Association of gene mutations with clinicopathologic features in patients with external auditory canal squamous cell carcinoma

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Abstract

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

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

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

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

Introduction

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 squamous cell carcinoma (SCC) being the most common histology [1, 2]. Surgical resection with or without postoperative radiotherapy (RT) is widely performed as the mainstay of treatment for EACSCC, and definitive chemoradiotherapy (CRT) may be appropriate for cases with unresectable tumors [1-3]. However, no evidence-based treatment strategy for EACSCC has been established to date due to the rarity of the disease, and no clinical trials investigating targeted therapies have been conducted. Thus, it is necessary to analyze the association between gene mutations and clinicopathologic features in EACSCC and explore potential molecular biomarkers for the development of novel therapeutic targets.

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

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

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

Material and methods

Ethics statement

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

Treatment strategy

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

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.

DNA extraction and quality assessment

The PAXgene Tissue-fixed, paraffin-embedded (PFPE) tissues were cut into 10-μm-thick sections with a microtome. Areas with higher proportion of tumor cells and less inflammatory cells were identified by hematoxylin and eosin staining, and the presence of at least 20% malignant cells in the 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 sections. DNA extraction was performed using the GeneRead DNA FFPE Tissue Kit (QIAGEN, Hilden, NRW, Germany). PFPE sample-derived DNA quantity and quality were assessed by calculating the QC score using GeneRead DNA QuantiMIZE Kits (QIAGEN, Hilden, NRW, Germany), according to the manufacturer's protocol. Samples with sufficient DNA that passed the quality control checks were then used for genetic analysis.

NGS

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

Analysis of NGS data

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

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

Analysis of gene expression by IHC staining

The specimens were embedded in paraffin and cut into 4-μm-thick sections. They were then deparaffinized in xylene, dehydrated through a graded series of alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous peroxidase activity. Antigen retrieval was performed using a 750 W 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 minutes at room temperature to prevent the non-specific binding of antibodies. The slides were then incubated with a p53 mouse monoclonal antibody (DO-7, Roche Diagnostics Ltd., Tokyo, Japan) in a humid chamber at 4 °C overnight. The sections were then incubated with a biotin-labeled 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 streptavidin-biotin horseradish peroxidase complex. The reaction products were observed by immersing the slides in a freshly prepared diaminobenzidine solution for 10 minutes and counterstaining them with hematoxylin before dehydration and mounting.

Assessment of IHC assay

All slides were examined by light microscopy scanning the entire tissue

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

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

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (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 differences were analyzed using the Mann-Whitney U-test for two independent groups, with a P value of less than 0.05 considered statistically 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 counted as an event, whereas the patient being alive at the latest contact, regardless of disease status, was counted as censored. The times of interest included the beginning of treatment, the last follow-up date and death. Survival status was updated in December 2021. Statistical differences were analyzed using the log rank test. Stepwise regression analysis was performed to build an appropriate model through the addition and removal of predictor variables. Multivariable analysis was performed using the Cox

proportional hazard model.

Results

Clinicopathological features

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 of necrosis, keratin, inflammatory infiltrates or hemorrhaging in tissue samples. Further, 4 patients were excluded due to an insufficient quantity and quality of tumor tissue-derived DNA. Finally, a total of 22 patients were enrolled in this analysis.

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

Somatic gene mutations in EACSCC

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

Relationships between somatic gene mutations and clinicopathologic 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 between *TP53* mutation status and the other parameters, such as age, gender, smoking status and alcohol consumption, N classification, and histopathological differentiation.

Survival analysis with regard to TP53 mutations and clinicopathologic features

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

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

Relationships between TP53 mutations and p53 expression phenotype

The p53-EN, -EP, and -NE phenotypes were found in 5 (22.7%), 7 (31.8%) and 10 (45.5%) of the EACSCC samples, respectively (Table 5 and Fig. 4a-f). With regard to the correlation between *TP53* mutation status and the p53 expression phenotype, *TP53* mutations were more frequently identified in p53-EN/EP cases than in p53-NE cases (P<0.001), with 2 patients with *TP53* nonsense mutations revealing a p53-EN phenotype. The 5-year OS rates for patients with the p53-EN/EP and p53-NE phenotypes were 75.0% and 46.9%, respectively (Fig. 5). The survival outcomes based on IHC findings were found to be similar to those based on NGS results.

