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An Ultra-Deep Targeted Sequencing Gene Panel Improves the Prognostic Stratification of Patients With Advanced Oral Cavity Squamous Cell Carcinoma

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Abstract: An improved prognostic stratification of patients with oral cavity squamous cell carcinoma (OSCC) and pathologically positive (pN+) nodes is urgently needed. Here, we sought to examine whether an ultra-deep targeted sequencing (UDT-Seq) gene panel may improve the prognostic stratification in this patient group.

A mutation-based signature affecting 10 genes (including genetic mutations in 6 oncogenes and 4 tumor suppressor genes) was devised to predict disease-free survival (DFS) in 345 primary tumor specimens obtained from pN+ OSCC patients. Of the 345 patients, 144 were extracapsular spread (ECS)-negative and 201 were ECS-positive. The 5-year locoregional control, distant metastases, disease-free, disease-specific, and overall survival (OS) rates served as outcome measures.

The UDT-Seq panel was an independent risk factor (RF) for 5-year locoregional control ($P = 0.0067$), distant metastases ($P = 0.0001$), DFS ($P < 0.0001$), disease-specific survival (DSS, $P < 0.0001$), and OS ($P = 0.0003$) in pN+ OSCC patients. The presence of ECS and pT3–4 disease were also independent RFs for DFS, DSS, and OS. A prognostic scoring system was formulated by summing up the significant covariates (UDT-Seq, ECS, pT3–4) separately for each survival endpoint. The presence of a positive UDT-Seq panel (n = 77) significantly improved risk stratification for all the survival endpoints as compared with traditional AJCC staging ($P < 0.0001$). Among ECS-negative patients, those with a UDT-Seq-positive panel (n = 31) had significantly worse DFS ($P = 0.0005$) and DSS ($P = 0.0002$). Among ECS-positive patients, those with a UDT-Seq-positive panel (n = 46) also had significantly worse DFS ($P = 0.0032$) and DSS ($P = 0.0098$).

Our UDT-Seq gene panel consisting of clinically actionable genes was significantly associated with patient outcomes and provided better prognostic stratification than traditional AJCC staging. It was also able to predict prognosis in OSCC patients regardless of ECS presence.

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Abbreviations: AJCC = American Joint Committee on Cancer, CCRT = concomitant chemoradiation, DFS = disease-free survival, DSS = disease-specific survival, ECS = extracapsular spread, MVA = multivariate analyses, OS = overall survival, OSCC = oral cavity squamous cell carcinoma, PFS = progression-free survival, pN+ = pathological neck node metastases, RF = risk factor, RT = radiotherapy, TCGA = The Cancer Genome Atlas, UDT-Seq = ultra-deep targeted sequencing, UVA = univariate analyses.

INTRODUCTION

Oral cavity squamous cell carcinoma (OSCC) is a common malignancy of the head and neck area that currently ranks sixth among all tumors in Taiwan. It is the most common form of cancer in Taiwanese males aged between 30 and 50 years because of their indulgence in risky oral habits (ie, betel quid chewing, cigarette smoking, and alcohol drinking).[1–3] Surgical excision remains the mainstay of treatment for OSCC, either with or without adjuvant therapy (depending on the presence of specific risk factors [RFs]). According to the Taiwanese official statistics (2004–2010, n = 23,360), the overall survival (OS) rates of OSCC patients critically depend on disease stage, being 80% for stage I, 70% for stage II, 57% for stage III, and 37% for stage IV.[1] In general, the presence of tumor relapse and distant metastases is associated with dismal outcomes. In this context, the prognostic stratification of OSCC patients continues to be largely based on traditional clinicopathological RFs (eg, American Joint Committee on Cancer [AJCC] staging and extracapsular spread [ECS]).

Pathological neck node metastases (pN+)—indicating the presence of a disease stage III–IV—are a major adverse prognostic factor in OSCC patients.[4–7] Although pN− OSCC patients have 5-year OS rates of 80%, only 45% of pN+ patients survive at 5 years.[8] However, OSCC patients with nodal metastases do not have a uniformly poor prognosis.[8,9]

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Because OSCC patients with similar clinicopathological RFs can have large differences in how their disease evolves over time, new insights into risk stratification, and, simultaneously, novel targeted therapies are eagerly awaited. In this scenario, novel sequencing techniques hold great promise for expanding our understanding of the molecular basis of OSCC.[10,11] In this retrospective study, we examined whether genetic mutations identified by ultra-deep targeted sequencing (UDT-Seq) (ie, molecular risk stratification) may improve traditional prognostic models based on common clinicopathological RFs in pN+ OSCC patients.[10]

PATIENTS AND METHODS

Patients and Clinical Management

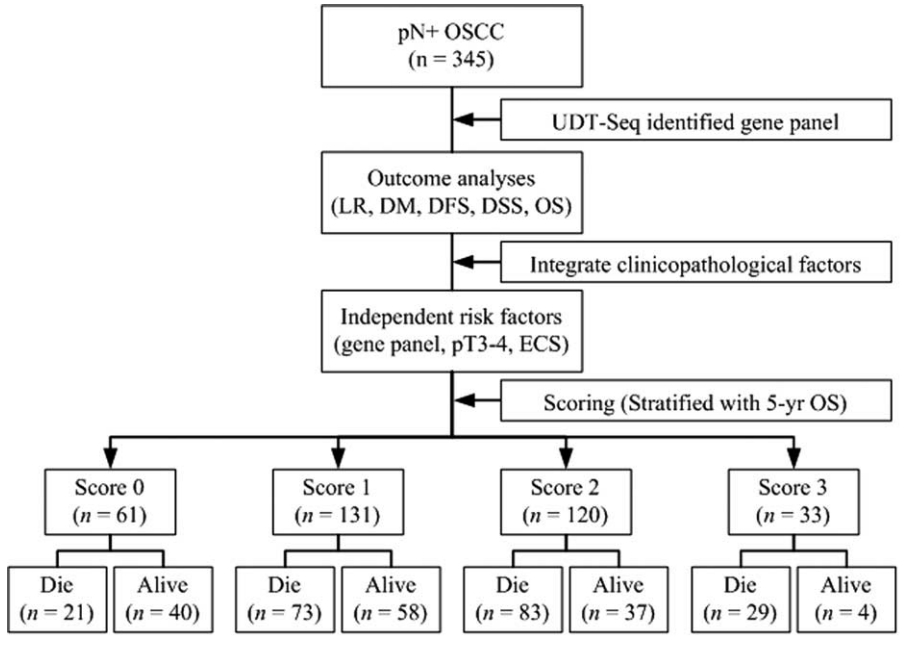
We retrospectively reviewed the records of 345 pN+ patients with previously untreated first primary OSCC who were referred for radical tumor excision and neck dissection between 1996 and 2011. The flow diagram of the patients through the study is depicted in Figure 1. All of the participants underwent an extensive evaluation before primary surgery.[7,8] Tumor staging was performed using the 1997 (5th edition) and 2010 (7th edition) AJCC staging criteria as previously described in detail.[2] Surgery and adjuvant therapy were performed in accordance with our institutional policy.[3] RFs were classified according to the National Comprehensive Cancer Network (NCCN)[12] guidelines before 2008 or according to our published criteria[3] thereafter. The indications for concomitant chemoradiation (CCRT, 66 Gy)[7,8,13] and the applied chemotherapy regimens[13,14] have been previously reported. The study was granted ethical approval by the Institutional Review Board of the Chang Gung Memorial Hospital (CGMH 101-4457B). The need for informed consent was waived because of the retrospective nature.

