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Author(s)	Morita, Shinya; Kano, Satoshi; Hatanaka, Kanako C.; Hatanaka, Yutaka; Suzuki, Takayoshi; Fukuda, Atsushi; Hoshino, Kimiko; Fujiwara, Keishi; Nakamaru, Yuji; Homma, Akihiro
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1       **Association of gene mutations with clinicopathologic features in patients**  
2               **with external auditory canal squamous cell carcinoma**

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6 Shinya Morita<sup>1</sup>, M.D., Ph.D., Satoshi Kano<sup>1</sup>, M.D., Ph.D., Kanako C.  
7 Hatanaka<sup>2</sup>, M.D., Ph.D., Yutaka Hatanaka<sup>2, 3</sup>, Ph.D., Takayoshi Suzuki<sup>1</sup>,  
8 M.D., Ph.D., Atsushi Fukuda<sup>1</sup>, M.D., Ph.D., Kimiko Hoshino<sup>1</sup>, M.D., Keishi  
9 Fujiwara<sup>1</sup>, M.D., Ph.D., Yuji Nakamaru<sup>1</sup>, M.D., Ph.D., Akihiro Homma<sup>1</sup>,  
10 M.D., Ph.D.

11  
12 **Affiliations:**

13 <sup>1</sup>Department of Otolaryngology - Head and Neck Surgery, Faculty of  
14 Medicine and Graduate School of Medicine, Hokkaido University, Sapporo,  
15 Japan

16  
17 <sup>2</sup>Center for Development of Advanced Diagnostics, Hokkaido University  
18 Hospital, Sapporo, Japan

19  
20 <sup>3</sup>Research Division of Genome Companion Diagnostics, Hokkaido Univer  
21 sity Hospital, Sapporo Japan

1

2 Corresponding author: Shinya Morita

3 Department of Otolaryngology - Head and Neck Surgery

4 Faculty of Medicine and Graduate School of Medicine, Hokkaido University.

5 Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

6 Tel: +81-(0)11-706-5958; Fax: +81-(0)11-717-7566

7 E-mail address: [shinyamorita@huhp.hokudai.ac.jp](mailto:shinyamorita@huhp.hokudai.ac.jp)

8

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13

1 **Abstract**

2 **Background:** External auditory canal squamous cell carcinoma (EACSCC) is  
3 a rare form of malignant tumor. Due to the extremely limited understanding  
4 of the genomic landscape in EACSCC, the association between gene  
5 mutations and clinicopathologic features remains unclear. This study aimed  
6 to explore somatic gene mutations associated with the clinicopathological  
7 features in patients with EACSCC, and to identify the candidate gene  
8 mutations for predicting survival outcome in EACSCC.

9 **Methods:** Twenty-two tissue samples obtained from patients with EACSCC  
10 were analyzed for genetic mutations based on targeted next-generation  
11 sequencing and genetic expression based on IHC staining to investigate the  
12 driver of tumorigenesis and/or the candidates of genes for predicting clinical  
13 outcome in EACSCC.

14 **Results:** Gene alterations were most frequently observed in *TP53* (59.1%),  
15 followed by *CREBBP* (9.1%). *TP53* mutations showed significant correlation  
16 with T classification ( $P=0.027$ ) and p53 expression phenotype ( $P<0.001$ ). The  
17 5-year overall survival (OS) rates for EACSCC patients with *TP53* mutations  
18 and wild-type *TP53* were 45.0% and 75.0%, respectively. Multivariable  
19 analysis using the Cox proportional hazards model demonstrated that *TP53*  
20 mutations were independent predictors of OS rates for EACSCC patients  
21 ( $P=0.007$ ).

22 **Conclusion:** This study has suggested that *TP53* mutations have potential  
23 for use as a biomarker for identifying individuals at high risk of developing  
24 tumors and for predicting survival outcome in EACSCC. IHC staining for

1 p53 might play a useful role as screening tool for detecting *TP53* mutations  
2 in patients with EACSCC.

3

4 ***Keywords:*** external auditory canal cancer; squamous cell carcinoma;  
5 next-generation sequencing; gene mutations; clinicopathologic features

6

## 1 **Introduction**

2 External auditory canal (EAC) cancer is a rare form of malignant tumor  
3 representing less than 0.2% of all head and neck (HN) cancers, with  
4 squamous cell carcinoma (SCC) being the most common histology [1, 2].  
5 Surgical resection with or without postoperative radiotherapy (RT) is widely  
6 performed as the mainstay of treatment for EACSCC, and definitive  
7 chemoradiotherapy (CRT) may be appropriate for cases with unresectable  
8 tumors [1-3]. However, no evidence-based treatment strategy for EACSCC  
9 has been established to date due to the rarity of the disease, and no clinical  
10 trials investigating targeted therapies have been conducted. Thus, it is  
11 necessary to analyze the association between gene mutations and  
12 clinicopathologic features in EACSCC and explore potential molecular  
13 biomarkers for the development of novel therapeutic targets.

14 The recent advances in next-generation sequencing (NGS) technologies  
15 have afforded remarkable progress in the identification of cancer-related  
16 genes, and have significantly transformed treatment modalities and survival  
17 in several lethal malignancies, such as mucosal HNSCC and cutaneous  
18 HNSCC [4-7]. Further, immunohistochemical (IHC) assays have been widely  
19 used as an affordable and straightforward tool for the analysis of gene  
20 expression. Based on the results of an IHC assay, a previous study reported  
21 that the expression of p53 and EGFR may be valuable biomarkers for  
22 developing tumors in EACSCC [8]. However, it is widely accepted that  
23 discrepancies in gene mutations based on NGS and gene expression based on  
24 IHC staining might affect the corresponding cancer risk [9]. EACSCC has

1 not been included in The Cancer Genome Atlas  
2 (<https://portal.gdc.cancer.gov>), and the understanding of its genomic makeup  
3 is extremely limited [10]. Thus, it remains unclear if the evidence from other  
4 HNSCC primary sites applies.

