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Prognostic Relevance of *TERT* Promoter Mutations in Tonsillar Squamous Cell Carcinoma in Association with Human Papillomavirus

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Abstract

Background: Telomerase Reverse Transcriptase (*TERT*) gene promoter mutation, leading to immortalization of cancer cells, is a potential candidate for pathogenesis and therapeutic target of Tonsillar Squamous Cell Carcinomas (TSCCs) in association with Human Papillomavirus (HPV). However, the prevalence of *TERT* promoter mutations and their clinical or prognostic relevance in patients with tonsil cancer in association with HPV under the new staging system is not clear.

Methods: We analyzed the *TERT* promoter mutations through real-time peptide nucleic acid-mediated PCR methods and detected the HPV status in 80 TSCC patients.

Results: The *TERT* promoter mutation was found in 7.5%, and HPV in 80.0% of the patients. We did not observe any association between *TERT* promoter mutation and the clinicopathological variables. However, *TERT* promoter mutation was identified as the independent prognostic factor for Disease-Free Survival (DFS) in TSCC patients. Interestingly, *TERT* promoter mutation had strong prognostic impacts on worse Overall Survival (OS) and DFS only in HPV-negative tumors. The HPV-negative and *TERT* promoter-mutated subgroup had the worst prognosis than other subgroups. In addition, the 8th American Joint Committee on Cancer (AJCC) staging system performed better in the discrimination and stratification of T, and N categories, and overall staging than the 7th edition.

Conclusion: Our results suggest that a combined analysis of HPV and *TERT* promoter mutation could define a representative subset of patients and prognostic groups.

Keywords: Tonsil; Squamous cell carcinoma; Human papillomavirus; *TERT* promoter mutation

Introduction

Tonsillar Squamous Cell Carcinomas (TSCC) is the most common malignant tumor among oropharyngeal cancers, representing 70% to 80% of the malignancies and the most prevalent sites of Human Papillomavirus (HPV) [1-3]. Although the incidence of TSCC is uncommon, HPV-related

oropharyngeal SCC has increased notably in young men [4]. Recently, the American Joint Committee on Cancer (AJCC) staging system for oropharyngeal cancer, published in 2017, has incorporated the HPV infection and Extranodal Extension (ENE), but whether it resulted in better stratification of these patients is unclear. Early detection of tonsil cancer, associated with a good prognosis, is not easy and usually incidental. However, a 5-year survival in early cases has been reported to be more than 90%, wherein this decreases to less than 20% in advanced tonsil cancer [5,6]. Because of the unique tonsillar anatomical features of the unnoticeable tumor spreading beneath the surface mucosa and abundant lymphatic vessel plexus around tonsils, the clinical course of TSCC is more aggressive with frequent advanced stage and early dissemination at initial diagnosis, compared with other oropharyngeal cancers [5,6]. Nevertheless, the hope remains that TSCC is relatively radiosensitive [5-7]. To date, concomitant chemotherapy (with 5-fluorouracil and cisplatin) and radiotherapy appear to be the most effective approaches for the treatment for tonsil cancers [8]; however, treatment failures develop unexpectedly as therapy-refractory recurrences. Therefore, it is important to identify molecular markers that are diagnostic and predictive of clinical outcomes to stratify patients to a more radical surgical approach or further adjuvant therapy, which ultimately reduces surgery-related morbidity and improves patient survival. Telomeres are multiplied, repeated DNA sequences located at the ends of linear eukaryotic chromosomes that serve to protect the ends against chromosomal fusion, recombination, and terminal DNA degradation [9,10]. It has been shown that DNA polymerase cannot replicate the ends of linear chromosomes, and the chromosomal ends shorten after each cell cycle in the absence of telomerase [9,10]. However, the Telomerase Reverse Transcriptase (*TERT*) gene encoding the catalytic reverse transcriptase subunit of telomerase maintains the telomere length and genomic integrity through the *de novo* synthesis of repeated telomere units at chromosomal ends [9]. Recently, highly recurrent somatic mutations in the promoter region of the *TERT* gene have been demonstrated as a driver mutation in head and neck SCC [11-15]. The *TERT* promoter, a critically important regulatory element for telomerase expression harboring binding sites for a number of transcriptional activators and repressors, contribute to increased telomerase activity that leads to immortalization of cells, which is one of the hallmarks of cancer [9,16]. The *TERT* promoter mutation, either alone or in association with the HPV oncogenes, has been shown to play an important role in the development and progression of oral and uterine cervical SCCs [12,17,18]. Two viral oncogenes, E6 and E7, expressed by high-risk HPV-associated cancers affect the oncogenic pathway related to cellular immortalization, typically activating the telomerase expression [7,17,19]. Therefore, therapy targeting the activation of telomerase could be effective against TSCC. However, the prevalence of *TERT* promoter mutations and their clinical or prognostic relevance in patients with tonsil cancer in association with HPV, the subsite of oropharyngeal cancers with the highest HPV-positive rate, remains unclear, because previous studies examined the multiple subsites of oral or oropharyngeal cancers and rarely focused on the specific TSCCs. Here, we investigated the frequencies of *TERT* promoter mutation and HPV infection in 80 Korean primary TSCC patients and analyzed their correlation with clinicopathological characteristics of patients s and evaluated their prognostic relevance. Furthermore, we assessed the performance of the 8th edition of the AJCC Cancer Staging Manual in TSCC patients compared to that of the 7th edition.