Ultra-Deep Targeted Sequencing Signature Gene Selection and Confirmation

In our previous study, a mutation-based signature involving 6 oncogenes and 4 tumor suppressor genes (HRAS, BRAF, FGFR3, SMAD4, KIT, PTEN, NOTCH1, AKT1, CTNNB1, and PTPN11) was identified from 45 cancer-related genes (29 oncogenes and 16 tumor suppressor genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNAS, HNF1A, HRAS, IGH1, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL) as a predictor of 5-year disease-free survival (DFS) in 345 primary tumor specimens obtained from pN+ OSCC patients.[10] Of the 345 OSCC patients, 77 were UDT-Seq-positive and 268 UDT-Seq-negative.

Validation of the UDT-Seq Gene Signature[15]

The prognostic value of the UDT-Seq signature has been internally validated by our group using 2 different resampling methods (randomization and enrollment period).[10] Here, we externally validated the signature by analyzing its association with clinical outcomes using the head and neck squamous cell carcinoma dataset from The Cancer Genome Atlas (TCGA). Mutation and survival data for the head and neck squamous cell carcinoma dataset were downloaded from the TCGA database using the cBioPortal (http://www.cbioportal.org/).[16] Only



DFS = disease-free survival, DM = distant metastases, DSS = disease-specific survival, LR = local regional control, OS = overall survival, OSCC = oral cavity squamous cell carcinoma, UDT-Seq = ultra-deep targeted sequencing.

FIGURE 1. Flow of the participants through the study.

samples with complete progression-free survival (PFS) information (n = 226) were used for the analysis.

Statistical Analysis

A total of 19 clinicopathological RFs were analyzed. The 5-year rates of locoregional control, distant metastases, DFS, disease-specific survival (DSS), and OS served as outcome measures. DFS was defined from the date of surgery to the date of local, regional, distant progression, or the date of last follow-up. DSS was calculated from the date of surgery to the date of death from OSCC or the last follow-up. OS was calculated from the date of surgery to the date of last follow-up or death. Survival curves were plotted using the Kaplan–Meier method and compared with the log-rank test. Cox regression models were used to identify the independent predictors of outcomes. All of the study variables were considered as potential predictors/covariates in both univariate analyses (UVA, log-rank test) and multivariate analyses (MVA, Cox regression models). A stepwise forward selection procedure was used to identify the independent variables after allowance for potential confounders in MVA. Model fit assessment and model improvement were performed with the −2log likelihood statistics. Stratification of risk groups was based on the score calculated using the sum of the predictors as a grouping factor, and comparisons were performed accordingly. Data were analyzed using the SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL). Two-tailed P values <0.05 were considered statistically significant.

RESULTS

Patients

The characteristics of the study patients and their clinicopathological RFs are reported in Table 1. The study cohort consisted of 345 OSCC patients (325 males and 20 females; age range: 27–89 years; mean age: 49.6 years). A history of preoperative alcohol drinking was identified in 246 patients (71%). In addition, 282 (82%) and 313 (91%) patients had a positive preoperative history of betel chewing and cigarette smoking, respectively. Pathological stages of III and IV were present in 85 (25%) and 260 (75%) patients, respectively. Twenty-five (7.2%) patients received surgery only. Surgery plus radiotherapy (RT) and surgery plus CCRT were utilized in 108 (31.3%) and 212 (61.4%) patients, respectively.

Clinical Course in the Entire Study Group

All of the participants were followed for at least 30 months after primary surgery or until death (mean: 55.0 months, median: 42.0 months, range: 1–211 months). At the end of the study period, 139 patients (40.3%) were alive, and 206 (59.7%) were dead. The patterns of recurrences and second primary tumors were as follows: local recurrence, 19.7% (n = 68); neck recurrence, 24.6% (n = 85); distant metastases, 25.8% (n = 89); and second primary tumors, 18.6% (n = 64). Salvage therapy was performed in 60 (48.4%) of the 124 patients with local and/or neck recurrences. Among the patients who were salvaged, 20 (33.3%) were still alive when the data were analyzed, whereas the remaining 40 (66.7%) were dead.

UDT-Seq-Identified Gene Panel and Clinical Outcomes

We found significant differences in terms of 5-year outcomes according to the presence or absence of the UDT-Seq panel, as follows: locoregional control, 47% versus 66%, $P = 0.0067$; distant metastases, 44% versus 23%, $P = 0.0001$; DFS, 31% versus 57%, $P < 0.0001$; DSS, 39% versus 64%, $P < 0.0001$; OS, 33% versus 53%, $P = 0.0003$ (Figure 2A–E).

External Validation of the UDT-Seq Gene Panel Using the TCGA Dataset

A total of 226 head and neck cancer patients were identified in the TCGA dataset (median follow-up: 13.6 months). We thus analyzed externally validated the UDT-Seq signature by examining its impact on PFS in the TCGA cohort. Sixty-two (27.4%) cases were event-positive and 33 (14.6%) tumors were positive for our gene signature. Kaplan–Meier analysis revealed that the median PFS for UDT-Seq(+) and UDT-Seq(−) patients was 25.7 and 53.1 months, respectively. Although the difference was marginally significant ($P = 0.0673$; Figure 3), there was a trend toward a poorer PFS for UDT-Seq(+) patients (hazard ratio [HR] = 1.826, 95% confidence interval [CI] = 0.958–3.479).

Univariate and Multivariate Analyses of 5-Year Outcomes

In the entire study cohort, we observed the following 5-year outcomes: locoregional control, 62%; distant metastases, 27%; DFS, 51%; DSS, 58%; and OS, 48%. We then examined the entire study cohort (n = 345) with respect to the ability of UDT-Seq panels and other clinicopathological RFs (sex, age, preoperative alcohol drinking, preoperative betel quid chewing, preoperative cigarette smoking, pT status, pN status, p-Stage, ECS, lymph node density [optimal cutoff value = 0.043], differentiation, tumor depth, margin status, perineural invasion, lymphatic invasion, vascular invasion, skin invasion, bone marrow invasion) to predict the study outcomes. Tables 1 and 2 show the results of UVA and MVA of 5-year outcomes in the entire study cohort. The results indicated that the UDT-Seq panel was independently associated with all of the 5 outcomes (locoregional control, distant metastases, DFS, DSS, and OS) even after allowance for traditional RFs (Table 2). The presence of ECS and pT3–4 disease were also independent RFs for distant metastases and all 3 survival endpoints.