Discussion

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

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

remains unclear due to its rarity. The gene mutational rates of mucosal 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, except for *TP53*, this analysis did not identify mutations in major cancer-related gene, such as *NOTCH1*, *FAT1*, and *PIK3CA*. Thus, the mutational patterns in EACSCC might differ slightly from those in mucosal HNSCC due to tissue-specific histopathological and environmental factors, as mentioned below: EACSCC arises from the skin of the EAC, whereas mucosal HNSCC occurs in the mucosa of the nasal cavity, paranasal sinus, oral cavity, pharynx or larynx. Chronic inflammation in the EAC induced by habitual ear picking and other mechanical stimuli causes EACSCC [1, 15], with smoking and alcohol unlikely to be major carcinogens of EACSCC.

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

affected by the exon coverage of the gene panels, mutations in unknown genes, the presence of epigenetic aberrations, or copy number variations. 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 the clinicopathological and prognostic implications due to the rarity of EACSCC. This analysis found that gene alterations in *TP53* in EACSCC tissues were significantly associated with T classification and survival outcome. *TP53* encodes a tumor suppressor protein p53 containing transcriptional activation, DNA binding, and oligomerization domains. The encoded p53 protein responds to diverse cellular stresses to regulate the expression of target genes and is involved in cell cycle regulation, proliferation and apoptosis [17]. The mutated *TP53* has a longer half-life and deactivates the trigger of p53-dependent apoptosis, resulting in tumor development and enlargement in a variety of human cancers [17, 18]. The gene alterations in *TP53* in other primary sites of HNSCC have been reported to correlate with T classification and survival outcome [19], which is in agreement with the results of this analysis of EACSCC.

It remains controversial whether the p53 expression detected by IHC staining affords a consistent prognostic biomarker of EACSCC [8]. Although several IHC evaluation systems for p53 have been proposed as surrogate methods for the molecular subtypes based on the gene expression patterns, the discrepancy between *TP53* mutations based on NGS and p53 expression based on IHC affects the corresponding assessment of cancer risk [9]. Missense mutations that lead to less aberrant functional proteins have been

shown to produce strong IHC staining, and nonsense mutations that lead to a complete absence of p53 protein expression have been shown to produce no 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 IHC findings into three groups: EN, EP, and NE [11]. They found that p53-EN or -EP was significantly associated with a poorer survival outcome than was p53-NE, and that combined p53-EN and -EP better predicted the survival outcome than either pattern alone. Furthermore, in their analysis of the *TP53* mutation status, the detected mutation types appeared to be related to the protein status, with a missense mutation corresponding to the p53-EP phenotype, and a nonsense mutation appearing to abrogate protein expression, manifesting as the p53-EN phenotype. This analysis demonstrated that the *TP53* mutation status was significantly correlated with the p53 expression phenotype in patients with EACSCC, which is more consistent with the results of the previous analysis [11]. *TP53* mutations as 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 affordable and straightforward assay to detect genetic expression in comparison with NGS. IHC staining might be a cost-effective screening tool for detecting *TP53* mutations by assigning p53-EN/EP as representing a *TP53* mutation and p53-NE as the wild-type *TP53*.

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

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

Conclusion

This study analyzed genetic mutations based on targeted NGS and genetic expression based on IHC staining in EACSCC tissue samples. Gene alterations were most frequently observed in *TP53*, followed by *CREBBP*. The mutated *TP53* was significantly correlated with T classification, ~~and~~ poor prognosis and p53 expression phenotype, suggesting that *TP53* mutations have potential for use as a biomarker for identifying individuals 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 screening tool for detecting *TP53* mutations in patients with EACSCC.

Disclosure statement

The authors have no conflicts of interest to declare.

