Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Mutation analysis of the TERT gene in ovarian cancer patients of the Turkish population by next generation sequencing method

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ARTICLE INFO

Original paper

Article history:
Received: April 15, 2023
Accepted: July 18, 2023
Published: November 15, 2023

Keywords:

Next-generation sequencing, ovarian cancer, molecular biology, IDH1, IDH2, MGMT, TERT, ATRX

ABSTRACT

Ovarian cancer (OC) ranks seventh among malignant tumors worldwide. As one of the most common gynecological malignancies, ovarian cancer has the second-highest mortality rate, after cervical and uterine cancer. Next-Generation Sequencing (NGS) technology has enhanced multi-gene panel analysis and its clinical utility for identifying cancer-causing gene mutations. This study aimed to determine the presence of significant and nonsense mutations in telomerase reverse transcriptase (TERT), alpha-thalassemia/mental retardation, X-linked (ATRX), O-6-methylguanine-DNA methyltransferase (MGMT), and isocitrate dehydrogenase 1 and 2 (IDH1/IDH2) genes using the Next-Generation Sequencing (NGS) method. A cohort of 33 patients diagnosed with ovarian cancer was included in this investigation, and peripheral blood samples were collected from all participants. Significant and nonsense mutations in TERT, ATRX, MGMT, IDH1, and IDH2 genes were detected using the Next-Generation Sequencing method. Bioinformatics analysis was conducted using the QIAGEN Clinical Insight system. Twenty-four patients exhibited seven different TERT mutations, occurring in both exonic and intronic regions. One patient displayed a c.699-3delC deletion in the intronic region of the IDH1 gene, and the c.532G > A (p.V178I) mutation observed in three patients was assessed as potentially harmful. Additionally, novel mutations c.881A > G and c.995A > G were observed in the ATRX gene. The heterozygous novel mutation identified in the ATRX gene was confirmed through Sanger sequencing. These mutations were not previously associated with ovarian cancer and are considered novel candidate markers for ovarian cancer susceptibility. Confirmation of these results through larger cohort studies or functional investigations will contribute to a better understanding of the molecular mechanisms underlying ovarian cancer.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.11.9

Introduction

Cancer is the leading cause of death worldwide and has a substantial impact on the quality of life. Ovarian cancer (OC) ranks seventh among malignant tumors affecting women worldwide. It is one of the most common types of gynecological cancer and has the highest mortality rate after cervical and uterine cancer (1). Due to its high prevalence, high mortality rate, economic burden, and significant impact on patients' quality of life ovarian cancer is recognized as a critical health issue that demands special attention (2). Age, reproductive history, family history, and genetic mutations are some of the risk factors for ovarian cancer (3). Next-Generation Sequencing (NGS) technology has significantly improved the analysis of multi-gene panels, making it a valuable tool for identifying cancer-causing gene alterations in clinical practice (4,5).

The TERT gene, which is unique to humans and consists of 16 exons and 15 introns, is essential for maintaining telomere length by adding "TTAGG" repeats to chromosome ends. Mutations in TERT can reactivate telomerase, which increases the probability of carcinogenesis. SNPs within the TERT gene have been linked to telomere length and telomerase reactivation (6). IDH mutations are commonly observed in primary and recurrent tumors. These mutations hinder normal cellular differentiation and promote tumor genesis through abnormal production of the oncometabolite 2-HG (7). The ATRX gene, a component of the SWI/SNF chromatin remodeling complex, is frequently mutated in various human cancers (8). The MGMT gene, crucial for DNA repair, plays a vital role in mitigating DNA damage caused by alkylating chemicals and suppressing oncogenesis. Extensive methylation of the MGMT gene's promoter region has been observed in different carcinomas, including ovarian cancer. This methylation directly affects the repair pathway, reducing DNA damage and subsequent cancer growth (9).

To date, the relationship between TERT, MGMT, ATRX, IDH1, and IDH2 gene mutations and ovarian cancer remains unknown. Understanding the impact of cancer-associated mutations identified in one cancer type on different types of cancer is critical. Our study aimed to utilize next-generation sequencing (NGS) to evaluate the presence of significant and nonsense mutations in telomerase reverse transcriptase (TERT), alpha-thalassemia/mental retardation, X-linked (ATRX), O-6-methylguanine-DNA methyltransferase (MGMT), and isocitrate dehydrogenase 1 and 2 (IDH1/IDH2) genes.

