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Association Analysis of TP53 Mutations in Codon R175 And G245 With Histopathological Grading Outcomes in OSCC Patients

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a malignant tumor lesion in the oral cavity area with less than 50% five-year survival rate for decades. Mutation of the TP53 gene in codons R175 and G245 is a reliable genetic diagnostic for prognosis and prediction of therapeutic response. However, the association of these mutations with the histopathological grading of the OSCC is still unknown. This study aimed to see the association between TP53 mutations in codon R175 and G245 with histopathological grading outcomes in OSCC patients. Forty-four FFPE samples were stained with hematoxylin-eosin to observe their histopathological grade. The mutation was tested through the PCR-RFLP method using Hha1 and Aci1 enzymes. Data recorded were analyzed using Cramer's V association. This study showed 43.2% of heterozygous mutations in codon R175 and 4.5% of heterozygous mutations in codon G245. Very strong significant associations were found between the histopathological grading result and the mutational status of both TP53 codons R175 (V=0.631; p=0.000) and codons G245 (V=0.303; p=0.044). To be concluded, there was a very strong significant association between the mutational status of TP53 on both codons R175 and G245 with the histopathological grading outcome in OSCC patients.

Keywords: Cancer; OSCC; G245; R175; RFLP; TP53

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a malignant tumor lesion in the oral cavity area [1]. It is a subset of head and neck squamous cell carcinoma (HNSCC) which placed sixth in the cancer incidence rate in the world. At least 354,864 OSCC cases are found worldwide every year, with 177,384 deaths observed [2]. There are several sites where OSCC can arise, such as the tongue, gingiva, buccal surface, palatal, floor of the mouth, and lips. Compared to other sites, the tongue area has the highest occurrence with the worst prognosis outcome [3].

Despite the advancing technology for the treatment and drug discovery in the cancer field, OSCC has kept its high death risk with less than 50% of five-year survival rate for decades [3,4]. To date, the prognosis and the treatment plan of OSCC are often decided through its location and TNM staging system [5].

However, several findings found some unsatisfactory results of OSCC treatment, with early-stage lesions may result in poor outcomes [6]. Thus, a reliable biomarker with better effectivity is needed to decide the prognosis of OSCC.

TP53 is a gene that codes p53 tumor suppressor protein, which plays a vital role in suppressing tumor formation and guarding the DNA damage through stimulating cell cycle arrest, DNA repair, or apoptosis [7,8]. Mutation of this gene leads to losing its function as a tumor suppressor. Instead, it can gain new function as a pro-oncogene and clinically increase the tumor progressivity [9]. Recent findings show the potential of TP53 mutation in codons R175 and G245 to be a reliable genetic diagnostic for prognosis and prediction of therapeutic response, as both mutations are strongly linked to increasing progressivity [9], poor survival [10], and tumor resistance to radiation and chemotherapy [5].

However, the association of these mutations with the histopathological grading of the OSCC is still unknown. Thus, this study aimed to see the association between TP53 mutations in codon R175 and G245 with histopathological grading outcomes in OSCC patients.

MATERIALS AND METHODS

This study was approved by the ethical committee of Dr. Soetomo General Hospital (No. 0111/KEPK/XII/2020). Forty-four formalin-fixed paraffin-embedded (FFPE) samples were obtained from the Department of Anatomical Pathology of Dr. Soetomo General Hospital, Surabaya, based on the data of OSCC patients who received treatment in the Department of Surgery from 2014 to 2020. Each FFPE sample was sliced using a rotary microtome (Leica Biosystems, IL, AS) into four slides: one slide for hematoxylin-eosin (HE) staining for histopathological grading examination, and the other three were subjected to TP53 mutational detection using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method.

Histopathological grading examination

The slide prepared for histopathological examination was subjected to HE staining [11]. Before the staining, the slides underwent deparaffinization and rehydration to allow good preparation for staining. The slides were then incubated with hematoxylin for 5 minutes and washed with distilled water. The step was continued by eosin incubation for 5 minutes and rewashed. After the staining process, the slides were cleared with xylene and mounted with cover glass using a mounting medium [11].