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

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

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

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

Fig. 5. The 5-year overall survival rates for external auditory canal squamous cell carcinoma patients with p53-EN/EP phenotype and p53-NE phenotype
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	*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*	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 3. Relationships between *TP53* mutations and clinicopathologic features

	n	*TP53* mutation (n, %)	*P* value
Age			
≤ 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

Moderate-Poor	6	5 (83.3%)

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

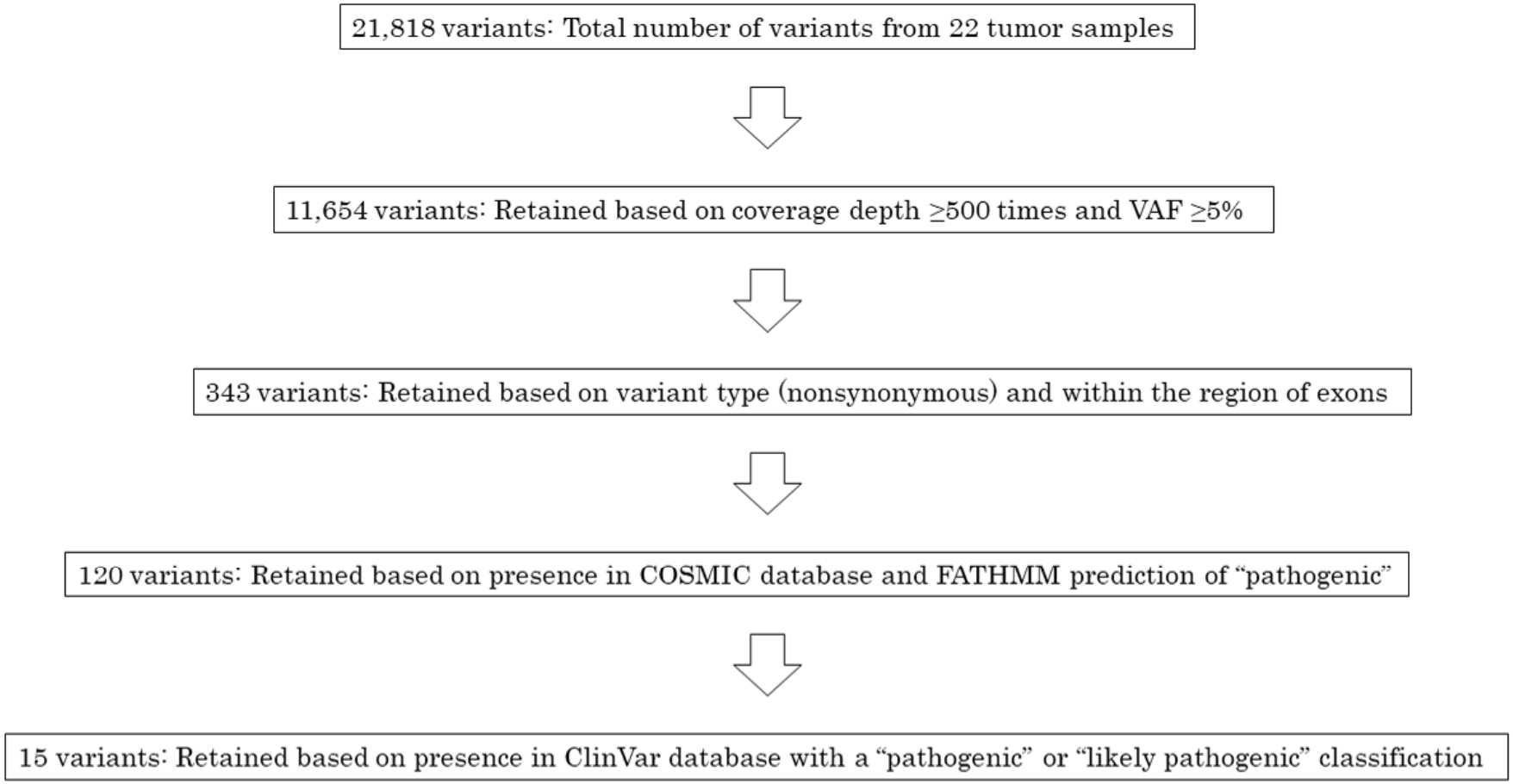
	5 year-OS rate (%)	Univariate analysis		Multivariate analysis	
		HR	*P* value	HR	*P* value
Age (years)					
≤ 65	65.6%	Ref.			
> 65	53.3%	1.56	0.60		
Gender					
Female	71.8%	Ref.			
Male	38.9%	1.97	0.37		
Smoking					
Never and former	62.2%	Ref.			
Current	56.3%	1.36	0.69		
Alcohol					
Never and former	68.6%	Ref.			
Current	44.4%	1.67	0.51		
T classification					
1-2	100%	Ref.			
3-4	30.8%	1.54×10^{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%	5.66×10^{10}	0.006	30.8	0.010

