FGFR4 polymorphism, *TP53* mutation, and their combinations are prognostic factors for oral squamous cell carcinoma

JUN-ICHI TANUMA¹, TOSHIYUKI IZUMO², MASATO HIRANO¹, YOSHITAKA OYAZATO¹, FUMIYA HORI¹, ERI UMEMURA¹, HAYASE SHISA², HIROSHI HIAI³ and MOTOO KITANO⁴

¹Department of Oral Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544; ²Department of Pathology, Saitama Cancer Center Research Institute, Ina, Saitama 362-0800; ³Shiga Medical Center, Moriyama, Shiga 520-8501; ⁴Department of Medical Research and Clinical Pathology, Nakatsu Municipal Hospital, Nakatsu 871-8511, Japan

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Abstract. The genotype of the fibroblast growth factor receptor 4 (FGFR4) gene and TP53 mutation have been reported as prognostic factors for cancers of the head and neck, bladder, breast and colon. To determine whether they are applicable for oral squamous cell carcinoma (OSCC), we investigated these two genes in OSCC samples from 150 patients who had undergone radical surgery and in 100 cancer-free individuals. In OSCC, the FGFR4 Gly388Arg polymorphism and the presence or absence of mutation in TP53 did not show a significant association with the clinicopathological features of the tumors at surgery. However, the FGFR4 Arg388 allele, as well as mutations in TP53, was found to be closely associated with poor prognosis. Moreover, these two parameters synergistically affected the survival of OSCC patients. During 60 months of observation after radical surgery, a majority of patients with homozygous Arg388 FGFR4 plus mutated TP53 died of cancer, whereas >90% patients carrying homozygous Gly388 FGFR4 plus wild-type TP53 survived. Therefore, the FGFR4 Gly388Arg polymorphism and TP53 mutations, as well as their combinations, are excellent predictors of the prognosis for OSCC patients.

Introduction

More than 500,000 people are diagnosed every year worldwide with head and neck cancer, including the cancers of the larynx, nasal passages/nose, oral cavity and pharynx (1). Oral squamous cell carcinoma (OSCC) is prone to local recurrence and distant metastasis. These properties are major hazards to establish an effective therapeutic strategy. To explore reliable molecular parameters to predict OSCC prognosis, we selected two genes. The mutations in the TP53 gene, which occur in >50% of OSCC, especially in advanced tumors (2,3), and the polymorphism in the FGFR4 gene, which is a member of the family of fibroblast growth factor receptors (FGFRs). FGFRs are shown to interact with >20 known ligands and to display multiple biological activities, including mitogenic and angiogenic activities. Dysregulation of this pathway has been demonstrated in several tumor types (4-6). In the human FGFR4 gene, a single nucleotide polymorphism in exon 9 results in an amino acid change (Gly388Arg) in the transmembrane domain. The FGFR4 Arg388 allele has been reported to be a promising prognostic factor in some human cancers, including cancers of the breast (6,7), colon (7), lung (8), prostate (9) and head and neck (10,11) and in soft tissue sarcomas (12). However, its significance is still controversial (13, 14).

To evaluate the prognostic significance of *FGFR4*, *Tp53* and their combinations in OSCC, we studied the genotypes of these genes in Japanese OSCC patients and control individuals, the clinicopathological parameters and the prognosis. Possible correlations among these parameters were analyzed.

Materials and methods

Patients. The study included patients suffering from OSCC who underwent surgery at Saitama Cancer Center Hospital and control patients without OSCC enrolled at the same Institution and the Kagoshima University Hospital. Clinicopathological data for all OSCC patients are summarized in Table I.

The group of OSCC consisted of 150 individuals (80 males and 70 females) aged 35 to 80 years (median 55.5) with primary cancers of the tongue (77), gingival (35), buccal mucosa (28) and floor of the mouth (10). The age of the 100 controls (55 males and 45 females) ranged from 31 to 85 years (median 60.2).

Genomic DNAs were extracted from the fresh tumor tissues and the peripheral lymphocytes in blood samples. For the control, genomic DNAs were obtained from the peripheral

Correspondence to: Dr Jun-ichi Tanuma, Department of Oral Pathology, Field of Oncology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan E-mail: tanuma@dent.kagoshima-u.ac.jp

Key words: fibroblast growth factor receptor 4, polymorphism, *TP53*, oral squamous cell carcinoma