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Materials and Methods

Study population and protocol

In this study, we examined a cohort of 33 patients diagnosed with ovarian cancer. Signed informed consent agreements were obtained from all participants, ensuring their voluntary participation in the study. Demographic profiles of the patients were meticulously recorded and carefully reviewed for future reference. Peripheral blood samples were collected from all consenting participants. The collection of blood samples was conducted in accordance with the ethical guidelines set by the Yeditepe University Faculty of Medicine Clinical Research Ethics Committee, as indicated by their decision number 1431, dated April 21, 2021. This ethical approval justified the inclusion of blood samples in our study, deeming it appropriate and ethically sound. The primary objective of this study was to comprehensively evaluate the TERT, ATRX, MGMT, IDH1, and IDH2 genes using Next-Generation Sequencing (NGS) technology. The aim was to identify potential pathological mutations and determine their impact on the disease.

Preparation of samples

Venous blood samples from the patients were collected using 5 cc EDTA tubes and stored at +4°C until DNA extraction. The iPrep DNA extraction robot, along with the iPrep genomic DNA isolation kits (iPrep gDNA Blood kit; Invitrogen, Thermo Fisher Scientific Inc), was employed for DNA extraction from the samples. This automated system has the capacity to isolate DNA from up to 13 samples in a single run. A volume of 350 µl of peripheral blood was utilized for each sample, employing a robotic system based on magnetic bead-based technology. The extraction process relies on the surface charge of the nucleic acid backbone and the pH of the buffer in the system. At low pH, the negatively charged nucleic acid backbone binds to the positively charged ChargeSwitch Technology®, enabling the removal of proteins and other impurities through a liquid wash buffer. To purify the nucleic acids, a low salt elution buffer is used to neutralize the charge on the bead surface, raising the pH to 8.5 and facilitating the elution of the isolated nucleic acids. Subsequently, the isolated DNA samples were transferred to appropriate containers and stored in a refrigerator at +4°C.

Measurement of DNA purity

The purity of DNA samples was determined using the Nanodrop 2000 device (Thermo Fisher Scientific, Wilmington, DE, USA). The optical density (OD) of 50 ng/l of double-stranded DNA at 260 nm wavelength was found to be 1. The OD260/OD280 ratio was used to evaluate the purity of DNA samples. Samples with an OD260/OD280 ratio of 1.7 to 1.9 were considered clean and acceptable for genotyping.

Design of ovarian cancer-associated gene panel

QIAseq-targeted DNA panels (Qiagen, Hilden, Germany) were utilized in conjunction with the Illumina NGS System for this study. A thorough literature review was conducted to create five gene panels associated with diverse types of cancer. These gene panels were selected based on their documented relevance and potential diagnostic utility. Moreover, the utilization of these five gene panels was found to be cost-effective for diagnostic purposes.

Library preparation and NGS

The Qiaseq Targeted DNA Panel kit (Qiagen) was employed to generate DNA libraries using 10 ng/µl DNA that was enzymatically fragmented with 1.25 µl of the fragmentation enzyme mix and 2.5 µl of the 10X fragmentation buffer. The fragmentation reaction was carried out for 1 minute at 4°C, 24 minutes at 32°C, and 30 minutes at 65°C. Subsequently, adapter ligation was performed in a total volume of 50 µl, consisting of 2.8 µl of interleukin-N7 barcoded adapter, 5 µl of DNA ligase, 10 µl of 5X ligation buffer, and nuclease-free water. The ligation reaction was conducted for 15 minutes at 4°C. To ensure the removal of all unbound barcoded adapters, each reaction underwent two purification steps using 1.8X and 0.9X Ampure beads (Beckman, Indianapolis, IN, USA).