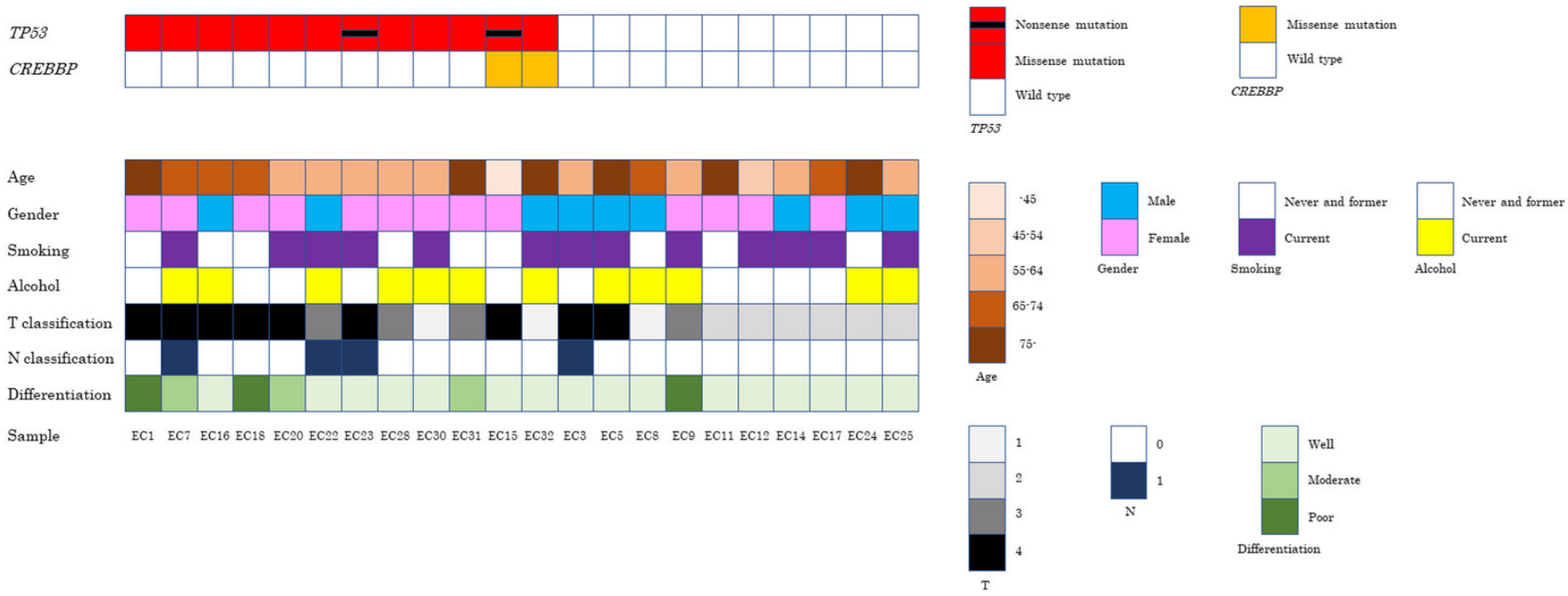
OS; overall survival, HR; hazard ratio, Ref.; reference (HR=1.0), CRT; chemoradiotherapy