5 In this study, genetic mutations based on targeted NGS and genetic  
6 expression based on IHC staining were analyzed using EACSCC tissue  
7 samples with the aim of exploring somatic gene mutations associated with  
8 the clinicopathological features in patients with EACSCC, and identifying  
9 candidate gene mutations for the prediction of survival outcome in EACSCC.

10

1 **Material and methods**

2 ***Ethics statement***

3 This study enrolled patients with previously untreated EACSCC who were  
4 treated with curative intent in the Department of Otolaryngology, Head and  
5 Neck Surgery, Hokkaido University Hospital between April 2016 and  
6 January 2021. All patients were instructed on the potential risks and  
7 benefits of the management program, and written informed consent for the  
8 use of their tissue samples and clinical data was obtained after a full  
9 explanation. This research adhered to the tenets of the Declaration of  
10 Helsinki and was approved by our Institutional Review Board (No.  
11 020-0373).

12

13 ***Treatment strategy***

14 All patients were initially evaluated by a multidisciplinary team  
15 consisting of otolaryngologists, radiation oncologists and medical oncologists.  
16 Staging was performed using the University of Pittsburgh modified TNM  
17 staging system [2]. Treatment selection based on the extent of the disease,  
18 performance status and/or comorbidities in each case. For patients with T1-2  
19 tumors, surgery was selected as the initial treatment choice. If histologic  
20 examination revealed positive surgical margins or more extensive  
21 involvement than suggested by preoperative imaging, postoperative RT was  
22 performed. For patients with T3-4 tumors, surgery followed by CRT was  
23 performed. Neck dissection was performed in cases clinically positive for  
24 neck lymph node metastasis. For locally advanced tumors with extension to



1 the pyramidal apex, posterior cranial fossa, middle cranial fossa or internal  
2 carotid artery, definitive CRT was selected.

### 3 4 *Tissue samples*

5 All tissue samples were obtained from pre-treatment biopsy specimens or  
6 surgical specimens from the primary tumor. All collected samples were  
7 immediately fixed using the PAXgene Tissue System (PreAnalytiX, QIAGEN,  
8 Hilden, NRW, Germany) and embedded in paraffin until DNA extraction,  
9 based on the manufacturer's protocol.

### 10 11 *DNA extraction and quality assessment*

12 The PAXgene Tissue-fixed, paraffin-embedded (PFPE) tissues were cut  
13 into 10- $\mu$ m-thick sections with a microtome. Areas with higher proportion of  
14 tumor cells and less inflammatory cells were identified by hematoxylin and  
15 eosin staining, and the presence of at least 20% malignant cells in the  
16 sample was determined. If the percentage of tumor cells was less than 20%, a  
17 macro-dissection technique was used to collect the malignant cells from the  
18 sections. DNA extraction was performed using the GeneRead DNA FFPE  
19 Tissue Kit (QIAGEN, Hilden, NRW, Germany). PFPE sample-derived DNA  
20 quantity and quality were assessed by calculating the QC score using  
21 GeneRead DNA QuantiMIZE Kits (QIAGEN, Hilden, NRW, Germany),  
22 according to the manufacturer's protocol. Samples with sufficient DNA that  
23 passed the quality control checks were then used for genetic analysis.

1 *NGS*

2 The GeneRead Human Comprehensive Cancer Panel (NGHS-501X®,  
3 QIAGEN, Hilden, NRW, Germany) was utilized to assess 160 clinically  
4 cancer-related genes. DNA amplification for library construction was  
5 performed by quantitative polymerase chain reaction using QIAseq DNA  
6 QuantiMIZE Assay Kits (QIAGEN, Hilden, NRW, Germany). The amplified  
7 DNA library was assessed using a QIAseq Library Quant Assay Kit  
8 (QIAGEN, Hilden, NRW, Germany), and was sequenced using a MiSeq  
9 sequencer (Illumina, San Diego, CA, USA).

10

11 *Analysis of NGS data*

12 The Qiagen web portal  
13 ([https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-o](https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page)  
14 [verview-page](https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page)) was utilized for the GeneRead Human Comprehensive Cancer  
15 Panel results and data analysis. The coverage depth was considered to be  
16 sufficient when an amplicon was sequenced 500 times or more. Variant allele  
17 frequency (VAF) was calculated as the number of variant reads divided by  
18 the total number of reads for the mutation position. High-confidence  
19 variants with a VAF of 5% or more were included in the further analysis. The  
20 Catalogue of Somatic Mutations in Cancer (COSMIC) database  
21 (<https://cancer.sanger.ac.uk/cosmic>), Functional Analysis through Hidden  
22 Markov Models (FATHMM) (<http://fathmm.biocompute.org.uk/cancer.html>)  
23 and ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar>) were used to  
24 ascertain the pathogenicity of the identified variants. The variants were

1 categorized as pathogenic, common polymorphisms or variants of uncertain  
2 significance. The pathogenic variants were selected and investigated for  
3 their association with clinicopathological features in this analysis.

#### 4 5 ***Analysis of gene expression by IHC staining***

6 The specimens were embedded in paraffin and cut into 4- $\mu$ m-thick sections.  
7 They were then deparaffinized in xylene, dehydrated through a graded series  
8 of alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous  
9 peroxidase activity. Antigen retrieval was performed using a 750 W  
10 microwave oven for 15 minutes in 10 mM sodium citrate buffer (10 mmol/L,  
11 pH 6.0). The sections were blocked with 10% normal goat serum for 30  
12 minutes at room temperature to prevent the non-specific binding of  
13 antibodies. The slides were then incubated with a p53 mouse monoclonal  
14 antibody (DO-7, Roche Diagnostics Ltd., Tokyo, Japan) in a humid chamber  
15 at 4 °C overnight. The sections were then incubated with a biotin-labeled  
16 goat anti-rabbit secondary antibody (Histofine Simple Stain MAX-PO (M) kit,  
17 Nichirei, Tokyo, Japan) for 30 minutes at 37 °C, followed by reaction with a  
18 streptavidin-biotin horseradish peroxidase complex. The reaction products  
19 were observed by immersing the slides in a freshly prepared  
20 diaminobenzidine solution for 10 minutes and counterstaining them with  
21 hematoxylin before dehydration and mounting.

