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Sequence Characterization of Anti Tumorigenic (IL12B) and Pro Tumorigenic (IL17A) Ovine Interleukins for an Immune Approach to Cancer Immunotherapy

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A B S T R A C T

Cancer is a complex illness characterized by uncontrolled cell growth and the spread of abnormal cells throughout the body. Immunotherapy is one of the cancer treatment modalities gained prominence in recent years. Immunotherapy is a type of bio-therapy that works by retraining the patient's immune system to recognize cancer, boosting selectivity, and decreasing side effects. Immune modulation is a complex process involving several cell types and factors. High-dose of IL-12 provides long-term responses in a subset of patients with metastatic melanoma and renal cell carcinoma. IL-17 has been identified as a frequent cytokine in the tumour microenvironment that can have dual roles in cancer growth and tumour eradication. In present study, ovine Interleukin 12B and 17A genes were amplified, cloned, and *in silico* analyzed for its potential in cancer treatment.

Introduction

Cancer is a collection of illnesses caused by the uncontrolled proliferation of abnormal cells in practically every organ or tissue of the body (Yadav and Mohite, 2020). Cancer is caused by the change of normal cells into tumor cells in a multi-step process that typically leads to the development of a malignant tumor (Poon and Ailles, 2022). Cancer begins with the uncontrolled proliferation of aberrant cells in many regions of the body, with DNA damage being the primary cause (Kay *et al.*, 2019). The condition impairs cellular metabolism and causes malignant cells to proliferate abnormally. The knowledge gained from the molecular studies of cancer is not yet complete which can give a comprehensive grasp of how cancer develops. Treatment modalities for cancer include 1) Surgery: to remove all underlying tumors and treat them with precision. 2) Radiation therapy: focused radiation energy on a specific organ, followed by surgery to stop the tumor from spreading further, 3) Chemotherapy: use of medicines to kill cancerous cells and 4) Biotherapy: harnesses the patient's immune system to target tumor cell killing (Debela *et al.*, 2021). Biotherapy is a previously untapped

technique for cancer treatment that prevents cancer mortality. Immunotherapy has now become a popular therapeutic option for a variety of cancers in the last decade (Quillien *et al.*, 2023). Immunotherapy aims to stop cancer from spreading and progressing by assisting the immune system in regaining its ability to combat malignant cells (Kraehenbuehl *et al.*, 2022). Immunotherapy, which would be based on the promise that the immune system may kill tumors via immune-surveillance, is now regarded a successful treatment for many cancers (Finck *et al.*, 2022). Several forms of immunotherapy have different mechanisms of action. Some immunotherapy treatments work by assisting the immune system in slowing or stopping cancer cell development. Others aid the immune system in the destruction of cancer cells or the prevention of cancer spreading to other parts of the body. Many factors viz. cancer's type, size, location, and extent of dissemination influence the cancer immunotherapy. Immunostimulatory genes like cytokines produce its effects by activating several cellular pathways that kill and remove cancer cells. Cytokines are important regulators of both innate and adaptive immunity, allowing immune cells to interact over short distances (Lee and Margolin, 2011). Cytokine therapy has been an important treatment option for cancer patients, and it continues to be a vital addition to contemporary clinical cancer research. Interferon alpha (IFN-α) is licensed as an adjuvant treatment for individuals with totally re-sected high-risk melanoma and a variety of refractory cancers (Eggermont and Robert, 2011). Cytokines have numerous pleiotropism that allows them to reveal distinct biological aspects, the therapeutic utility of cytokines is limited. When generated in high quantities, cytokines can penetrate the bloodstream and exert an endocrine effect far from their source. Several animal tumor model studies have shown that cytokines have wide anti-tumor activity, which has led to the development of a number of cytokine-based cancer therapies (Berraondo *et al.*, 2019). Interleukins also promote cancer catenation and proliferation by their pleiotropic capacity, which encodes the blockade of a signaling molecule, suppression of ligand-receptor interaction, and cancer initiation by promoting activity in a particular cell region. Knowing the exact mechanism of action of interleukins in the immune system and their precise immunological responses to certain cancers might help to build successful therapeutics. Immunotherapy works by increasing the patient's immune system while also destroying malignant cells. In the development of the immune system, host defense, and tumor immunobiology, cytokines play complicated and frequently contradictory functions. IL12 is a cytokine that is implicated in both innate and adaptive immune responses. Tumor development and dissemination are linked to host immune responses, and it's known that cytokines from the IL-12 family can control tumor progression (Mirlekar and Pylayeva-Gupta, 2021). IL-17 is a cytokine that plays a role in inflammatory and autoimmune disorders and known as pro-tumor cytokines (Song and Qian, 2013). Understanding the biological activities and mechanisms of action of these components is therefore critical for the development of cytokine-based immunotherapy for cancer treatment. Present work has been designed with the objective to analyze IL12 and IL17 genes from ovine origin cancer immunotherapy.

