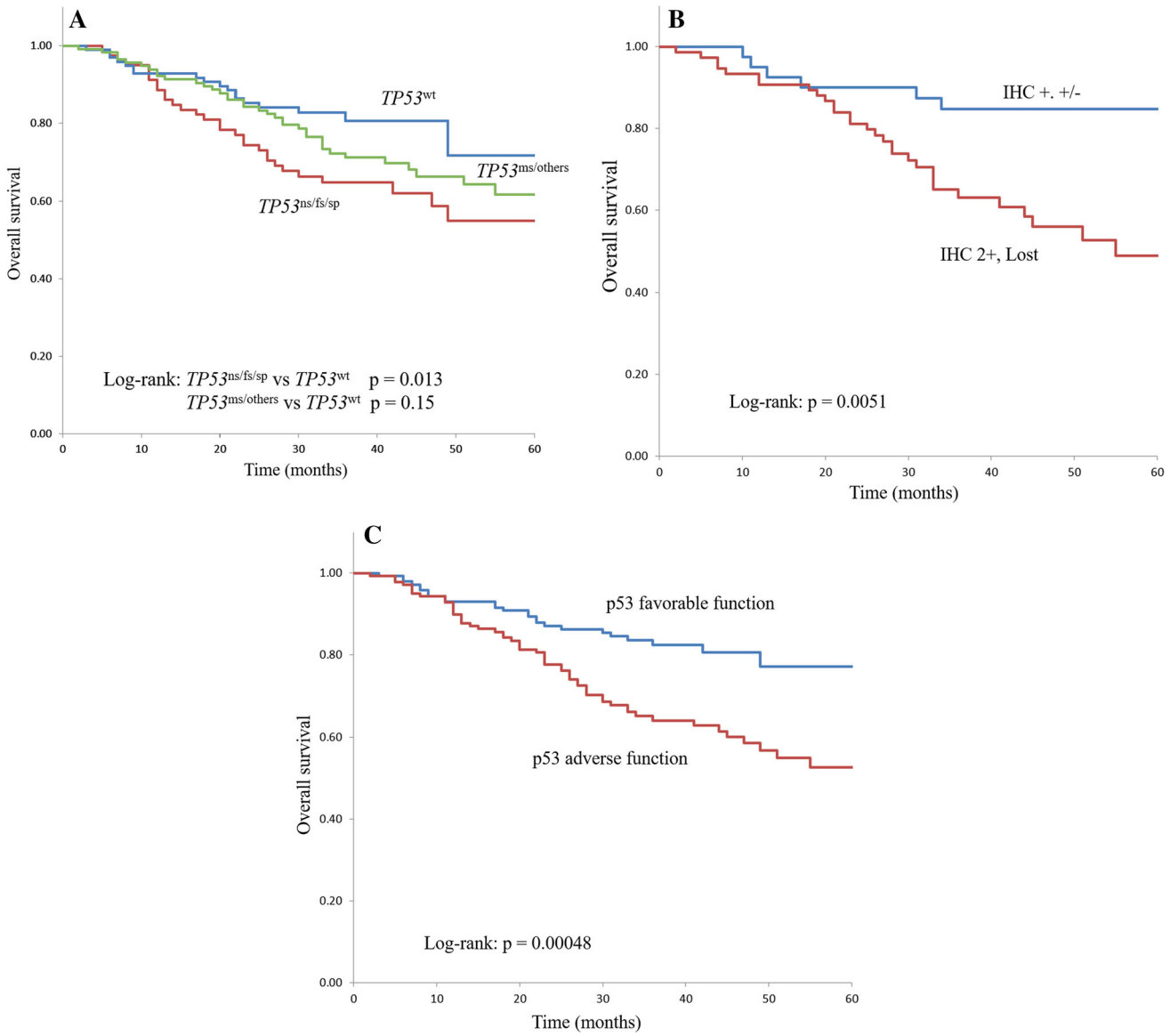


FIG. 2 Kaplan–Meier estimates of overall survival among patients with head and neck squamous carcinoma: **a** Patients with *TP53* nonsense/frameshift/splice mutations had worse OS rates than those with wild-type *TP53*. However, there was no statistical difference in survival between the *TP53* missense mutation/in-frame deletion/intron variant and wild-type *TP53* groups; **b** Among patients with *TP53* missense mutation/in-frame deletion/intron variant, those with p53 immunohistochemistry scores of 2+ or lost had significantly poorer OS rates than those with +/− or +; **c** "p53 adverse function" was associated with significantly decreased overall survival rates compared with the findings for "p53 favorable function"