The UDT-Seq Panel Improves the 5-Year Prognostic Stratification When Compared With AJCC Staging

A prognostic scoring system was formulated by summing up the 3 significant covariates identified in multivariate analysis: 0 for UDT-Seq negative and 1 for UDT-Seq positive; 0 for without ECS and 1 for with ECS; 0 for pT1–2 disease and 1 for pT3–4 disease. We then constructed the Kaplan–Meier curves to examine the 5-year distant metastases and survival rates according to the prognostic scoring system (from score 0 to score 3) (Figure 4A–D). The results demonstrated that the presence of a positive UDT-Seq panel (n = 77) significantly improved risk stratification with wider ranges of 4 subgroups in curves for 5-year distant metastases and survival rates seen with the use of the prognostic scoring system compared with the traditional AJCC staging ($P < 0.0001$). We used the −2log likelihood statistics to assess model fit and improvement. The P values for the −2log likelihood tests (multivariate Cox regression models for predicting DFS) were 6.4×10^{-5}, 1.1273×10^{-7}, and 9.5195×10^{-11} for AJCC staging, pT3–4/ECS, and UDT-Seq/pT3–4/ECS,

TABLE 1. Univariate Analyses of Risk Factors for 5-Year Outcomes in OSCC Patients (n = 345)

Risk Factors	5-yr Locoregional Control (%, n Event)	*P*	5-yr Distant Metastases (%, n Event)	*P*	5-yr Disease-Free Survival (%, n Event)	*P*	5-yr Disease-Specific Survival (%, n Event)	*P*	5-yr Overall Survival (%, n Event)	*P*
UDT-Seq panel		0.0067		0.0001		<0.0001		<0.0001		0.0003
No (268, 77.7)	66 (90)		23 (58)		57 (119)		64 (99)		53 (148)	
Yes (77, 22.3)	47 (34)		44 (31)		31 (53)		39 (47)		33 (58)	
Sex		0.7163		0.9021		0.5547		0.1809		0.3646
Male (325, 94.2)	62 (118)		27 (84)		51 (164)		58 (141)		48 (197)	
Female (20, 5.8)	67 (6)		26 (5)		58 (8)		74 (5)		53 (9)	
Age of disease onset, yr		0.6773		0.4970		0.6203		0.5073		0.2079
<65 (307, 89.0)	62 (112)		27 (81)		51 (155)		58 (132)		49 (179)	
≥65 (38, 11.0)	63 (12)		27 (8)		50 (17)		63 (14)		43 (27)	
Alcohol drinking		0.4843		0.3268		0.1833		0.1565		0.0401
No (99, 28.7)	67 (34)		24 (22)		58 (44)		65 (36)		55 (52)	
Yes (246, 71.3)	60 (90)		29 (67)		48 (128)		56 (110)		46 (154)	
Betel quid chewing		0.3350		0.5904		0.9445		0.6622		0.5538
No (63, 18.3)	57 (25)		23 (14)		52 (30)		60 (24)		45 (38)	
Yes (282, 81.7)	63 (99)		28 (75)		51 (142)		58 (122)		49 (168)	
Cigarette smoking		0.5404		0.5653		0.7173		0.5327		0.4876
No (32, 9.3)	49 (13)		23 (7)		41 (17)		64 (12)		55 (16)	
Yes (313, 90.7)	63 (111)		28 (82)		52 (155)		58 (134)		48 (190)	
pT-status		0.0189		0.0014		<0.0001		<0.0001		<0.0001
pT1–2 (153, 44.3)	69 (49)		18 (28)		64 (59)		71 (45)		62 (73)	
pT3–4 (192, 55.7)	56 (75)		35 (61)		41 (113)		48 (101)		37 (133)	
pN-status		0.0008		0.0001		<0.0001		<0.0001		0.0042
pN0–1 (123, 35.7)	74 (33)		15 (18)		66 (44)		74 (36)		60 (66)	
pN2 (222, 64.3)	56 (91)		35 (71)		43 (128)		50 (110)		42 (140)	
Pathological stage		0.0062		0.0003		<0.0001		<0.0001		0.0009
III (85, 24.6)	76 (23)		12 (10)		71 (27)		79 (21)		67 (41)	
IV (260, 75.4)	57 (101)		33 (79)		44 (145)		52 (125)		42 (165)	
Extracapsular spread		0.0411		<0.0001		<0.0001		<0.0001		<0.0001
No (144, 41.7)	68 (48)		12 (18)		63 (56)		74 (42)		63 (71)	
Yes (201, 58.3)	58 (76)		39 (71)		42 (116)		47 (104)		38 (135)	
Lymph node density		<0.0001		0.0006		<0.0001		<0.0001		0.0030
<0.043 (141, 40.9)	75 (34)		18 (24)		65 (49)		72 (43)		57 (73)	
≥0.043 (204, 59.1)	53 (90)		34 (65)		41 (123)		49 (103)		42 (133)	
Differentiation		0.1856		0.0001		0.0167		0.0049		0.0890
Well/moderate (289, 83.8)	64 (102)		24 (64)		53 (138)		61 (115)		50 (171)	
Poor (56, 16.2)	53 (22)		47 (25)		38 (34)		44 (31)		41 (35)	
Tumor depth, mm*		0.9129		0.0038		0.0210		0.0012		0.0120
<10 (113, 32.8)	65 (44)		17 (19)		62 (48)		72 (35)		59 (59)	
≥10 (231, 67.2)	61 (80)		33 (70)		45 (124)		51 (111)		43 (147)	