Materials and Methods

Patient characteristics

The Formalin-Fixed, Paraffin-Embedded (FFPE) tissues obtained from 80 TSCC patients who underwent primary resection, with no prior treatment and available complete medical records at our institution between 1997 and 2018 were used in the present study. Clinical information was analyzed using the medical records and radiological study results. Smoking history was measured in pack-years, and patients were classified into two categories using 20 pack-years as a cut-off value with heavy smoking defined by >20 pack-years [20]. Alcohol consumption was divided into two categories using 14 drinks/week as the cut-off value, and heavy alcohol consumption was defined by >14 drinks/week [20]. Of these 80 patients, 11 patients underwent postoperative radiotherapy, 2 patients had chemotherapy, and 39 patients had chemo-radiotherapy following the surgical resection. The remaining 28 patients were treated with surgery alone. Radiation doses ranged from 5040 cGy to 7200 cGy/36 fractions over 8 weeks. Diagnosis and histological differentiation were evaluated according to the World Health Organization classification [3]. The patient data set was re-staged according to the 8th edition of the AJCC/UICC TNM classification [20]. All patients provided informed consent before study participation, and the study was approved by the Institutional Ethics Committee.

DNA extraction and detection of *TERT* promoter mutation

Genomic DNA was extracted from 10-μm-thick sections of 10% neutral FFPE tumor tissue blocks using Maxwell® 16 FFPE Tissue LEV DNA Purification Kit for DNA (Promega, USA). Mutational analysis of *TERT* promoter gene variants flanking the C228 and C250 loci (C228 and C250) was performed by PNAClamp™ *TERT* Mutation Detection Kit (PANAGENE, Daejeon, South Korea), following the manufacturer's instructions [16]. The standard Ct values were 36 and 33 for C228, and C250 PNA mix, respectively. The *TERT* promoter gene was considered to be mutated when the ΔCt1 values were more than 2.0. When the ΔCt1 values were between 0 and 2, a ΔCt2 value was considered to be mutated if the calculated ΔCt2 value was ≤ 6. PCR amplification was optimal when non-PNA mix Ct values fell within a range of 22<Ct<30 for *TERT* promoter gene. Subsequently, the *TERT* promoter mutation analyses were confirmed by directional sequencing of PCR fragments amplified from genomic DNA. The primers used for *TERT* promoter were as follows: forward, 5′- AGTGGATTCGCGGGCACAGA -3′; and reverse, 5′-AGCACCTCGCGGTAGTGG-3′, which amplified a 346 bp fragment. The PCR amplification was carried out in a reaction volume of 30 μl containing 100 ng of template DNA, 10 × PCR buffer, 0.25 mM dNTPs, 10 pmol primers, and 1.25 U Taq DNA polymerase (Solgent, Korea). The thermal cycling conditions were: an initial denaturation step at 95°C for 3 min, followed by 10 cycles of 95°C denaturation for 30 sec, 60°C annealing for 30 sec, and 68°C elongation for 1 min. This was followed by 30 cycles under the same settings, except for the elongation step that was modified to continue for an additional 5 sec in each cycle. The PCR was completed with a final elongation step at 68°C for 7 min. PCR products were electrophoresed on 2% agarose gels and were purified with a Solgent PCR purification kit (Solgent). All amplification products were sequenced bi-directionally using an automated sequencer (ABI 3130xl genetic analyzer; Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator v1.1 kit (Applied Biosystems) and the appropriate forward and reverse primers. All sequences were replicated in duplicate, and the results

Table 1: Association between HPV and *TERT* promoter mutation and patient characteristics.

