



# Association of Polymorphism (RS1800896) of IL-10 Gene and IL-10 Gene Expression in Ovarian Cancer Patients From Georgia

Pavithra Balakrishnan,<sup>1</sup> Jahnvi Shah,<sup>1</sup> Sandro Surmava,<sup>1</sup> Eka Kvaratskhelia,<sup>1,2</sup> Elene Abzianidze,<sup>1,3</sup> Nino Vardiashvili,<sup>4</sup> Ketevani Kankava<sup>1</sup>

## Abstract

**Background/Aim:** Ovarian cancer is one of the most important causes of tumour-associated mortality and morbidity in women. Some genetic alterations, determining predisposition to ovarian cancer have already been identified, but these are mostly syndrome-associated cases, most ovarian tumours are still regarded as sporadic. The aim of this research was to identify new predisposing factors that might increase ovarian cancer risk. Genetic variants of IL-10 gene in patients with ovarian cancer was analysed.

**Methods:** Forty-eight patients with ovarian cancer along with 48 age-matched controls were included in the study. Single nucleotide polymorphism (SNP) genotyping and gene expression assays for IL-10 were performed using TaqMan assay (*Thermo Scientific*, USA). The selected SNP was rs1800896 upstream of IL-10 gene (IL-10-1082). All statistical analyses were performed by GraphPad Prism 9.3.1 for Mac.

**Results:** The genotype distributions of IL-10 gene polymorphisms among cancer and control groups were all according to the expected Hardy-Weinberg equilibrium. There was no statistically significant difference in frequency of genotypes and alleles between the two study groups ( $p > 0.05$ ). In another analysis, the samples were grouped according to the polymorphic variant IL-10 (-1082) A/G. Subjects having the homozygous variant (A/A) had lower IL-10 mRNA levels than those with the homozygous wild (G/G) genotype in both, ovarian cancer patients and controls,  $p < 0.05$ . mRNA levels on IL-10 were different among cases and controls ( $p < 0.05$ ). Patients with ovarian cancer had higher level of mRNA for IL-10.

**Conclusions:** These results support the theory that IL-10 gene expression levels differ in patients with and without ovarian cancer. Polymorphic variant IL-10 (-1082) A/G couldn't be confirmed to explain this difference in gene expression levels.

**Key words:** Ovarian cancer; Single nucleotide polymorphism (SNP); IL-10, Tumour microenvironment.

1. Department of Molecular and Medical Genetics, Tbilisi State Medical University, Tbilisi, Georgia.
2. Bakhutashvili Institute of Medical Biotechnology, Tbilisi State Medical University, Tbilisi, Georgia.
3. Ivane Beritashvili Centre of Experimental Biomedicine, Tbilisi, Georgia.
4. Inova Medical Centre, Tbilisi, Georgia.

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### Corresponding author:

EKA KVARATSKHELIA  
E: e.kvaratskhelia@tsmu.edu  
T: +995599298168

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## Introduction

Being one of the most lethal malignancies, ovarian cancer represents the 3rd most common gynaecological cancer and the 8th most common cancer in women worldwide with a poor 5-year

survival rate of 17%.<sup>1,2</sup> Over 3.1 million new cases and over 207,000 deaths were reported worldwide in 2020 alone.<sup>2</sup> Often it is diagnosed at late stage making it hard to achieve a good progno-

sis. Ovarian cancer is considered to demonstrate multifactorial causes including risk factors such as age, parity, obesity, smoking, etc. However, increasing evidence suggests that genetic factors contribute significantly to the incidence of ovarian cancer.<sup>3</sup> Most common mutations seen in ovarian cancer are in *TP53*, *BRCA1*, *BRCA2*, *KRAS* and *PIK3CA* genes.<sup>4</sup>

Tumour microenvironment (TME) plays a vital role in tumour cell survival and cancer progression, as well as determining the efficiency of administered chemotherapies and immunotherapies and consecutively the prognosis of the patients. Especially in ovarian cancer, due to a highly immunosuppressive microenvironment, the first line platinum-based drugs showed high recurrence rates and consequently became chemo-resistant. This leads to significant challenges in treatment modalities and patient recovery.<sup>5</sup> Multiple players of immune microenvironment of tumours have been studied in context of cancer risk or different biological behaviour of tumours. One of them is IL-10. Both, gene characteristic and protein expression levels of this cytokine are found to be altered in some tumour tissues of cancer patients. The IL-10 gene is located on chromosome 1q32.1 with 5 exons. About 40 SNPs have been described in the promoter region.<sup>7</sup>