The slides were observed under a light microscope (Olympus, Tokyo, Japan) for the histopathological grading examination at 100x magnification. The grading criteria followed the previous study[12], which classified the OSCC into low-grade malignancy and high-grade malignancy.

DNA extraction

Hematoxylin eosin-stained slides were used as the guide to cut the tumor cell for the DNA extraction tissue preparation. The area of tumor in the HE slide was outlined and drawn on paper, and the slides subjected to PCR-RFLP were cut out using a scalpel following the marking area[13]. The cut tissues were put into microtubes. The DNA extraction was conducted using Qiamp DNA FFPE Tissue Kit (ref 56404; Qiagen, Hilden, Germany) following the manufacturer instruction, except the proteinase K incubation, which was conducted for overnight[14].

At the end of the DNA extraction process, 100 µL of DNA samples were obtained. The DNA samples were then subjected to Polymerase Chain Reaction (PCR), followed by Restriction Fragment Length Polymorphism (RFLP) examination to detect the possible TP53 mutations in codons R175 and G245.

PCR-RFLP examination

The DNA amplification process was conducted using the same PCR reaction of 20 µL mixture containing: 12,5 µL master mix (Cat#M7122; Promega, Madison, USA), 3 µL genomic DNA templates, 2,5 µL nuclease-free water (NFW) (Promega, Madison, USA) 1 µL (10 pM) each for forward and reverse primers. The forward and reverse primers to examine PCR-RFLP codons R175 and G245 were made in Integrated DNA Technologies (IDT), Singapore, with the sequence details in Table 1. The PCR program used was pre-denaturation at 94 °C for 3 min, followed by 45 amplifications of denaturation at 94 °C for 30s, annealing following the specific temperature on Table1 for 30s, and extension at 72 °C for 1 min. The final extension ended the protocol at 72 °C for 5 min. The PCR amplified products were electrophoresed on 2.5% agarose gels stained with ethidium bromide for 30 min at 100volt to confirm the amplification successful by observing the specific band length (Table 1) under UV transilluminator (ATTO, Tokyo, Japan). For results showing the presence of non-specific bands were subjected to gel purification using QIAquick Gel Extraction Kit (ref. 29704; Qiagen, Hilden, Germany).

The RFLP process was conducted following the previous studies with slight modifications [15,16]. The PCR and gel purification products were processed in 20µl RFLP reaction mixture with different ratios for each codon examination. To examine mutation in codon R175, the mixture containing 12µL of PCR products, 2µL of buffer 10X, 5µL of NFW, and 1µL (20 unit) of restriction enzyme HHa1 was put into a microcentrifuge tube and incubated at 37 °C for 150 min. To examine mutation in codon G245, the mixture containing 12µL of PCR products, 2µL of buffer 10X, 5.5µL of NFW, and 0.5µL (5 unit) of restriction enzyme Aci1 was put into a microcentrifuge tube and incubated overnight at 37°C. The results of the RFLP process were observed under UV light with the visualization of two bands for wild-type codons, three bands for heterozygous mutation in respective codons, and one band with the length of original PCR product for homozygous mutation. For the length of the respective bands, see Table 1.

TABLE 1: Primer, annealing temperature, RFLP enzyme, and band lengths

No.	Codon	Primer*	Annealing (°C)	RFLP Enzyme	Length (bp)
1	R175	F 5'-GCA CCC GCG TCC TCG CCA TG-3' R 5'-GCT CCA CCA TCG CTA TCT GAG CAT CG-3'	65	Hha1	PCR result 106 After cleaved 64+42
2	G245	F 5'- TAG GTT GGC TCT GAC TGT ACC A -3' R 5'- TGT GAT GAG AGG TGG ATG GGT A -3'	58	Aci1	PCR result 236 After cleaved 174+62

*Primer for codon R175 was cited from Srividya et al[15] and for codon G245 was cited from Almeida et al[32]

Data analysis

The recorded data were analyzed using SPSS ver. 21 (IBM, New York, USA). The frequencies were descriptively tabulated, and the association between mutational status for each codon and the histopathological grading was tested through Cramer's V. To know the association strength, the V values were compared to the association scale in Table 2 [17], while the significant value was $p<0.05$.

