Research Article

RELA mRNA Expression in Epithelial Ovarian Cancer: Correlation with rs11820062 Gene Variant

Ekspresi mRNA RELA pada Kanker Ovarium Epitelial : Korelasinya dengan Varian Gen rs11820062

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Abstract

Objective: To examine the distribution of RELA rs11820062 and its correlation to i mRNA expression in low-grade and high-grade EOC's patients from Dr. Cipto Mangunkusumo General hospital, Indonesia.

Methods: This study is cross-sectional with a total of 65 healthy subjects and 80 ovarian biopsies (15 ovarian cysts as expression calibrators, 36 low-grade EOC, and 29 high-grade EOC) were used in this study. The distribution of genotypes and alleles was analyzed using ARMS PCR. The mRNA expressions of RELA were determined by real-time polymerase chain reaction (qPCR) analysis.

Results: There was no significant difference between genotype and allele distributions for RELA rs11820062 in normal and case group. RELA relative mRNA expression was significantly higher in low-grade and high-grade EOC compared to in ovarian cysts ($p<0.01$). RELA rs11820062 CC genotype correlated to higher RELA mRNA relative expression and the TT genotype of RELA rs11820062 correlated with lower RELA mRNA relative expression in low-grade and high-grade EOC.

Conclusion: C allele in rs11820062 caused an increased expression of RELA mRNA, which individuals with CC genotype correlated with higher RELA expression in low-grade and high-grade EOC. In contrast, individuals with the T allele of RELA rs11820062 had a protective effect against EOC risk because the RELA TT genotype tended to have a lower RELA mRNA expression in EOC.

Keywords: epithelial ovarian cancer, NF-kB, RELA, rs11820062.

Abstrak

Tujuan: Mengetahui distribusi RELA rs11820062 dan korelasinya dengan ekspresi mRNA RELA pada pasien EOC low-grade dan high-grade di Rumah Sakit Dr. Cipto Mangunkusumo, Indonesia.

Metode: *Penelitian ini merupakan peneliatan potong lintang terhadap 65 sampel darah perempuan normal dan total 80 biopsi kanker ovarium dengan rincian: 15 kista ovarium sebagai kalibrator ekspresi, 36 EOC low-grade, dan 29 EOC high-grade. Distribusi genotipe dan alel dianalisis menggunakan ARMS PCR dan ekspresi mRNA RELA dikuantifikasi menggunakan teknik qPCR.*

Hasil: *Tidak terdapat perbedaan distribusi genotipe dan alel antara kelompok normal dengan kasus EOC. Ekspresi relatif mRNA RELA meningkat secara signifikan pada kelompok EOC low-grade dan high-grade. Individu dengan genotipe RELA rs11820062 homozigot CC memiliki ekspresi mRNA yang lebih tinggi dibandingkan genotipe lain. Sebaliknya individu dengan genotipe TT memiliki korelasi dengan ekspresi mRNA RELA yang lebih rendah pada tipe low-grade dan high-grade EOC.*

Kesimpulan: *Alel C pada RELA rs11820062 menyebabkan peningkatan ekspresi mRNA RELA pada pasien EO yang dilihat dari individu dengan genotipe CC cenderung memiliki ekspresi mRNA RELA yang lebih tinggi pada tipe EOC low-grade dan high-grade. Sebaliknya, individu dengan alel T RELA rs11820062 diduga memiliki efek protektif terhadap risiko EOC karena adanya korelasi antara genotipe TT dengan ekspresi mRNA RELA yang lebih rendah pada EOC.*