On univariate analysis, pT, pN, vascular invasion, lymphatic invasion, perineural invasion, and p53 functional classification were significantly associated with OS. On multivariate analysis, pT, pN, perineural invasion, and p53 functional classification remained independent prognosticators of OS (Table 2).

Moreover, LFFS and DMFS were also analyzed according to the p53 functional classification. Multivariate analysis revealed that p53 functional classification is also an independent predictor of LFFS and DMFS (Table 2).

Local Control and Surgical Margin Analysis

In our analysis, pathological complete resection was performed in 222 patients. We compared the local control rate according to primary tumor location, surgical margin distance, and p53 functional classification. After excluding 29 patients who underwent postoperative radiotherapy, we analyzed 193 patients (117 and 76 patients with oral cavity and pharyngeal/laryngeal cancer, respectively).

First, for oral cavity cancer, using ROC curve analysis, the optimal cutoff associated with local recurrence was

TABLE 2 Hazard rations for overall survival, local failure-free survival and distant metastasis-free survival according to patient group

Covariate	Univariate model hazard ratio (95% CI)	*p* value	Multivariate model hazard ratio (95% CI)	*p* value
Overall survival				
Sex (male vs female)	1.09 (0.64–1.87)	0.74	-	-
Age (> 75 y/o vs < 75 y/o)	1.58 (0.98–2.55)	0.061	–	–
Smoking status (smokers vs nonsmokers)	1.23 (0.78–1.95)	0.37	–	–
Drinking status (drinkers vs nondrinkers)	1.55 (0.99–2.41)	0.051	–	–
Primary tumor site (Oral cavity vs Pharynx/ Larynx)	0.75 (0.48–1.17)	0.20	–	–
pT classification (pT3/pT4 vs pT1/T2)	3.16 (2.02–4.93)	4.52E−07	2.10 (1.30–3.40)	0.0025
pN classification (pN2/N3 vs pN0/N1)	3.17 (2.04–4.92)	2.75E−07	1.97 (1.14–3.40)	0.015
Vascular invasion (v1/v2 vs v0)	2.79 (1.78–4.35)	6.56E−06	1.34 (0.79–2.29)	0.28
Lymphatic invation (Ly1/Ly2 vs Ly0)	3.05 (1.96–4.74)	8.30E−07	1.22 (0.70–2.12)	0.49
Perineural invasion (n1/n2 vs n0)	2.99 (1.87–4.79)	4.81E−06	1.73 (1.02–2.94)	0.042
p16 (Negative vs Positive)	2.28 (0.72–7.24)	0.16	–	–
p53 functional classification (adverse vs favorable)	2.27 (1.42–3.65)	0.00067	2.27 (1.41–3.66)	0.00077
PIK3CA (mutant vs wild type)	0.93 (0.40–2.13)	0.86	–	–
AKT (mutant vs wild type)	1.10 (0.40–3.01)	0.85	–	–
HRAS (mutant vs wild type)	0.92 (0.23–3.74)	0.91	–	–
Local failure-free survival				
Sex (male vs female)	0.81 (0.46–1.43)	0.46	–	—
Age (> 75 y/o vs < 75 y/o)	1.54 (0.88–2.66)	0.13	–	–
Smoking status (smokers vs nonsmokers)	0.78 (0.47–1.29)	0.33	–	–