Risk Factors	5-yr Locoregional Control (%, n Event)	*P*	5-yr Distant Metastases (%, n Event)	*P*	5-yr Disease-Free Survival (%, n Event)	*P*	5-yr Disease-Specific Survival (%, n Event)	*P*	5-yr Overall Survival (%, n Event)	*P*
Margin status*		0.0143		0.0068		0.0002		0.0025		0.0120
≤4 (43, 12.6)	41 (19)		40 (17)		28 (30)		41 (25)		51 (172)	
>4 (298, 87.4)	66 (101)		25 (71)		55 (138)		62 (117)		31 (30)	
Perineural invasion		0.7225		0.0109		0.1790		0.1018		0.1485
No (168, 48.7)	61 (66)		20 (33)		55 (80)		62 (65)		52 (100)	
Yes (177, 51.3)	64 (58)		34 (56)		47 (92)		55 (81)		45 (106)	
Lymphatic invasion		0.0828		0.1117		0.0014		0.0039		0.0230
No (301, 87.2)	65 (105)		26 (74)		54 (141)		61 (119)		51 (173)	
Yes (44, 12.8)	43 (19)		37 (15)		28 (31)		39 (27)		30 (33)	
Vascular invasion		0.9090		0.8639		0.8990		0.8031		0.8678
No (327, 94.8)	62 (118)		27 (84)		51 (163)		58 (139)		49 (196)	
Yes (18, 5.2)	59 (6)		29 (5)		46 (9)		61 (7)		42 (10)	
Skin invasion		0.2231		0.1187		0.0253		0.0090		0.0081
No (308, 89.3)	64 (109)		26 (76)		53 (148)		61 (123)		51 (177)	
Yes (37, 10.7)	51 (15)		39 (13)		34 (24)		40 (23)		29 (29)	
Bone marrow invasion		0.3997		0.0631		0.1936		0.0404		0.0106
No (274, 79.4)	64 (97)		25 (65)		53 (133)		61 (109)		51 (155)	
Yes (71, 20.6)	56 (27)		35 (24)		44 (39)		48 (37)		38 (51)	
Treatment modality		0.0115		0.1951		0.0236		0.0182		0.0238
Surgery (25, 7.2)	44 (13)		33 (8)		38 (16)		41 (15)		32 (18)	
Surgery + RT/CCRT (320, 92.8)	64 (111)		27 (81)		52 (156)		60 (131)		50 (188)	

CCRT = concomitant chemoradiation, RT = radiotherapy, UDT-Seq = ultra-deep targeted sequencing.
* Unknown data: depth, n = 1; margins, n = 4.

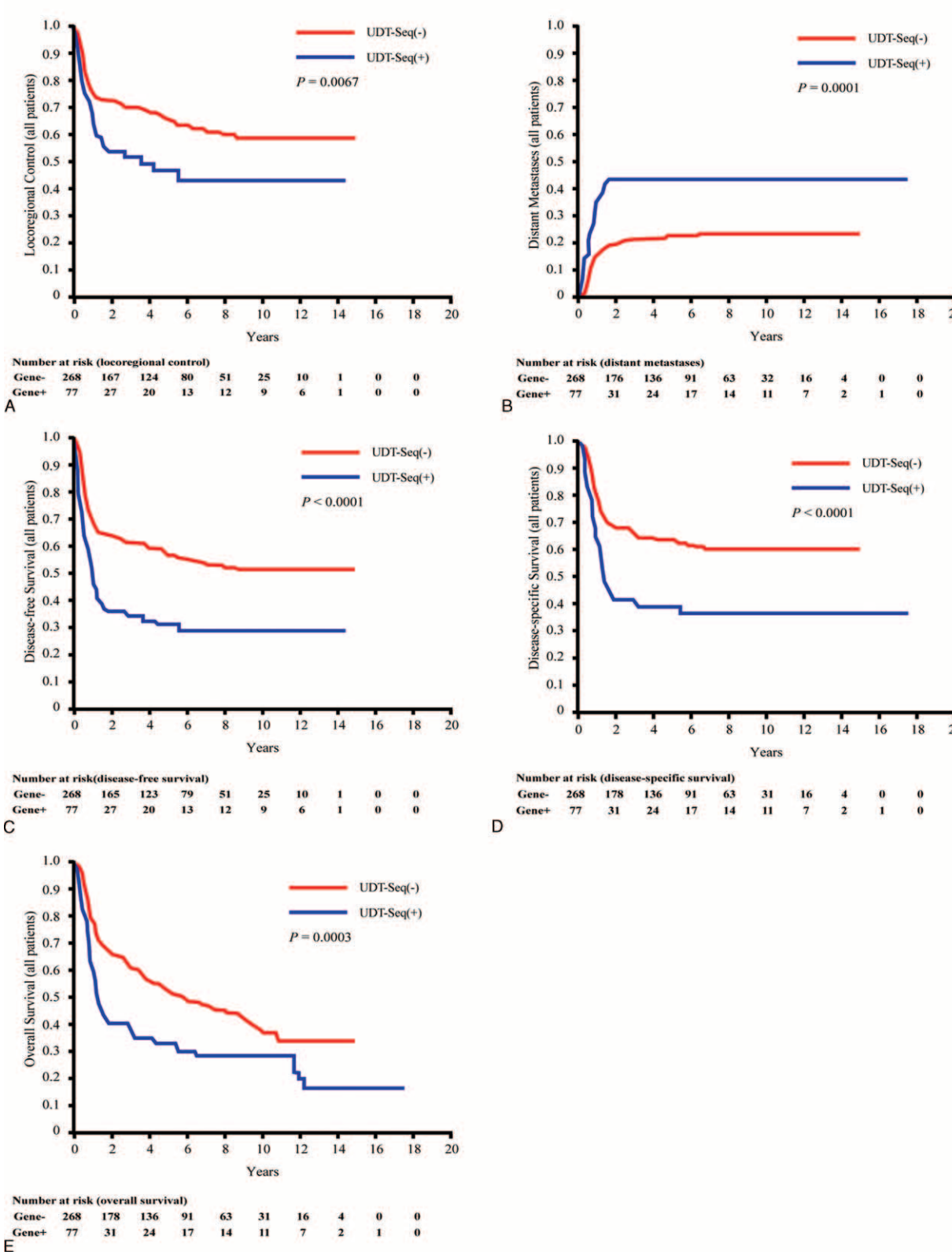


FIGURE 2. Five-year Kaplan–Meier estimates for all OSCC patients with ([+]) and without ([−]) a positive UDT-Seq panel. (A) Locoregional control, (B) distant metastases, (C) disease-free survival, (D) disease-specific survival, (E) overall survival.