Parameter	Total	HPV			*TERT* gene promoter		
		Positive	Negative	*P*	Mutated	Wild type	*P*
	N=80 (%)	n=64 (80.0%)	n=16 (20.0%)		n=6 (7.5%)	n=74 (92.5%)	
Gender				1.000			1.000
Male	70 (87.5)	56 (87.5)	14 (87.5)		6 (100)	64 (86.5)	
Female	10 (12.5)	8 (12.5)	2 (12.5)		0 (0)	10 (13.5)	
Age (y)				0.010*			1.000
≤ 60	52 (65.0)	46 (71.9)	6 (37.5)		4 (66.7)	48 (64.9)	
>60	28 (35.0)	18 (28.1)	10 (62.5)		2 (33.3)	26 (35.1)	
Smoking				0.263			0.392
Light	33 (41.3)	32 (50.0)	5 (31.2)		1 (16.7)	32 (43.2)	
Heavy	47 (58.7)	32 (50.0)	11 (68.8)		5 (83.3)	42 (56.8)	
Alcohol				0.021*			0.667
Light	50 (62.5)	44 (68.8)	6 (37.5)		3 (50.0)	47 (63.5)	
Heavy	30 (37.5)	20 (31.2)	10 (62.5)		3 (50.0)	27 (36.5)	
Tumor location				0.173			0.651
Right side	47 (58.7)	40 (62.5)	7 (43.8)		3 (50.0)	44 (59.5)	
Left side	33 (41.3)	24 (37.5)	9 (56.2)		3 (50.0)	30 (40.5)	
pT category				0.088			0.556
T1-T2	49 (61.3)	36 (56.3)	13 (81.3)		3 (50.0)	46 (62.2)	
T3-T4	31 (38.7)	28 (43.7)	3 (18.7)		3 (50.0)	28 (37.8)	
Nodal status				<0.001*			0.333
N0	17 (21.3)	8 (12.5)	9 (56.2)		0 (0)	17 (23.0)	
N1-3	63 (78.7)	56 (87.5)	7 (43.8)		6 (100)	57 (77.0)	
AJCC stage (8^{th})				0.020*			0.624
I-III	60 (75.0)	52 (81.2)	8 (50.0)		4 (66.7)	56 (75.7)	
IV	20 (25.0)	12 (18.8)	8 (50.0)		2 (33.3)	18 (24.3)	
HPV status				-			1.000
Positive	64 (80.0)	-	-		5 (83.3)	59 (79.7)	
Negative	16 (20.0)	-	-		1 (16.7)	15 (20.3)	
BOT invasion				0.263			0.407
Present	43 (53.8)	32 (50.0)	5 (31.2)		4 (66.7)	33 (44.6)	
Absent	37 (46.2)	32 (50.0)	11 (68.8)		2 (33.3)	41 (55.4)	
Soft palate invasion				0.154			0.423
Present	28 (35.0)	25 (39.1)	3 (18.7)		3 (50.0)	25 (33.8)	
Absent	52 (65.0)	39 (60.9)	13 (81.3)		3 (50.0)	49 (66.2)	
Ipsilateral LN meta				0.004*			1.000
Present	58 (72.5)	51 (79.7)	7 (43.8)		5 (83.3)	53 (71.6)	
Absent	22 (27.5)	13 (20.3)	9 (56.2)		1 (16.7)	21 (28.4)	
Contralateral LN meta				1.000			1.000
Present	12 (15.0)	10 (15.6)	2 (12.5)		1 (16.7)	11 (14.9)	
Absent	68 (85.0)	54 (84.4)	14 (87.5)		5 (83.3)	63 (85.1)	
ENE				0.485			0.409
Present	51 (63.8)	42 (65.6)	9 (56.2)		5 (83.3)	46 (62.2)	
Absent	29 (36.2)	22 (34.4)	7 (43.8)		1 (16.7)	28 (37.8)	
P53 expression				0.540			1.000
Positive	21 (26.3)	18 (28.1)	3 (18.7)		1 (16.7)	20 (27.0)	
Negative	59 (73.7)	46 (71.9)	13 (81.3)		5 (83.3)	54 (73.0)	

HPV: Human Papillomavirus; LN: Lymph Node; BOT: Base of Tongue; AJCC: American Joint Committee on Cancer; ENE: Extranodal Extension
*Statistically significant, $P<0.05$

were marked as mutation-positive if a mutation was detected in both the forward and reverse DNA strand.

Detection of HPV

HPV status was evaluated by PANA RealTyper™ HPV Kit (PANAGENE) according to the manufacturer's instructions. Briefly, real-time PCR assays were performed in a 25-μl reaction mixture containing 19 μl of HPV mix, 1 μl of Taq DNA polymerase, and 5 μl of extracted DNA, positive control, or negative control. PCR was performed using the following conditions: 1 cycle of incubation at 50°C for 2 min and Taq activation at 95°C for 15 min; 45 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 45 sec, and extension at 72°C for 15 sec; and a melting curve step at 95°C for 5 min, 35°C for 5 min, followed by increase in temperature from 3°C to 80°C for 5 min, with a gradual increment of 0.5°C (every 5 sec) to achieve fluorescence in all the four channels (FAM, HEX or VIC, ROX, and Cy5).

Immunohistochemistry

Immunohistochemistry was carried out using 4-μm-thick FFPE sections in a BenchMark XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's protocol. After antigen retrieval, sections were incubated with the primary antibody, p53 (1:500; Novocastra, Newcastle, UK) for 40 min at 37°C, and a secondary antibody of Univeral HRP Multimer for 8 min at 37°C, followed by diaminobenzidine chromogen (Ventana Ultraview DAB Kit, Ventana) and counterstaining with hematoxylin. For assessment of p53, only nuclear staining more than 10% of tumor cells was considered positive.

Statistical analysis

Analyses of the correlations between the *TERT* promoter mutation and clinicopathological variables were carried out using the Chi-squared (χ^2) test or two-tailed Fisher's exact test. Factors found to be significant in univariate analysis were included in subsequent binary logistic regression analysis to identify independent variables associated with *TERT* promoter mutation. Survival analyses were performed using the Kaplan-Meier method and were compared using a log-rank test. Overall Survival (OS) was defined as the interval from the first day of surgery until death or the end of the follow-up period. Disease-Free Survival (DFS) was defined as the interval from the first day of surgery until tumor progression, death, or end of the follow-up period. OS and DFS were analyzed until February 2019. Univariate and multivariate analyses using the Cox proportional hazard regression model were applied to determine the Hazard Ratio (HR) and 95% Confidence Intervals (CI) for specific variables related to OS and DFS. SPSS version 18 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. A P value <0.05 was considered statistically significant.

