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Prognostic significance of actionable next-generation sequencing multigene panel in esophageal cancer treatment

Z. TEKE[1], A. BISGIN[2,3], C. ORUC RENCUZOGULLARI[3], K. EREN ERDOGAN[4], C.K. PARSAK[5], S. KALKANLI TAS[6]

[1]Department of Gastroenterological Surgery, University of Health Sciences, Başakşehir Çam and Sakura City Hospital, Istanbul, Turkey
[2]Department of Medical Genetics, Faculty of Medicine, Çukurova University, Adana, Turkey
[3]Adana Genetic Diseases Diagnosis and Treatment Center (AGENTEM), Faculty of Medicine, Çukurova University, Adana, Turkey
[4]Department of Pathology, Faculty of Medicine, Çukurova University, Adana, Turkey
[5]Department of General Surgery, Faculty of Medicine, Çukurova University, Adana, Turkey
[6]Department of Immunology, Hamidiye Faculty of Medicine, University of Health Sciences, Istanbul, Turkey

Abstract. – OBJECTIVE: Next-generation sequencing (NGS) has been offered as a large-scale and effective genomic analyzing tool. In this research, we seek to examine the possible benefits of an actionable mutation panel in association with clinical and pathological features in the treatment of esophageal cancer.

PATIENTS AND METHODS: In our study, 85 cases whose diagnosis of carcinoma was confirmed histopathologically either by endoscopic biopsy or esophageal surgery between 2010 and 2020 were identified from the hospital database. In formalin-fixed, paraffin-embedded tumor samples, a total of 20 genes of *AKT1*, *ALK*, *BRAF*, *DDR*, *EGFR*, *ERBB2*, *ERBB3*, *ESR1*, *FGFR1*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *NTRK*, *PDGFRA*, *PIK3CA*, *PTEN*, *RICTOR* and *ROS1* were analyzed *via* NGS for actionable mutations.

RESULTS: Of 85 cases, 47 patients (55.3%) were men and 38 (44.7%) were women, and the mean age of the patients was 58.01±11.45 years. There were substantial distinctions in the variables of pathogenicity of variant, operation type, stage, and both lymphovascular and perineural invasion ($p<0.05$). Most of the primary tumors were situated in the lower thoracic esophagus ($n=23$; 27%). *PIK3CA* variant was the highest in number among the variant types ($n=17$) and was detected in 41.2% of the lower thoracic tumors. The increases in mutation numbers of ≥2 were especially concentrated in the lower thoracic esophageal carcinomas.

CONCLUSIONS: The utility of an actionable multigene panel revealed the value of a well-designed NGS workflow in the practical use of clinical outcomes *via* the prediction of responsiveness to therapeutic agents or indications for novel treatment modalities in addition to the estimation of prognosis.

Key Words:
Next-generation sequencing, Esophageal cancer, Multigene panel.

Introduction

Esophageal carcinoma (EC) is the 18th most commonly diagnosed cancer, and it represents 1% of all newly diagnosed cancer cases[1]. EC is not significant in terms of diagnosis or global burden, but it is increasing in incidence faster than other cancers in some Western countries. According to GLOBOCAN 2020 data, EC has the 7th highest incidence of all cancers and is the 6th most common cause of death, and approximately 0.6 million new EC cases were diagnosed in 2020 and 0.54 million deaths happened as a result of this disease[2]. Despite many new-generation technological chemotherapeutic agents and surgical procedures, the 5-year survival ratios of EC still remain low and are found to be 47% in adenocarcinoma (AC) and 37% in squamous cell carcinoma (SCC)[3]. Therefore, methods that will be useful in the management of this disease are urgently needed.

A brief review of revolution of the sequencing technology is firstly presented. In 1977, Frederick Sanger was the first to sequence the complete

Corresponding Author: Zafer Teke, MD, Ph.D; e-mail: zafer.teke@sbu.edu.tr

deoxyribonucleic acid (DNA) genome of a bacteriophage, and then he invented DNA sequencing with chain-terminating inhibitors[4]. Leroy Hood invented the first semi-automated DNA sequencing machine in 1986, and this became a crucial means for mapping and sequencing genetic material. In 1987, Applied Biosystems commercialized ABI370, the first automated sequencing machine, and this was a vast advance for mapping the human genome. In 2000, Lynx Therapeutics Company introduced "Massively Parallel Signature Sequencing" (MPSS), marking the debut of next-generation sequencing (NGS) technologies[5]. In 2004, 454 Life Sciences merchandized Roche GS20, a new generation pyrosequencing technology, and this was the first NGS platform in the industry and revolutionized DNA sequencing by producing 20 million base pairs[6]. Illumina introduced HiSeq X Sequencer in 2014, and these platforms produced almost all publicly available human DNA sequencing data[7]. In 2022, Illumina announced the launch of NovaSeq X Series, which generates 20,000 whole genomes per year.