Drinking status (drinkers vs nondrinkers)	0.68 (0.39–1.19)	0.18	–	–
Primary tumor site (Oral cavity vs Pharynx/ Larynx)	1.10 (0.66–1.83)	0.73	–	–
pT classification (pT3/pT4 vs pT1/T2)	1.60 (0.96–2.67)	0.072	–	–
pN classification (pN2/N3 vs pN0/N1)	0.89 (0.49–1.63)	0.71	–	–
Vascular invasion (v1/v2 vs v0)	0.70 (0.40–1.24)	0.23	–	–
Lymphatic invasion (Ly1/Ly2 vs Ly0)	1.68 (0.99–2.86)	0.055	–	–
Perineural invasion (n1/n2 vs n0)	1.07 (0.54–2.11)	0.85	–	–
Surgical margin (positive vs negative)	2.55 (1.52–4.29)	0.00038	2.54 (1.51–4.29)	0.00048
p16 (negative vs positive)	1.63 (0.51–5.20)	0.41	–	–
p53 functional classification (adverse vs favorable)	2.48 (1.44–4.27)	0.0011	2.42 (1.39–4.23)	0.0019
PIK3CA (mutant vs wild type)	0.38 (0.09–1.55)	0.18	–	–
AKT (mutant vs wild type)	0.33 (0.05–2.39)	0.27	–	–
HRAS (mutant vs wild type)	2.06 (0.64–6.58)	0.22	–	–
Distant metastasis-free survival				
Sex (male vs female)	1.41 (0.59–3.39)	0.44	–	–
Age (> 75 y/o vs < 75 y/o)	0.73 (0.30–1.76)	0.49	–	–
Smoking status (smokers vs nonsmokers)	1.73 (0.83–3.58)	0.14	–	–
Drinking status (drinkers vs nondrinkers)	2.16 (1.12–4.17)	0.022	0.73 (0.33–1.66)	0.46
Primary tumor site (Oral cavity vs Pharynx/ Larynx)	0.35 (0.17–0.71)	0.0036	0.74 (0.32–1.71)	0.48
pT classification (pT3/pT4 vs pT1/T2)	3.54 (1.79–7.00)	0.00027	1.65 (0.79–3.43)	0.18
pN classification (pN2/N3 vs pN0/N1)	7.61 (3.74–15.5)	2.29E−0.8	5.91 (2.30–15.2)	0.00022
Vascular invasion (v1/v2 vs v0)	4.33 (2.13–8.76)	4.63E−05	2.04 (0.90–4.65)	0.090

TABLE 2 continued

Covariate	Univariate model hazard ratio (95% CI)	*p* value	Multivariate model hazard ratio (95% CI)	*p* value
Lymphatic invation (Ly1/Ly2 vs Ly0)	3.86 (2.00–7.47)	5.97E−05	0.97 (0.44–2.17)	0.95
Perineural invasion (n1/n2 vs n0)	2.23 (1.07–4.65)	0.033	1.04 (0.44–2.45)	0.93
p16 (negative vs positive)	1.52 (0.36–6.37)	0.57	–	–
p53 functional classification (adverse vs favorable)	3.09 (1.45–6.58)	0.0034	3.55 (1.60–7.90)	0.0019
PIK3CA (mutant vs wild type)	1.04 (0.32–3.39)	0.95	–	–
AKT (mutant vs wild type)	0.61 (0.08–4.46)	0.63	–	–
HRAS (mutant vs wild type)	0.92 (0.12–6.71)	0.93	–	–

determined to be 6 mm (area under the curve 0.7) (Fig. 3a). For local recurrence, this cutoff was compared according to p53 functional classification (Fig. 3b). In patients with surgical margin > 6 mm, the "p53 favorable function" group showed a significantly lower local recurrence rate than the "p53 adverse function" group (0 vs 14%, $p = 0.044$). However, in patients with surgical margin ≤ 6 mm, no statistical difference was observed in the local recurrence rate between the groups (23 vs 31%, $p = 0.34$) (Fig. 3c).