Materials and Methods

RNA preparation and complementary DNA (cDNA) synthesis

Total RNA was extracted from blood using the TRIzol reagent (Sigma) following manufacturer's instructions. The purity of the RNA sample was determined using agarose gel electrophoresis and optical density was measured with a micro volume spectrophotometer. Complementary DNA was synthesized using cDNA preparation kit (Applied Biosystems) following manufacturer instructions.

Amplification and cloning of pro- (IL17A) and anti-tumorigenic interleukins (IL12B)

Gene specific primers were designed from the sheep gene sequences obtained from Ensembl genome

browser using Primer3 (Primer3web version 4.1.0). PCR reaction was run in a thermal cycler (BIORAD) with the steps: 96 °C for 3 minutes and a 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 57°C for 30 sec for the Interleukin-17A, whereas, Tm for IL-12B was 58°C amplification at 72° C for 30 sec and a final extension at 72°C for 5 minutes. The PCR products were resolved on agarose (3%) gel electrophoresis with a 100 bp DNA ladder side by side to estimate PCR product size. The desired PCR product was purified using the Gel extraction kit (Thermo Scientific) following manufacturer's instructions. The purified PCR products were ligated in the pJET vector supplied with the pJET cloning kit (Thermo Scientific). The *Escherichia coli* strain DH5α was used for transformation of ligated PCR products using Transform Aid Bacterial Transformation kit (Thermo Scientific). Colony PCR was done to identify true clones. Plasmid containing gene inserts in bacterial cells was isolated using the GeneJET plasmid miniprep kit (Thermo Scientific) following manufacturer's instructions.

In-silico analysis of nucleotide sequences

Gene sequences were aligned with similar sequences available in NCBI database using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and exact CDS were determined. Expasy (https://web.expasy.org/translate/) tool was used to produce the protein sequences. MEGA 11 tool was used for phylogeny analysis of DNA/protein sequences to reveal the evolutionary relationship with other animal sequences, as well as a protein sequence and structure analysis (Tamura *et al.*, 2021). Protein modeling, visualization and validation with primary and secondary structure analysis have been done using computational tools (Jiang *et al.*, 2001).

Homology modeling is used to search the conformation space while causing the least amount of disruption to the existing solutions, i.e. the structures that have been experimentally solved. In the SWISS-MODEL, IL17 and IL-12 protein data obtained from ovine sequences are supplied as a data input. The template for IL-12 is 5mxa.1.B whereas, for IL-17 is 5n7w.1.E were used. The SWISS-MODEL template library (SMTL version 2022-05-25, PDB release 2022-05-20) was searched with BLAST and HHblit for evolutionary related structures matching the target sequence. IL-12: Overall 16159 templates were found. Only 50 results are considered, A further 10,061 templates were found which were considered to be less suitable for modeling than the filtered. IL-17: Overall 82 templates were found. Only 8 results are considered, A further 42 templates were found which were considered to be less suitable for modeling than the filtered. PSIPRED server was used to predict the secondary structure of both IL-12 and IL-17, which is based on the analysis of PSI-BLAST (Position Specific Iterated-BLAST) output by two feed-forward neural networks. The protein 3d structure was designed by using SWISS-MODEL. The structures were visualized by using *pymol* visualization tool. The Ramachandran plot of the model generated was visualized using PROCHECK.

Results and Discussion

The two interleukin genes IL12 (anti-tumor) and IL17 (pro-tumor) were amplified using gene-specific primers (table 1). The amplified mRNA for the IL-12 gene is 1107 bp with 5’ UTR region (40 bp), coding region (41..1024bp; 984 bp) and 3’UTR region (83 bp) whereas, the amplified mRNA for the IL-17 gene is 630 bp with 5’ UTR region (49bp), coding region (50…511 bp; 462 bp) and 3’UTR region (119 bp). Ovine IL12 and ovine IL17 gene sequences of Malpura sheep has been submitted to NCBI GenBank with accession numbers ON131071 and ON131072, respectively. The CDS were *in silico* translated using expasy translation tools. The coding region of the IL-12B and IL-17A gene yielded a 327 and 153 amino-acid polypeptide chain, respectively. Expasy's ProtParam (Table 2) revealed that the IL12 Protein has 327 amino acid residues with an estimated molecular weight of 36996.75. The theoretical pI (pH at which a protein