Results

Patient characteristics

Of the 80 patients, 70 (87.5%) were predominantly male, and 10 (12.5%) were female, with the median age at diagnosis of 55 years (range 36 to 80 years). Forty-seven patients (58.7%) were heavy smokers. Thirty patients (37.5%) were heavy alcohol drinkers. Right tonsil (n=47, 58.7%) was slightly prevalent subsite compared to left tonsil (n=33, 41.3%). According to the 8th edition staging system of AJCC, 18 (22.5%) tumors were classified as T1, 31 (38.8%) as T2, 20 (25.0%) as T3 and 11 (13.7%) as T4. Of the 80 patients, 17 (21.3%) were categorized as N0, 37 patients (46.3%) as N1, 11 (13.7%) as N2, and 15 (18.7%) as N3. Combining the T and N categories the overall stage of 31 patients were diagnosed as stage I (38.8%), 18 (22.5%) as stage II, 11 (13.7%) as stage III, and 20 (25.0%) as stage IV. The median follow-up period was 64 months (range, 3 to 136 months). The 5-year OS and DFS were 53.2% and 43.8%, respectively.

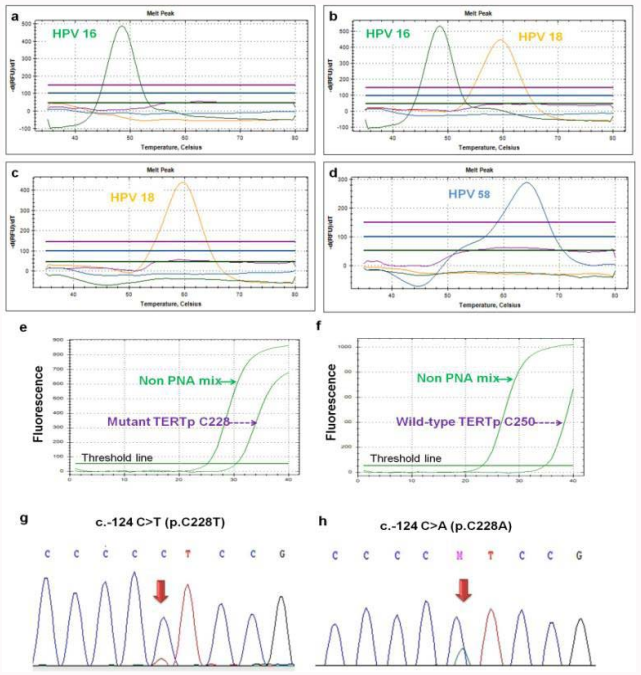

Figure 1: (a-c) HPV genotypes detected in tonsillar squamous cell carcinomas: HPV 16 (a), concurrent HPV 16 and HPV 18 (b), and HPV 18 (c). (d) HPV-negative tonsil cancers. PNA Clamp Real-Time PCR detected *TERT* promoter mutation on C228 locus (e) but not on C250 (f) in tonsillar squamous cell carcinomas. Sequence chromatography demonstrated *TERT* promoter c.-124 C>T (p. C228T) mutation (g) and a *TERT* promoter c.-124 C>A (p. C228A) mutation (h).

Frequencies of HPV and *TERT* promoter mutation and their histological findings

Only high-risk HPV genotypes were detected in 64 (80.0%) of the 80 patients. The genotypes included HPV 16 (54/64, 84.4%), HPV 18 (4/64, 6.2%), HPV 58 (1/64, 1.6%), and concurrent HPV 16 and HPV 18 (5/64, 7.8%). The real-time quantitative PCR with PNA-mediated clamping method identified six mutations in the C228 locus in the *TERT* promoter gene in 80 TSCC patients. Direct sequencing analysis also confirmed C228 locus mutations in 6 (7.5%) out of the 80 tumors with five c.-124 C>T alterations (C228T) and one c.-124 C>A alteration (C228A). However, no *TERT* promoter mutation was identified in the C250 locus and the tandem GG>AA mutation, a hallmark of ultraviolet-induced mutagenesis in melanomas [21], was also not detected. Co-existence of HPV and *TERT* promoter mutation occurred in 6.3% of the cases: a total of 5 (83.3%) among all 6 *TERT* promoter mutations were found exclusively in HPV 16-positive tonsil cancers. Representative HPV genotypes and *TERT* promoter mutations detected in the study are summarized in Figure 1. In histological review (Figure 2), HPV$^+$/*TERT* promoter wild-type (*TERTp*^{wt}) TSCC seemed to be arranged as thick trabecular sheets with mild to moderate lymphocyte infiltration predominantly in the stroma and mild tumor-lymphocyte infiltration. HPV$^-$/*TERTp*^{wt} tumors seemed to be composed of infiltrative bordered tumor nests with abundant desmoplastic stroma with scant lymphocyte

Table 2: Univariate and Multivariate analyses of overall survival and disease-free survival of patients with tonsillar squamous cell carcinoma by univariate and multivariate analyses.