#### 22 23 ***Assessment of IHC assay***

24 All slides were examined by light microscopy scanning the entire tissue

1 specimen under low magnification ( $\times 40$ ) and then confirmed under high  
2 magnification ( $\times 200$  and  $\times 400$ ). Two researchers including one pathologist  
3 blinded to the clinical data assessed the staining of each specimen.

4 For p53 IHC, the expression patterns were classified into three groups in  
5 accordance with the methods described by Boyle et al. as follows: extreme  
6 negative (EN), complete confluent negativity of staining; extreme positive  
7 (EP), strong diffuse confluent positivity; and non-extreme (NE), all  
8 intermediate expression of any intensity [11].

#### 9 10 *Statistical analysis*

11 Statistical analyses were performed using GraphPad Prism software  
12 (version 6.0, GraphPad Software Inc., La Jolla, CA, U.S.A.) and JMP  
13 software (version 16.0; SAS Institute Inc.; Cary, NC, U.S.A.). Statistical  
14 differences were analyzed using the Mann-Whitney U-test for two  
15 independent groups, with a  $P$  value of less than 0.05 considered statistically  
16 significant. A Kaplan-Meier time-to-event method was used to calculate the  
17 overall survival (OS) rates. For the calculation of survival rates, death was  
18 counted as an event, whereas the patient being alive at the latest contact,  
19 regardless of disease status, was counted as censored. The times of interest  
20 included the beginning of treatment, the last follow-up date and death.  
21 Survival status was updated in December 2021. Statistical differences were  
22 analyzed using the log rank test. Stepwise regression analysis was  
23 performed to build an appropriate model through the addition and removal  
24 of predictor variables. Multivariable analysis was performed using the Cox

1 proportional hazard model.

2

1 **Results**

2 *Clinicopathological features*

3 The PFPE tissue samples were obtained from 32 patients with EACSCC.  
4 Of these, 6 patients were ineligible for genetic analysis due to the high levels  
5 of necrosis, keratin, inflammatory infiltrates or hemorrhaging in tissue  
6 samples. Further, 4 patients were excluded due to an insufficient quantity  
7 and quality of tumor tissue-derived DNA. Finally, a total of 22 patients were  
8 enrolled in this analysis.

9 Patient profiles are summarized in Table 1. Regarding T classification, 9  
10 patients were categorized as early T stage (T1-2) and 13 patients as  
11 advanced T stage (T3-4). Four patients had clinically positive lymph node  
12 metastasis. The histopathological diagnoses consisted of well-differentiated  
13 SCC in 16 patients (72.7%) and moderately- or poorly-differentiated SCC in 6  
14 patients (27.3%). Surgical resection was performed in 3 patients with T1, 6  
15 with T2, and 2 with T3 disease. Of the 6 patients with T2 disease, 3 with  
16 pathologically positive surgical margins received postoperative RT. All T3  
17 patients receiving surgery were followed by postoperative CRT. Two patients  
18 with T3 and 9 patients with T4 disease underwent definitive CRT using  
19 platinum-based regimens.

20

1 *Somatic gene mutations in EACSCC*

2 The flowchart for NGS data analysis is summarized in Fig. 1. The  
3 nonsynonymous single nucleotide variants and indels, with or without  
4 frameshifts, in the exon regions were assigned as somatic variants. One  
5 hundred twenty somatic variants were categorized as pathogenic according  
6 to the COSMIC database. Of these, 105 variants were categorized as  
7 “benign”, “not provided”, “not reported” or “of uncertain significance” in the  
8 ClinVar database (see also Table 1S in the electronic supplementary  
9 material). Finally, this analysis considered *TP53* (c.422G>A, c.916C>T,  
10 c.742C>T, c.527G>T, c.743G>A, c.752T>G, c.517G>T, c.586C>T, c.736A>G,  
11 c.451C>A, c.856G>A) and *CREBBP* (c.4337G>A) as significant mutated  
12 genes in patients with EACSCC (Table 2). Gene alterations were most  
13 frequently observed in *TP53* (59.1%), followed by *CREBBP* (9.1%). The rates  
14 of each *TP53* status were 40.9% for wild-type, 50% for missense mutations,  
15 and 9.1% for nonsense mutations. Case EC22 carried *TP53* mutations at 2  
16 different positions (c.752T>G, c.517G>T). Eight of the patients showed no  
17 significant genetic mutations among the analyzed genes.

18

1 ***Relationships between somatic gene mutations and clinicopathologic***  
2 ***features***

3 Figure 2 depicts a visual representation of the relationships between gene  
4 mutations and clinical parameters in EACSCC patients. Genetic mutations  
5 appeared to be more frequent among female patients, those with a more  
6 advanced T and N staging, and those with poorer differentiation.

7 Table 3 shows that *TP53* mutations were correlated significantly with T  
8 classification ( $P=0.027$ ). Meanwhile, there were no significant correlations  
9 between *TP53* mutation status and the other parameters, such as age,  
10 gender, smoking status and alcohol consumption, N classification, and  
11 histopathological differentiation.

12  
13 ***Survival analysis with regard to TP53 mutations and clinicopathologic***  
14 ***features***

15 Based on NGS results, the 5-year OS rates for EACSCC patients with  
16 *TP53* mutations and wild-type *TP53* were 45.0% and 75.0%, respectively (Fig.  
17 3). Univariable analysis indicated that T classification, *TP53* mutations and  
18 treatment modality ( $P=0.003$ ,  $P=0.046$  and  $P=0.006$ , respectively) were  
19 significant prognostic factors for EACSCC (Table 4). Meanwhile, other  
20 clinicopathologic features, such as age, gender, smoking status and alcohol  
21 consumption, N classification, and histopathological differentiation, were not  
22 significantly correlated with survival outcomes. Stepwise regression analysis  
23 and multivariable analysis using the Cox proportional hazards model  
24 demonstrated that *TP53* mutations and treatment modality ( $P=0.007$  and



1  $P=0.010$ , respectively) were independent predictors of OS rate.