is stable) was calculated to be 6.10, which is lower than pI=7, indicating that the protein is acidic in nature. The IL17 protein has 153 amino acids, has a molecular weight of 17222.88, and has a theoretical pI of 9.24. This estimate can be used to create a buffer system for the protein in question. The total number of positive (R+K) and negative (D+E) residues was found to be 38 and 42, respectively. Total numbers of negatively charged residues (Asp + Glu) were 12 and total numbers of positively charged residues (Arg + Lys) were 18 for IL-12, respectively. The half life is the time it takes for a protein to decay to half its original concentration after it has been 45 synthesized. For both IL-12 and IL-17, the half lives were calculated to be 30 hours for mammalian reticulocytes in vitro, 20 hours for yeast in vivo, and more than 10 hours for *E. coli* in vivo in three model species (mammalian, yeast, and *E. coli*). PSIPRED data have shown that random coils have a higher proportion than alpha helices and beta sheets, with beta sheets having the least structural influence. The protein 3d structure designed by using SWISS-MODEL and visualized using *pymol* visualization tool (figure 1). The Ramachandran plot of the model generated using PROCHECK show the residues are occurring under the core region of both the interleukin protein IL-12 and IL-17 Model which shows that the model is stereo chemically stable (figure 2).

Table.1 List of primers used for amplification of ovine Interleukin gene

Gene	Primer	Length (5' to 3')	Tm	Product size
1	IL12B	F-5'TTTCAGACCCAGCGAACTCT-3' R-5'GGAGGTCTGTTCCGTCATGT-3'	57.7	1107 bp
2	IL17A	F-5'CACAGCGAGCACAAGTTCAT-3' R-5'TGCCCCAAAGTTATTTCAGG-3'	57	630bp

Table.2 Expasy ProtParam analysis of various physical and chemical parameters of ovine IL-12 and IL-17 interleukins.

PARAMETERS	*IL-12*	*IL-17*
Molecular weight	36996.75	17222.88
Theoretical pI	6.10	9.24
Number of positive residues	38	18
Number of negative residues	42	12
Half life mammalian reticulocytes (in vitro)	30	30
Half life yeast (in vivo)	20	20
Half life E. coli (in vivo)	10	10
Extinction coefficient	74870	14355
Aliphatic index	73.88	84.64
Instability index	36.35	64.84
GRAVY index	-0.465	-0.335

Fig.1 Prediction of ovine IL-12 and IL-17 protein 3D structure using protein structure homology-modelling server (SWISS-MODEL) with PyMOL molecular visualization system.

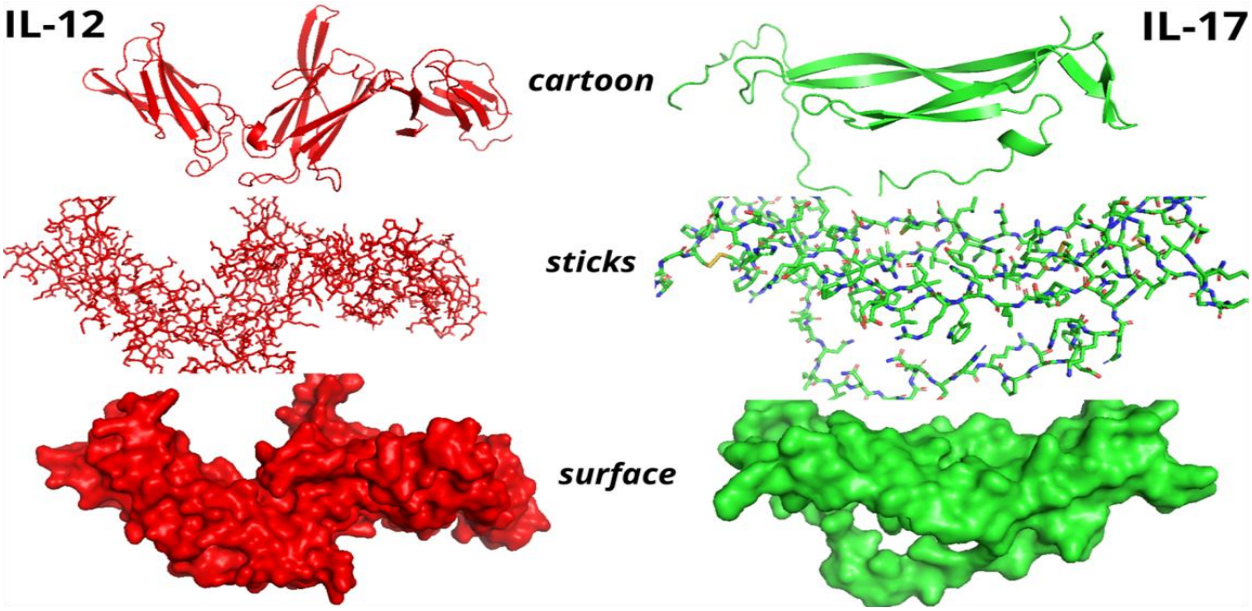
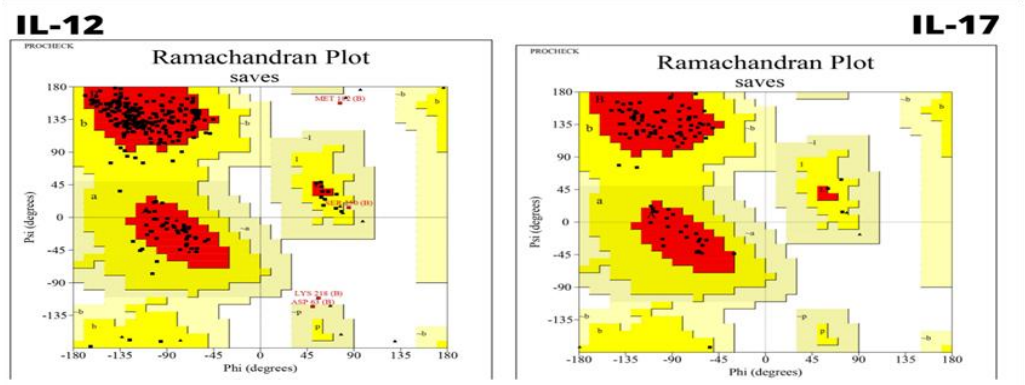