NGS platforms are divided into two categories based on clonal template generation: short-read approaches and long-read sequencing. Short-read sequencing approaches fall under two broad categories: 1) sequencing by ligation: SOLiD 5500 xl (Thermo Fisher Scientific, Life Technologies Co., Carlsbad, CA, USA) and BGISEQ-500 FCL (Complete Genomics, BGI Group, Yantian, Shenzhen, China), and 2) sequencing by synthesis: a) cyclic reversible termination: HiSeq X (Illumina Inc., San Diego, CA, USA) and GeneReader (Qiagen Inc., Hilden, Germany), and b) single-nucleotide addition: 454 pyrosequencing [454 GS FLX (Roche 454 Life Sciences, Branford, CT, USA)] and Ion Torrent [ION S5 540 (Thermo Fisher Scientific, Life Technologies Co., Carlsbad, CA, USA)].

Currently, there are two main types of long-read technologies. 1) Single-molecule real-time sequencing approaches: a) single-molecule real-time (SMRT), Pacific Biosciences RS II or Sequel (PacBio) (Pacific Biosciences, Menlo Park, CA, USA), and b) MinION or PromethION (ONT) (Oxford Nanopore Technologies, Oxford Science Park, Oxford, UK), and 2) synthetic approaches: a) Illumina synthetic long-read sequencing platform, and b) 10X Genomics emulsion-based system. Now, the most widely used long-read platform is the single-molecule real-time (SMRT) sequencing approach used by Pacific Biosciences.

NGS technologies have revolutionized genomic medicine by allowing high-throughput, parallel sequencing of the human genome[8]. NGS allows simultaneous, accurate, and reliable operation of multiple gene panels, making it the gold standard for identifying patient-specific treatment protocols for detecting somatic mutations. With genomic medicine, individual risks can be identified, and individualized treatment is possible. Specific genes in the cell signaling pathways should be the target of potential therapies in the patient-specific treatment. Today, however, a large proportion of clinical NGS enterprises are supported by larger academic institutions with shared access to established genomic and bioinformatics research infrastructures, and routine clinical implementation of NGS is complicated by mitigating factors, such as clinical performance, laboratory expertise, lengthy turn-around times, and cost[10]. Thus, we investigated clinically relevant mutations in esophageal malignancies that generated superquality sequencing information from formalin-fixed paraffin-embedded (FFPE) tumor tissues. This NGS panel is a multiplex Polymerase Chain Reaction (PCR)-based library preparation method that encompasses mutational hotspots of 20 genes related to solid tumors, including EC, which are selectively amplified and sequenced.

The burden of EC is heterogeneous across regions and countries in terms of sex, age, and socio-demographic indices; steps towards NGS may be effective in expanding our knowledge of additional factors in the etiopathogenesis of EC to develop more detailed prevention and intervention strategies. In the present study, we seek to analyze the potential benefits of an actionable mutation panel for EC with reference to clinical features and pathological characteristics.

Patients and Methods

Eighty-five patients who underwent either endoscopic biopsy or operation with the intent of curation or palliation for confirmed EC by histopathological examination between January 2010 and June 2020 were identified from the Çukurova University (Adana, Turkey) hospital database. FFPE tumor specimens were examined for targeted mutations in 20 genes by NGS. Detected variants were divided into four categories as pathogenic, non-pathogenic, probably pathogenic, and variants of unknown clinical significance (VUS). The informed consent was read and signed by all participants. This research was approved by the Institutional Review Board of Çukurova Univer-

sity, Adana, Turkey (IRB No. 28/100/05.06.2020). All procedures involving human participants in this study adhered to the ethical standards of the Institutional Review Board and the Helsinki Declaration.

Sampling

Tumor samples from 85 cases whose molecular testing was requested by staff surgeons were studied in daily routine practice. Data were compiled in a prospective manner. DNA was derived from FFPE tumor specimens after macrodissection of the tumor field by using the QIAamp FFPE tissue kit (Qiagen, Heidelberg, Germany). The hematoxylin and eosin-dyed microscopic slides from the same piece examined by a pathologist earlier who ascertained the tumor field and calculated both the percentage of tumor and the percentage of tumor necrosis were utilized as a landmark for the somatic variant interpretation. The DNA extracted was measured using the Qubit® fluorometer in conjunction with the Qubit® dsDNA HS assay kit (Life Technologies, Gent, Belgium).