	Overall survival				Disease-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
TERT gene promoter	0.984	0.983			3.879	0.007*	3.216	0.021*
Wild type *vs.* mutated	(0.234–4.146)				(1.449–10.388)		(1.197–8.644)	
HPV	0.580	0.195			0.802	0.567		
Absent *vs.* Present	(0.255–1.321)				(0.377–1.707)			
Sex	0.454	0.282			0.719	0.533		
Male *vs.* Female	(0.108–1.910)				(0.255–2.029)			
Age (y)	2.744	0.006*	4.467	<0.001*	1.813	0.075		
<60 *vs.* ≥60	(1.327–5.674)		(2.037–9.793)		(0.943–3.488)			
Tonsil side	1.396	0.363			0.917	0.794		
Rt *vs.* Lt	(0.680–2.863)				(0.478–1.758)			
Alcohol	1.371	0.394			1.064	0.852		
Light *vs.* heavy	(0.663–2.834)				(0.554–2.045)			
Smoking	1.853	0.105			1.358	0.353		
Light *vs.* heavy	(0.878–3.908)				(0.712–2.591)			
pT stage	2.745	0.007*	3.152	0.016*	2.549	0.004*	1.556	0.257
T1-2 *vs.* T3-4	(1.321–5.708)		(1.244–7.988)		(1.340–4.850)		(0.725–3.341)	
pN stage	2.651	0.110			2.821	0.050		
N0 *vs.* N1-3	(0.801–8.773)				(0.998–7.968)			
BOT invasion	2.579	0.015*	1.770	0.222	2.951	0.002*	1.823	0.159
Absent *vs.* Present	(1.206–5.513)		(0.707–4.426)		(1.504–5.791)		(0.790–4.206)	
Soft palate invasion	1.775	0.116			2.513	0.005*	1.445	0.356
Absent *vs.* Present	(0.867–3.630)				(1.323–4.771)		(0.662–3.156)	

HR: Hazard Ratio; CI: Confidence Interval; HPV: Human Papillomavirus; Rt: Right; Lt: Left; BOT: Base of Tongue
*statistically significant, *P*<0.05

infiltration and mild tumor-lymphocyte infiltration. In contrast, both HPV$^+$/*TERT* promoter mutated (*TERTp*^{mt}) and HPV$^-$/*TERTp*^{mt} tonsil cancers seemed to have abundant tumor-lymphocyte infiltration within tumor nests. Central necrosis within the tumor nests and abundant inflammatory cells in the stroma were often seen in the HPV$^+$/*TERTp*^{mt} tumors.

Correlation status of HPV and *TERT* promoter mutation with clinicopathological features of TSCC patients

We analyzed the associations of HPV or *TERT* promoter mutation with clinical and pathological features of 80 TSCCs (Table 1). The presence of HPV was more frequently associated with younger age (≤ 60 years), low alcohol drinker, pN-positive status, lower AJCC stage, and presence of ipsilateral lymph node metastasis than older age (>60 years) (*P*=0.010), heavy alcohol drinker (*P*=0.021), pN0 status (*P*<0.001), advanced AJCC stage (*P*=0.020), and absence of ipsilateral nodal metastasis (*P*=0.004). However, HPV positivity was not associated with *TERT* promoter mutation. On the other hand, there were no statistical associations between *TERT* promoter mutation and clinicopathologic features of TSCCs.

Comparisons of overall survival between the AJCC 8th and AJCC 7th staging systems

We performed Kaplan–Meier survival analyses of OS in 65 patients who had the previous TNM information according to AJCC 8th *vs.* AJCC 7th staging system (Figure 3). We compared the T category, N category, and overall stages assigned by the 8th and 7th editions of the AJCC staging system. As shown in Figure 3, the 7th edition of the AJCC staging system performed poorly with respect to the discrimination and stratification of N category and overall stages (*P*=0.063 and *P*=0.471, respectively). Only the T category was well discriminated according to clinical outcomes (*P*=0.043). In contrast, the 8th edition of the AJCC staging system for survival provided statistically significant stratification for all the T category, N category, and overall staging (*P*=0.041, *P*<0.001, and *P*<0.001, respectively). Therefore, the 8th edition of the AJCC staging system was applied in our study.

Relationship between *TERT* promoter mutation, overall survival, and disease-free survival

In Kaplan–Meier survival analyses, the patients with HPV-negative TSCCs showed poorer OS or DFS rates than those with HPV-positive TSCCs (OS, mean 51 months *vs.* 107 months; DFS, 46 months *vs.* 77 months). However, the differences were not statistically significant (*P*=0.188 and *P*=0.564, respectively) (Figure 4A,4B). *TERT* promoter mutation was found to be statistically associated with shorter DFS rates than those of *TERT* promoter wild-type (*P*=0.003), whereas no statistical difference was observed for OS between *TERT* promoter mutation and *TERT* promoter wild-type (*P*=0.983) (Figure 4C,4D). We analyzed the OS and DFS through univariate and multivariate analyses (Table 2). In the univariate analyses, older age (*P*=0.006), higher T category (*P*=0.007), and Base of Tongue (BOT)