Second, regarding laryngeal and pharyngeal cancers, ROC curve analysis could not determine an optimal cutoff for surgical margin distance (4-mm cutoff, area under the curve 0.5) (Fig. 3d). However, in patients with surgical margin > 4 mm, the "p53 favorable function" group demonstrated a significantly lower local recurrence rate than the "p53 adverse function" group (0 vs 34%, $p = 0.0028$) (Fig. 3c, e).

DISCUSSION

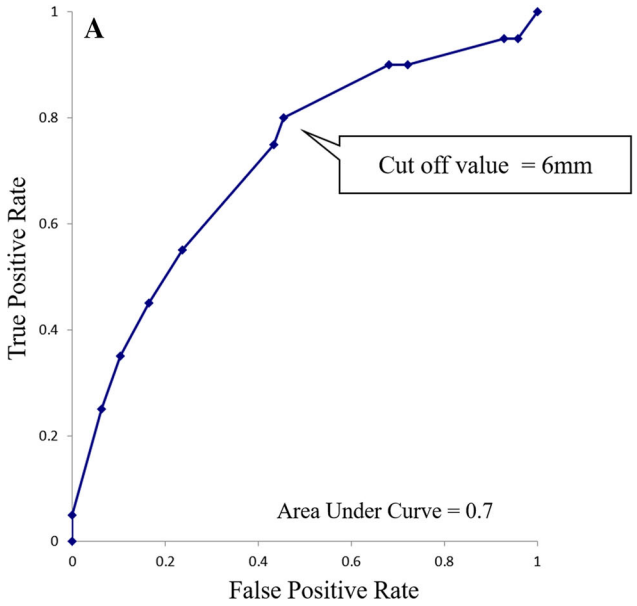
In this study, we accurately predicted clinical outcomes using FFPE biopsy or surgical specimens. Moreover, we created 24 amplicons and performed deep reads of each amplicon for full-coverage sequencing of all exons of the *TP53* gene.

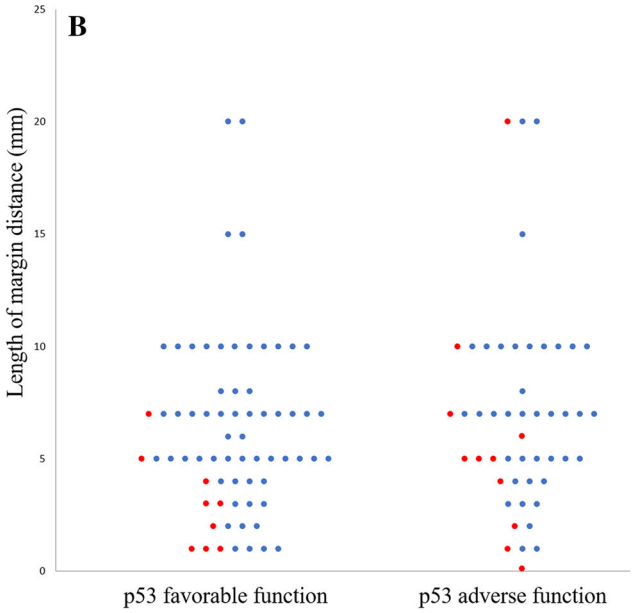
The analysis of amino acid replacement due to missense mutation and its location is shown in Supplementary Fig. 3. Almost all mutations occurred within the central core DNA-binding domain (amino acid residues 100–300), as previously reported. Regarding prognosis, mutation within the L2 or L3 binding domains (codons 163–195 or 236–251) was reported to lead to poorer prognostic outcomes compared with mutation in the DNA-binding domain.[10] However, in our analysis, poor prognoses were noted both in patients with L2 or L3 binding domain mutations and in those with DNA-binding domain mutations except L2 or L3 regions. The EAp53 scoring system has also been used to predict poorer prognostic outcome,[5] but it was not effective in our cohort.

