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## 1 Abstract

2	<b>Background:</b> 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	<i>Methods:</i> 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	<i>Results:</i> Gene alterations were most frequently observed in <i>TP53</i> (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	( <i>P</i> =0.007).

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

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

3

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

## 1 Introduction

 $\mathbf{2}$ External auditory canal (EAC) cancer is a rare form of malignant tumor representing less than 0.2% of all head and neck (HN) cancers, with 3 squamous cell carcinoma (SCC) being the most common histology [1, 2]. 4 Surgical resection with or without postoperative radiotherapy (RT) is widely  $\mathbf{5}$ performed as the mainstay of treatment for EACSCC, and definitive 6 chemoradiotherapy (CRT) may be appropriate for cases with unresectable 7tumors [1-3]. However, no evidence-based treatment strategy for EACSCC 8 has been established to date due to the rarity of the disease, and no clinical 9 10 trials investigating targeted therapies have been conducted. Thus, it is necessary to analyze the association between gene mutations and 11 12clinicopathologic features in EACSCC and explore potential molecular 13biomarkers for the development of novel therapeutic targets. 14The recent advances in next-generation sequencing (NGS) technologies 15have afforded remarkable progress in the identification of cancer-related 16 genes, and have significantly transformed treatment modalities and survival in several lethal malignancies, such as mucosal HNSCC and cutaneous 17HNSCC [4-7]. Further, immunohistochemical (IHC) assays have been widely 18used as an affordable and straightforward tool for the analysis of gene 19expression. Based on the results of an IHC assay, a previous study reported 2021that the expression of p53 and EGFR may be valuable biomarkers for developing tumors in EACSCC [8]. However, it is widely accepted that 2223discrepancies in gene mutations based on NGS and gene expression based on 24IHC staining might affect the corresponding cancer risk [9]. EACSCC has

 $\mathbf{5}$ 

1 not been included in The Cancer Genome Atras

2 (<u>https://portal.gdc.cancer.gov</u>), 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.

### 1 Material and methods

## 2 Ethics statement

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

12

## 13 Treatment strategy

All patients were initially evaluated by a multidisciplinary team 14consisting of otolaryngologists, radiation oncologists and medical oncologists. 1516 Staging was performed using the University of Pittsburgh modified TNM staging system [2]. Treatment selection based on the extent of the disease, 17performance status and/or comorbidities in each case. For patients with T1-2 18 19tumors, surgery was selected as the initial treatment choice. If histologic examination revealed positive surgical margins or more extensive 20involvement than suggested by preoperative imaging, postoperative RT was 2122performed. For patients with T3-4 tumors, surgery followed by CRT was 23performed. Neck dissection was performed in cases clinically positive for 24neck lymph node metastasis. For locally advanced tumors with extension to the pyramidal apex, posterior cranial fossa, middle cranial fossa or internal
 carotid artery, definitive CRT was selected.

3

## 4 Tissue samples

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

10

## 11 DNA extraction and quality assessment

12The PAXgene Tissue-fixed, paraffin-embedded (PFPE) tissues were cut into 10-µm-thick sections with a microtome. Areas with higher proportion of 13 14tumor cells and less inflammatory cells were identified by hematoxylin and 15eosin 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 macro-dissection technique was used to collect the malignant cells from the 17sections. DNA extraction was performed using the GeneRead DNA FFPE 18Tissue Kit (QIAGEN, Hilden, NRW, Germany). PFPE sample-derived DNA 19 quantity and quality were assessed by calculating the QC score using 20GeneRead DNA QuantiMIZE Kits (QIAGEN, Hilden, NRW, Germany), 2122according to the manufacturer's protocol. Samples with sufficient DNA that 23passed the quality control checks were then used for genetic analysis.

24

## **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
14	verview-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	( <u>https://cancer.sanger.ac.uk/cosmic</u> ), Functional Analysis through Hidden
22	Markov Models (FATHMM) ( <u>http://fathmm.biocompute.org.uk/cancer.html</u> )
23	and ClinVar database ( <u>https://www.ncbi.nlm.nih.gov/clinvar</u> ) 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

The specimens were embedded in paraffin and cut into 4-µm-thick sections. 6 7They were then deparaffinized in xylene, dehydrated through a graded series of alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous 8 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, pH 6.0). The sections were blocked with 10% normal goat serum for 30 11 12minutes at room temperature to prevent the non-specific binding of 13antibodies. The slides were then incubated with a p53 mouse monoclonal antibody (DO-7, Roche Diagnostics Ltd., Tokyo, Japan) in a humid chamber 1415at 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, Nichirei, Tokyo, Japan) for 30 minutes at 37 °C, followed by reaction with a 17streptavidin-biotin horseradish peroxidase complex. The reaction products 1819were observed by immersing the slides in a freshly prepared diaminobenzidine solution for 10 minutes and counterstaining them with 2021hematoxylin before dehydration and mounting. 22