Characteristics No. of patients (%) Gly/Gly Gly/Arg Arg/Arg Yes ^a All patients (%) 150 (100) 69 (46.0) 53 (35.3) 28 (18.7) 55 (36.7) Age (yrs) 33 25 13 25 >55 79 (52.7) 36 28 15 30	No 95 (63.3) 46 49 725 47 48	
All patients (%) 150 (100) 69 (46.0) 53 (35.3) 28 (18.7) 55 (36.7) Age (yrs) 33 25 13 25 >55 71 (47.3) 33 25 13 25 >55 79 (52.7) 36 28 15 30	95 (63.3) 46 49 725 47 48	
Age (yrs)<55	46 49 725 47 48	
<5571 (47.3)33251325>5579 (52.7)36281530	46 49 725 47 48	
>55 79 (52.7) 36 28 15 30	49 725 47 48	
	47	
P-value 0.991 0.	47	
Gender	47	
Male 80 (53.3) 36 24 20 33	10	
Female 70 (46.7) 33 31 8 22	40	
P-value 0.078 0.1	0.213	
Smoking status		
Current 50 (33.3) 20 16 14 15	35	
Former 75 (50.0) 35 30 10 30	45	
Never 25 (16.7) 14 7 4 10	15	
P-value 0.238 0.	0.487	
Tumor site		
Tongue 77 (51.3) 32 30 15 34	43	
Another region 73 (48.7) 37 23 13 21	52	
P-value 0.516 0.	061	
T classification		
T1 + T2 49 (32.7) 29 13 7 20	29	
T3 + T4 101 (67.3) 40 40 21 35	66	
P-value 0.078 0.	462	
Mode of invasion		
Grade 1 + 2 45 (30.0) 25 14 6 21	24	
Grade 3 + 4 105 (70.0) 44 39 22 34	71	
P-value 0.275 0.	101	
Vascular invasion		
Positive 59 (39.3) 25 22 12 22	37	
Negative 91 (60.7) 44 31 16 33	58	
P-value 0.767 0.	898	
Lymph node involvement		
Positive 51 (34.0) 25 16 10 23	28	
Negative 99 (66.0) 44 37 18 32	20 67	
P-value 0.766 0.	124	

Table I. Correlation among the FGFR4	polymorp	phism, TP53 mutation and	d clinicopathologica	al features of OSCC	patients
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^aYes means the number of cases carrying any of the TP53 mutations.

lymphocytes in blood samples or stored specimens fixed in formalin and embedded in paraffin using the QIAamp Tissue Extraction Kit (Qiagen, Chatsworth, CA, USA).

The present study was performed under approval of the Ethics Committees of both the Saitama Cancer Center (No. 49) and the Kagoshima University Dental School (No. 1).

Genotyping. Samples were automatically genotyped for the FGFR4 Gly388Arg polymorphism (rs351855) employing

the ABI Prism SNaPshot Multiplex kit according to the manufacturer's recommendations (ABI, Foster City, CA, USA). Briefly, the genomic DNA flanking the SNP was amplified with PCR with forward primer AGG TGT GGG TGC CTG GGA CT and reverse primer GGG AAC TCC CAT AGT GGG TCG. The PCR mix contained 25 ng of genomic DNA, 1 μ l of 10X PCR Gold buffer, 200 μ M dNTPs, 3 mM MgCl₂, 0.5 U of AmpliTaq Gold Polymerase (ABI) and 1 pM of each primer. PCR was carried out as follows: 10 min at 95°C for 1 cycle

Genotype	No. of patients	No. of patients who died	Harzard ratio ^a	95% CI	P-value
FGFR4 at amino acid 388					
Gly/Gly	69	18	1		
Gly/Arg	53	25	1.15	0.58-2.49	0.048
Arg/Arg	28	17	1.43	1.15-3.01	0.025
TP53					
Wild-type	95	25	1		
Mutant	55	35	1.38	1.21-2.85	0.031
FGFR4: TP53					
Gly/Gly: Wild-type	38	3	1		
Gly/Gly: Mutant	31	15	1.29	0.68-2.93	0.067
Gly/Arg: Wild-type	40	15	1.31	0.99-2.56	0.065
Gly/Arg: Mutant	13	10	1.39	1.11-2.64	0.046
Arg/Arg: Wild-type	17	7	1.41	1.21-2.96	0.028
Arg/Arg: Mutant	11	10	1.69	0.87-2.87	0.001

Table II. Hazard ratios of death for the FGFR4 polymorphism and TP53 mutation status in OSCC patients.

and 35 cycles at 95°C for 30 sec, 58°C for 40 sec and 72°C for 1 min, followed by 1 cycle at 72°C for 7 min. After amplification, the PCR products were treated with 1 unit each of shrimp alkaline phosphatase (SAP) (Roche) and exonuclease I (USB Corporation) at 37°C for 60 min and 72°C for 15 min to purify the amplified products. One μ l of the purified amplification products was added to SNaPshot Multiplex Ready reaction mixture containing 0.15 pmol of genotyping primer CTG CCC TCG ATA CAG CC for primer extension reaction. The primer extension reaction was carried out for 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec. The reaction products were treated with 1 unit of SAP at 37°C for 1 h and 72°C for 15 min to remove excess fluorescent dye terminators. One μ l of the final reaction samples containing the extension products was added to 9 μ l of Hi-Di formamide (ABI). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by electrophoresis in ABI 310. Results were analyzed using GeneScan analysis software (ABI).