To generalize these diverse *TP53* missense mutations, we categorized missense mutations based on the protein phenotype. The efficacy of p53 IHC in predicting oncological outcomes was reported previously.[11,12] However, there is no consensus regarding the classification of p53 IHC because of the biological uncertainty. Boyle et al. reported that patients with breast cancer and extremely positive or negative p53 IHC expression have worse OS than those with nonextreme expression.[11]

In our analysis, p53 immunostaining status was classified as +/−, +, 2+, and lost. We hypothesized that mild to moderate p53 structural changes due to *TP53* missense mutation/in-frame deletion/intron variant might lead to p53 accumulation in the nucleus, resulting in strong or weak staining. By contrast, severe p53 structural changes due to *TP53* nonsense/frameshift/splice mutations might result in deletion at mRNA or protein level, leading to negative stating. The association between protein phenotype and mutation showed high sensitivity but low specificity in 2+, +, and missense mutation/in-frame deletion/intron variant, whereas low sensitivity and high specificity were observed for lost and nonsense/frameshift/splice mutations, in line with previous findings.[13] There is a limitation in predicting the effects of mutations via immunophenotyping from preoperative biopsy specimens; however, the results of this study reveal that the prognosis of patients with *TP53* mutations was accurately stratified via p53 protein phenotype.

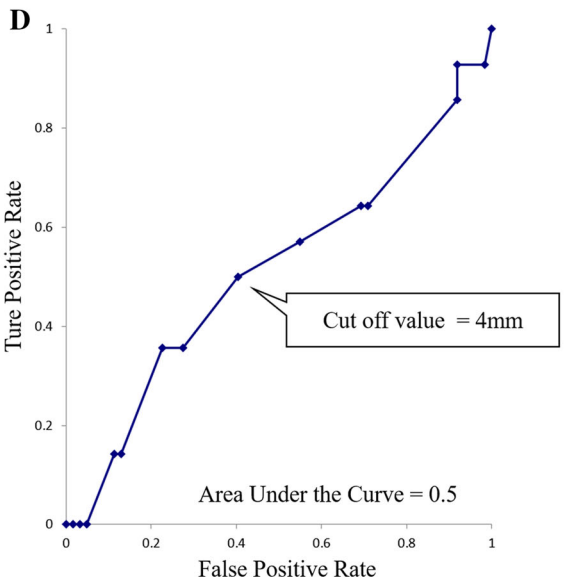
In surgical treatment of HNSCC, even when the surgical margin is diagnosed as cancer free via histopathological examination, the local recurrence rate is still 10–30%.[7] Possible explanations for this finding are limited margin surveillance by the pathologist and the existence of tumor-related mucosal precursor lesions.[14,15] The most common distance taken to indicate clear pathological margins on microscopic evaluation is ≥ 5–7 mm.[16–23] The surgical experience of the surgeon is an important factor for a clear surgical margin. In our institute, all surgeons are board certified, thus securing an optimal quality of the surgical procedure. We compared the local control rate according to

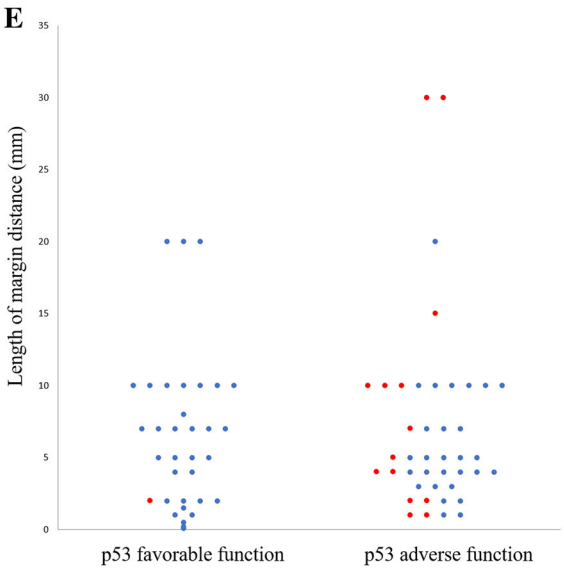




C Local recurrence rate after pathological complete resection

Site	Margin definition	No. of patients	Local recurrence rate (p53 favorable vs p53 adverse)	*P*-value
Oral cavity	>6mm	57	0% vs 14%	0.044
	≤6mm	60	23% vs 31%	0.34
Pharynx / Larynx	>4mm	44	0% vs 34%	0.0028
	≤4mm	32	38% vs 32%	0.11





the primary tumor location, surgical margin distance, and p53 functional classification. The results showed that the local recurrence rate after adequate surgical margin was higher, and a longer surgical margin distance was required for patients with "p53 adverse function."