2

3 ***Relationships between TP53 mutations and p53 expression phenotype***

4 The p53-EN, -EP, and -NE phenotypes were found in 5 (22.7%), 7 (31.8%)  
5 and 10 (45.5%) of the EACSCC samples, respectively (Table 5 and Fig. 4a-f).

6 With regard to the correlation between *TP53* mutation status and the p53  
7 expression phenotype, *TP53* mutations were more frequently identified in  
8 p53-EN/EP cases than in p53-NE cases ( $P<0.001$ ), with 2 patients with *TP53*  
9 nonsense mutations revealing a p53-EN phenotype. The 5-year OS rates for  
10 patients with the p53-EN/EP and p53-NE phenotypes were 75.0% and 46.9%,  
11 respectively (Fig. 5). The survival outcomes based on IHC findings were  
12 found to be similar to those based on NGS results.

13

## 1 Discussion

2 Patients with early-stage EACSCC have good survival outcomes, whereas  
3 advanced-stage patients have a poor prognosis [1-3, 12]. In cases with  
4 unresectable or recurrence tumors, in particular, the treatment options,  
5 including targeted therapies, have been limited [3, 12]. This study has  
6 confirmed that treatment modality based on T classification is an  
7 independent prognostic factor, which is consistent with the results of  
8 previous analyses of EACSCC [1-3, 12]. Meanwhile, no studies are yet to  
9 focus on genetic mutations of EACSCC and their relationship to  
10 clinicopathological features. Thus, this study analyzed genetic mutations  
11 based on a targeted NGS and genetic expression based on IHC staining to  
12 investigate the driver of tumorigenesis and/or the candidates of genes for  
13 predicting clinical outcome in EACSCC. In patients with EACSCC, gene  
14 mutations were most frequently observed in *TP53* (59.1%), followed by  
15 *CREBBP* (9.1%). *TP53* is the most commonly mutated tumor suppressor  
16 gene in human cancers, which is consistent with the results of previous  
17 comprehensive genetic studies of mucosal and cutaneous HNSCC [13].  
18 *CREBBP* encodes the CBP protein containing a transcriptional cofactor with  
19 lysine acetyltransferase activity and close homology to p300, which is  
20 associated with tumor progression [14]. *CREBBP* mutations are frequently  
21 identified across a range of different cancers, such as lymphomas and  
22 bladder, esophageal, and cutaneous SCC [14].

23 Although the gene mutations in EACSCC were expected to mostly  
24 resemble those in mucosal HNSCC [10], the genomic landscape of EACSCC

1 remains unclear due to its rarity. The gene mutational rates of mucosal  
2 HNSCC have been reported to be 44% in *TP53*, 20% in *NOTCH1*, 19% in  
3 *FAT1*, and 14% in *PIK3CA*, according to the COSMIC database. However,  
4 except for *TP53*, this analysis did not identify mutations in major  
5 cancer-related gene, such as *NOTCH1*, *FAT1*, and *PIK3CA*. Thus, the  
6 mutational patterns in EACSCC might differ slightly from those in mucosal  
7 HNSCC due to tissue-specific histopathological and environmental factors,  
8 as mentioned below: EACSCC arises from the skin of the EAC, whereas  
9 mucosal HNSCC occurs in the mucosa of the nasal cavity, paranasal sinus,  
10 oral cavity, pharynx or larynx. Chronic inflammation in the EAC induced by  
11 habitual ear picking and other mechanical stimuli causes EACSCC [1, 15],  
12 with smoking and alcohol unlikely to be major carcinogens of EACSCC.

13 It is noteworthy that *CREBBP* mutations have been identified in patients  
14 with EACSCC. *CREBBP* mutations are common in cutaneous SCC and  
15 contribute to the tumorigenesis of epidermal keratinocytes [14, 16].  
16 Mutations in *NOTCH1*, being one of the major cancer-related genes of  
17 cutaneous SCC, frequently develop under ultraviolet irradiation, whereas  
18 *CREBBP* mutations occur in epidermal keratinocytes that are less exposed  
19 to sunlight [14]. Thus, the gene mutational patterns in EACSCC, in which  
20 the tissue of origin receive less exposure to sunlight than other cutaneous  
21 sites of HN, might resemble those in cutaneous SCC in less sun-exposed skin  
22 rather than mucosal HNSCC. However, among the genes analyzed using the  
23 GeneRead Human Comprehensive Cancer Panel, 45.5 % of patients with  
24 EACSCC presented no genetic mutations. This analysis might have been

1 affected by the exon coverage of the gene panels, mutations in unknown  
2 genes, the presence of epigenetic aberrations, or copy number variations.  
3 Further analysis is required to explore the genomic landscape of EACSCC.

4 To date, there is little in the way of convincing genetic data in relation to  
5 the clinicopathological and prognostic implications due to the rarity of  
6 EACSCC. This analysis found that gene alterations in *TP53* in EACSCC  
7 tissues were significantly associated with T classification and survival  
8 outcome. *TP53* encodes a tumor suppressor protein p53 containing  
9 transcriptional activation, DNA binding, and oligomerization domains. The  
10 encoded p53 protein responds to diverse cellular stresses to regulate the  
11 expression of target genes and is involved in cell cycle regulation,  
12 proliferation and apoptosis [17]. The mutated *TP53* has a longer half-life and  
13 deactivates the trigger of p53-dependent apoptosis, resulting in tumor  
14 development and enlargement in a variety of human cancers [17, 18]. The  
15 gene alterations in *TP53* in other primary sites of HNSCC have been  
16 reported to correlate with T classification and survival outcome [19], which is  
17 in agreement with the results of this analysis of EACSCC.

18 It remains controversial whether the p53 expression detected by IHC  
19 staining affords a consistent prognostic biomarker of EACSCC [8]. Although  
20 several IHC evaluation systems for p53 have been proposed as surrogate  
21 methods for the molecular subtypes based on the gene expression patterns,  
22 the discrepancy between *TP53* mutations based on NGS and p53 expression  
23 based on IHC affects the corresponding assessment of cancer risk [9].