The ovarian cancer patients showed significantly higher concentration of IL-10 cytokine in the ascites fluid and in the serum compared to the control group.<sup>8</sup> Even tumour biopsies showed a similar increase in IL-10 in tumour samples compared to normal ovarian tissue and normal ovaries.<sup>9</sup> The high expression of IL-10 is considered as a bad omen as it decreases the survival rates<sup>10</sup> and is shown to promote cancer cell migration which results in local spread and metastasis if present in ascites,<sup>11</sup> while some polymorphism in IL-10 gene promoter region showed to give advantageous results by providing optimal tumour debulking and disease free survival rates<sup>12</sup> and support tumour rejection by helping NK cells to activate against the tumour cells.<sup>7</sup>

This may suggest the possibility of IL-10 acting differently based on the presence of different cytokines and the result of which would reflect based on those other cytokines as well. Thus, knowledge about IL-10 could bear a new torch to understand the mechanisms of TME.

In the present study the single nucleotide polymorphism (SNP) rs1800896 also mentioned as

the IL-10-1082A/G polymorphism in the IL-10 gene was analysed and the expression of IL-10 in the peripheral blood of ovarian cancer patients to check the extent of the role of this SNP in the ovarian cancer patients in Georgia.

## Methods

The study design was a case-control study. Ovarian cancer patients who were consecutively admitted to the oncology department of Madison Hospital and Inova Medical Centre in Tbilisi, Georgia were recruited. Age-matched women who were regularly involved in cancer screening in an outpatient clinic in Tbilisi and were healthy, were asked to volunteer as control group members. Clinical information of patients was collected from medical notes.

The eligibility criteria for the cases were: a) diagnosis of ovarian carcinoma; b) ability to understand the purpose of the study and provide informed consent; c) being ethnically Georgian. Eligibility criteria for controls were as followed: no diagnosis of ovarian cancer and minor illnesses were acceptable (eg common cold, headache). The rest of the criteria (2-3) were identical to that of the cases. In total 48 patients were involved in the study along with 48 healthy controls.

Blood samples were collected in a vacutainer tube containing ethylenediaminetetraacetic acid (EDTA). Genomic DNA and RNA were extracted from the whole blood using DNA and RNA purification kits (*Qiagen*, USA). DNA and RNA concentrations were measured using the fluorometer-based method (*Qubit*, *Thermo Scientific*, USA).

## Genotyping

SNP genotyping was performed using TaqMan assay (*Thermo Scientific*, USA). The selected SNP was rs1800896 SNP upstream of IL-10 gene (IL-10-1082). Each TaqMan SNP genotyping assay contained sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and 2 TaqMan minor groove binder (MGB) probes with nonfluorescent quenchers (NFQ): One VIC-labelled probe to detect allele 1(A) sequence and one FAM-labelled probe to detect allele 2(G) sequence. Real time-PCR was performed based on standard protocols. The reaction mix, including the TaqMan Master Mix (5.00 uL) and the 20X Assay Working Stock (0.50 uL),

totalled 5.50  $\mu\text{L}$ . Together with the DNA sample (4.50  $\mu\text{L}$ ), the final reaction volume was 10  $\mu\text{L}$ . PCR conditions for amplification included polymerase activation at 95 °C for 10 min (hold), denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min (cycle 40). The real-time PCR instrument software plots the results of the allelic discrimination data as a plot of allele 1 (VIC dye) versus allele 2 (FAM dye). The allelic discrimination (AD) plot represents each sample well as an individual point on the plot. A typical AD plot shows homozygote clusters, a heterozygote cluster and the no-template controls. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters.

### Reverse transcription

The reverse transcription was carried out by using *Thermo Fisher High-Capacity* cDNA reverse transcription kits. The final volume of each reaction well was 20  $\mu\text{L}$  of which 10  $\mu\text{L}$  was contributed by RNA sample and 10  $\mu\text{L}$  was contributed by 2X RT master mix. The 2X RT master mix was prepared as per the protocol which consisted of 2  $\mu\text{L}$  of RT random primers, 1  $\mu\text{L}$  of *Multiscribe transcriptase*, 0.8  $\mu\text{L}$  of dNTP nucleotide mix, 2  $\mu\text{L}$  of RT buffer and 4.2  $\mu\text{L}$  of nuclease free water to fit each well of 20  $\mu\text{L}$  reaction.

After loading, the PCR conditions for reverse transcription included 10 min of incubation period at 25 °C, 120 mins of second stage at 37 °C and

5 min of final step at 85 °C. The obtained cDNA samples were stored at 4 °C for further analysis.

### Gene expression

Gene expression assays for IL-10 was performed using TaqMan gene expression assays (*Thermo Scientific*, USA). The final volume of PCR reaction of each well was 20  $\mu\text{L}$  which contained 1  $\mu\text{L}$  of gene expression assay, 10  $\mu\text{L}$  of gene expression master mix, 4  $\mu\text{L}$  of cDNA template and 5  $\mu\text{L}$  of nuclease free water. The PCR settings for amplification required polymerase activation at 95 °C for 10 min (hold), denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min (cycle 40).