◀ **FIG. 3** Surgical margin analysis: **a** Receiver operating characteristic (ROC) curve of the surgical margin and local recurrence in patients with oral cavity cancer. The area under the ROC curve was 0.7. Based on the ROC curve, the optimal cutoff associated with local recurrence was 6 mm; **b** Surgical margin distance among patients with oral cavity cancer and pathological complete resection plotted according to p53 functional classification. Red dot, patients with local recurrence; blue dot, patients without local recurrence; **c** Local recurrence rate after adequate surgical margin was higher for patients with "p53 adverse function"; **d** ROC curve of surgical margin and local recurrence in patients with pharyngeal and laryngeal cancer. Area under the ROC curve 0.5. Based on the ROC curve, the optimal cutoff associated with local recurrence was 4 mm; **e** Surgical margin distance among patients with pharyngeal and laryngeal cancers with pathological complete resection plotted based on p53 functional classification. Red dot, patients with local recurrence; blue dot, patients without local recurrence

Regarding postoperative treatment for local control, presence of positive margins, vascular/lymphatic/perineural invasion, or pT3 or T4 primary tumors are the established indications for postoperative chemotherapy/radiotherapy.[16,24–30] However, the management of patients with close surgical margins remains controversial, and the p53 functional classification might help clarify the indication for postoperative radiotherapy. Conversely, among patients who developed local recurrence even when the clear pathological margin was longer than 30 mm, it might be difficult to achieve tumor control via surgical resection alone. Currently, we are repeating this analysis in patients treated via radiotherapy. Predicting radiosensitivity and personalized therapy might be possible for such patients.

In HPV-negative HNSCC, a subgroup of patients with activating mutations of *HRAS* and *PIK3CA* coupled with inactivating mutations of *TP53* have favorable clinical outcomes.[3] However, we failed to detect any prognostic impact of these mutations.

In conclusion, whole-exon sequencing of the *TP53* gene and p53 protein phenotype analysis can accurately predict clinical outcomes for HNSCC using FFPE specimens. Except for patients with HPV-associated OPC, p53 functional classification remained an independent prognostic factor for OS, LC, and DMFS in the enrolled patients. Moreover, the results of this work also reveal that the local recurrence rate was higher even in patients with adequate surgical margin, and a longer surgical margin distance was required for patients with "p53 adverse function." Further technological innovation in genomic analysis may help predict tumor biological characteristics using preoperative microsamples. Such information may help strategize personalized surgical treatment and permit surgical margin distance determination.

The main limitation of this study is selection bias because of the retrospective setting and inclusion of only surgically treated patients. However, the exclusion criteria and treatment strategy were consistent, all surgeons were board certified, and FFPE specimens were well annotated to the clinical information. Finally, this was a single-center study that used semiquantitative analyses such as IHC; additionally, institutional-specific factors may potentially limit its generalizability. Further research is warranted to verify the findings of this study.

ACKNOWLEDGEMENTS We acknowledge Yuki Saito, MD, PhD for his substantial contribution to critical manuscript revisions. The authors wish to thank Mr. Kensuke Suzuki (Illumina Inc.) for technical support. We also thank Ms. Sachiko Miura, Ms. Toshiko Sakaguchi, and Ms. Chizu Kina for skillful technical assistance.

FUNDING This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant nos. 16K20232 to K.K. and 17K08710 to T.M.

DISCLOSURE All authors declare that they have no conflict of interest.

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