Next-Generation Sequencing

An optimized NGS workflow diagram was carried out as defined earlier[11]. Concisely, 40 ng of DNA was richened by PCR so as to sequence actionable hotspot zones in 20 genes involving *AKT1*, *ALK*, *BRAF*, *DDR*, *EGFR*, *ERBB2*, *ERBB3*, *ESR1*, *FGFR1*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *NTRK*, *PDGFRA*, *PIK3CA*, *PTEN*, *ROS1*, and *RICTOR*. Then, specimens tagged with specimen-specific barcodes and libraries were prepared for the sequencing stage. Quality control (QC) was performed using capillary gel electrophoresis at the end of each stage. Immediately after ending the workflow, prepared libraries were sequenced with the Gene-Reader NGS system (Qiagen, Heidelberg, Germany). The raw data and QC of sequenced data were assessed before variant interpretation. Specimens with suitable sequencing data went through somatic mutation examination by utilizing QCI-A software (Qiagen, Heidelberg, Germany). All sequenced specimens had at least 500× coverage. In the variant list acquired, we regarded a variant as authentic if the variant coverage was at least 500×, which allows us to determine somatic variants with lower frequency[10]. Bioinformatics tests were carried out for all actionable variants in the QCI-I bioinformatics tool. Various databases and guidelines were used during interpretations such as the Catalogue of Somatic Mutations in Cancer (COSMIC), National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO), US Food and Drug Administration (FDA), American Society for Clinical Oncology (ASCO), European Medicines Agency (EMA) and Pharmaceuticals and Medical Devices Agency (PMDA).

Statistical Analysis

Categoric variables were shown as numbers and percentages, whereas continuous variables were expressed as mean and median. The Chi-square exact test was applied to match categoric variables between the groups. The normality of statistical distribution for continuous variables was validated with the Shapiro-Wilk test. For comparing the continuous variables between the two groups, the Student's *t*-test or Mann-Whitney U test was preferred. All tests were carried out by using the IBM SPSS Statistics Version 20.0 statistical software package (IBM Corp., Armonk, NY, USA). The statistical level of importance for all tests was considered to be 0.05.

Results

Demographic and Operative Data

A total of 85 patients who received either endoscopic biopsy or operation with the intent of curation or palliation for confirmed EC by histopathological examination were included in the research. Table I shows the demographic variables, pathologic findings, and operational features of the cases. There were 47 men and 38 women, and the mean age of the patients was 58.01±11.45 years. Tumor pathology was 75.3% SCC and 24.7% AC. The stage distribution of the cases was defined as 16% stage 0, 4% stage 1A, 4% stage 1B, 4% stage 1C, 20% stage 2A, 20% stage 2B and 32% stage 3B. The lymphovascular invasion positivity rate was 84.2%, whereas perineural invasion was 53.8%. While 60 patients (70.6%) underwent endoscopic biopsy, 25 patients (29.4%) received elective surgery with curative or palliative intent. Ten patients underwent transhiatal esophagectomy, 3 patients underwent McKeown procedure, 5 underwent Ivor-Lewis procedure, and 7 patients required feeding gastrostomy or jejunostomy.

Overview of Identified Variants

The number of mutations per tumor ranged between 0 to 4. In 43 cases (50.6%), no variants were found in any of the investigated regions. In the majority of the cases (39/85; 45.9%), only

Table I. Demographical, pathological, and surgical characteristics of the patients.

		n	%
Sex	Male	47	55.3%
	Female	38	44.7%
Sampling method	Surgical specimen	25	29.4%
	Endoscopic biopsy	60	70.6%
Surgery	Orringer procedure	11	30.6%
	McKeown procedure	5	13.9%
	Ivor-Lewis procedure	9	25.0%
	Feeding tube	11	30.6%
Variant	Variant (+)	43	50.6%
	Variant (-)	42	49.4%
Tumor histology	ESCC	64	75.3%
	EAC	21	24.7%
Pathogenicity of variant	Non-pathogenic	45	52.9%
	Pathogenic	40	47.1%
Tumor localization	Cervical	19	23.5%
	Upper thoracic	7	8.6%
	Middle thoracic	18	22.2%
	Lower thoracic	21	25.9%
	Abdominal	16	19.8%
uT stage (endosonography)	T2	2	13.3%
	T3	11	73.3%
	T4	2	13.3%
uN stage (endosonography)	N0	4	26.7%
	N1	11	73.3%
TNM stage	0	4	16.0%
	1A	1	4.0%
	1B	1	4.0%
	1C	1	4.0%
	2A	5	20.0%
	2B	5	20.0%
	3B	8	32.0%
	4A	0	0.0%
Lymphovascular invasion	(-)	3	15.8%
	(+)	16	84.2%
Perineural invasion	(-)	6	46.2%
	(+)	7	53.8%