For target enrichment PCR, the solution comprised 5 µl of target primer, 0.8 µl of interleukin-forward primer, 5X TEPCR buffer, and 0.8 µl of HotStarTaq DNA polymerase in a 20 µl volume. Ampure beads were employed for the purification of the target primer (10). The universal PCR was performed in a total volume of 20 µl, consisting of 13.4 µl of enriched DNA, 1 µl of HotStarTaq DNA polymerase, 4 µl of universal primer PCR buffer, and 1.6 µl of nuclease-free water.

Sequencing and analysis

The sequencing of the libraries was performed using the FASTQ application (Illumina, Hayward, CA, USA) to read the sequence data. To achieve deep sequencing coverage, the Unique Dual Index option was utilized in the QIAseq Targeted DNA Panel Library (Qiagen). The libraries were sequenced on the Illumina NGS System (Illumina). The output readings from the GeneReader were then subjected to variant detection analysis using the QIAGEN Clinical Insight system.

DNA Sanger Sequencing

To validate the heterozygous variation in the ATRX gene, Polymerase Chain Reaction (PCR) and Sanger sequencing were employed. Purification of the PCR products was performed using the NucleoFast® 96 PCR Clean-up kit (Macherey-Nagel GmbH and Co., Düren, Germany), following the manufacturer's instructions. Sequencing procedures were conducted using the BigDye® Terminator v1.1 Cycle Sequencing kit with 2 µl of purified PCR product (Applied Biosystems, Foster City, CA, USA). Prior to electrophoresis, the sequencing reaction products were purified using the MontageTM SEQ96 Sequencing Reaction kit (EMD Millipore Corp., Billerica, MA, USA). Electrophoresis of the sequencing products was carried out using the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems).

Results

The median age of the patients was 53.36±12.79 (range: 18-76). Among the patients, 7 (21.2%) were premenopausal, while 26 (78.8%) were postmenopausal. Alcohol use was reported in 1 (3.03%) patient, and cigarette use was reported in 6 (18.1%) patients. When examining the stages of the patients, 13 (39.3%) were classified as

stage 3. The distribution of other stages was as follows: 8 (24.2%) patients in stage 1, 5 (15.1%) patients in stage 2, and 7 (21.2%) patients in stage 4. Among the patients, 28 (84.9%) had epithelial cell ovarian cancer, 2 (6.1%) had sex cord-stromal type ovarian cancer, and 3 (9.1%) had germ cell ovarian cancer.

In our study, we identified 7 different mutations in the TERT gene among 24 out of 33 patients. The TERT mutations observed were as follows: c.2850-3039C>T (p.H950H, p.H1013H) mutation; c.2517G>A (p.T839T) mutation; c.2031C>T (p.G677G) mutation; c.835G>A (p.A279T) mutation; c.1392C>T (p.F464F) mutation; c.2995-3184G>A (p.A1062T, p.A999T) mutation; and c.915G>A (p.A305A) mutation. The mutations were classified and the translation effect and results from various in silico prediction programs are presented (Table 1). The mutations were classified as Benign or Likely Benign. The translation effect in patients was identified as missense, and the pathogenicity effect was evaluated as tolerable/benign based on SIFT Function Prediction and PolyPhen-2 Function Prediction databases.

Furthermore, we observed 5 mutations in the ATRX gene, 3 mutations in the IDH1 gene, 1 mutation in the IDH2 gene, and 6 mutations in the MGMT gene (Table 2). In one patient, a c.699-3delC deletion was observed in the intronic region of the IDH1 gene, and the c.532G > A (p.V178I) mutation detected in 3 patients was considered potentially harmful. Additionally, a novel mutation, c.881A > G; c.995A > G, was detected in the ATRX gene. The heterozygous novel mutation identified in the ATRX gene was confirmed by Sanger sequencing (Figure 1).

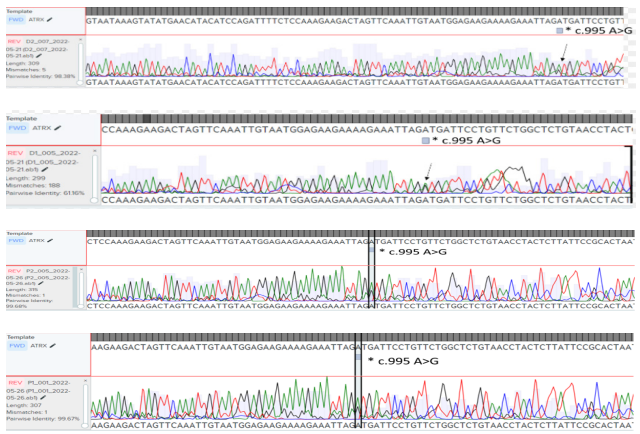

Figure 1. ATRX c.995A>G (NM_000489) = c.881A>G (NM_138270) Forward image and reverse image confirmation by Sanger sequencing.