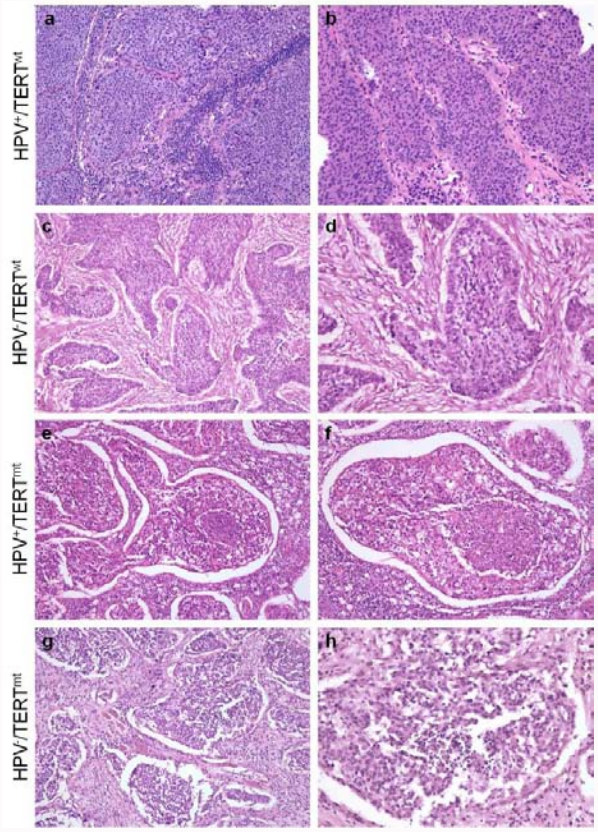

Figure 2: Representative H&E images of HPV^{+}/*TERTp*^{wt} (a), HPV^{-}/*TERTp*^{wt} (b), HPV^{+}/*TERTp*^{mt} (c), HPV^{-}/*TERTp*^{mt} (d) tonsillar squamous cell carcinomas (a, c, e, g: Magnifications, x40; b, d, f, g: Magnifications, x200).

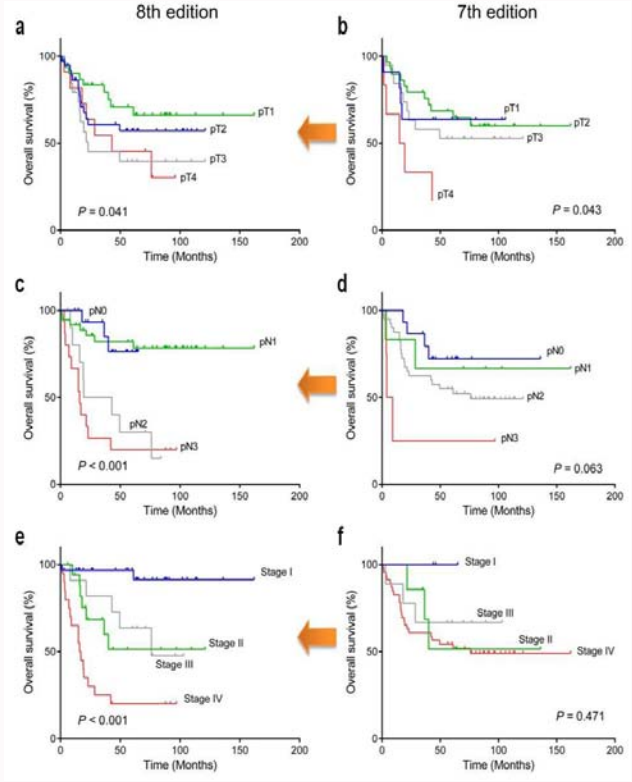

Figure 3: Overall survival analyses of tonsillar squamous cell carcinomas according to newly-revised pT category (a), pN category (c), and AJCC stage 8th (e) compared to previous pT (7th edition) (b), pN (7th edition) (d), and AJCC stage 7th (f).

invasion (*P*=0.015) were associated with shorter OS rates, while *TERT* promoter mutation (*P*=0.007), higher T category (*P*=0.004), BOT invasion (*P*=0.002), and soft palate invasion (*P*=0.005) were associated with shorter DFS rates. Multivariate analyses analyzed the variables that showed significant correlation with survival in the univariate analyses and confirmed that older age and higher T category were the independent negative prognostic factors for shorter OS of patients with TSCCs (*P*<0.001, HR: 4.467, 95% CI: 2.037–9.793; *P*=0.016, HR: 3.152, 95% CI: 1.244–7.988, respectively). On the other hand, *TERT* promoter mutation was identified to be the only independent prognostic factor for DFS in tonsil cancers (*P*=0.021, HR: 3.216, 95% CI: 1.197–8.644).