Kata kunci: *kanker ovarium epitelial, NF-kB, RELA, rs11820062.*

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INTRODUCTION

In terms of gynecological cancers, ovarian cancer has the greatest mortality rate. Epithelial Ovarian Cancer (EOC) accounts for more than 90% of all ovarian cancer cases.[1] In 2018, GLOBOCAN recorded 295,414 new cases of ovarian cancer with a mortality rate of 184,799 people.[1] Meanwhile, the incidence of new cases of ovarian cancer in Indonesia in the same year was 14,896, with a total death cases of 9,581.[2] The high mortality from ovarian cancer caused by this cancer is asymptomatic, and no diagnostic method detects the ovaries at an early stage. Therefore, current research on ovarian cancer centers on a new approach or biomarker with diagnostic, prognostic, and predictive potential.[3] Knowledge of the etiology of various histologic types of ovarian cancer might open new pathways in basic research and clinical studies to develop screening and diagnostic methods for ovarian cancer. EOC based on their progressivity, molecular characteristic, and histologic type into low-grade and high-grade EOC. Generally, the low-grade type has a better prognosis characterized by slow progression and a lower proliferation rate than the high-grade type.[4]

Chronic infections and the following inflammatory response have been estimated to be responsible for 15% of the world's cancer burden.[5] EOC is frequently associated with a number of disease that causes an inflammatory response, including endometriosis, pelvic inflammatory disease, and polycystic ovary syndrome.[6] In areas of inflammation, epithelial cells are exposed to greater concentrations of inflammatory mediators, such as reactive oxygen species, cytokines, prostaglandins, and growth hormones that hasten cell division and genetic and epigenetic changes.[7] Later, these inflammatory mediators, particularly pro-inflammatory cytokines like TNF, IL-1, and IL-6, would cause a group of transcription factors, called nuclear factor kappa-κB (NF-κB) to become activated. When TNF and IL-1 are secreted, they interact with premalignant cells to activate NF-κB, which then triggers the production of genes that inhibit apoptosis and promote angiogenesis and proliferation to promote malignancy.[8] RelA, RelB, c-Rel, p105/p50 (NF-kB1), and p100/52 are the five members of the NF-κB family (NF-κB2). The NF-κB p65 subunit is regarded to be the family's most potent transcriptional activator.[9]

RelA is a subunit of NF-κB, which has a transcription activation domain (TAD) at the C-terminal end. TAD is a domain that will interact with the basal promoter region of various downstream genes from NF-κB. During inflammation, the classical pathway NF-κB is activated with a dimer in RelA with its p50 subunit pair.[10] These dimer pairs then translocate into the cell nucleus and regulate the expression of various related genes. Therefore, increased expression of the RelA subunit is often associated with multi types of cancer, such as lung, prostate, breast, and other solid cancers, including ovarian cancer.[11] This makes the gene encoding RelA, *RELA* (11q13), a potential proto-oncogene.[12] In many cancer cases, increased *RELA* expression is associated with poor prognosis and low life expectancy.[13] Thus, research on the relationship between *RELA* expression and ovarian cancer is essential.

Numerous disorders, including cancer, have been linked to single nucleotide polymorphisms (SNPs) in the NF-κB signaling pathway genes.[14] Most risk alleles predispose to disease are in the non-coding region, with the mechanism of their function largely unknown. Information regarding the association of variants in this gene with the risk of epithelial ovarian cancer is essential, especially since research in Indonesia has never been done.[15] The variation to be studied in this study is rs11820062 which is in the first intron, almost 5' untranslated region (5' UTR), of *RELA* gene. The position of this variant was predicted to have a transcription factor binding site. In addition, due to its location near the five ′ end of *RELA,* this mutation can alter transcription factors' binding and regulation.[16]. This study aimed to evaluate the correlation of the 5′ most intron variants of *RELA,* rs11820062 C/T, with the susceptibility for EOC with its possible mechanism at the transcriptional level. In addition, the present study aimed to analyze the connection between this genetic variant and the dualistic model of EOC.

METHODS

This cross-sectional study analyzed the relationship between genetic variant and mRNA expression of *RELA* with the risk of low-grade and high-grade EOC. As NF-κB plays major role in cancer development and progression, we hypothesize that *RELA* mRNA expression is increased in both low- and grade- EOC and individuals with genetic variation rs11820062 are at greater risk for EOC.