24 Missense mutations that lead to less aberrant functional proteins have been

1 shown to produce strong IHC staining, and nonsense mutations that lead to  
2 a complete absence of p53 protein expression have been shown to produce no  
3 IHC staining [19]. Boyle et al. focused on the IHC expression patterns of p53  
4 and predicted the prognosis in patients with breast cancer by classifying the  
5 IHC findings into three groups: EN, EP, and NE [11]. They found that  
6 p53-EN or -EP was significantly associated with a poorer survival outcome  
7 than was p53-NE, and that combined p53-EN and -EP better predicted the  
8 survival outcome than either pattern alone. Furthermore, in their analysis of  
9 the *TP53* mutation status, the detected mutation types appeared to be  
10 related to the protein status, with a missense mutation corresponding to the  
11 p53-EP phenotype, and a nonsense mutation appearing to abrogate protein  
12 expression, manifesting as the p53-EN phenotype. This analysis  
13 demonstrated that the *TP53* mutation status was significantly correlated  
14 with the p53 expression phenotype in patients with EACSCC, which is more  
15 consistent with the results of the previous analysis [11]. *TP53* mutations as  
16 well as p53-EN/EP were significantly correlated with poor survival outcome  
17 in patients with EACSCC. The advantage of IHC is that it offers an  
18 affordable and straightforward assay to detect genetic expression in  
19 comparison with NGS. IHC staining might be a cost-effective screening tool  
20 for detecting *TP53* mutations by assigning p53-EN/EP as representing a  
21 *TP53* mutation and p53-NE as the wild-type *TP53*.

22 The results of this analysis might have been affected by the small cohort  
23 size, quantity and quality of samples, use of a targeted panel in NGS,  
24 potential confounding factors during statistical analysis, the sensitivity of

1 antibodies, antigen retrieval methods, incubation time, detection system and  
2 the IHC assessment methods. Although extraction of high-quality DNA from  
3 tissue samples is inherently difficult, stringent sample storage and quality  
4 control checks were applied to ensure that only cases with high-quality DNA  
5 were included in this analysis. All EACSCC samples were stored as PFPE  
6 tissues, which have been reported to preserve proteins and nucleic acids in a  
7 more native state compared to formalin-fixed paraffin-embedded tissues [20].  
8 Another limitation is the ambiguity with regard to the classification of  
9 variants as pathogenic. There were 105 missense variants categorized as  
10 pathogenic in the COSMIC database, whereas those variants were  
11 categorized as “benign”, “not provided”, “not reported” or “of uncertain  
12 significance” in the ClinVar database. This analysis did not assign those  
13 variants as pathogenic mutations, and might, therefore, have  
14 underestimated the prevalence of pathogenic mutations.  
15

1 **Conclusion**

2 This study analyzed genetic mutations based on targeted NGS and genetic  
3 expression based on IHC staining in EACSCC tissue samples. Gene  
4 alterations were most frequently observed in *TP53*, followed by *CREBBP*.  
5 The mutated *TP53* was significantly correlated with T classification, and  
6 poor prognosis and p53 expression phenotype, suggesting that *TP53*  
7 mutations have potential for use as a biomarker for identifying individuals  
8 at high risk of developing tumors and as well as for predicting survival  
9 outcome in EACSCC. IHC staining for p53 might play a useful role as a  
10 screening tool for detecting *TP53* mutations in patients with EACSCC.

11

1 **Disclosure statement**

2 The authors have no conflicts of interest to declare.

3



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4

## 1 **References**

- 2 1. Yin M, Ishikawa K, Honda K, et al (2006) Analysis of 95 cases of squamous  
3 cell carcinoma of the external and middle ear. *Auris Nasus Larynx*.  
4 33:251-257
- 5 2. Moody SA, Hirsch BE, Myers EN (2000) Squamous cell carcinoma of the  
6 external auditory canal: an evaluation of a staging system. *Am J Otol*.  
7 21:582-588
- 8 3. Morita S, Homma A, Nakamaru Y, et al (2016) Outcomes of surgery and  
9 chemoradiotherapy for temporal bone cancer. *Otol Neurotol*. 37:1174-1182
- 10 4. Stransky N, Egloff AM, Tward AD, et al (2011) The mutational landscape  
11 of head and neck squamous cell carcinoma. *Science*. 333:1157-1160
- 12 5. Mountzios G, Rampias T, Psyrris A (2014) The mutational spectrum of  
13 squamous-cell carcinoma of the head and neck: targetable genetic events and  
14 clinical impact. *Ann Oncol*. 25:1889-1900
- 15 6. Li YY, Hanna GJ, Laga AC, et al (2015) Genomic analysis of metastatic  
16 cutaneous squamous cell carcinoma. *Clin Cancer Res*. 21:1447-1456
- 17 7. Zilberg C, Lee MW, Yu B, et al (2018) Analysis of clinically relevant  
18 somatic mutations in high-risk head and neck cutaneous squamous cell  
19 carcinoma. *Mod Pathol*. 31:275-287
- 20 8. Morita S, Nakamaru Y, Homma A, et al (2017) Expression of p53, p16,  
21 cyclin D1, epidermal growth factor receptor and Notch1 in patients with  
22 temporal bone squamous cell carcinoma. *Int J Clin Oncol*. 22:181-189
- 23 9. Zambetti GP (2007) The p53 mutation “gradient effect” and its clinical  
24 implications. *J Cell Physiol*. 213:370-373