PCR-SSCP analysis and DNA sequencing. Single-stranded conformation polymorphism (SSCP) analysis was used to analyze tumor samples for mutations within exons 4-10 of the *TP53* gene as described (15,16). Cases displaying an altered electrophoretic mobility were reamplified and analyzed by direct sequencing of both strands to confirm and characterize the nature of the mutation.

Statistical analysis. Molecular genetic analysis was performed without knowledge of the clinical data, which were disclosed after completion of the marker analysis. Logistic regression was used to compute odds ratios (ORs) and 95% confidence intervals (CIs). The power of the study design was calculated according to Dupont and Plummer (17). The Kaplan-Meier

product limit method and the log-rank test were used to estimate survival functions (18,19). Hardy-Weinberg equilibrium was tested by the Chi-square method (20). Based on the extracted binary data, odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated for the association between the *FGFR4* polymorphism and *TP53* mutations. Statistical procedures were carried out with SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software (21).

Results

Genotype of FGFR4 and TP53 in OSCC patients. The allele types of FGFR4 amino acid 388 in 150 OSCC patients were Arg/Arg (15.3%), Arg/Gly (38.7%) and Gly/Gly (46.0%), while those in 100 control individuals were Arg/Arg (10.0%), Arg/Gly (48.0%) and Gly/Gly (42.0%). The odds ratio for the OSCC patients vs. control for the Arg388 allele was 1.15 (95% CI, 0.5-2.1). Therefore, the risk for OSCC development did not show an association with the FGFR4 genotype. In addition, the FGFR4 Gly388Arg genotypes did not show any obvious correlation with the clinicopathological parameters at the time of initial treatment (Table I).

On the other hand, the mutations of the *TP53* gene were detected by SSCP in 55 of 150 OSCC samples (36.7%). There were eight types of mutations of the *TP53* gene in these samples: 9 cases in exon 5 (TGC/TAC transition at codon 135, leading to Cys/Phe substitution), 8 in exon 5 (CCG/CTG at codon 152, Pro/Leu substitution), 5 in exon 5 (CGC/CAC at codon 17, Arg/His substitution), 4 in exon 6 (TTG/TAG at codon 201, Leu/stop codon), 10 in exon 7 (CGC/AGC at codon 245, Gly/Ser substitution), 8 in exon 8 (CGT/TGT at codon 273, Arg/Cys substitution), 10 in exon 9 (CAG /TAG at codon 317, Gly/stop codon). Although these mutations



Figure 1. Kaplan-Meier survival curves in OSCC patients. The solid lines represent wild-type *TP53* and the broken lines, mutant *TP53*. (A) *FGFR4* allele at codon 388 Gly/Gly. Log-rank analysis indicates significant differences between the patients with wild-type *TP53* and those with mutant *TP53* mutations (P=0.030). (B) *FGFR4* allele, Gly/Arg. The difference between wild-type and mutant *TP53* was significant (P=0.035). (C) *FGFR4* allele, Arg/Arg. The difference between wild-type and mutant *TP53* was significant (P=0.012). The difference between the patients having the *FGFR4* allele Gly/Gly and wild-type *TP53* and those having the *FGFR4* allele Arg/Arg and mutant *TP53* was highly significant (P=0.001).

caused amino acid substitutions or the generation of a stop codon, none of them was specifically associated with the clinicopathological parameters at the initial treatment.

Prognosis of OSCC patients by genotypes. Of the 150 patients who underwent a radical operation, 60 (40.0%) died of OSCC recurrence within the 60 month follow-up period. The Cox proportional hazard analysis of survival, adjusted for gender, smoking and age at diagnosis (in decennia) showed that the patients carrying FGFR4 allele Gly/Arg or Arg/Arg at amino acid 388 exhibited a much higher hazard ratio (HR) than the patients with FGFR4 allele Gly/Gly (Table II). The median survival period for Arg allele carriers was 34 months, whereas that for Gly/Gly OSCC patients was 78 months. Table II also shows that the patients with OSCC carrying the TP53 mutation had higher HR (1.38) than those with wild-type TP53. There were minor differences in the survival rate among the types of TP53 mutation, but the number of each type was insufficient for statistical analysis. In this study, therefore, all the TP53 mutations were handled as a single group.