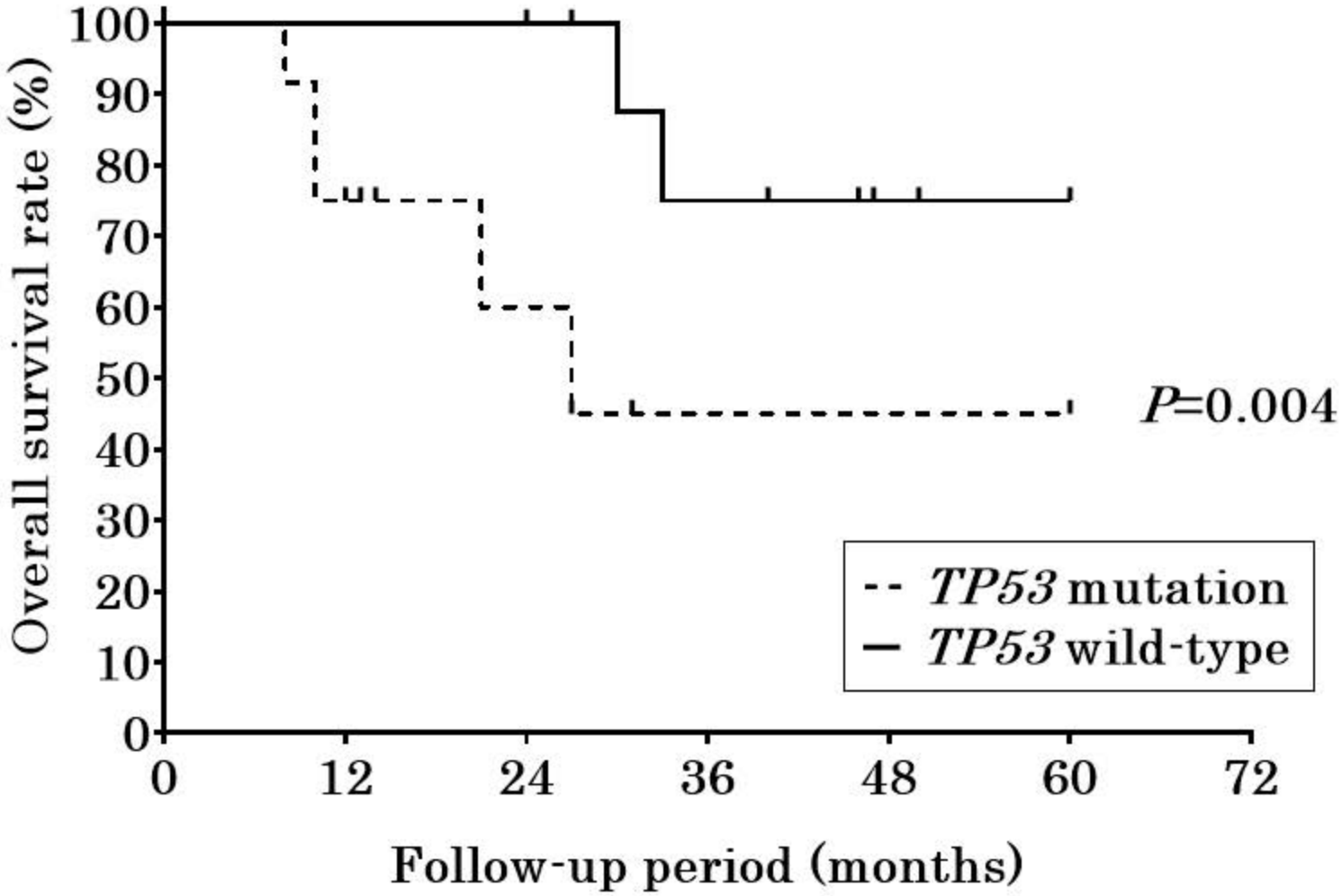
Table 5. Correlation between p53-expression phenotype and *TP53* status in external auditory canal squamous cell carcinoma