## 23 Assessment of IHC assay

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

specimen under low magnification (×40) and then confirmed under high 1  $\mathbf{2}$ magnification (×200 and ×400). Two researchers including one pathologist 3 blinded to the clinical data assessed the staining of each specimen. For p53 IHC, the expression patterns were classified into three groups in 4 accordance with the methods described by Boyle et al. as follows: extreme  $\mathbf{5}$ negative (EN), complete confluent negativity of staining; extreme positive 6 7(EP), strong diffuse confluent positivity; and non-extreme (NE), all intermediate expression of any intensity [11]. 8

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 software (version 16.0; SAS Institute Inc.; Cary, NC, U.S.A.). Statistical 1314differences were analyzed using the Mann-Whitney U-test for two 15independent 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 overall survival (OS) rates. For the calculation of survival rates, death was 17counted as an event, whereas the patient being alive at the latest contact, 1819 regardless of disease status, was counted as censored. The times of interest included the beginning of treatment, the last follow-up date and death. 2021Survival status was updated in December 2021. Statistical differences were 22analyzed using the log rank test. Stepwise regression analysis was 23performed to build an appropriate model through the addition and removal 24of predictor variables. Multivariable analysis was performed using the Cox

1 proportional hazard model.

 $\mathbf{2}$ 

## 1 Results

## 2 Clinicopathological features

3 The PFPE tissue samples were obtained from 32 patients with EACSCC. Of these, 6 patients were ineligible for genetic analysis due to the high levels 4 of necrosis, keratin, inflammatory infiltrates or hemorrhaging in tissue  $\mathbf{5}$ samples. Further, 4 patients were excluded due to an insufficient quantity 6 7and quality of tumor tissue-derived DNA. Finally, a total of 22 patients were enrolled in this analysis. 8 9 Patient profiles are summarized in Table 1. Regarding T classification, 9 patients were categorized as early T stage (T1-2) and 13 patients as 10

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 <i>TP53</i> (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 <i>CREBBP</i> (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*

Figure 2 depicts a visual representation of the relationships between gene
mutations and clinical parameters in EACSCC patients. Genetic mutations
appeared to be more frequent among female patients, those with a more
advanced T and N staging, and those with poorer differentiation.
Table 3 shows that *TP53* mutations were correlated significantly with T
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

## Survival analysis with regard to TP53 mutations and clinicopathologic features

Based on NGS results, the 5-year OS rates for EACSCC patients with 15TP53 mutations and wild-type TP53 were 45.0% and 75.0%, respectively (Fig. 163). Univariable analysis indicated that T classification, TP53 mutations and 17treatment modality (P=0.003, P=0.046 and P=0.006, respectively) were 18significant prognostic factors for EACSCC (Table 4). Meanwhile, other 19clinicopathologic features, such as age, gender, smoking status and alcohol 20consumption, N classification, and histopathological differentiation, were not 21significantly correlated with survival outcomes. Stepwise regression analysis 2223and multivariable analysis using the Cox proportional hazards model 24demonstrated that TP53 mutations and treatment modality (P=0.007 and

- 1 *P*=0.010, respectively) were independent predictors of OS rate.
- $\mathbf{2}$

## 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.

### 1 Discussion

 $\mathbf{2}$ Patients with early-stage EACSCC have good survival outcomes, whereas advanced-stage patients have a poor prognosis [1-3, 12]. In cases with 3 unresectable or recurrence tumors, in particular, the treatment options, 4 including targeted therapies, have been limited [3, 12]. This study has  $\mathbf{5}$ confirmed that treatment modality based on T classification is an 6 7independent prognostic factor, which is consistent with the results of previous analyses of EACSCC [1-3, 12]. Meanwhile, no studies are yet to 8 focus on genetic mutations of EACSCC and their relationship to 9 10 clinicopathological features. Thus, this study analyzed genetic mutations based on a targeted NGS and genetic expression based on IHC staining to 11 12investigate the driver of tumorigenesis and/or the candidates of genes for predicting clinical outcome in EACSCC. In patients with EACSCC, gene 1314mutations were most frequently observed in *TP53* (59.1%), followed by 15CREBBP (9.1%). TP53 is the most commonly mutated tumor suppressor 16 gene in human cancers, which is consistent with the results of previous comprehensive genetic studies of mucosal and cutaneous HNSCC [13]. 17CREBBP encodes the CBP protein containing a transcriptional cofactor with 1819lysine acetyltransferase activity and close homology to p300, which is associated with tumor progression [14]. *CREBBP* mutations are frequently 2021identified across a range of different cancers, such as lymphomas and 22bladder, esophageal, and cutaneous SCC [14]. Although the gene mutations in EACSCC were expected to mostly 23