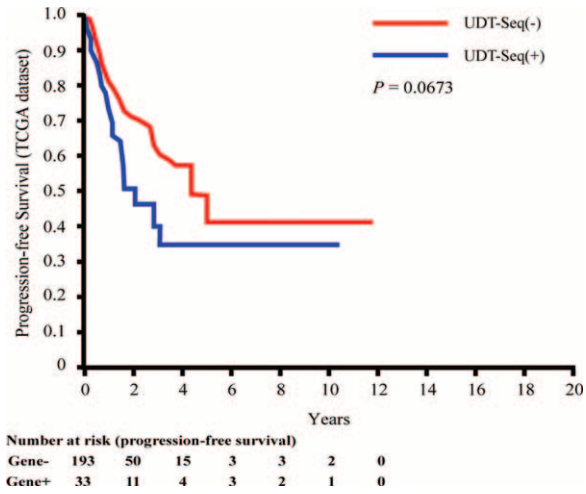


FIGURE 3. Five-year Kaplan–Meier estimates of progression-free survival for head and neck cancer patients in the TCGA dataset.

respectively. These results indicate that the UDT-Seq panel improves the prognostic prediction offered by the pT3–4/ECS model. Both the pT3–4/ECS and UDT-Seq/pT3–4/ECS models were prognostically superior to the AJCC staging. To further analyze the prognostic improvement offered by the UDT-Seq panel, we formulated another 3-point scoring system based on ECS and pT3–4 only (ie, without the inclusion of UDT-Seq panel). Table 3 summarizes the outcome comparisons of the UDT-Seq/pT3–4/ECS vs pT3–4/ECS scoring systems vs AJCC staging. The UDT-Seq/pT3–4/ECS scoring systems having stronger *P* values and hazard ratios compared with either pT3–4/ECS scoring systems or AJCC staging. Among pN+ patients who presented with p-Stage IV disease (n = 260), the presence of a positive UDT-Seq panel (n = 62) also significantly improved risk stratification for DFS and DSS as compared with traditional AJCC staging ($P < 0.0001$, Figure 4E and F).

Prognostic Value of the UDT-Seq Panel in Relation to the Presence of ECS

Of the 345 patients, 144 were ECS-negative and 201 were ECS-positive. Among ECS-negative patients (n = 144), those with a UDT-Seq-positive panel (n = 31) had significantly worse DFS and DSS ($P = 0.0005$ and $P = 0.0002$ respectively, Figure 5A and B). Among ECS-positive patients (n = 201), those with a UDT-Seq-positive panel (n = 46) had significantly worse DFS and DSS ($P = 0.0032$ and $P = 0.0098$ respectively, Figure 5C and D).

DISCUSSION

An improved prognostic stratification of pN+ OSCC patients is urgently needed to devise tailored treatment strategies and optimize clinical outcomes. Molecular classifications of OSCC have been introduced to identify subsets of patients that share common biological features.[17] In this study, we used UDT-Seq with the goal of identifying genetic mutations specifically associated with 5-year clinical outcomes. Notably, the selection of genes for UDT-Seq was focused on oncogenes and tumor suppressor gene that could serve as potential therapeutic targets. We also aimed at investigating whether UDT-Seq-identified genetic mutations might improve risk stratification beyond traditional clinicopathological RFs.

TABLE 2. Stepwise Multivariate Analyses of 5-Year Outcomes in the Entire Cohort of pN+ OSCC Patients (n = 345)

Risk Factor/(n)	Locoregional Control *P*, HR (95% CI)	Distant Metastases *P*, HR (95% CI)	Disease-Free Survival *P*, HR (95% CI)	Disease-Specific Survival *P*, HR (95% CI)	Overall Survival *P*, HR (95% CI)
UDT-Seq panel (n = 77)	0.023	0.001	0.001	0.001	0.009
	1.597 (1.067–2.388)	2.145 (1.366–3.368)	1.727 (1.235–2.414)	1.849 (1.289–2.654)	1.518 (1.109–2.078)
Extracapsular spread (n = 201)		<0.001	0.008	<0.001	<0.001
		3.070 (1.781–5.293)	1.598 (1.127–2.266)	2.084 (1.417–3.065)	1.937 (1.434–2.616)
pT3–4 disease (n = 192)		0.013	0.001	<0.001	<0.001
		1.783 (1.129–2.818)	1.725 (1.243–2.393)	2.073 (1.441–2.980)	1.838 (1.370–2.467)
Lymph node density	<0.001	0.007	<0.001	0.001	
≥0.043 (n = 204)	2.168 (1.455–3.230)	1.951 (1.198–3.179)	2.055 (1.452–2.910)	1.838 (1.269–2.662)	
Poor differentiation (n = 56)		0.001	0.044	0.008	
		2.298 (1.428–3.698)	1.487 (1.010–2.189)	1.741 (1.156–2.621)	
Margin status ≤4 mm (n = 43)	0.034		0.013		
	1.705 (1.041–2.795)		1.676 (1.116–2.517)		

CI = confidence interval, HR = hazard ratio, UDT-Seq = ultra-deep targeted sequencing.