ESCC: esophageal squamous cell carcinoma; EAC: esophageal adenocarcinoma; u: ultrasonic (endoscopic ultrasonography).

single or double mutations were detected. Three patients showed triple mutations, 2 in the lower thoracic group and 1 in the cervical group. Quadruple mutation was only detected in one patient from the lower thoracic group. Table II shows the forms of single or multiple variants considering the site of tumor. Most of the single mutation were detected in the lower thoracic tumors (n=8) with a rate of 34.8%, double mutations were found in the abdominal tumors with a rate of 37.5% (n=6), triple mutations were seen with a rate of 5.3% in the cervical tumors (n=1) and 4.3% in the lower thoracic tumors (n=6), and the quadruple mutation was only detected in the lower thoracic tumor with a rate of 4.3% (n=1) (Table III). No statistically significant correlation was detected between the number of mutations and the tumor location (p=0.340).

Table III summarizes the relationship between the number of mutations and the tumor site. Of sequenced cases, a total of 61 mutations of *PIK3CA* (n=17), *KRAS* (n=7), *EGFR* (n=5), *EGFR-VUS* (n=5), *ERBB2* (n=4), *FGFR1* (n=3), *MET* (n=3), *NRAS* (n=3), *FBXW7* (n=2), *ALK-VUS* (n=1), *ERBB3-VUS* (n=1), *ERBB4* (n=1), *ERBB4-VUS* (n=1), *FGFR2* (n=1), *FGFR3* (n=1), *KRAS-VUS* (n=1), *MAP2K2* (n=1), *NOTCH1* (n=1), *SMAD4* (n=1), and *SOD2-VUS* (n=1) were identified in 85 patients. Twenty-five cases (29.4%) had only one mutation. Double mutations were predominantly

detected in the abdominal esophagus group (*n*=6; 37.5%) (*p*=0.02). Triple mutations (*n*=3) were detected in both cervical EC (*n*=1) and lower thoracic EC (*n*=1). Quadruple mutation (*n*=1) was also identified in the lower thoracic EC.

When the pathogenicity of the variants was examined, pathogenic was 55% (*n*=22), VUS was 12.5% (*n*=5), and probable pathogenic was 7.5% (*n*=3). We found that 71% of the total 61 variants were in the SCC group, and 29% were in the AC group. It was determined that the *PIK3CA* variant, which was the highest in number among the variant types (*n*=17), was seen in 41.2% of the lower thoracic tumors, 35.3% of the abdominal tumors, and 17.7% of cervical tumors, respectively. Of the total 26 variants, 42.3% are in the Orringer procedure group, 26.9% in the feeding tube group, 10.23% in the Ivor-Lewis procedure group, and 11.5% in the McKeown procedure group. In total, 52.6% of the 19 variants were in the stage 3B and 26.3% were in the stage 2B.

Polymorphisms were also detected in our study population. We observed VUS in a total of 11 genes. There were 7 male and 4 female patients in the VUS group. Of the 11 VUS genes, 8 were detected in SCC patients and 3 in AC patients.

Table II. Types of single or multiple mutation according to the location of tumor.