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  $\mathbf{2}$ HNSCC have been reported to be 44% in TP53, 20% in NOTCH1, 19% in FAT1, and 14% in PIK3CA, according to the COSMIC database. However, 3 except for TP53, this analysis did not identify mutations in major 4 cancer-related gene, such as NOTCH1, FAT1, and PIK3CA. Thus, the  $\mathbf{5}$ mutational patterns in EACSCC might differ slightly from those in mucosal 6 7HNSCC due to tissue-specific histopathological and environmental factors, 8 as mentioned below: EACSCC arises from the skin of the EAC, whereas mucosal HNSCC occurs in the mucosa of the nasal cavity, paranasal sinus, 9 10 oral cavity, pharynx or larynx. Chronic inflammation in the EAC induced by habitual ear picking and other mechanical stimuli causes EACSCC [1, 15], 11 12with smoking and alcohol unlikely to be major carcinogens of EACSCC. 13It is noteworthy that *CREBBP* mutations have been identified in patients with EACSCC. CREBBP mutations are common in cutaneous SCC and 14contribute to the tumorigenesis of epidermal keratinocytes [14, 16]. 1516 Mutations in *NOTCH1*, being one of the major cancer-related genes of cutaneous SCC, frequently develop under ultraviolet irradiation, whereas 17CREBBP mutations occur in epidermal keratinocytes that are less exposed 1819to sunlight [14]. Thus, the gene mutational patterns in EACSCC, in which the tissue of origin receive less exposure to sunlight than other cutaneous 20sites of HN, might resemble those in cutaneous SCC in less sun-exposed skin 2122rather than mucosal HNSCC. However, among the genes analyzed using the 23GeneRead Human Comprehensive Cancer Panel, 45.5 % of patients with 24EACSCC presented no genetic mutations. This analysis might have been

1 affected by the exon coverage of the gene panels, mutations in unknown  $\mathbf{2}$ genes, the presence of epigenetic aberrations, or copy number variations. 3 Further analysis is required to explore the genomic landscape of EACSCC. To date, there is little in the way of convincing genetic data in relation to 4 the clinicopathological and prognostic implications due to the rarity of  $\mathbf{5}$ EACSCC. This analysis found that gene alterations in *TP53* in EACSCC 6 7tissues were significantly associated with T classification and survival 8 outcome. TP53 encodes a tumor suppressor protein p53 containing transcriptional activation, DNA binding, and oligomerization domains. The 9 10 encoded p53 protein responds to diverse cellular stresses to regulate the expression of target genes and is involved in cell cycle regulation, 11 12proliferation and apoptosis [17]. The mutated TP53 has a longer half-life and 13deactivates the trigger of p53-dependent apoptosis, resulting in tumor 14development and enlargement in a variety of human cancers [17, 18]. The 15gene alterations in TP53 in other primary sites of HNSCC have been 16 reported to correlate with T classification and survival outcome [19], which is in agreement with the results of this analysis of EACSCC. 17It remains controversial whether the p53 expression detected by IHC 18staining affords a consistent prognostic biomarker of EACSCC [8]. Although 19several IHC evaluation systems for p53 have been proposed as surrogate 2021methods for the molecular subtypes based on the gene expression patterns, 22the discrepancy between *TP53* mutations based on NGS and p53 expression 23based on IHC affects the corresponding assessment of cancer risk [9]. 24Missense mutations that lead to less aberrant functional proteins have been