TABLE 2: Interpretation of Cramer's V

Cramer's V	Interpretation
>0.25	Very strong
>0.15	Strong
>0.10	Moderate
>0.05	Weak
>0	No or very weak

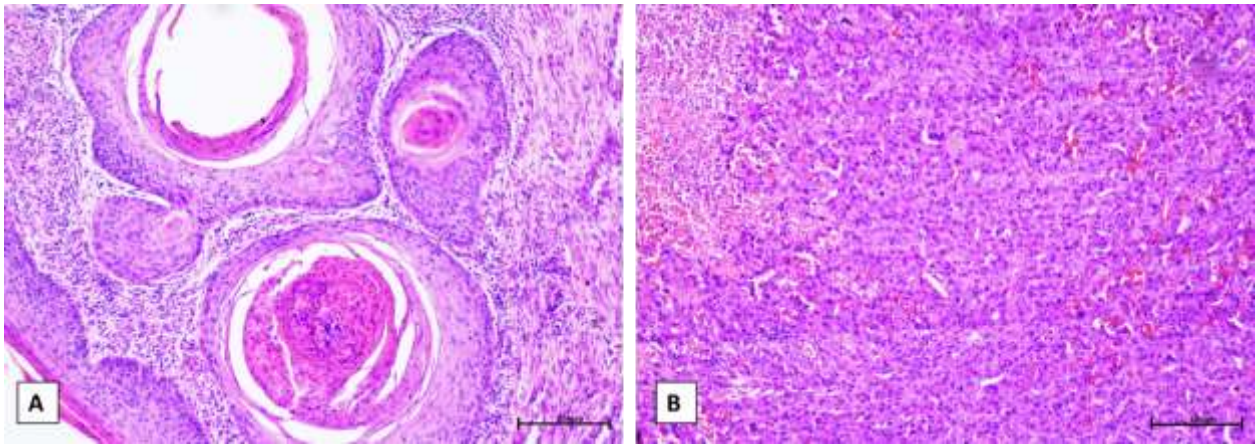

FIGURE 1: The histopathological grading of OSCC at 100x magnification.
(A) Low-grade malignancy showed well-differentiated OSCC with keratin pearl formation inside the epithelial island.
(B) High-grade malignancy with poorly differentiated manifestation showed no keratinization.

RESULTS

After being classified based on the grading criteria from previous study[12], there were 29 cases considered low-grade malignancy, which were incorporated by well-differentiated OSCC. The rest 15 cases were high-grade malignancy with poor differentiation of OSCC (Figure 1).

For the mutational status, from the visualization under UV light, only wild-type and heterozygous mutation was found in codons R175 and G245 (See Figure 2). No homozygous mutation observed in both codons. There are 43.2% of heterozygous mutation of codon R175. For codon G245, only 4.5% heterozygous mutation observed (Table 3).