		Cervical (n:19) (%)	Upper thoracic (n:7) (%)	Middle thoracic (n:20) (%)	Lower thoracic (n:23) (%)	Abdominal (n:16) (%)	*p*
1st Mutation	***PIK3CA***	3 (15.8%)	0 (0%)	1 (5%)	4 (17.5%)	4 (25%)	
	MET	2 (10.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	EGFR	1 (5.3%)	1 (14.3%)	1 (5%)	0 (0%)	0 (0%)	
	FGFR1	0 (0%)	1 (14.3%)	1 (5%)	0 (0%)	0 (0%)	
	NOTCH1	0 (0%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	
	***SOD2* (VUS)**	0 (0%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	
	KRAS	0 (0%)	1 (14.3%)	0 (0%)	3 (13.1%)	1 (6.25%)	
	***ERBB2* (VUS)**	0 (0%)	0 (0%)	1 (5%)	0 (0%)	1 (6.25%)	
	***ALK* (VUS)**	0 (0%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	
	FBXW7	0 (0%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	
	FGFR3	0 (0%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	
	SMAD4	0 (0%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	
	***EGFR* (VUS)**	0 (0%)	0 (0%)	1 (5%)	1 (4.3%)	0 (0%)	
	MAP2K2	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	ERBB2	0 (0%)	0 (0%)	0 (0%)	2 (8.7%)	2 (12.5%)	
	FGFR2	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	***ERBB4* (VUS)**	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	None	13 (68.4%)	2 (28.5%)	11 (55%)	10 (43.5%)	8 (50%)	0.340* 0.374**
2nd Mutation	***EGFR***	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)	1 (6.25%)	
	***EGFR* (VUS)**	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	FBXW7	0 (0%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	
	KRAS	0 (0%)	0 (0%)	1 (5%)	1 (4.3%)	0 (0%)	
	NRAS	0 (0%)	0 (0%)	1 (5%)	2 (8.7%)	0 (0%)	
	FGFR2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	***KRAS* (VUS)**	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	PIK3CA	0 (0%)	0 (0%)	0 (0%)	2 (8.7%)	2 (12.5%)	
	MET	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (6.25%)	
	FGFR1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (6.25%)	
	SMAD4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (6.25%)	
	None	17 (89.4%)	6 (85.7%)	18 (90%)	17 (74%)	10 (62.5%)	
3rd Mutation	***PIK3CA***	0 (0%)	0 (0%)	0 (0%)	1 (4.35%)	0 (0%)	
	ERBB4	0 (0%)	0 (0%)	0 (0%)	1 (4.35%)	0 (0%)	
	***EGFR* (VUS)**	1 (5.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	None	18 (94.7%)	7 (100%)	20 (100%)	21 (91.3%)	16 (100%)	
4th Mutation	***ERBB3* (VUS)**	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	None	19 (100%)	7 (100%)	20 (100%)	22 (95.7%)	16 (100%)	

*Pearson's Chi-square *p*-value. **Likelihood ratio *p*-value. VUS: variants of unknown clinical significance.

Table III. Number of mutations in relation to the location of the tumor.

		Cervical (n:19) (%)	Upper thoracic (n:7) (%)	Middle thoracic (n:20) (%)	Lower thoracic (n:23) (%)	Abdominal (n:16) (%)	*p*
Number of mutation	0	13 (68.4%)	2 (28.6%)	11 (55%)	10 (43.5%)	7 (43.7%)	0.340* 0.374**
	1	4 (21%)	4 (57.1%)	6 (30%)	8 (34.8%)	3 (18.8%)	
	2	1 (5.3%)	1 (14.3%)	3 (15%)	3 (13.1%)	6 (37.5%)	
	3	1 (5.3%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	
	4	0 (0%)	0 (0%)	0 (0%)	1 (4.3%)	0 (0%)	

*Pearson's Chi-square *p*-value. **Likelihood ratio *p*-value.

When we look at the distribution of VUS genes in terms of tumor site, we can state that the maximum number of VUS genes (n=4; 36.3%) were found in the lower thoracic ECs. The VUS genes were detected in six patients as polyvariant. For example, *ERBB3* c.3353G>A p.(R1118Q) variant was the 4th mutation as VUS in a female patient with SCC located in the lower esophageal segment. We observed a novel clinically uncertain significant variant of c.1453 G>A (p.G485S) in an *EGFR* gene in a patient with liver metastasis from lower thoracic SCC.

Discussion

This study showed that the variant profile in the lower thoracic EC was distinct from the variant profile in the cervical, upper thoracic, middle thoracic, and abdominal ECs *via* NGS. Although not statistically significant, our NGS results may explain why distal ECs behave more aggressively. This condition was assessed with both the mutation profile and the number of mutations. In addition, this research can provide critical data on chemotherapeutic resistance and sensitivity to modify therapy. The identified polymorphisms also provide an idea about the increased risk of EC and prognostic biomarkers for which close follow-up protocols are required.

First-generation NGS machines were capable of reading short fragments, between 300 and 500 base pairs in length. The technology was based on the chain-termination method developed by Sanger in 1977[11]. This method was based on the use of dideoxynucleotides (ddNTPs), which block DNA polymerization and do not contain hydroxyl groups. These nucleotides perform DNA sequencing by staining with the specific fluorochrome dye and forming peaks of the nucleotide-specific wavelength. The primary benefits of first-generation NGS technologies are their high overall sequencing output and impressive accuracy, reaching 99%. However, this form of sequencing is considered high cost and low throughput, both of which have a significant impact on large-scale applications. The ABI PRISM 310 sequencers[12] (Applied Biosystems, Foster City, CA, USA) allowed the simultaneous sequencing of hundreds of samples and were used to help draft the human genome as part of the Human Genome Project[13].