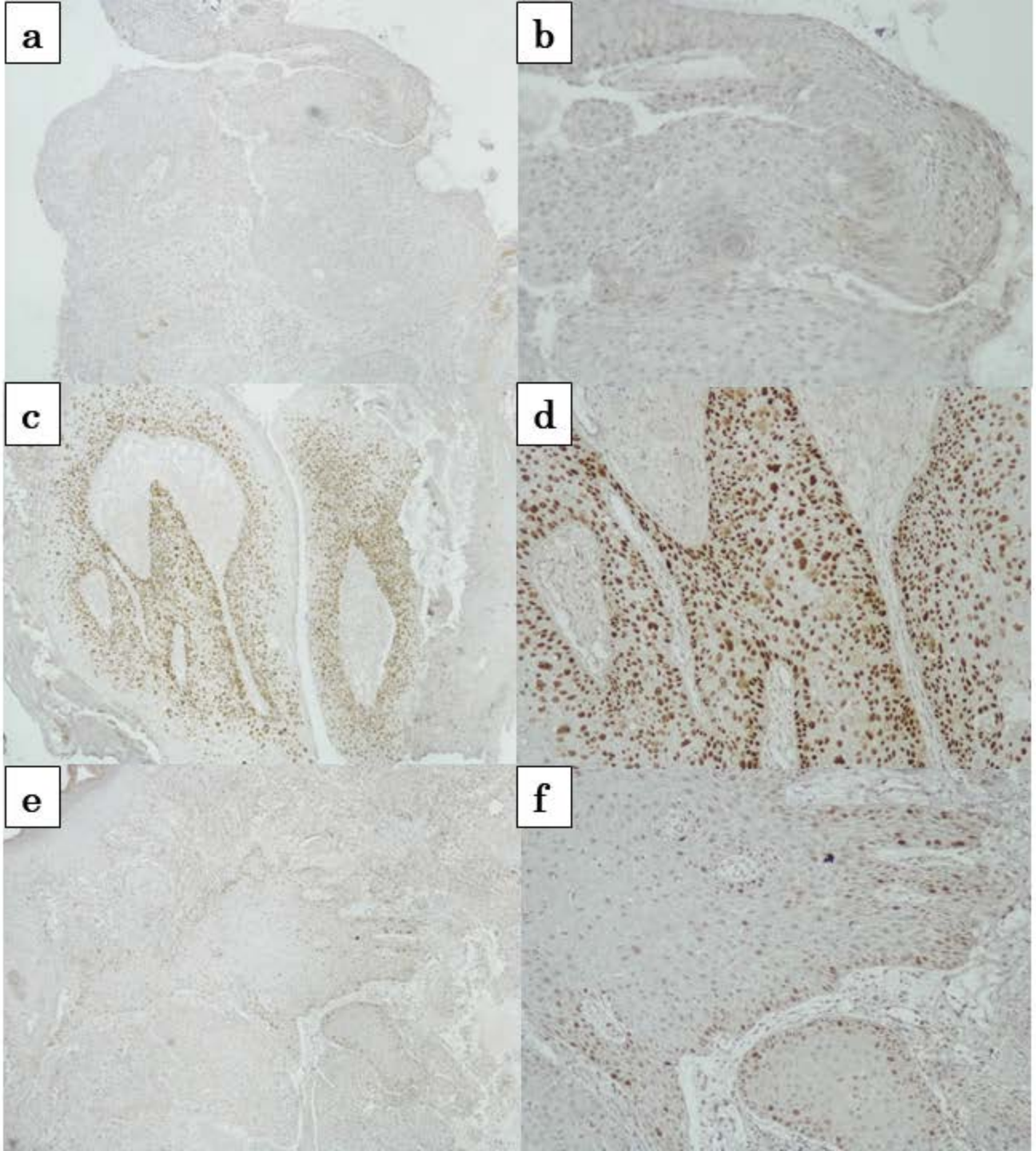
p53-expression phenotype	*TP53* status		
	Wild-type	Mutation	
		Missense	Nonsense
EN	0	3	2
EP	1	6	0
NE	9	1	0
Total	10	10	2