Combined prognostic effects of FGFR4 and TP53. The genotype combinations of both markers showed stronger association with HR (Table II) and survival rates (Fig. 1) than the genotype of each marker. Fig. 1 shows the survival curves of patients with FGFR4 of (A) Gly/Gly allele, (B) Gly/Arg, or (C) Arg/Arg allele. In all three groups, the patients having a TP53 mutation showed a significantly lower rate of survival than those with wild-type TP53 by Kaplan-Meier analysis. The most striking difference was seen between the patients with FGFR4 Arg/Arg plus TP53 mutations (median survival time = 20.5 months) and the patient group with FGFR4 Gly/Gly plus wild-type TP53 (median survival time = 34.7 months).

Discussion

The present study demonstrated that two genetic parameters, the *FGFR4* Gly388Arg polymorphism and mutations in *TP53* and their combinations are significant predictors for the fate of OSCC patients after radical surgery.

The mechanism by which FGFR4 affects cancer progression is poorly understood. FGFR4 is implicated in signaling cascades with regard to cell-matrix adhesion and angiogenesis. These functions include the up-regulation of a urokinase-type plasminogen activator (uPA), a proteolytic enzyme, required for cell migration (22) and the association with N-CAM in cell adhesion to the extracellular matrix (23). Not surprisingly, abnormal expression of *FGFR4* has been suggested as a potential mechanism in the progression of cancers in several organs (24,25). On the other hand, its single nucleotide polymorphism at codon 388 has been reported to be relevant to cancer prognosis. Bange et al (6) reported that the progression, tumor cell motility, metastasis and reduced disease-free survival of breast cancer patients are associated with the FGFR4 Arg388 allele, although this finding remains controversial (7,14,23). In prostate cancer (9), lung cancer (8), colorectal cancer (7), head and neck cancers (10,11) and soft tissue sarcomas (12), the presence of the FGFR4 Arg388 allele has been shown to indicate a poor

prognosis. The present data confirmed that it is a promising determinant for the prognosis of an OSCC patient. Although there was no significant correlation between the genotype of *FGFR4* and the clinicopathological parameters of OSCC at the first treatment, the presence of the *FGFR4* Arg388 allele reduced the patient survival rate in a dose-dependent manner.

The *FGFR4* Gly388Arg polymorphism results in an amino acid change in the transmembrane domain, a highly conserved region for receptor tyrosine kinase. Analogous missense mutations in the transmembrane domain in the *FGFR3* gene, resulting from a Gly to Arg substitution at codon 380, were proposed to result in constitutive activation of *FGFR3* signaling (26). In general, activated *FGFR3* mutations are associated with favorable disease characteristics, such as low stage and grade, low recurrence rate and a lower mortality rate (27,28). Therefore, the *FGFR4* Arg388 allele could augment the aggressive behavior of cancer cells in OSCC, although it remains obscure if *FGFR4* Arg388 is more activated than its Gly388 form.

It has been previously noted that the *FGFR4* Arg388 allele increases *FGFR4* expression (6). Streit *et al* (10) reported that *FGFR4* expression *per se* was not associated with a worse prognosis in head and neck SCC. However, among the patients with high *FGFR4* expression, the overall survival was significantly more reduced in those with the Arg388 allele than in those with the Gly allele. Although we did not evaluate the expression of *FGFR4* in the present series, further study should be conducted to determine whether the role of the *FGFR4* polymorphism in OSCC progression is the result of an activated function of the receptor, increased expression, or their combination.

We also showed the *TP53* mutations increased the hazard ratio and reduced the survival rate of OSCC patients. The importance of the *TP53* tumor suppressor gene in the process of carcinogenesis is well established. *TP53* controls several key pathways that protect cells from malignant transformation. Approximately 40-50% of OSCC has alternations in *TP53* (16), which regulates the cellular stress response pathway that has been shown to be critical for the maintenance of genomic integrity. In particular, a high incidence of the *TP53* mutation has been demonstrated in tobacco-related cancers (29-31).

The most striking finding of this study is that these two predictors act jointly to reduce the survival rate of OSCC patients. The observation supports the hypothesis in which the *FGFR4* and *TP53* gene mutations may represent alternative genetic pathways in the progression of OSCC. The joint action of the *FGFR4* genotype and *TP53* has also been reported in bladder cancer patients (32). In this case, neither the *FGFR4* polymorphism nor the *TP53* mutation status was an independent predictor of prognosis, but a combination of *FGFR4* Gly388 plus *TP53* mutation is associated with a poor probability of survival. The apparently reverse function of *FGFR4* alleles on cancer progression suggests that their biological action may be organ-specific.

In conclusion, the *FGFR4* polymorphism and *TP53* mutation status are useful prognostic factors for OSCC. Their combination, in particular, is a highly reliable and useful predictor in clinical situations.

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