Second-generation NGS used a luminescent method for measuring pyrophosphate synthesis, a process that converts pyrophosphate into ATP. This approach was used to infer DNA sequence by measuring the pyrophosphate produced by each nucleotide. This pyrosequencing technique was pioneered by Pal Nyren[14], and these machines greatly increased the amount of DNA that could be sequenced at once. Second-generation NGS machines [SOLiD[15] (Thermo Fisher Scientific, Life Technologies Co., Carlsbad, CA, USA), 454 GS FLX+ (Roche 454 Life Sciences, Branford, CT, USA), NextSeq 550Dx (Illumina Inc., San Diego, CA, USA), Revolocity Supersequencer[16] (Beijing Genomics Institute, Yantian, Shenzhen, China)] were introduced and immediately began to drive the genomics revolution. They increased the yield of sequencing efforts massively and allowed researchers to completely sequence a whole genome much more rapidly and cheaply than Sanger sequencing could. Recently, Illumina sequencers could be considered to have made the greatest contribution to second-generation NGS.

Third-generation NGS technologies[17] are capable of sequencing single molecules without the requirement for DNA amplification. Helicos Biosciences commercialized single-molecule sequencing in 2009, but this was slow, expensive, and produced short reads. Today, single molecule sequencing platforms [SMRT (Pacific Biosciences, Menlo Park, CA, USA), ONT (Oxford

Nanopore Technologies, Oxford Science Park, Oxford, UK)] show huge promise for the future of genomic medicine[18]. These technologies offer extremely long read lengths, at high accuracies, in regions of the genome that are mostly inaccessible by short-read platforms. Complex gene loci and extreme guanine and cytosine (G+C) content regions can easily be investigated, opening novel clinical ways that can be implemented in cancer, neurological disorders, and pharmacogenomics.

The significance of DNA sequencing has provoked researchers to invest a great amount of time and funds into developing and improving NGS machines. Innovation over the years has given rise to increased technical capabilities of sequencing while decreasing the costs to allow the reading of thousands of DNA base pairs within hours. NGS has a rich history, and understanding this history can provide insights into what future sequencing enterprises may be to come.

The advantages of next-generation DNA sequencing platforms include faster turnaround times for samples with high volumes and increased productivity through sample multiplexing. They are capable of simultaneously sequencing hundreds of samples and picking up low-frequency variations. Huge amounts of data are produced from the same amount of DNA input. Despite many benefits, NGS has several key disadvantages. First, although NGS presents information on countless molecular deviances, clinical implications of discovered anomalies are still unknown. Second, NGS requires high-priced yet sophisticated bioinformatics systems, rapid data processing infrastructures, and immense data storage capabilities. Third, despite the fact that NGS can be utilized to sequence a whole DNA sequence, clinicians can only use data from approximately 3% of the genome. Hence, NGS has far more potential for research purposes than it does for therapeutic implementations.

NGS technologies have revolutionized the ability to examine genomic deviations in cancerous tissues[19]. Over the past decade, this technological innovation has become more affordable, leading to larger-scale, collective whole-genome cancer research[20]. The resultant information has improved our understanding of the genes and pathways that drive tumor development, provided a rational basis for drug development and treatment strategies, and identified potential biomarkers for cancer categorization, diagnosis, and prognosis[21]. Consequently, the number of specific gene deviations being requested for testing is rapidly increasing. There is an urgent need for validation of NGS technology in the clinical setting. With the quickly growing number of laboratory biomarkers, multiple tests are often asked for an individual patient specimen, and consequently, there is a greater need for multiplexing with a range of tests that are frequently particular to some cancer types. In this sense, NGS is increasingly being recognized as being suitable for meeting these requirements[22]. NGS is versatile since it can easily determine insertions, deletions, or single-nucleotide variants. Furthermore, it can detect definitive mutant allele rates and also provide new sequence mutations.

Personal varieties in the risk of cancer have been correlated with particular mutation alleles (polymorphisms) of distinct genes that exist in a substantial percentage of the general population[23]. Polymorphisms are genetic changes that are also observed in healthy individuals and do not explain the disease on their own. However, in some diseases and/or cases, they may have an impact on the course of the disease. For example, a polymorphism may increase susceptibility to a disease or be considered a good/poor prognostic factor. Current research has revealed that genetic polymorphisms may elucidate the reasons and incidents that play a crucial role in the carcinogenesis of EC[24]. Various genes may be related to the carcinogenesis of esophageal malignancies, like genes taking part in cell cycle control, oncogenes, DNA repair, carcinogen metabolism, folate metabolism, and alcohol metabolism[25].