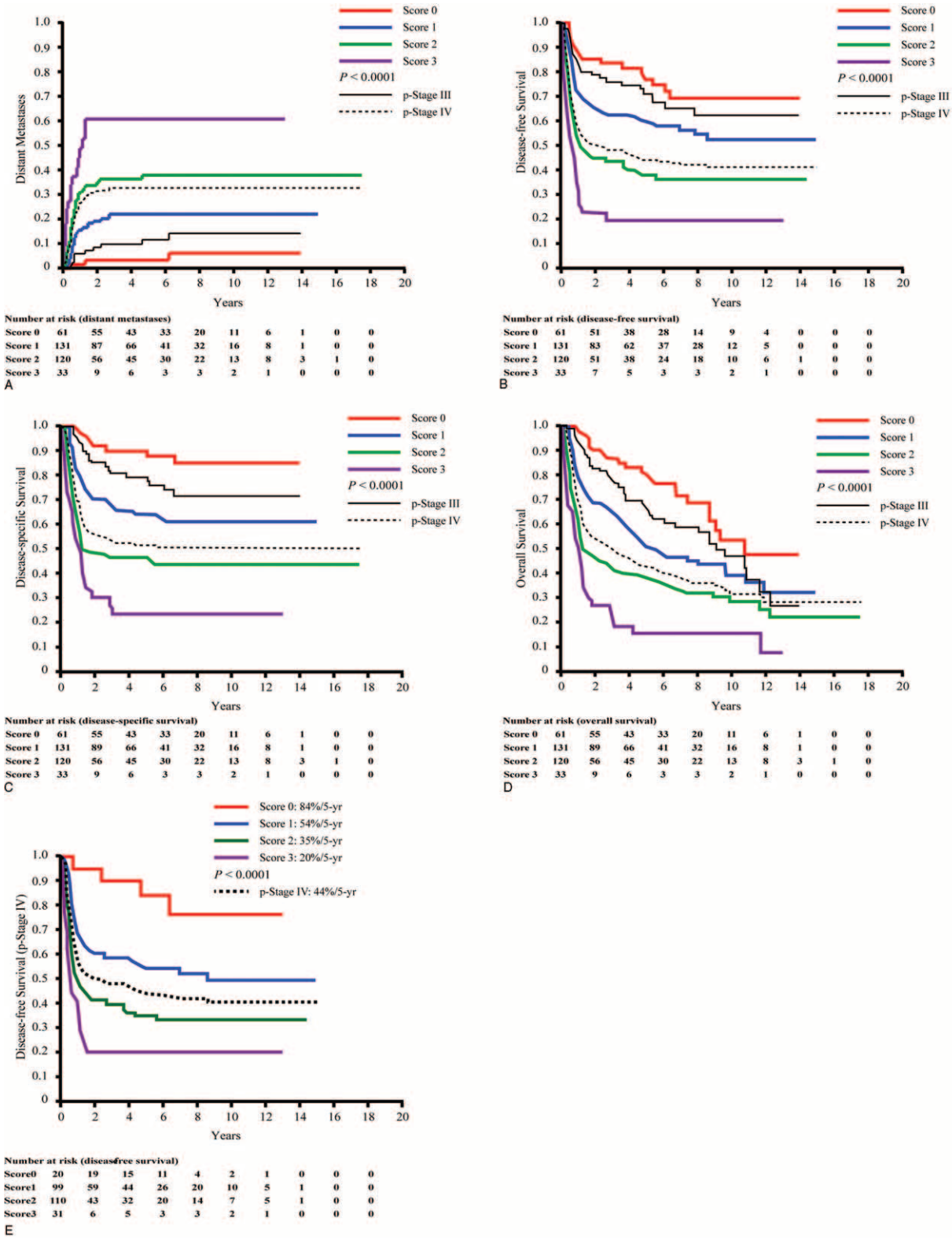


FIGURE 4. Five-year Kaplan–Meier estimates for OSCC patients according to prognostic scoring system and the AJCC p-Stage III or IV. (A) distant metastases, (B) disease-free survival, (C) disease-specific survival, (D) overall survival; according to prognostic scoring system and the AJCC p-Stage IV, (E) disease-free survival.

TABLE 3. Prognostic Value of the AJCC Staging and Different Scoring Systems (pT3–4/ECS and UDT-Seq/pT3–4/ECS)

AJCC Staging/ Risk Grouping	p-Stage III–IV/ Score	Distant Metastases (*P*; HR [95% CI])	*Disease-Free Survival (*P*; HR [95% CI])	Disease-Specific Survival (*P*; HR [95% CI])	Overall Survival (*P*; HR [95% CI])
AJCC	p-Stage III	Reference	Reference	Reference	Reference
	p-Stage IV	0.001	<0.001	<0.001	0.001
		3.166 (1.639–6.117)	2.262 (1.499–3.413)	2.519 (1.586–4.001)	1.765 (1.253–2.487)
pT3–4/ECS	0	Reference	Reference	Reference	Reference
	1	0.063	0.028	0.007	0.033
		2.095 (0.960–4.571)	1.699 (1.060–2.725)	2.195 (1.243–3.878)	1.566 (1.036–2.368)
	2	<0.001	<0.001	<0.001	<0.001
		5.399 (2.558–11.395)	3.210 (2.016–5.111)	4.610 (2.643–8.041)	3.090 (2.054–4.648)
UDT-Seq/pT3–4/ECS	0	Reference	Reference	Reference	Reference
	1	0.009	0.021	0.002	0.004
		4.863 (1.475–16.035)	1.923 (1.103–3.352)	3.359 (1.589–7.103)	2.040 (1.255–3.316)
	2	<0.001	<0.001	<0.001	<0.001
		9.534 (2.949–30.822)	3.496 (2.034–6.007)	6.327 (3.035–13.191)	3.186 (1.972–5.148)
	3	<0.001	<0.001	<0.001	<0.001
		19.741 (5.802–67.171)	6.174 (3.302–11.544)	10.962 (4.914–24.454)	5.867 (3.335–10.323)

AJCC = American Joint Committee on Cancer, CI = confidence interval, ECS = extracapsular spread, HR = hazard ratio, UDT-Seq = ultra-deep targeted sequencing.
*Statistical significance for the prediction of disease-free survival: AJCC staging ($P = 6.4 \times 10^{-5}$), pT3–4/ECS scoring ($P = 1.1273 \times 10^{-7}$), UDT-Seq/pT3–4/ECS scoring ($P = 9.5195 \times 10^{-11}$).

Among the 45 genes submitted to UDT-Seq, we identified mutations in 10 genes as significantly associated with 5-year DFS.[10] We have previously performed an internal validation of the UDT-Seq panel using 2 different resampling methods (randomization and enrollment period).[10] Here, the results obtained in the external validation using the TCGA dataset indicated a borderline statistical significance ($P = 0.0673$) in the prediction of PFS. The possible reasons for the different prognostic impact of the gene panel in distinct cohorts include, but are not limited to, the following reasons: different types of malignancies (oral SCC in our study vs head and neck cancers in TCGA), different risky oral habits (betel nut chewing in endemic in our country but not in patients enrolled in TCGA), and ethnicity (Asian countries vs Western countries). In this study, we further analyzed different outcomes using 19 clinicopathological RFs identified before as potential covariates (Table 1).[7,18] Our results indicated that the UDT-Seq panel not only predicted 5-year DFS, but also other clinical outcomes including locoregional control, distant metastases, DSS, and OS. Importantly, after allowance for potential confounders, we found that UDT-Seq panel was 1 of the 3 independent factors (the remaining 2 being pT3–4 and ECS) significantly associated with all of the survival endpoints (Table 2). ECS is widely recognized as a major adverse prognostic factor in OSCC patients.[12,19] The results of the present study indicate that the UDT-Seq gene panel combined with other independent RFs (pT3–4 and ECS) significantly improved the prognostic stratification provided by traditional AJCC staging in both pN+ patients and p-Stage IV patients (Table 3). Of note, UDT-Seq panel also identified patients with poor outcomes in both the ECS-negative and ECS-positive subgroups.

The genes signature identified in this study can be classified into 3 major pathways, that is, the RTK-RAS-MAPK pathway, the PI3K-AKT-mTOR pathway, and the NOTCH1-TGF-beta-Wnt signaling pathways. The genes in the RTK-RAS-MAPK signaling pathway include FGFR3, KIT, HRAS, and BRAF. KIT encodes for a stem cell factor receptor involved in the regulation cell shape, motility, and adhesion via cytoskeletal changes.[20] KIT mutations have been frequently reported in patients with primary adenoid cystic carcinoma of the salivary glands, but rarely in oral cavity cancer.[21] FGFR3—encoding the fibroblast growth factor receptor 3—plays a key role in mitogenesis and differentiation.[22] BRAF is a protein kinase that mediates intracellular signaling through the MAPK pathway and acts downstream to the RAS protein.[23] Although HRAS mutations are common in OSCC, BRAF gene mutations are generally found in approximately 3% of such tumors.[24] All of these genes encode for tyrosine kinase receptors that are targeted by pharmacological agents already available or currently under development.[25]