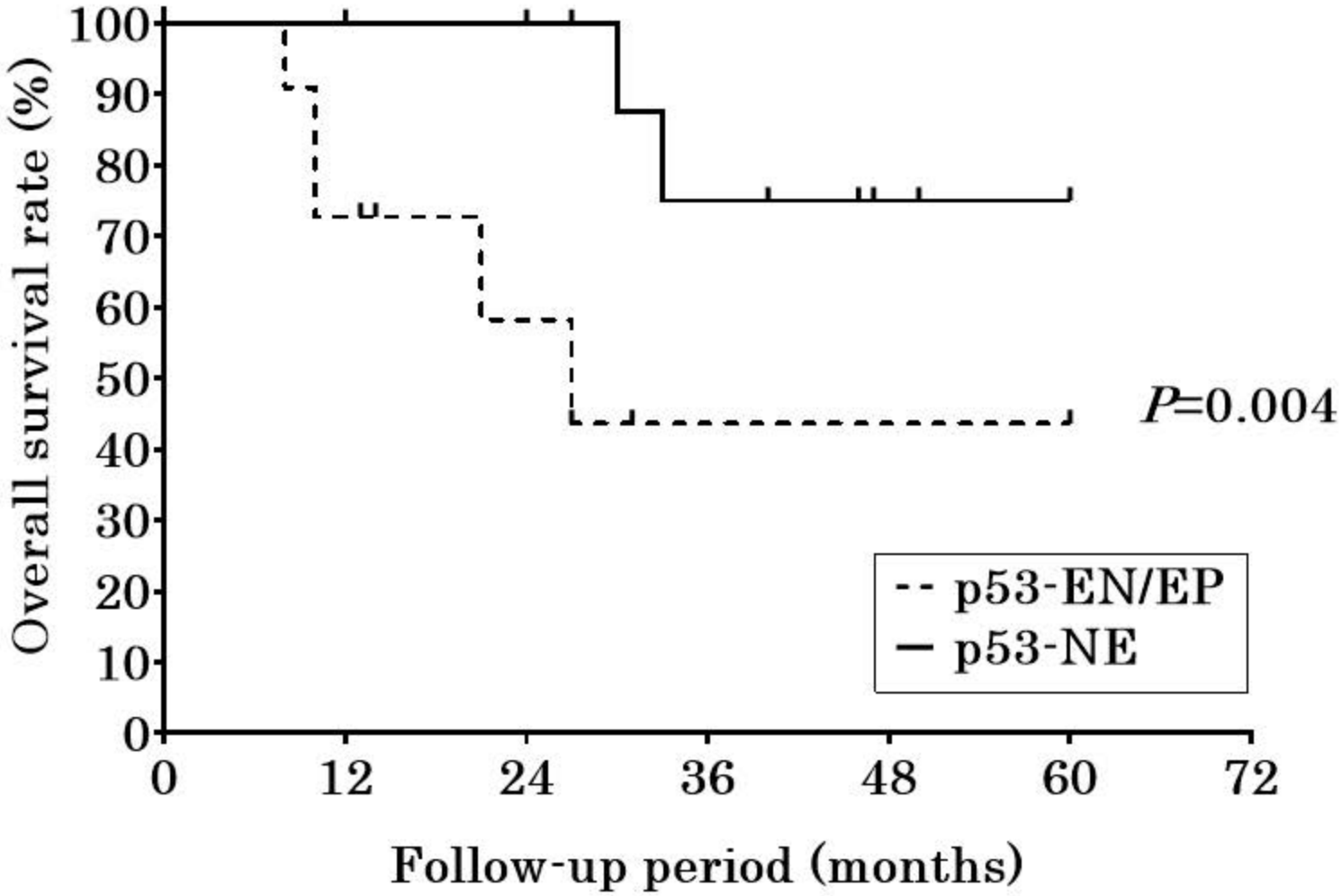
EN; extreme negative, EP; extreme positive, NE; non-extreme


21,818 variants: Total number of variants from 22 tumor samples
11,654 variants: Retained based on coverage depth ≥500 times and VAF ≥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


TP53
CREBBP
Age
Gender
Smoking
Alcohol
T classification
N classification
Differentiation
Sample
EC1 EC7 EC16 EC18 EC20 EC22 EC23 EC28 EC30 EC31 EC15 EC32 EC3 EC5 EC8 EC9 EC11 EC12 EC14 EC17 EC24 EC25
Nonsense mutation
Missense mutation
Wild type
TP53
Missense mutation
Wild type
CREBBP
·45
45-54
55-64
65-74
75·
Age
Male
Female
Gender
Never and former
Current
Smoking
Never and former
Current
Alcohol
1
2
3
4
T
0
1
N
Well
Moderate
Poor
Differentiation


Overall survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
Follow-up period (months)
P=0.004
-- TP53 mutation
— TP53 wild-type


a
b
c
d
e
f


Overall survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
60
72
Follow-up period (months)
P=0.004
-- p53-EN/EP
— p53-NE