Fig.2 Prediction of possible conformation of the peptides i.e. ovine IL-12 and IL-17 by Ramachandran plot.



IL-12

Plot statistics

Residues in most favoured regions [A,B,L]	246	89.5%
Residues in additional allowed regions [a,b,l,p]	25	9.1%
Residues in generously allowed regions [~a,~b,~l,~p]	2	0.7%
Residues in disallowed regions	2	0.7%
Number of non-glycine and non-proline residues	275	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	15	
Number of proline residues	13	
Total number of residues	305	

IL-17

Plot statistics

Residues in most favoured regions [A,B,L]	95	92.2%
Residues in additional allowed regions [a,b,l,p]	8	7.8%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	103	100.0%
Number of end-residues (excl. Gly and Pro)	1	
Number of glycine residues (shown as triangles)	4	
Number of proline residues	10	
Total number of residues	118	

The models generated for IL-12 and IL-17 were showed good overall stereo chemical quality as expected for modeled with high sequence identity with the template. The IL-12 model had 0.7% residues in the disallowed region, while 89.5% of residues lied in the favored region, and IL-17 had 92.2% of its residues in the favored region, while no residues were found in the outlier region.

The area of cancer immunotherapy is quickly expanding and it will undoubtedly be a part of cancer therapies in the future. Immunotherapy has a lot of potential to improve the efficacy of existing chemotherapeutic and courses of therapy. This approach may be used for both precancerous and cancerous cells. Although interleukin or interleukin-targeted therapy still has a decent road to go before having reached the clinic, the decade of intensive foundational research on interleukin in cancer biology has helped us better understand the mechanisms of interleukin therapy and thus contributed to the development of new strategies.

In addition, there is a plethora of new preclinical and clinical trials on interleukin treatment for cancer in the pipeline that will offer fascinating results in the future years. IL-17 is a cytokine that plays a role in inflammatory and autoimmune disorders. Pro-tumor cytokines are the ones that cause cancer cells to multiply. The expression of this gene may provide a significant approach to utilize in cancer patient treatments. New immunotherapeutic techniques for the treatment of cancers that target IL-17 might be developed. The delivery of anticancer inhibition should target the IL- 17 gene to limit their growth by evaluating their influence on cancer and normal cells. As innate and adaptive immune responses would both be regulated by cytokines IL-12, it can control tumor progression. Although IL-12 appears to be potential options for treating a variety of malignancies, their side effects and functions in controlling the local vs systemic immune system still need to be explored. Blocking the tumor cell cycle, inducing apoptosis, and preventing tumor cell growth, as well as enhancing effectors immune responses against cancer cells, are all possible with treatments concentrating on the IL-12 family of cytokines. Localized IL-12 might be employed as a neoadjuvant to resection in cancer patients at an earlier stage. Combining localized IL-12 with ablation for inoperable tumors may aid boost local and distant tumor control. The genes characterized in our study would be induced in the cancer cells to delineate its molecular events in cancer progression and control. It is anticipated that the future prospects for the work would be encouraging.

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