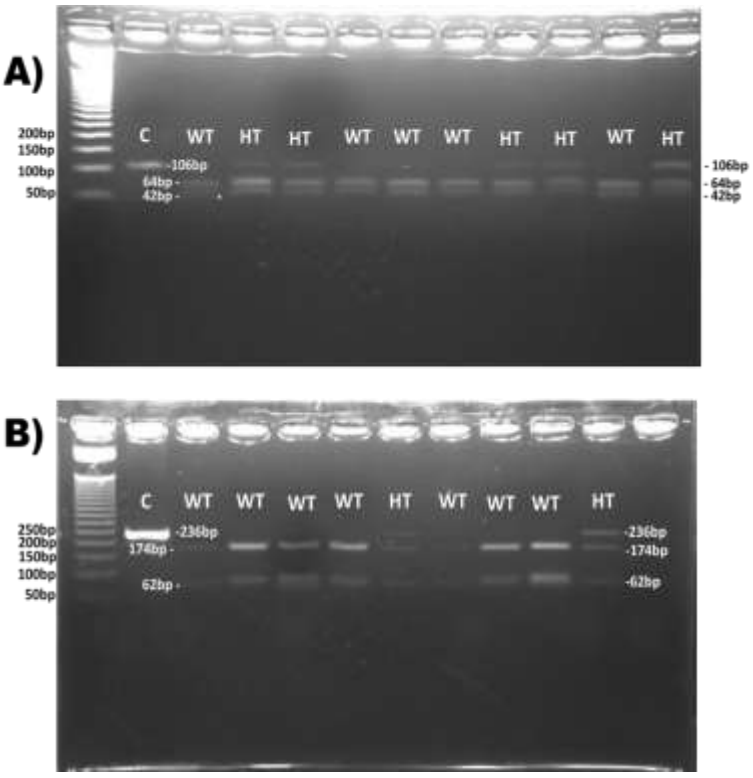

FIGURE 2: The UV visualization of RFLP examination result of TP53 mutation for codon R175 (A) and codon G245 (B). The C denotes control without enzyme addition, WT for wild-type, and HT for heterozygous mutation.

The obtained data were then processed to determine the associations between mutational status in each codon to historical grading of the OSCC and the V value were compared to the association scale (Table 2). The interpretation showed that there was a very strong significant associations (p<0.05) between the mutational status of TP53 codons R175 and the histopathological grading. The similar result was found between the mutational status of TP53 codons G245 and the histopathological grading, although with lower value (p,0.05). The analysis data could be seen on Table 3.

TABLE 3: The Cramer's V association analysis between mutational status and histopathological grading outcome

TP53 Codon		Histopathological grading: Low-grade malignancy n (%)	Histopathological grading: High-grade malignancy n (%)	Total n (%)	Cramer's V	p
R175	Wildtype	23 (52.3%)	2 (4.5%)	**25 (56.8%)**	0.631	0.000*
	Mutation	6 (13.6%)	13 (29.6%)	**19 (43.2%)**		
Total		**29 (65.9%)**	**15 (34.1%)**	**44 (100%)**		
G245	Wildtype	29 (65.9%)	13 (29.6%)	**42 (95.5%)**	0.303	0.044*
	Mutation	0 (0%)	2 (4.5%)	**2 (4.5%)**		
Total		**29 (65.9%)**	**15 (34.1%)**	**44 (100%)**		

*denotes significant by p<0.05

DISCUSSION

The TP53 gene has the highest mutation occurrence rate in OSCC for about 65-85% of all cases [5]. The mutation of TP53 gene mostly happens in the DNA Binding Domain (DBD) in the form of missense mutation [18]. There are six hotspot codons in TP53 (R248, R273, G245, R175, R282, H179), and those mutations are known to increase tumorigenesis and progressivity of the disease [10]. The missense mutation in those six codons produces mutated p53 protein with conformation alteration, which leads to transcriptional dysfunction and inadequate pro-apoptosis ability [9]. Of those six hotspot codons mutation, only mutation in codons R175 dan G245 are well studied [9,10,19,20]. Thus, this study was further concerned with the mutational status of both codons.

This study showed that 43.2% of the OSCC cases have a mutation in codon R175. The number was a bit higher but similar to a previous study by Srividya *et al.*[15], which showed 30% mutation of the same codons out of 85 cases in cancer in general, not limited to OSCC. For mutation in codon G245, only 4.5% mutation was found, and the number was much lower than in the previous study (21%) [15]. However, in general, this result with higher mutation incidence in codon R175 compared to in codon G245 is in accordance with sequencing analysis done by The Cancer Genome Atlas (TCGA) in 510 cases of HNSCC, where the mutation in codon R175 placed third of the most frequently mutated codon and codon G245 rank lower in the ninth place [9].