Primary samples were ovarian cancer biopsies from women that underwent surgery in Dr. Cipto Mangunkusumo Hospital from 2016-2021. Samples were obtained from Biobank Research IMERI-FMUI and Department of Pathological Anatomy FMUI as formalin-fixed paraffin-embedded (FFPE) block. The total ovarian biopsies used in this study were 85 blocks, divided into two groups: 15 ovarian cysts, 36 low-grade EOC, and 29 low-grade EOC. This study also used healthy blood samples from women that underwent blood transfusion at Palang Merah Jakarta Pusat aged 40-70, with no history of ovarian cyst, cancer, endometriosis, or PCOS, and cancer-free in a family for at least three generations. Blood samples from women that match the criteria be a normal group for rs11820062 genotype and allele distribution analysis. The University approved this study by Indonesia's Ethical Committee on Medical Research (No. KET-689/UN2.F1/ETIK/PPM.00.02/2020), and the participants were informed of the study's purpose for normal control. Each participant signed written informed consent forms.

In genotyping analysis, there are two types of DNA sources: whole blood for normal subjects and FFPE biopsies for cases. To isolate DNA from whole blood, the salting-out method was applied, which includes red blood lysis by a red lysis blood solution (RBCs), cell and nuclei lysis by cell lysis solution (Tris HCl 1M, EDTA 0.5M, and 10% SDS) and protein precipitation by ammonium acetate 5M. For DNA and RNA extraction from FFPE ovarian biopsies, the paraffin block had already been cut into 6x5µm and undergone microdissection to separate cancer tissue from other types. Samples were deparaffinized using xylene to dissolve the paraffin, followed by a rehydration process with ethanol. After the deparaffination process, samples followed the gSYNC™ DNA Extraction Kit protocols. The purity of the isolated DNA was measured using NanoDrop (Maestrogen) at a wavelength of 260/280nm. Pure DNA has expected ratios of 1.7–1.9.

RELA Rs11820062 were detected using T-Arms PCR with primers designed using the Primer1 application from http://primer1.soton.ac.uk. The reference sequence used is from NCBI. The primers were then BLAST (NCBI) to determine the specificity of the primers. The primers that were used in this study are listed in Table 1. The expected size of the PCR products is below 200 bp because of the nature of DNA obtained from FFPE, which was heavily degraded. From our experienced, PCR products above 200 could not be amplified (not included).

After the primers were obtained, T-ARMS PCR optimization was performed to get the right PCR settings. The amplification uses three different tubes, each consisting of a pair of primers: forward outer and reverse outer for internal control, forward inner and reverse outer for wild type allele, and forward outer and reverse inner for the alternate allele. The master mix composition (25 uL) consisted of 12.5 µL MyTaq Red Mix 2x, 1 µL of forward and reverse primers, 100 ng of template DNA, and nuclease-free water. Special for amplifying the C allele, 5% DMSO was added into the tube. DNA samples were amplified for up 35 cycles, beginning with 5 minutes pre-denaturation temperature of 94°C, followed by a process of denaturation at 94 °C for 30 seconds, annealing at 57°C for 15 seconds, and elongation at 72°C for 10 seconds. The extension time was extended by 72°C for 7 minutes at the end of the cycle. The amplification results of the PCR products were mixed in 1 tube and visualized using 3% agarose gel electrophoresis with the Hyper Ladder™ 50bp ladder. The primers' sequence and estimated results to be obtained are shown in Table 1.