- 1 10. Sato K, Komune N, Hongo T, et al (2020) Genetic landscape of external  
2 auditory canal squamous cell carcinoma. *Cancer Sci.* 111:3010-3019
- 3 11. Boyle DP, McArt DG, Irwin G, et al (2014) The prognostic significance of  
4 the aberrant extremes of p53 immunophenotypes in breast cancer.  
5 *Histopathology.* 65:340-352
- 6 12. Takenaka Y, Cho H, Nakahara S, et al (2015) Chemoradiation therapy  
7 for squamous cell carcinoma of the external auditory canal: A meta-analysis.  
8 *Head Neck.* 37:1073-1080
- 9 13. Lawrence MS, Sougnez C, Lichtenstein L, et al (2015) Comprehensive  
10 genomic characterization of head and neck squamous cell carcinomas.  
11 *Nature.* 517:576-582
- 12 14. Watt SA, Purdie KJ, den Breems NY, et al (2016) CREBBP mutation in  
13 human cutaneous squamous cell carcinoma. *Exp Dermatol.* 25:650-651
- 14 15. Tsunoda A, Sumi T, Terasaki O, et al (2017) Right dominance in the  
15 incidence of external auditory canal squamous cell carcinoma in the  
16 Japanese population: Does handedness affect carcinogenesis? *Laryngoscope*  
17 *Investig Otolaryngol.* 2:19-22
- 18 16. Ichise T, Yoshida N, Ichise H (2019) CBP/p300 antagonises  
19 EGFR-Ras-Erk signalling and suppresses increased Ras-Erk  
20 signalling-induced tumour formation in mice. *J Pathol.* 249:39-51
- 21 17. Levine AJ, Momand J, Finlay CA (1991) The p53 tumor suppressor gene.  
22 *Nature.* 351:453-456
- 23 18. Marchetti P, Cannita K, Ricevuto E, et al (2003) Prognostic value of p53  
24 molecular status in high-risk primary breast cancer. *Ann Oncol.* 14:704-708

- 1 19. Karpathiou G, Monaya A, Forest F, et al (2016) p16 and p53 expression  
2 status in head and neck squamous cell carcinoma: a correlation with  
3 histological, histoprognostic and clinical parameters. *Pathology*. 48:341-348
- 4 20. Kap M, Smedts F, Oosterhuis W, et al (2011) Histological assessment of  
5 PAXgene tissue fixation and stabilization reagents. *PLoS One*. 6:e27704
- 6

1 **Table headings**

2 Table 1. Characteristics of patients with external auditory canal squamous  
3 cell carcinoma

4

5 Table 2. Genetic mutations based on next-generation sequencing in tissue  
6 samples of external auditory canal squamous cell carcinoma

7

8 Table 3. Relationships between *TP53* mutations and clinicopathologic  
9 features

10

11 Table 4. Univariate and multivariate analyses of various potential prognostic  
12 factors for overall survival

13

14 Table 5. Correlation between p53-expression phenotype and *TP53* status in  
15 external auditory canal squamous cell carcinoma

16

17 Supplementary Table 1S. Somatic variants categorized as "pathogenic"  
18 according to the COSMIC database but as "benign", "not provided", "not  
19 reported" or "of uncertain significance" according to the ClinVar database

20

1 **Figure legends**

2 Fig. 1. Flowchart for the next-generation sequencing data analysis

3

4 Fig. 2. Summary of the associations between somatic gene mutations and  
5 clinical features in patients with external auditory canal squamous cell  
6 carcinoma

7

8 Fig. 3. The 5-year overall survival rates for external auditory canal  
9 squamous cell carcinoma patients with mutated and wild-type *TP53*

10

11 Fig. 4a-f. Examples of p53-EN (a, b), p53-EP (c, d) and p53-NE (e, f) in  
12 external auditory canal squamous cell carcinoma samples under low (×40)  
13 and high magnification (×200)

14 EN; extreme negative, EP; extreme positive, NE; non-extreme

15

16 Fig. 5. The 5-year overall survival rates for external auditory canal  
17 squamous cell carcinoma patients with p53-EN/EP phenotype and p53-NE  
18 phenotype

19 EN; extreme negative, EP; extreme positive, NE; non-extreme

Table 1. Characteristics of patients with external auditory canal squamous cell carcinoma

Parameters	
Age (years)	
range	39 - 85
median	68
Gender (n)	
Female	13
Male	9
Smoking (n)	
Never and former	9
Current	13
Alcohol (n)	
Never and former	10
Current	12
T classification (n)	
1	3
2	6
3	4
4	9
N classification (n)	
0	18
1	4
Differentiation (n)	
Well	16
Moderate	3
Poor	3
Treatment modality (n)	
Surgery alone	6
Surgery + (C)RT	5
Definitive CRT	11
Follow-up period (months)	
range	13 - 60
median	37
RT; radiotherapy, CRT; chemoradiotherapy	

Table 2. Genetic mutations based on next-generation sequencing in tissue samples of external auditory canal squamous cell carcinoma

Sample	Gene name	Transcript ID	Codon change	Amino acid change	Type of nonsynonymous substitution	Variant clinical significance	
						COSMIC(FATHMM prediction)	ClinVar
EC1	<i>TP53</i>	NM_000546.5	c.422G>A	p.C141Y	Missense	Pathogenic	Pathogenic
EC7	<i>TP53</i>	NM_000546.5	c.451C>A	p.P151T	Missense	Pathogenic	Likely pathogenic
EC15	<i>TP53</i>	NM_000546.5	c.916C>T	p.R306*	Nonsense	Pathogenic	Pathogenic
EC15	<i>CREBBP</i>	NM_004380.2	c.4337G>A	p.R1446H	Missense	Pathogenic	Likely pathogenic
EC16	<i>TP53</i>	NM_000546.5	c.742C>T	p.R248W	Missense	Pathogenic	Pathogenic
EC18	<i>TP53</i>	NM_000546.5	c.527G>T	p.C176F	Missense	Pathogenic	Likely pathogenic
EC20	<i>TP53</i>	NM_000546.5	c.743G>A	p.R248Q	Missense	Pathogenic	Pathogenic
EC22	<i>TP53</i>	NM_000546.5	c.752T>G	p.I251S	Missense	Pathogenic	Likely pathogenic
EC22	<i>TP53</i>	NM_000546.5	c.517G>T	p.V173L	Missense	Pathogenic	Pathogenic
EC23	<i>TP53</i>	NM_000546.5	c.586C>T	p.R196*	Nonsense	Pathogenic	Pathogenic
EC28	<i>TP53</i>	NM_000546.5	c.736A>G	p.M246V	Missense	Pathogenic	Pathogenic
EC30	<i>TP53</i>	NM_000546.5	c.743G>A	p.R248Q	Missense	Pathogenic	Pathogenic
EC31	<i>TP53</i>	NM_000546.5	c.451C>A	p.P151T	Missense	Pathogenic	Likely pathogenic
EC32	<i>TP53</i>	NM_000546.5	c.856G>A	p.E286K	Missense	Pathogenic	Pathogenic
EC32	<i>CREBBP</i>	NM_004380.2	c.4337G>A	p.R1446H	Missense	Pathogenic	Likely pathogenic