There was a very strong significant association between the mutational status of TP53 codons R175 and the histopathological grading, which means the cases with mutated codons R175 showed more high-grade OSCC compared to those without mutation. This result was in accordance with *in vitro* and *in vivo* studies conducted by Neskey *et al.*[19], which showed that culture cells expressing mutation R175H *in vitro* had more invasive growth, while *in vivo,* it was more prone to metastasis compared to p53 wild-type, p53 null, and other low-risk mutation. Mutation in codon R175 causes conformation changes in the DNA binding interface, which has a role in identifying and binding the DNA target [21]. Because of its poor DNA binding interface, the p53-R175H lacks the transactivating function of wild-type p53 and causes the loss of function (LOF). In addition, R175H mutation causes gain of function (GOF) through several mechanisms. One of the mechanisms is to modulate various gene expressions by binding to various gene promotors, with or without the aid of transcription factors, and stimulate a variety of downstream oncogenic pathways, such as guanine nucleotide exchange factor-H1 (GEF-H1), growth-regulated oncogene (GRO) 1, methyltransferases and acetyltransferase, whose expressions required in tumor growth [20]. A recent study reported that p53-R175H promotes the expression of heterogeneous nuclear ribonucleoproteins (hnRNPK), a splicing regulator, leading to GTPase-activating protein isoforms (GAPs). These GAP isoforms have lost their ability to bind and inactivate mutant Kirsten rat sarcoma viral oncogene homolog (KRAS), resulting in the activation of KRAS downstream signaling cascades, which contribute to tumor formation [22]. Those findings support previous in vivo study in HNSCC murine in the knock-in murine models expressing p53R172H (equivalent to R175H in human) and oncogenic KRAS, stimulating tumor growth in the oral cavity and promoting tumor progressivity to be carcinoma [23]. R175H mutation accompanied by E2F1 binds to Inhibitor of Differentiation 4 (ID4) promoter and stimulates ID4 protein expression, which will bind and stabilize mRNA IL8 and GRO-α, pro-angiogenesis factor [20]. Besides R175H, another mutation in codon R175 with a similar frequency rate is R175G [24]. The mutation of R175G causes TP53 transactivation activity to decrease in culture cells, thus is predicted to cause LOF [25].

The association between the mutational status of TP53 codons G245 and the histopathological grading showed a similar very strong significant association result. Similar to codon R175 mutation, codon G245 mutation is one of three mutations promoting the invasive growth of HNSCC [26]. One frequent form of mutation in codon G245, G245D, was reported to cause more tumorigenesis and had a higher proclivity for distant metastases in cell culture [19]. Another study showed that G245D mutation could significantly decrease gene target expression of p53, namely p21, Notch1, and B-cell translocation gene 2 (BTG2) compared to low-risk mutation TP53 and p53 WT. Notch1 and BTG2 play a role as tumor suppressors, especially in malignancy [27,28]. G245D mutation was also reported to decrease p21 expression after cisplatin induction, resulting in cisplatin

resistance in vitro and was related to animal model death in vivo study [10]. Another mutation form in codon G245, G245V, was previously reported to decrease both TP53 transactivation and trans repression activity and have lower growth suppression ability compared to TP53 WT in cell culture [29]. According to two other previous researches, each identifying mutation in lung cancer and gastric cancer, patients with G245V mutation had low differentiation on its histopathological manifestation, resulting in high-grade malignancy [30,31].

The limitation of this study was that only two codons were observed in this study. Thus, there was a possibility of high-grade malignancy with wild-type mutational status, possibly due to other mutations in other codons.

CONCLUSIONS

There was a very strong significant association between the mutational status of TP53 on both codons R175 and G245 with the histopathological grading outcome in OSCC patients.

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