The PI3K-AKT-mTOR pathway is a prototypic survival pathway that is frequently activated in several malignancies, including OSCC.[26] Two genes involved in this pathway (AKT1 and PTEN) are significantly associated with OSCC relapse. AKT1 encodes a protein kinase that regulates apoptosis and cell cycle progression.[27] PTEN encodes a lipid phosphatase which normally suppresses activation of the PI3K-AKT-mTOR pathway and is frequently inactivated in cancer.[28] Moreover, PTEN genetic mutations have been shown to predict prognosis in patients with head-and-neck squamous cell carcinoma undergoing postoperative RT.[29]

Three differentiation-related genes (NOTCH1, SMAD4, and CTNNB1) were associated with poor survivals. NOTCH1 encodes a transcription factor which plays an important role in

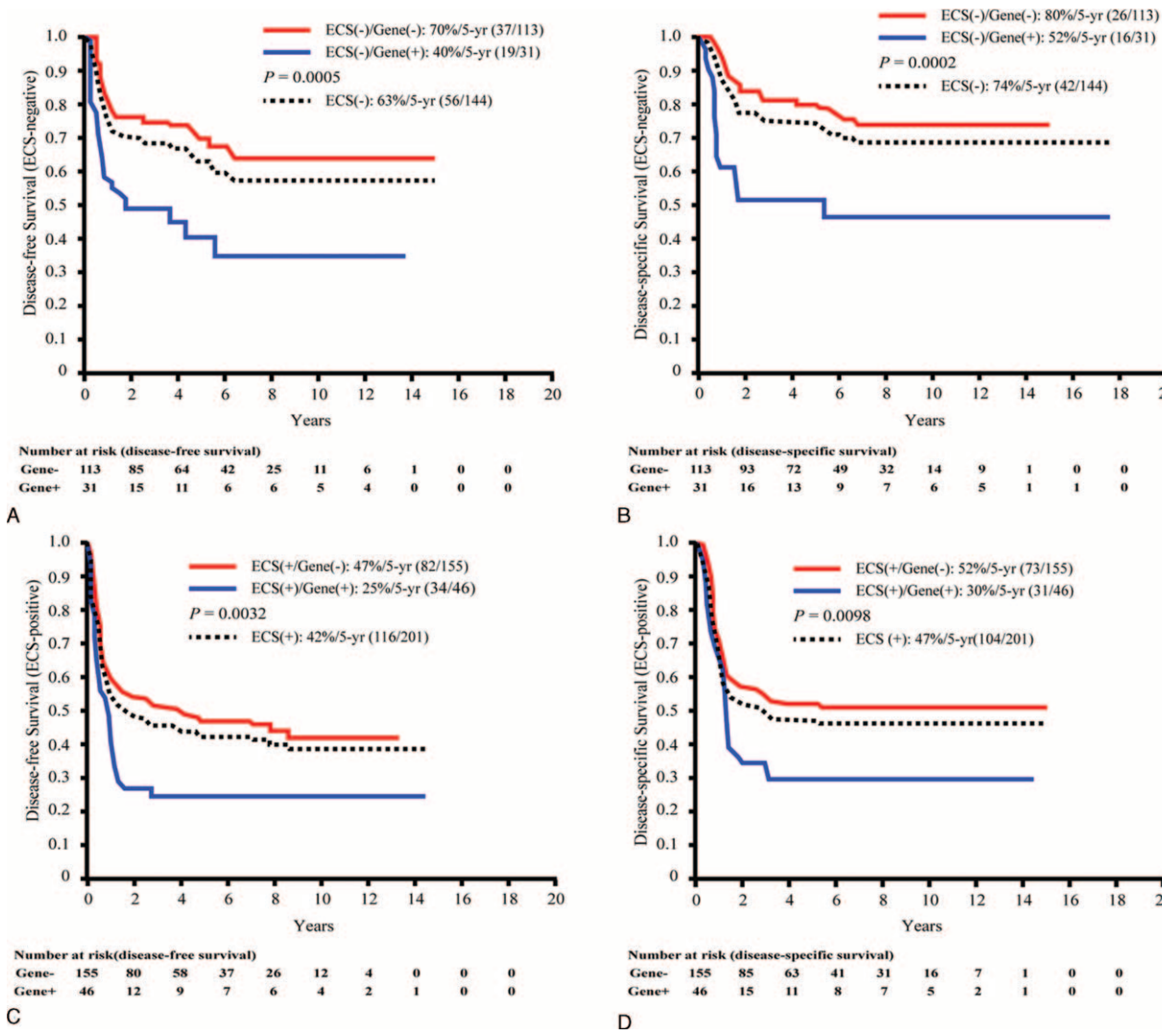


FIGURE 5. Five-year Kaplan–Meier estimates for ECS-negative patients with ([+]) and without ([−]) a positive UDT-Seq panel. (A) Disease-free survival, (B) disease-specific survival; for ECS-positive patients with ([+]) and without ([−]) a positive UDT-Seq panel, (C) disease-free survival, (D) disease-specific survival.

promoting the differentiation of squamous cells.[30] SMAD4 is a critical component of the TGF-beta signaling pathway which suppresses cell proliferation and induces cell differentiation.[31] CTNNB1 encodes a transcription factor that acts as a key mediator of the canonical Wnt signaling pathway.[32] We thus hypothesize that OSCC carcinogenesis may be linked to a dysregulated Wnt signaling (due to CTNNB1 activation and/or SMAD4 and NOTCH1 inactivation). Our results are consistent with the notion that an altered keratinocyte differentiation is critical for OSCC development.[33,34]

Strengths of our study include the large sample size of patients treated in a homogenous manner (radical surgery plus RT/CCRT for high-risk patients) and the long follow-up time. However, we acknowledge three main limitations to our report. First, our study has a long enrollment period. Consequently, the RFs used to select patients for RT/CCRT, level I–III or level I–V neck dissections, or RT techniques might have changed over time. The existence of a selection or treatment bias should not therefore be excluded. Second, the single-center, retrospective nature of the study limits the generalizability of our results. Finally, the study participants were enrolled in a betel quid chewing endemic area. Therefore, the question as to whether our findings can be applied to other populations remains open.

Resected OSCC patients sharing similar clinicopathological features may show marked differences in terms of clinical outcomes. Consequently, improved risk stratification strategies informed by novel sequencing techniques are eagerly awaited. To date, only small sized, single-center studies have searched for specific gene mutations of prognostic significance. Moreover, only a gene expression panel has been identified as having predictive value for clinical outcomes.[17] In this study, a UDT-Seq identified mutation panel was proven to predict several distinct endpoints. Notably, our panel was mainly consisted of clinically actionable genes that may serve as therapeutic targets. Importantly, the UDT-Seq-identified molecular mutations improved the prognostic stratification beyond that provided by traditional AJCC staging. Further external validation is

warrant by independent research groups to confirm the clinical usefulness of our UDT-Seq panel for OSCC targeted therapy.