Most interestingly, 11 (8 SCC and 3 AC) out of 85 patients had clinically uncertain significant variants in *EGFR*, *ERBB2*, *ERBB3*, *ERBB4*, *KRAS*, *ALK*, and *SOD2* genes in our series. Four VUS genes, *KRAS* deletion (in the SCC group), *ERBB2* c.821A>G p.(Y274C) (in the AC group), *EGFR* c.1458G>C p.(Q486H) (in the SCC group) and *ALK* c.2577G>C p.(E859D) (in the AC group) were all in the double mutations group. Besides, the VUS gene of *EGFR* c.1553C>T p.(P518L) (in the SCC group) was in the triple mutation group, and the VUS gene of *ERBB3* c.3353G>A p.(R1118Q) was in the quadruple mutation group. Since these patients had ≥2 mutations in their tumors, we are of the opinion that these VUS variants may be related to a worse prognosis. Moreover, one of our patients who had liver metastasis had a novel clinically uncertain significant variant of c.1453 G>A (p.G485S) in an *EGFR* gene. Since this patient had liver metastasis at the time of diagnosis, we consider that this variant may be associated with poor prognosis.

In the current study, we present a large patient series of EC whose tumor samples were analyzed using actionable NGS. Of 85 consecutive EC cases, the molecular analysis of the tumor was accomplished for 100%. The molecular examination of esophageal malignancies provided in this study is comparable to that presented in the medical literature and public databases[26]. We were able to determine potentially actionable mutations for 85 cases, most of them harboring *PIK3CA* (*n*=17), *KRAS* (*n*=7), *EGFR* (*n*=5), *EGFR-VUS* (*n*=5), *ERBB2* (*n*=4), *FGFR1* (*n*=3), *MET* (*n*=3), *NRAS* (*n*=3), and *FBXW7* (*n*=2) mutations. We noticed that the rates of mutations ranging between 0 to 4 were especially concentrated in the lower thoracic esophageal group. According to The Cancer Genome Atlas (TCGA) statistics, the *EGFR* pathway was stimulated by amplification or mutation in 19% of cancers, and phosphoinositide 3-kinase catalytic subunit-α (*PIK3CA*) was triggered in 13% of malignancies. These signaling pathways have been successfully targeted using inhibitors of tyrosine kinase that are presently authorized for the treatment of other different types of cancers[27]. Current oncologic guidelines revealed that the existence of mutation in the *RAS* gene is a contradiction to anti-*EGFR* inhibitors[28]. However, the detection of specific mutations in rare genes can lead to alteration in the therapy by incorporating the patient into a clinical study or compassionate use program.

The microbiome, chromosomal traits, molecular features, and clinical characteristics of SCC and AC have been reported to be different[29]. Embryology does not play a pivotal role in the differences observed in the prognosis of EC. Gene expression disparities have been published between the normal esophagus and esophageal diseases[30]. Biological evidence and clinical proof support that SCC and AC follow different molecular pathways of carcinogenesis. TCGA network conducted a large genome-scale analysis of EC samples, which revealed significant biological differences between SCC and AC; SCCs resembled squamous carcinomas of other organs more than they did ACs. ACs have highly resemblance to the chromosomally unstable variant of gastric AC, suggesting that these malignancies could be considered a single disease entity. Nevertheless, certain molecular characteristics, which include DNA hypermethylation, occurred disproportionally in ACs[26]. When we look at the distribution of genetic alterations observed in our cases with regards to the tumor site, we can state that the maximum number of pathogenic variants [*PIK3CA* (*n*=7); 41.2%] and the highest number of mutations (1 mutation: 34.8%; 2 mutations: 13.1%; 3 mutations: 4.3%; and 4 mutations: 4.3%) were found in the lower thoracic ECs. This may explain why distally located ECs (AC) behave more aggressively.

The gene of *PIK3CA* encodes for the catalytic subunit p110α of class IA phosphatidylinositol 3-kinases (PI3Ks)[31] and mutations of this gene are found in 2.2% to 11.8% of SCC cases[32]. *PIK3CA* mutations and AKT signaling pathways play a crucial role in human tumors[33]. Jiang et al[34] reported that PI3K-Akt signaling pathway is vital for tumor angiogenesis. The mutations of *PIK3CA* have been related to poor prognosis in colorectal[35] and lung cancers[36]. Although the results of our study are not statistically significant, they align with existing knowledge of poor prognosis that have been seen in colorectal and lung cancers, contributing to the ongoing scientific dialogue about EC. However, favorable prognoses have been shown between *PIK-3CA* mutations and breast cancer[37]. Recently, Shigaki et al[38] have investigated the prognostic effect of the mutations of *PIK3CA* in SCC, and they have shown that the mutations of *PIK3CA* in SCC are linked with longer lifespans and favorable prognoses, confirming its function as a prognostic marker.