Table 1. Mutations of the TERT gene found in patients and their characteristics.

dbSNP ID	Transcript Variant	Protein Variant	Translation Impact	Classification	SIFT Function Prediction / PolyPhen-2 Function Prediction
35719940	c.2995G>A;n.2856G>A; c.3184G>A; n.2892G>A	p.A1062T; p.A999T	missense	Benign	Tolerated/Benign
33954691	c.2850C>T; n.2711C>T; c.3039C>T; n.2747C>T	p.H950H; p.H1013H	synonymous	Benign	
140124989	n.2414G>A; n.2378G>A; c.2517G>A	p.T839T	synonymous	Likely Benign	
33956095	c.2031C>T; n.2110C>T	p.G677G	synonymous	Benign	
186596886	c.1392C>T; n.1471C>T	p.F464F	synonymous	Likely Benign	
2736098	c.915G>A; n.994G>A	p.A305A	synonymous	Benign	
61748181	n.914G>A; c.835G>A	p.A279T	missense	Benign	Tolerated/Benign

Table 2. ATRX, MGMT, IDH1 and IDH2 genes mutations and features.

Chromosome	dbSNP ID	Gene Symbol	Transcript Variant	Protein Variant
2	-	IDH1	c.699-3delC	
2	34218846	IDH1	c.532G>A	p.V178I
2	11554137	IDH1	c.315C>T	p.G105G
10	16906252	MGMT	c.66C>T; c.-28C>T	p.R22R
10	1803965	MGMT	c.159C>T; c.252C>T	p.L84L; p.L53L
10	12917	MGMT	c.250C>T; c.343C>T	p.L115F; p.L84F
10	2308321	MGMT	c.427A>G; c.520A>G	p.I143V; p.I174V
10	2308318	MGMT	c.478G>A; c.571G>A	p.G160R; p.G191R
10	2308327	MGMT	c.626A>G; c.533A>G	p.K178R; p.K209R
15	61737003	IDH2	c.996C>T; c.840C>T; c.606C>T	p.S280S; p.S332S; p.S202S
X	45439799	ATRX	c.5465A>G; c.5579A>G	p.N1822S; p.N1860S
X	3088074	ATRX	c.2785C>G; c.2671C>G; c.2785G>C *; c.2671G>C *	p.Q891E; p.Q929E; p.E929Q; p.E891Q
X	143413618	ATRX	c.2606G>A; c.2720G>A	p.R869Q; p.R907Q
X	61752455	ATRX	c.2481C>G; c.2595C>G	p.H865Q; p.H827Q
X	-	ATRX	c.881A>G; c.995A>G	p.D332G; p.D294G

Discussion

Today, the incidence and prevalence of cancer are increasing, alongside the extension of the human lifespan, which can be attributed to changing living conditions. Consequently, research focusing on genetic and environmental factors that potentially influence diverse cancer types is gaining momentum.

In this study, multiple gene variant analysis was conducted employing NGS technology to detect significant and nonsense mutations associated with ovarian cancer. Notably, these mutations have not been linked to ovarian cancer in previous investigations. The rs2736098 variant in the TERT gene was observed in 19 out of 24 patients with the detected variant. The rs2736098 (G > A) polymorphism, one of the extensively studied variants in TERT, has been associated with an augmented risk of cancer in numerous tumor types (11). A comprehensive study concentrated on two polymorphisms, rs2736100 and rs2736098, in TERT, which represent the most widely investigated polymorphisms associated with multiple cancer types. The rs2736098 variant exhibited a correlation with cancer risk in all population analyses, categorized by cancer type, and was found to heighten the risk of bladder and lung cancer (12).