Table 1. Primer used in ARMS PCR

Primer	Sequence (5′→3′)	TM (°C)	Amplicon Size (bp)
RELA (rs11820062)			
Forward-Outer	AGAAACACCTGCTTCTTGAGGGA	63	
Reverse-Outer	AACGCATCTGATTCAGTTTCCTCTCTG	63	151
Forward-Inner (T allele)	GGCCTGTTGTACTTTCTTAAGGAACAT	63	81
Reverse-Inner (C allele)	TGGGGCGTGCCCTCCCTAAG	66	115

RNA was extracted from samples who had been deparaffinized before using and following protocol from Quick-RNA Miniprep Plus Kit (ZymoResearch) for FFPE. RNA was and100 ng RNA was made into cDNA using a kit from ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo). cDNA template was used as a qPCR template. RELA gene expression was measured relative to GAPDH gene expression. qPCR master mix preparation was done in a cold rack and prepared per manufacturer instruction (SensiFAST SYBR Lo-ROX Mix®, Meridian Bioscience). The primers for RELA were designed via Primer Quest™ Tool (IDT DNA) with the following sequence: F: 5′- AAGAAGAGTCCTTTCAGCGG -3′; R: 5′- GACGTAAAGGGATAGGGCTG -3′ and GAPDH with the following sequence: F: 5′- GAAATCCCATCACCATCTTCCAGG-3′ and R: 5′-GAGCCCCAGCCTTCTCCATG-3′. The amplification was done in a 7500 Fast Real-Time PCR System and conducted twice for each sample. The relative expression level was quantified by the Livak method.

The chi-square was used to assess the connection between genotype and Fisher's exact test for allotype frequency in the EOC group. The difference in *RELA* mRNA relative expression in each group was counted statistically using the nonparametric independent test Kruskal Wallis as the distribution was not normal. The correlation between genotype and mRNA relative expression of *RELA* was done using a Pearson correlation test. All results counted as significant if the p-value <0.05 This statistical study was conducted using version 25 of the SPSS (Statistical Package for the Social Sciences) software.

RESULTS

In this study, the number of samples used was 36 samples for the low-grade epithelial ovarian cancer case group, 29 for the high-grade ovarian cancer case group, and 15 for ovarian cyst tissue as the control group. In addition, blood samples from normal women were also used to see the difference in distribution between the normal group and the EOC cases.

The non-serous histological type predominated in the low-grade EOC group, while in the high-grade EOC, the majority was the HGSC type (Table 2). The average age of subjects at the time of diagnosis for the low-grade EOC group was 49.4 years, and 50 years for the high-grade EOC group. At the same time, the usual group subjects had an average age of 51.1 years.

Table 2. Histological Types of EOCs Subjects

Histological types	N (%)
Low-grade	36 (100)
Endometrioid carcinoma	14 (38.89)
Mucinous carcinoma	9 (25)
Clear Cell Carcinoma	11 (30.56)
Low-grade Serous Carcinoma	2 (5.56)
High grade	29 (100)
High-grade Serous Carcinoma	27 (93.1%)
Carcinosarcoma	2 (6.9%)

Based on the results of the Chi-square test (Table 1), there was no significant difference between the distribution of the genotypes (p = 0.273) and al o types (p = 0.172) *RELA* rs11820062 (Table 3). The most common genotype found in both the control and case groups was CT, while the CC genotype was the genotype with minor frequency in the normal group and TT in the case groups. The alternative allele T has an allele frequency of 53.8% in the control group and 44.6% in the case group. T allele had a protective effect with an odds ratio of 0.690 (0.424-1.125).

Table 3. Genotype and Alleles Frequencies of rs11820062 in the RELA

Rs11820062	Total	Normal	Case	*P-value*
	(n = 130)	(n=65)	(n=65)	
Genotype				
CC	27.7	24.6	30.8	
CT	46.2	43.1	49.2	0.273
TT	26.2	32.3	20.0	
Allele				
C	50.8	46.2	55.4	0.172
T	49.2	53.8	44.6	

Additionally, both the normal and case groups obtained HWE p values > 0.05, indicating that the individuals' alleles were in Hardy-Weinberg equilibrium (HWE) and that their population had little to no random mating and a limited inflow of new genetic material (Table 4).