PIK3CA variants were identified in 17/85 samples (20%). This ratio is a little higher in comparison to those of earlier studies on ECs[32], but is similar to the Shigaki et al's result (21%)[38]. This distinction might result from a discrepancy in the methods or patient cohorts used to evaluate the mutation of *PIK3CA*. Of the 17 *PIK3CA* variants in our series, 15 were definitely pathogenic, while 10 of them were amplifications. The most common pathogenic mutation was the c.1624G>A p.(E542K), which was present in 3 tumors (1 AC and 2 SCCs), followed by c.1357G>A p.(E453K) mutation (1 SCC) and c.1633G>A p.(E545K) mutation (1 SCC). On the other hand, 2 mutations, namely c.2873A>G p.(Q958R) and c.320A>G p.(N107S), were probably pathogenic.

Here, we present proof for the oncogenicity of p110α E542K, E453K, E545K, Q958R, and N107S and identify the *PIK3CA* mutants as oncoproteins. Bader et al[39] showed that these mutants induced malignant cell growth and tumor angiogenesis in an avian tumor model. The three mutations of *PIK3CA* we determined at p.E542K, p.E543K, and p.E545K are common hotspot mutations that have earlier been detected in several cancers[39,40]. These mutations change interactions with other regulatory proteins that lead to an activation of downstream Akt signaling, which regulates apop-

tosis, proliferation, and cell survival[41]. Some latest clinical studies[38,40] showed that the mutations of *PIK3CA* are linked to improved prognosis in SCC patients. Therefore, the mutational condition of *PIK3CA* may be used as a novel biomarker to detect cases that will have a favored clinical outcome. The mutations of *PIK3CA* have been shown to interact with anti-*EGFR* therapy[42], and identifying any mutation before chemotherapy or immunotherapy may save many patients from the adverse effects of drug treatments that have been made ineffective by the mutations.

The molecular mechanisms by which the variants obtain enzymatic function and, therefore, gain oncogenic potential still remain unknown. Given the well-known roles of the Akt signaling pathway in tumor proliferation, invasion, and survival[43], one could expect that the mutations of *PIK3CA* would imply poor clinical outcomes. Although this research study had a limitation of a small cohort size (n=85), our research study suggests that *PIK3CA* variants are related to poor outcomes in EC, supporting its role as a prognostic biomarker. Future studies are necessary to elucidate the biologic mechanisms by which *PIK3CA* mutation activation affects EC behavior.

NGS platforms have started to find place in daily routine clinical practices in recent years, and they have taken a major step forward in terms of individual therapy, especially in the field of oncology. Planning adjuvant therapy regimens in view of the consequences of molecular genetic analysis and close follow-up of cases identified as high-risk may be linked with better clinical outcomes. NGS technologies enable the feasibility of personalized therapies through medical diagnostics and the establishment of correlations.

Conclusions

In conclusion, the application of an actionable multigene panel demonstrated the value of a well-structured NGS workflow in effectively utilizing clinical results, enabling the prediction of chemotherapy drug responsiveness, identification of new therapeutic options, and prognosis forecasting. This study shows how NGS technology can drive advancements toward personalized cancer treatment and how it is increasingly utilized to guide individualized therapy decisions, aligning with the ultimate goal of integrating personalized medicine into routine oncological clinical practice.

Conflict of Interest
The authors declare that they have no conflict of interest to disclose.

AI Disclosure
The authors disclose that they have not used any form of generative artificial intelligence to write the manuscript.

Ethics Approval
The study was conducted following the Helsinki Declaration and was approved by the Ethics Committee of Çukurova University, Adana, Turkey (approval number: 28/100, date of approval 05.06.2020).

Informed Consent
All subjects provided informed consent.

Data Availability
The data associated with the paper are available from the corresponding author upon reasonable request.

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ORCID ID
Zafer Teke: 0000-0001-8869-6476
Atil Bisgin: 0000-0002-2053-9076
Cagla Rencuzogullari: 0000-0003-4113-6820
Kivilcim Eren Erdogan: 0000-0002-4951-8703
Cem Kaan Parsak: 0000-0002-6515-8365
Sevgi Kalkanli Tas: 0000-0001-5288-6040

Authors' Contributions
Conceptualization, methodology, writing the original draft: Z.T., A.B.; data curation, resources, writing the original draft: Z.T., C.R., K.E.; Project administration, validation, supervision: Z.T., A.B., S.K.T.; conceptualization, methodology, project administration: Z.T., A.B., S.K.T.; investigation, validation, visualization: Z.T., A.B., C.K.P., S.K.T.

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