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REFERENCES

1. Cancer registry annual report, 2011 Taiwan. Available from: http://www.bhp.doh.gov.tw/. Accessed October 20, 2015.
2. Liao CT, Wallace CG, Lee LY, et al. Clinical evidence of field cancerization in patients with oral cavity cancer in a betel quid chewing area. *Oral Oncol.* 2014;50:721–731.
3. Liao CT, Wang HM, Ng SH, et al. Good tumor control and survivals of squamous cell carcinoma of buccal mucosa treated with radical surgery with or without neck dissection in Taiwan. *Oral Oncol.* 2006;42:800–809.
4. Shah JP, Candela FC, Poddar AK. The patterns of cervical lymph node metastases from squamous carcinoma of the oral cavity. *Cancer.* 1990;66:109–113.
5. Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol.* 2006;42:229–239.
6. Bernier J, Cooper JS, Pajak TF, et al. Defining risk levels in locally advanced head and neck cancers: a comparative analysis of concurrent postoperative radiation plus chemotherapy trials of the EORTC (#22931) and RTOG (#9501). *Head Neck.* 2005;27:843–850.
7. Liao CT, Hsueh C, Lee LY, et al. Neck dissection field and lymph nodes density predict prognosis in patients with oral cavity cancer and pathological node metastases treated by adjuvant therapy. *Oral Oncol.* 2012;48:329–336.
8. Liao CT, Huang SF, Chen IH, et al. Outcome analysis of patients with pN2 oral cavity cancer. *Ann Surg Oncol.* 2010;17:1118–1126.
9. Patel SG, Amit M, Yen TC, et al. Lymph node density in oral cavity cancer: results of the International Consortium for Outcomes Research. *Br J Cancer.* 2013;109:2087–2095.
10. Chen SJ, Liu H, Liao CT, et al. Ultra-deep targeted sequencing of advanced oral squamous cell carcinoma identifies a mutation-based prognostic gene signature. *Oncotarget.* 2015;6:18066–18080.
11. Peng CH, Liao CT, Ng KP, et al. Somatic copy number alterations detected by ultra-deep targeted sequencing predict prognosis in oral cavity squamous cell carcinoma. *Oncotarget.* 2015;6:19891–19906.
12. National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology, Head and Neck Cancers, V.I.2015. Available from: http://www.nccn.org/. Accessed October 22, 2015.
13. Wang HM, Wang CS, Chen JS, et al. Cisplatin, tegafur, and leucovorin: a moderately effective and minimally toxic outpatient neoadjuvant chemotherapy for locally advanced squamous cell carcinoma of the head and neck. *Cancer.* 2002;94:2989–2995.
14. Tsan DL, Lin CY, Kang CJ, et al. The comparison between weekly and three-weekly cisplatin delivered concurrently with radiotherapy for patients with postoperative high-risk squamous cell carcinoma of the oral cavity. *Radiat Oncol.* 2012;7:215.
15. Harrell FE Jr, Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. *Stat Med.* 1996;15:361–387.
16. cBioPortal for Cancer Genomics. Available from: http://www.cbioportal.org/. Accessed December 7, 2015.
17. Méndez E, Houck JR, Doody DR, et al. A genetic expression profile associated with oral cancer identifies a group of patients at high risk of poor survival. *Clin Cancer Res.* 2009;15:1353–1361.
18. Kassouf W, Agarwal PK, Herr HW, et al. Lymph node density is superior to TNM nodal status in predicting disease-specific survival after radical cystectomy for bladder cancer: analysis of pooled data from MDACC and MSKCC. *J Clin Oncol.* 2008;26:121–126.
19. Liao CT, Lee LY, Huang SF, et al. Outcome analysis of patients with oral cavity cancer and extracapsular spread in neck lymph nodes. *Int J Radiat Oncol Biol Phys.* 2011;81:930–937.
20. Kijima T, Maulik G, Ma PC, et al. Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells. *Cancer Res.* 2002;62:6304–6311.
21. Vila L, Liu H, Al-Quran SZ, et al. Identification of c-kit gene mutations in primary adenoid cystic carcinoma of the salivary gland. *Mod Pathol.* 2009;22:1296–1302.
22. Webster MK, Donoghue DJ. Enhanced signaling and morphological transformation by a membrane-localized derivative of the fibroblast growth factor receptor 3 kinase domain. *Mol Cell Biol.* 1997;17:5739–5747.
23. Morrison DK, Cutler RE. The complexity of Raf-1 regulation. *Curr Opin Cell Biol.* 1997;9:174–179.
24. Koumaki D, Kostakis G, Koumaki V, et al. Novel mutations of the HRAS gene and absence of hotspot mutations of the BRAF genes in oral squamous cell carcinoma in a Greek population. *Oncol Rep.* 2012;27:1555–1560.
25. Montagut C, Settleman J. Targeting the RAF-MEK-ERK pathway in cancer therapy. *Cancer Lett.* 2009;283:125–134.
26. Freudlsperger C, Burnett JR, Friedman JA, et al. EGFR-PI3K-AKT-mTOR signaling in head and neck squamous cell carcinomas: attractive targets for molecular-oriented therapy. *Expert Opin Ther Targets.* 2011;15:63–74.
27. Hemmings BA. Akt signaling: linking membrane events to life and death decisions. *Science.* 1997;275:628–630.
28. Pezzolesi MG, Zbuk KM, Waite KA, et al. Comparative genomic and functional analyses reveal a novel cis-acting PTEN regulatory element as a highly conserved functional E-box motif deleted in Cowden syndrome. *Hum Mol Genet.* 2007;16:1058–1071.
29. Snietura M, Jaworska M, Mlynarczyk-Liszka J, et al. PTEN as a prognostic and predictive marker in postoperative radiotherapy for squamous cell cancer of the head and neck. *PLoS ONE.* 2012;7:e33396.
30. Ohashi S, Natsuizaka M, Yashiro-Ohtani Y, et al. NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. *Gastroenterology.* 2010;139:2113–2123.
31. Zhou S, Buckhaults P, Zawel L, et al. Targeted deletion of Smad4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. *Proc Natl Acad Sci USA.* 1998;95:2412–2416.
32. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell.* 2009;17:9–26.
33. Sakamoto K, Fujii T, Kawachi H, et al. Reduction of NOTCH1 expression pertains to maturation abnormalities of keratinocytes in squamous neoplasms. *Lab Invest.* 2012;92:688–702.
34. Yoshida R, Nagata M, Nakayama H, et al. The pathological significance of Notch1 in oral squamous cell carcinoma. *Lab Invest.* 2013;93:1068–1081.