### Statistics

The study and control groups were analysed separately. All statistical analyses were performed by GraphPad Prism 9.3.1 for Mac (*GraphPad Software*, San Diego, California USA).

Statistical significance for differences in genotype frequencies was determined by Chi-square and Fisher's exact test and the level of significance was put at  $p < 0.05$ . An unpaired t-test was used to compare the difference in mean proliferative activity of patients with A/A genotype and patients having the G allele (A/G and G/G). To evaluate associations between the SNPs and the risk of cancer odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated using unconditional logistic regression analysis.

## Results

### Association between promoter polymorphisms of IL-10 gene and ovarian cancer

The genotype distributions of IL-10 gene polymorphisms among cancer and control groups were all according to the expected Hardy-Weinberg equilibrium. IL-10 (-1082) allele and genotype distributions of both ovarian cancer patients and controls are illustrated in Table 1. There was no statistically significant difference in frequency of genotypes and alleles between the two groups ( $p > 0.05$ ).

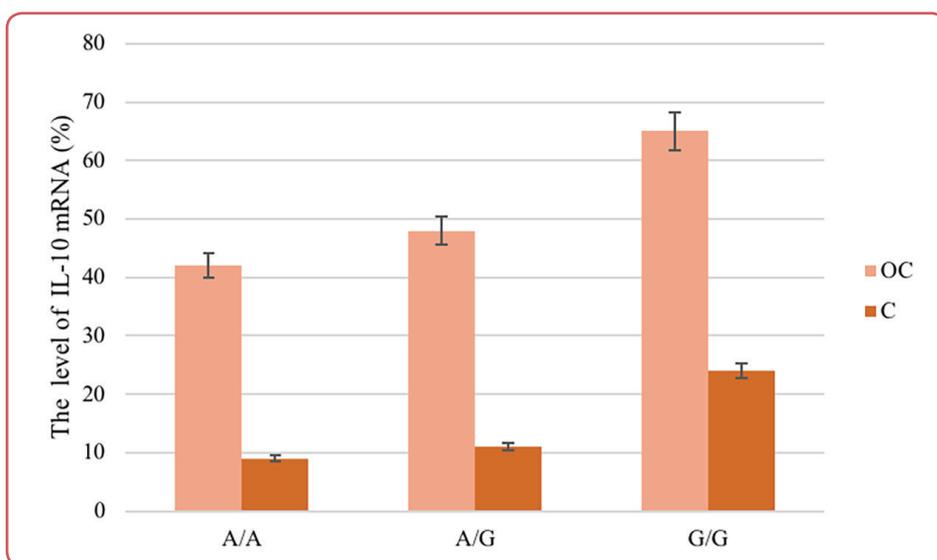
### Relationship between IL 10 (-1082) SNP and expression levels of IL-10 in ovarian cancer

In another analysis, the ovarian cancer samples and controls were grouped according to genotypes to the polymorphic variant IL 10 (-1082) A/A, A/G and G/G. Subjects having the homozygous variant (A/A) had lower IL-10 mRNA levels than those with the homozygous wild (G/G) genotype in both, ovarian cancer patients and controls,  $p < 0.05$ . In addition, mRNA levels on IL-10 were different among cases and controls ( $p < 0.05$ ). Patients with ovarian cancer had higher level of mRNA for IL-10 in all three groups (Figure 1).

**Table 1:** Distribution of IL-10 -1082 A/G genotype and allele frequency in ovarian cancer patients and controls

Genotypes or alleles	Cases N (%)	Controls N (%)	OR (95 % CI)	p-value
A/A	11 (22.9 %)	14 (29.2 %)	1.00 (ref)	
A/G	28 (58.3 %)	27 (56.2 %)	1.06 (0.57-1.74)	0.835
G/G	9 (18.8 %)	7 (14.6 %)	0.84 (0.38-2.36)	0.648
A allele	50 (52.1 %)	55 (57.3 %)	1.00 (ref)	
G allele	46 (47.9 %)	41 (42.7 %)	0.46 (0.21-1.43)	0.461

OR: odds ratio; CI: confidence interval;



**Figure 2:** Relative gene expression levels of the IL-10 in the ovarian cancer and control groups. OC: ovarian cancer patients group; C: control group;

## Discussion

Cytokines involved in ovarian carcinoma play a very important role in survival rate, prognosis, progression free survival and probability of metastasis. Higher concentration of IL-10 protein leads to higher chances of ascites in ovarian cancer patients.<sup>8</sup> As ovarian carcinoma has a very high tendency to metastasise through transcoelomic spread, presence of ascites makes the prognosis even worse as cytokines and chemokines of malignant ascites enhance weakening of cellular tight junctions, transmesothelial migration and epithelial-to-mesenchymal transition.<sup>13</sup> This process fastens the tumour cell seeding throughout the abdominal cavity and promotes tumour growth, thereby reducing the efficiency of debulking surgeries.