Table 3. Relationships between *TP53* mutations and clinicopathologic features

	n	<i>TP53</i> mutation (n, %)	<i>P</i> value
<b>Age</b>			
≤ 65 years	11	6 (54.5%)	>0.99
> 65 years	11	6 (54.5%)	
<b>Gender</b>			
Female	13	9 (69.2%)	0.19
Male	9	3 (33.3%)	
<b>Smoking</b>			
Never and former	9	6 (66.7%)	0.41
Current	13	6 (46.2%)	
<b>Alcohol</b>			
Never and former	10	5 (50.0%)	>0.99
Current	12	7 (58.3%)	
<b>T classification</b>			
1-2	9	2 (22.2%)	0.027
3-4	13	10 (76.9%)	
<b>N classification</b>			
0	18	9 (50.0%)	0.59
1	4	3 (75.0%)	
<b>Differentiation</b>			
Well	16	7 (43.8%)	0.16

Moderate-Poor	6	5 (83.3%)
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Table 4. Univariate and multivariate analyses of various potential prognostic factors for overall survival

	5 year-OS rate (%)	Univariate analysis		Multivariate analysis	
		HR	<i>P</i> value	HR	<i>P</i> value
Age (years)					
≤ 65	65.6%	Ref.			
> 65	53.3%	1.56	0.60		
Gender					
Female	71.8%	Ref.			
Male	38.9%	1.97	0.37		
Smoking					
Never and former	62.2%	Ref.			
Current	56.3%	1.36	0.69		
Alcohol					
Never and former	68.6%	Ref.			
Current	44.4%	1.67	0.51		
T classification					
1-2	100%	Ref.			
3-4	30.8%	1.54x10 <sup>9</sup>	0.003	1.33	0.81
N classification					
0	71.3%	Ref.			
1	0%	4.61	0.064		
Differentiation					
Well	65.6%	Ref.			
Moderately - Poor	41.7%	2.63	0.23		
<i>TP53</i>					
Wild-type	75.0%	Ref.			
Mutation	45.0%	5.30	0.046	20.2	0.007
Treatment modality					
Surgery (+ CRT)	100%	Ref.			
Definitive CRT	29.1%	5.66x10 <sup>10</sup>	0.006	30.8	0.010

OS; overall survival, HR; hazard ratio, Ref.; reference (HR=1.0), CRT; chemoradiotherapy

Table 5. Correlation between p53-expression phenotype and *TP53* status in external auditory canal squamous cell carcinoma

p53-expression phenotype	<i>TP53</i> status		
	Wild-type	Mutation	
		Missense	Nonsense
EN	0	3	2
EP	1	6	0
NE	9	1	0
Total	10	10	2

EN; extreme negative, EP; extreme positive, NE; non-extreme

21,818 variants: Total number of variants from 22 tumor samples



11,654 variants: Retained based on coverage depth  $\geq 500$  times and VAF  $\geq 5\%$



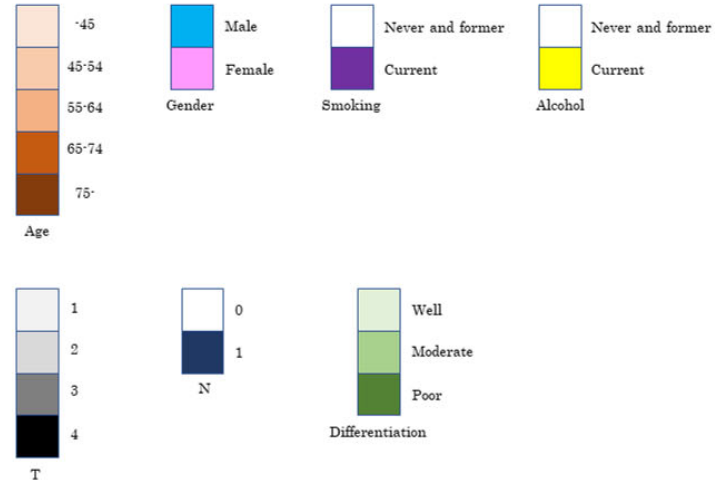
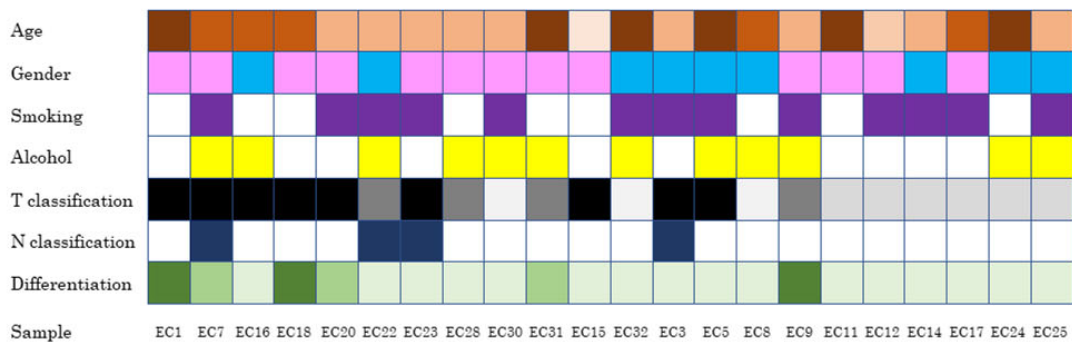
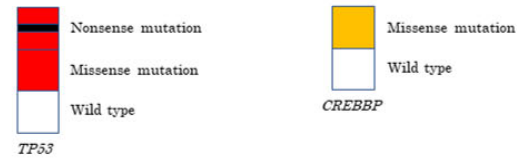
343 variants: Retained based on variant type (nonsynonymous) and within the region of exons