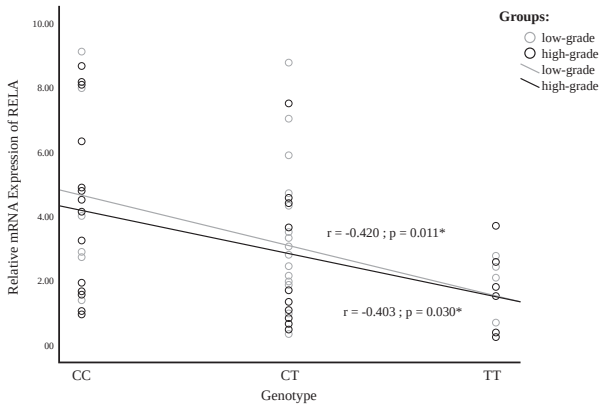
Table 4. HWE Analysis of rs11820062 in the RELA gene

		Normal			Case		
	Genotype	**Expected**	**Observed**	***P-value***	**Expected**	**Observed**	***P-value***
Rs11820062	CC	13.85	16	0.56114	19.94	20	0.99952
	CT	32.31	28		32.12	32	
	TT	18.85	21		12.94	13	

RELA's mRNA expression is significantly higher in EOCs (p=0.002) than in the cyst, but there is no significant difference between expression in low-grade and high-grade EOCs. The ovarian cyst group had a medium of 1.146 (0.330– 2,977), low-grade 2.814 (0.340 – 9.166), and high-grade 2 1.927 (0.274 – 8.724).

The relationship between the rs11820062 RELA genotype and the RELA mRNA expression level can be determined by performing a correlation test. Based on the normality test, it showed that the rs11820062 *RELA* genotype and RELA mRNA expression levels in low-grade and high-grade epithelial ovarian cancer patients were normally distributed (P value> 0.05), so the Pearson correlation statistical test was performed.

The data shown in Figure 2 shows that the relationship between the rs11820062 RELA genotype with the T allele and the level of RELA mRNA expression in both low- and high-grade EOC has a negative relationship. The correlation between the rs11820062 genotype and the *RELA* mRNA relative expression level in both groups falls in the medium criteria (Figure 1).



*p-value significant at <0.05 level

Figure 1. Correlation of the RELA rs11820062 genotype with *RELA* mRNA expression levels in the *low*- and *high*-grade EOC.

DISCUSSION

Like other types of cancer, NF-kB plays a huge role in every step of EOC tumorigenesis, from initiation to metastasis. During inflammation, NF-KB is more regulated in a classical manner than the alternative manner by forming RelA and p50 dimers.[17] RelA or p65 subunit of NF-κB, encoded by *RELA,* has a transcription activation domain (TAD), which will later regulate many gene targets, including genes that regulate cell proliferation.[18] *RELA* is a proto-oncogene and is the family's most potent transcriptional activator.[19] Low patient survival is associated with high RelA/p65 expression, which activates the NF-κB pathway. Enhanced p65 phosphorylation and NF-κB activity during tumor growth in a mouse model of OC are a result of increased M2 macrophage infiltration.19

There is no significant difference in rs11820062 genotype distribution between normal subjects and EOC cases. Homozygote CT was the most frequent genotype in both normal (43.1%) and cases subjects (49.2%). Similar to the result in this study, found that the most frequent genotype for both normal and ovarian case groups was CT, with 49.3% for the normal group and 49.7% for cases in the Han-Chinese population. In that study, the rs11820062 genotype alone did not correlate with EOC [20] Failure to detect the correlation of the RELA rs11820062 with epithelial ovarian cancer may also occur because only a single variant was detected in this study. In some cases, the protective or predisposition effect appears when an extreme mRNA expression cannot be detected only by a single genetic marker. [21] In accordance, found a correlation of *RELA* rs11820062 with EOC susceptibility by forming diplotype CC-CC with *RELA* rs7119750. The minor allele in normal groups was the C allele, with a frequency of 46.2%, and the T allele for case groups, with a frequency of 44.6%.20 However, there have been no other studies regarding the distribution of rs11820062 in other populations for epithelial ovarian cancer or other types of cancer. Therefore, information regarding the correlation of this allele with the risk of ovarian cancer is still very limited to support this hypothesis.