Prognostic implications of *TERT* promoter mutation in patients with concurrent HPV status

We further analyzed the prognostic impact of *TERT* promoter mutations on OS and DFS according to the HPV status. It was observed that the *TERT* promoter mutations were strongly correlated with both decreased OS and DFS in patients with HPV-negative TSCCs (*P*<0.001 and *P*<0.001, respectively), wherein showed correlation only with DFS but not with OS in patients with HPV-positive TSCCs (*P*=0.026 and *P*=0.562, respectively). We subdivided HPV and *TERT* promoter mutation status into four subgroups: HPV^{-}/*TERTp*^{mt} (n=1, 1.3%); HPV^{-}/*TERTp*^{wt} (n=15, 18.8%); HPV^{+}/*TERTp*^{wt} (n=59, 73.7%); and HPV^{+}/*TERTp*^{mt} (n=5, 6.2%). The survival analyses of the subgroups for HPV and *TERT* promoter mutation showed statistically significant survival differences for both OS and DFS (*P*=0.007 and *P*=0.007, respectively) (Figure 4E,4F). Among the four subgroups, patients in the HPV^{-}/*TERTp*^{mt} subgroup had drastically worst prognosis in terms of both OS and DFS than those in the HPV^{-}/*TERTp*^{wt} or HPV^{+}/*TERTp*^{wt} subgroup. Patients in the HPV^{-}/*TERTp*^{mt} subgroup showed shorter DFS (median 7 months) than those in the HPV^{-}/*TERTp*^{wt} subgroup (median 25 months; *P*<0.001) and the HPV^{+}/*TERTp*^{wt} subgroup (median 55 months; *P*<0.001). Taken together, the multivariate analyses revealed that the co-occurrence of HPV^{-}/*TERTp*^{mt} was a worse independent prognostic factor for both OS (*P*=0.017, HR: 17.053, 95% CI: 1.669–174.207) and DFS (*P*=0.040, HR: 10.343, 95% CI: 1.114–96.003).

Discussion

Our study aimed to investigate the prevalence of *TERT* promoter mutation in TSCCs and their role in prognosis in association with HPV, applying the newly introduced AJCC 8th staging system. In the present study, 7.5% of the 80 TSCCs exhibited *TERT* promoter mutation, all of which occurred in hot-spot C228 locus. The frequency of *TERT* promoter mutation seems to be relatively heterogeneous in the head and neck SCCs, which could be due to the ultraviolet signature of *TERT* promoter mutation in chronic sun-exposed areas [22]. *TERT* promoter mutation has been reported in 17% to 65% oral cavity SCCs [12,15,23,24]; while a single study has reported a very rare *TERT* promoter mutation in oropharyngeal SCCs [23]. We also identified that the *TERT* promoter mutations were rare in TSCCs, and our observations were more comparable to oropharyngeal SCCs rather than oral cavity SCCs. Prognostic implication of *TERT* promoter mutation in head and neck cancers appears variable depending on the regions. Our data identified the *TERT* promoter mutation as an independent prognostic factor for DFS in TSCC patients. Qu et al. [25] also reported that *TERT* promoter mutations are significant

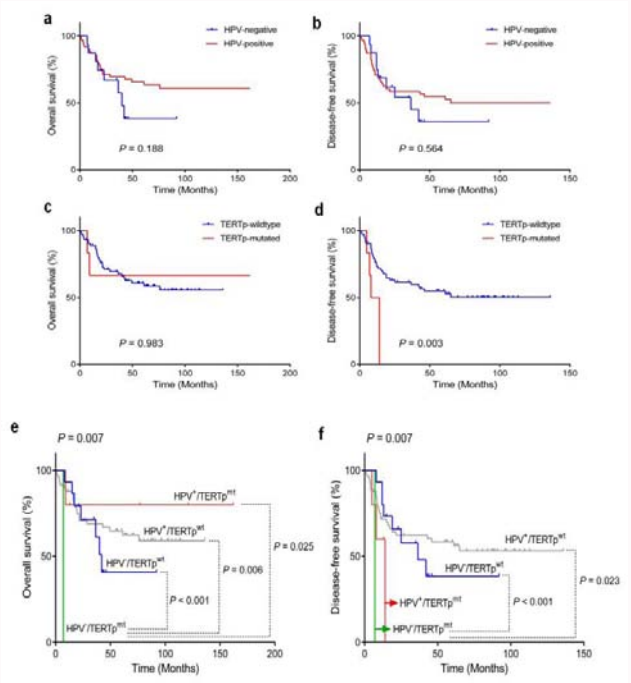

Figure 4: Human PapillomaVirus (HPV) status is not associated with overall survival (a) and disease-free survival (b) in tonsil cancers. *TERT* promoter mutation is not related to overall survival (c), whereas it has a strong prognostic impact on shorter disease-free survival (d). Analyses of combinations of HPV and *TERT* promoter mutation showed that the HPV-negative/*TERT* promoter-mutated (HPV-/*TERTp*^{mt}) subgroup has the worst survival outcomes of both overall survival (e) and disease-free survival (f) than the other three subgroups.