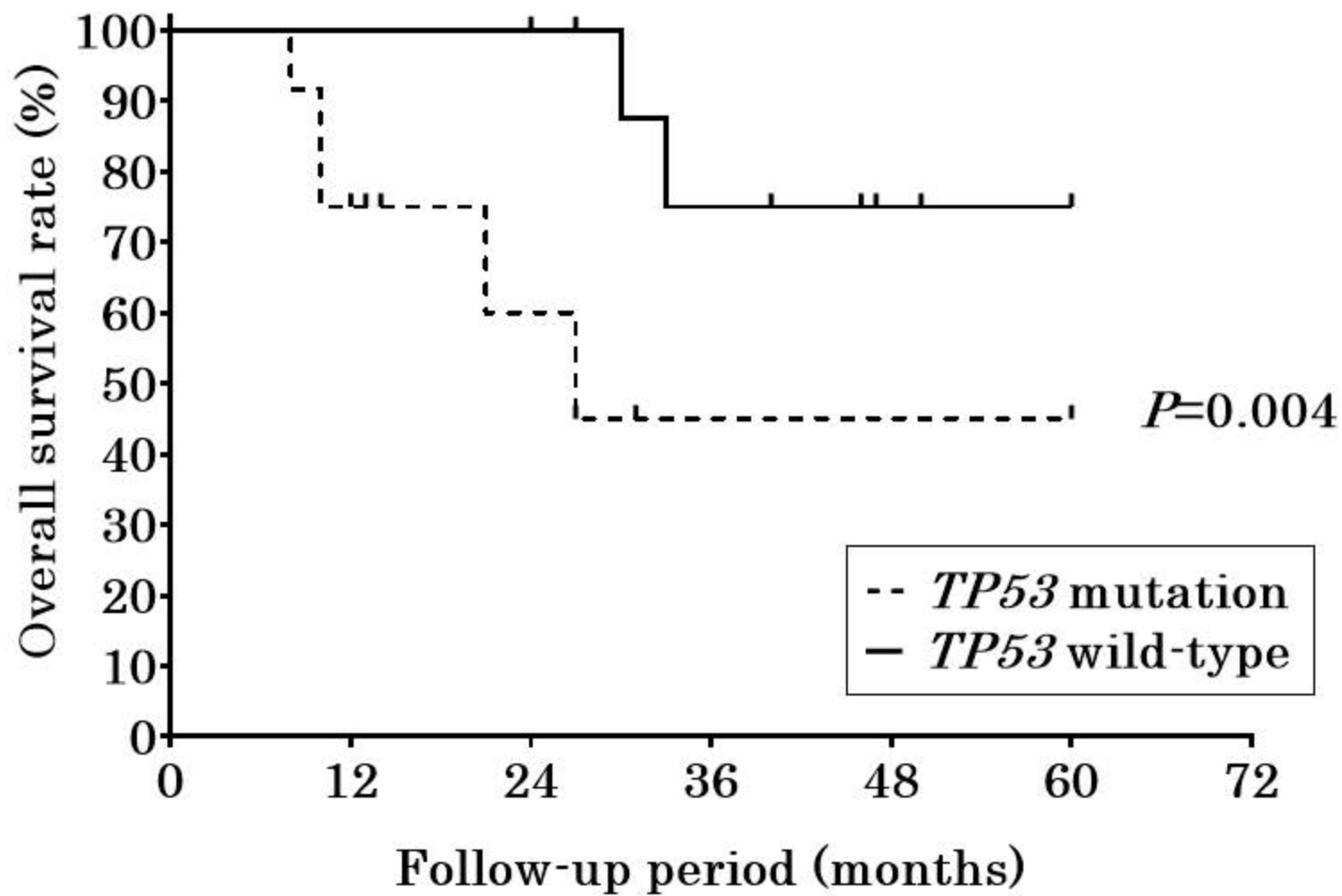


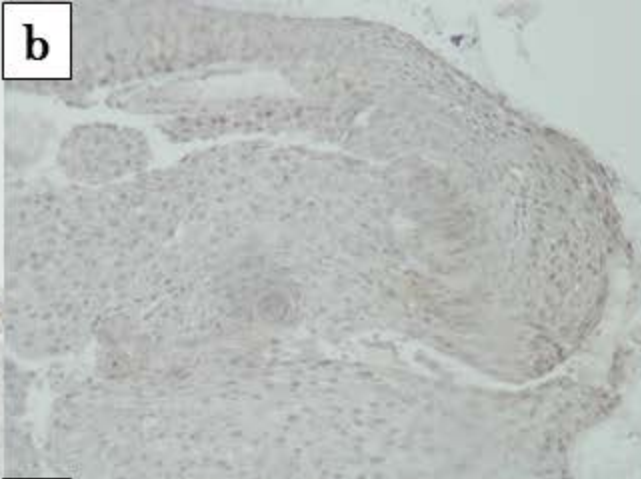
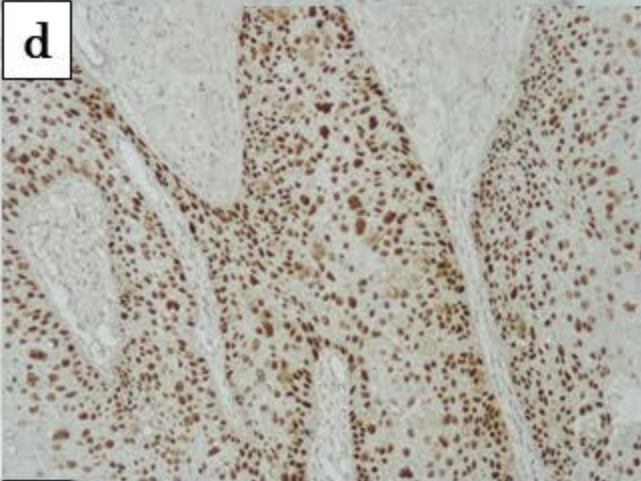
120 variants: Retained based on presence in COSMIC database and FATHMM prediction of "pathogenic"



15 variants: Retained based on presence in ClinVar database with a "pathogenic" or "likely pathogenic" classification





**a****b****c****d****e****f**