Conversely, another study aimed to identify genetic markers for lung cancer, confirming their presence. By analyzing 2910 genetic variants in 754 different genes or chromosome loci, a meta-analysis of 1018 publications was conducted. The TERT rs2736098 polymorphism emerged as a significant risk factor for lung cancer (13,14). Notably, the TERT rs2736098 polymorphism exhibited an association with increased cancer risk across all demographic groups. Subgroup analysis revealed distinct cancer-promoting properties for various cancer types. In five genetic lung cancer models, the TERT rs2736098 (G > A) polymorphism heightened cancer susceptibility. Furthermore, a study found that the homozygous TERT rs2736098 AA genotype was linked to an escalated risk of lung cancer in both smokers and nonsmokers, as observed in a hospital-based study involving patients with and without cancer (15). Additionally, other studies employed assignment and subset-based association analyses among various cancer types and identified multiple independent risk loci in the TERT region. Within their research, genome-wide association studies (GWAS) have mapped risk alleles for at least ten different cancer types to a specific small region on the chromosome where the TERT genes are located. The rs2736098 variant was classified as a risk variant for lung, bladder, prostate, ovarian, and breast cancers (16).

To investigate the association of ovarian cancer, we incorporated mutations in the ATRX, MGMT, IDH1, and IDH2 genes. Our study identified a total of four variants in the IDH genes. The variant c.532G > A (p.V1788I) (rs34218846) in the IDH1 gene was determined as potentially harmful based on the PolyPhen-2 Prediction of Function databases in three patients. Furthermore, a deletion c.699-3delC was observed in the intronic region of the IDH1 gene in one patient. Moreover, both univariate and multivariate analyses revealed that the IDH1 rs11554137 variant serves as an unfavorable prognostic factor for overall survival in acute myeloid leukemia (17,18,19). In previous studies, the variants rs2308318 c.478G > A and c.571G > A (p.G160R, p.G191R) of the MGMT gene, which encodes a DNA repair protein, have not been associated with ovarian cancer. The significance of these variants in the context of cancers and tumors remains debatable.

In a previous study conducted in 2010, the relationship between MGMT polymorphisms and head and neck squamous cell carcinoma was investigated. The variant rs2308318 was identified; however, its significance in this context remained unknown (20).

Regarding the ATRX gene, a novel mutation, namely c.881A > G and c.995A > G (p.D332G, p.D294G), was identified. The clinical significance of these mutations is currently unknown. Computational evidence suggests that they may have a negative impact on the gene or its product, as indicated by a Combined Annotation Dependent Depletion (CADD) score of 22.4. These mutations were located in exon 8 and were not observed in the general population, indicating a prevalence of 0%. Sanger sequencing was employed to validate the presence of heterozygous mutations in patients 15 and 32. Sanger sequencing confirmed that the mutations detected were indeed heterozygous. Based on these findings, we propose that this newly confirmed mutation may serve as a potential candidate mutation for ovarian cancer.

Conclusions

To the best of our knowledge, this study is the only one demonstrating the association of mutations in the TERT, ATRX, MGMT, IDH1, and IDH2 genes with ovarian cancer. However, further research with a larger patient population is warranted to obtain more comprehensive results regarding the treatment of ovarian cancer and to determine the specific effects of these mutations. This study has several limitations, including small sample size, the absence of a control group, and the use of blood tissue samples. Therefore, we believe that future studies should include individuals both with and without ovarian cancer to strengthen the observed correlations between the identified variants and ovarian cancer.

Conflicts of Interest

The authors declare no conflicts of interest regarding the materials, methods, or findings presented in this study.

Authors' Contributions

The study was conceived and designed by Betul Capar Gorali and Turgay Isbir. Data collection was performed by Betul Capar Gorali. Analysis and interpretation of the results were carried out by Seda Gulec Yilmaz, Fatma Tuba Akdeniz, and Rukset Attar. The initial draft of the article was prepared by Betul Capar Gorali, Seda Gulec Yilmaz, and Fatma Tuba Akdeniz. Turgay Isbir and Rukset Attar provided supervision throughout the study. All authors reviewed the results, contributed to the final version of the article, and approved its submission.

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