predictors of poor prognosis for laryngeal cancer patients. However, no such relationship with survival in oral SCCs has been reported [15]. A meta-analysis based on published articles and a cohort study reported that the *TERT* promoter mutation is associated with patient age, gender, tumor stage, or tumor grades, and tumor metastasis status, suggesting that *TERT* promoter mutation serves as an adverse prognostic factor in individuals with cancers [26]. The results of the present study suggest that the *TERT* promoter mutation could be used to monitor tumor recurrence or serve as a poor prognostic marker in tonsil cancer. We found no association between *TERT* promoter mutation and the clinicopathological variables, including HPV, specifically in TSCCs. The lack of specific clinicodemographic features related to *TERT* promoter mutation has been mainly suggested in SCCs occurred in head and neck or other sites including skin, lung, and uterine cervix [12,24,25,27]. A few studies have shown the positive correlation of *TERT* promoter mutation with increased T category in oral or head and neck SCCs, but they failed to show a significant association between HPV infection and *TERT* promoter mutation [12,24]. In the present study, although the majority (83.3%) of *TERT* promoter mutations occurred in the HPV16-positive tonsil cancers, HPV positivity was not associated with *TERT* promoter mutation. This phenomenon was also reported in the oral, oropharyngeal, and uterine cervical cancers, where all HPV16-positive oral SCCs and 70% of HPV-positive uterine cervical cancers highly harbored *TERT* promoter mutations, however, were not statistically significant [24]. A previous study showed that the TERT promoter mutation is related to HPV negativity in uterine cervical cancers and *TERT* promoter mutation in the concurrent presence of HPV has shown poorly and moderately differentiated histology [12]. Likewise, in our study, coincident HPV^{+}/*TERTp*^{mt} tonsil cancers showed non-keratinizing, poorly-differentiated squamous cell nests with abundant tumor-lymphocyte infiltration, central necrosis within the tumor nests, and abundant inflammatory cells in the stroma. The oncoproteins E6 and E7 of high-risk HPV are highly immunogenic and would be expected to induce an antitumor immune response [28]. A patient with chemotherapy-refractory, lung SCC had an exceptional clinical response to nivolumab that can quickly reset and sustain host immunity against *TERT* promoter mutation as a tumor-associated antigen [29]; indicating that *TERT* promoter mutation is also strongly immunogenic and *TERT* promoter mutation might reinforce the induction of an antitumor immune response in the HPV-associated cancers. Hence, several therapeutic approaches targeting TERT, including immunotherapies utilizing TERT as a tumor-associated antigen, and *TERT* promoter-directed cytotoxic molecules under development, would be effective in *TERT* promoter-mutated tonsil cancers [30]. Prognostic impact of *TERT* promoter mutation in HPV-positive cancers has been controversial. In the present study, we found a statistically significant association between *TERT* promoter mutation and worse OS and DFS only in HPV-negative tonsil cancers, implying that *TERT* promoter mutation might be an important driver event in HPV-negative tonsil cancers less likely affecting the prognostic effect in HPV-positive tonsil cancers. It has been shown that the HPV E6 via interactions with Myc protein cooperatively activates the *TERT* promoter and induces *TERT* mRNA transcription in genital keratinocytes, thereby increasing the cellular telomerase activity [7,19]. Wherein, HPV E7 plays a role in the maintenance of telomerase activity in stable cell lines and augments acute E6-induced *TERT* promoter activity [17], indicating that both E6 and E7 lead to increased telomerase activity. However, the HPV-induced *TERT* promoter activations seem to be prerequisite for wild-type or intact *TERT* promoter and are likely to be functionally different from the consequences of *TERT* gene promoter mutation. Usually, transcriptional repressors of *TERT* are bound to cis-elements in its core promoter, blocking transcription. Two E box cis-elements in the core promoter flank the transcriptional start site of *TERT*, and if these E boxes are mutated, TERT expression and telomerase activity are dramatically reduced [9,10]. The consequences of HPV-induced *TERT* promoter activations in uterine cervical cancers have been investigated to a large extent [7,17-19], but less information is available on downstream targets of the activated *TERT* promoter mutation in HPV-prevalent oropharyngeal cancers. Further studies are needed to validate the molecular association between *TERT* promoter mutation and HPV. The T stage, nodal status, histological grade, and pattern of invasion have been demonstrated to correlate with prognosis in oropharyngeal cancers [31,32]. Advanced T category (T3-4) has been reported to be positively correlated with decreased recurrences and survival in TSCCs [8,33]. In the present study, higher T category and older age were the independent negative prognostic factors for shorter OS of TSCC patients. We also confirmed that the 8th staging system had improved the stratification of the early and advanced stages and between T or N category concerning overall survival than the 7th edition. However, HPV infection has not been considered as an independent prognostic factor for OS and DFS in the 8th edition, a possible explanation could be their incorporation into T and N categories of the staging system, and the effect of HPV might have been shadowed by the impact of the modification of stage.

Conclusion

The present study revealed a relatively low frequency

of *TERT* promoter mutations in tonsil cancers, but the prognostic impact of *TERT* promoter mutation was strongly affected, especially in HPV-negative tonsil cancers. The combined analyses of HPV and *TERT* promoter mutation indicated that high-risk patients would substantially have treatment failure in tonsil cancers. The comparative analysis revealed that the 8th edition of the AJCC staging system for oropharyngeal cancers provided better prognostic stratifications than the 7th edition.

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