The paradox is that IL-10 also plays a crucial role

in tumour suppression. IL-10 can suppress HLA expression on the tumour cell surface which activates NK cells against those tumour cells.<sup>7</sup> Another study showed that PEGylated IL-10 activates systemic immunity by CD8<sup>+</sup> T cell invigoration, polyclonal T cell expansion; increase the levels of INF- $\gamma$ , granzyme B in cancer patients and promotes tumour cell regression.<sup>14</sup> There are various trials and studies which showed various modified IL-10 such as, PEG IL-10, PVC-Ag coated IL-10, adenoviral-mediated expression of IL-10 have showed results favouring anti-tumour properties of IL-10.<sup>15</sup>

Presented study showed no clinical significance of IL-10 SNP variant (-1082) when it comes to G/G vs A/G\*A/A polymorphisms. It reflects the results described earlier.<sup>12</sup> Analysis was also ex-

tended with IL-10 expression. This showed that homozygous variant (A/A) had lower IL-10 mRNA levels than those with the homozygous wild (G/G) genotype in both, ovarian cancer patients and controls, ( $p < 0.05$ ). Further analysis showed that patients with ovarian cancer had higher level of mRNA for IL-10.<sup>16</sup>

Furthermore, a study among colorectal cancer patients revealed that the GG genotype of -1082A/G polymorphism in IL-10 were higher in controls compared to colorectal cancer patients.<sup>17</sup> A case control study showed that similar results among breast cancer patients by proving AA genotype shows increased risks towards the development of the breast cancer.<sup>18</sup> While this SNP and corresponding gene expression have been analysed in a number of articles for separately separate groups of population, more studies are needed of rs1800896 SNP of IL-10 gene and IL-10 gene expression in ovarian cancer patients.

IL-10 along with other cytokines play an important role in inducing the immunosuppressive TME. Induced or adapted subset of regulatory T cells (Treg) and tumour cells produce immunosuppressive cytokines such as IL-10, TGF-B, etc. IL-10 in turn makes dendritic cells (DC) dysfunctional which further provides the positive feedback loop to induce and expand Treg cells further by stimulating their proliferation. Treg cells are known to induce tumour specific tolerance and immunosuppression.<sup>5</sup> The IL-10 treated DCs demonstrated antigen specific anergic conditions in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well.<sup>6</sup> But, IL-10 is also believed to be acting with anti tumourigenic properties.

Attempts were made to analyse both rs1800896 SNP of IL-10 gene and IL-10 gene expression in the same group of population. Yet, presented study has some limitations, one of which is the smaller sample size. Seeing some tendency despite this relatively small number of studied cases increases the value of the results and demonstrates importance of further research in this direction. Another significant improvement to study results could be brought by excluding the possibility of other polymorphism in the IL-10 promoter region influencing the gene expression.

## Conclusion

Presented results support the theory that IL-10 gene expression levels differ in patients with and without ovarian cancer. Polymorphic variant IL-10 (-1082) A/G couldn't be confirmed to explain this difference in gene expression levels.

## Ethics

The study was approved by the Ethics Committee of Tbilisi State Medical University, Tbilisi, Georgia (decision No N7-2021/91, dated 6 October 2021). Signed informed consent was collected from each of the study participants.

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## Conflicts of interest

The authors declare that there is no conflict of interest.

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## Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

## Author ORCID numbers

Pavithra Balakrishnan (PB):  
0000-0002-7718-6769  
Jahnvi Shah (JS):  
0000-0003-4373-4402  
Sandro Surmava (SS):  
0009-0006-1446-0990  
Eka Kvaratskhelia (EK):  
0000-0002-1200-0230  
Elene Abzianidze (EA):  
0000-0002-1140-7688  
Nino Vardiashvili (NV):  
0009-0005-0047-1253  
Ketevani Kankava (KK):  
0000-0001-6944-971X

## Author contributions

Conceptualisation: PB, EK  
Methodology: PB, JS, SS, EK  
Validation: SS  
Formal analysis: NV  
Investigation: PB, JS, SS, EK  
Resources: JS, NV  
Data curation: SS  
Writing - original draft: PB  
Writing - review and editing: EK, EA, KK  
Supervision: KK  
Project administration: EK, EA  
Funding acquisition: KK

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