1 shown to produce strong IHC staining, and nonsense mutations that lead to  $\mathbf{2}$ 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 and predicted the prognosis in patients with breast cancer by classifying the 4 IHC findings into three groups: EN, EP, and NE [11]. They found that  $\mathbf{5}$ p53-EN or -EP was significantly associated with a poorer survival outcome 6 than was p53-NE, and that combined p53-EN and -EP better predicted the 7survival outcome than either pattern alone. Furthermore, in their analysis of 8 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 p53-EP phenotype, and a nonsense mutation appearing to abrogate protein 11 12expression, manifesting as the p53-EN phenotype. This analysis 13demonstrated that the TP53 mutation status was significantly correlated 14with the p53 expression phenotype in patients with EACSCC, which is more 15consistent with the results of the previous analysis [11]. TP53 mutations as 16 well as p53-EN/EP were significantly correlated with poor survival outcome in patients with EACSCC. The advantage of IHC is that it offers an 17affordable and straightforward assay to detect genetic expression in 1819 comparison with NGS. IHC staining might be a cost-effective screening tool for detecting TP53 mutations by assigning p53-EN/EP as representing a 2021TP53 mutation and p53-NE as the wild-type TP53. 22The results of this analysis might have been affected by the small cohort 23size, 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  $\mathbf{2}$ the IHC assessment methods. Although extraction of high-quality DNA from 3 tissue samples is inherently difficult, stringent sample storage and quality control checks were applied to ensure that only cases with high-quality DNA 4 were included in this analysis. All EACSCC samples were stored as PFPE  $\mathbf{5}$ tissues, which have been reported to preserve proteins and nucleic acids in a 6 7more native state compared to formalin-fixed paraffin-embedded tissues [20]. Another limitation is the ambiguity with regard to the classification of 8 variants as pathogenic. There were 105 missense variants categorized as 9 10 pathogenic in the COSMIC database, whereas those variants were categorized as "benign", "not provided", "not reported" or "of uncertain 11 12significance" in the ClinVar database. This analysis did not assign those variants as pathogenic mutations, and might, therefore, have 1314underestimated the prevalence of pathogenic mutations. 15

## 1 Conclusion

 $\mathbf{2}$ This study analyzed genetic mutations based on targeted NGS and genetic expression based on IHC staining in EACSCC tissue samples. Gene 3 alterations were most frequently observed in TP53, followed by CREBBP. 4 The mutated TP53 was significantly correlated with T classification, and  $\mathbf{5}$ 6 poor prognosis and p53 expression phenotype, suggesting that TP53 mutations have potential for use as a biomarker for identifying individuals 78 at high risk of developing tumors and as well as for predicting survival outcome in EACSCC. IHC staining for p53 might play a useful role as a 9 screening tool for detecting TP53 mutations in patients with EACSCC. 10 11

## 1 Disclosure statement

2 The authors have no conflicts of interest to declare.

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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 <i>TP53</i> mutations and clinicopathologic						
9	features						
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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	
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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

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	у

Table 1. Characteristics of patients with 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	TP53	$NM_{000546.5}$	c.422G>A	p.C141Y	Missense	Pathogenic	Pathogenic
EC7	TP53	$NM_{000546.5}$	c.451C>A	p.P151T	Missense	Pathogenic	Likely pathogenic
EC15	TP53	$NM_{000546.5}$	c.916C>T	p.R306*	Nonsense	Pathogenic	Pathogenic
EC15	CREBBP	NM_004380.2	c.4337G>A	p.R1446H	Missense	Pathogenic	Likely pathogenic
EC16	TP53	NM_000546.5	c.742C>T	p.R248W	Missense	Pathogenic	Pathogenic
EC18	TP53	NM_000546.5	c.527G>T	p.C176F	Missense	Pathogenic	Likely pathogenic
EC20	TP53	NM_000546.5	c.743G>A	p.R248Q	Missense	Pathogenic	Pathogenic
EC22	TP53	$NM_{000546.5}$	c.752T>G	p.I251S	Missense	Pathogenic	Likely pathogenic
EC22	TP53	$NM_{000546.5}$	c.517G>T	p.V173L	Missense	Pathogenic	Pathogenic
EC23	TP53	$NM_{000546.5}$	c.586C>T	p.R196*	Nonsense	Pathogenic	Pathogenic
EC28	TP53	NM_000546.5	c.736A>G	p.M246V	Missense	Pathogenic	Pathogenic
EC30	TP53	$NM_{000546.5}$	c.743G>A	p.R248Q	Missense	Pathogenic	Pathogenic
EC31	TP53	NM_000546.5	c.451C>A	p.P151T	Missense	Pathogenic	Likely pathogenic
EC32	TP53	$\rm NM\_000546.5$	c.856G>A	p.E286K	Missense	Pathogenic	Pathogenic
EC32	CREBBP	NM_004380.2	c.4337G>A	p.R1446H	Missense	Pathogenic	Likely pathogenic

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

# Table 3. Relationships between TP53 mutations and clinicopathologic features

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

	5 year-OS rate (%)	Univariate analysis		Multivariate analysis	
		HR	Pvalue	HR	Pvalue
Age (years)					
$\leq 65$	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%	$1.54 x 10^{9}$	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		
TP53					
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%	$5.66 \mathrm{x10^{10}}$	0.006	30.8	0.010
OS; overall survival, HR; hazard ratio, Ref.; reference (HR=1.0), CRT;					
chemoradiotherapy					

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

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

	TP53 status					
p53-expression phenotype	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						



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