In this study, the distribution of *RELA* rs11820062, both in normal and case groups were in the HWE. Allele and genotype frequencies can be calculated using Hardy-Weinberg assumptions. When the ratios of homozygous

and heterozygous genotypes dramatically deviate from what would be expected based on HWE assumptions, it may be a sign of genotyping errors, batch effects, population stratification, or much less frequent association.

In this study, *RELA* relative mRNA expression is significantly higher in EOCs than in ovarian cysts. Rel A/p65 exhibits substantial transactivation potential, as seen by its constitutive activation in various human malignancies.[22] Compared to normal tissues, human tissues from colon adenocarcinoma, gastric carcinoma, lung carcinoma, and pancreatic adenocarcinoma exhibit greater nuclear translocation of RelA and NF-κB-DNA-binding activity.[23] However, there is no significant difference between the expression of RELA mRNA in low-grade and high-grade EOC. Similar results were obtained for other types of cancer where the relative expression of RELA mRNA was known to increase significantly in samples of colorectal tumor tissue compared to normal mucosa as the standard group. In that study, the relative expression of RELA mRNA did not correlate with clinicopathological characteristics, including tumor progression and AJCC staging classification.[24] Moreover, *low-grade* EOCs consist of diverse histological types of EOC, such as mucinous, endometrioid, clear cell carcinoma, and low-grade serous carcinoma, with different gene regulations and mutation characteristics. In high-grade EOC, the sample histological type is more uniform, as it mainly consists of high-grade serous carcinoma. Often the division based on the dualistic model is not correlated with the patient's prognosis and progressivity as it does not count the stage of cancers.[25] In some cases, non-serous low-grade EOC, but in stage III or IV, tend to be more resistant to chemotherapy than high-grade serous carcinoma. [26] The expression of *RELA* mRNA and p65 protein is higher in ovarian cancer cells (A2780CP) resistant to cisplatin than in ovarian cancer cells, which are sensitive to cisplatin and have higher mRNA expression than normal. [26]

This research shows a strong negative correlation between the rs11820062 genotype and RELA-relative mRNA activity, where TT groups had the lowest mRNA expression compared to CT groups, especially CC groups. Rs11820062 is located in first intron, almost to 5′ of *RELA.* As 5′-most intron variation, *RELA* rs1182062 is linked to mRNA expression levels and can initiate transcription from in silico analysis. That silico genotype-gene expression research revealed that this SNP could affect *RELA* mRNA expression and binding to the androgen receptor in immortalized B cells. In that study, the mutant type is the T, shown to be mainly linked to lower *RELA* transcriptional activity.[16] The decrease in RELA mRNA expression in the T allele could explain the odds ratio of the T allele in this study which was below 1 (OR=0.69), indicating that the T allele is protective against epithelial ovarian cancer. [14] In contrast, the C allele may predispose to EOC due to the tendency to high RELA mRNA expression in individuals with the CC genotype. Although correlated with the relative expression of *RELA* mRNA in low-grade and high-grade epithelial ovarian cancer, the Chi-square test shows that rs11820062 has no significant difference in distribution. However, rs11820062 has the potential to be studied further by looking at its interactions with other variants at different loci to be able to produce phenotypes such as epithelial ovarian cancer.

CONCLUSIONS

There was no significant difference between the distribution of RELA rs11820062's genotype and allele in the normal and EOC case groups. *RELA* mRNA relative expression is significantly higher in low-grade and high-grade EOC than in ovarian cysts. However, there is no significant difference between low-grade and high-grade relative mRNA expression of *RELA* allegedly because of the heterogeneity of low-grade EOC's histological subtype and grades. The high *RELA* mRNA expression in EOC due to genetic variation rs11820062 CC genotype. In contrast, individuals with the TT genotype tended to have lower expression of RELA in both low-grade and high-grade of cancer. This result corresponds to the T allele's odds ratio of 0.62 which might indicate the protective effects of T allele from EOC.

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