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GENOMIC ANALYSIS OF SLC9A7 REVEALS A NOVEL SEQUENCE VARIANT ASSOCIATED WITH X-LINKED INTELLECTUAL DISABILITY IN A CONSANGUINEOUS PASHTUN FAMILY

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Abstract

Intellectual disability is characterized by the development of motor, cognitive, language, and social skills. It is a complex condition influenced by either environmental or genetic factors. In this study, we have utilized whole exome sequencing (WES) to identify the genetic causes of Intellectual disability in a consanguineous family from Peshawar, Khyber Pakhtunkhwa, Pakistan. Resultantly, we have identified a novel hemizygous missense variant c.1207T>A (p.S403T) in the SLC9A7 gene on X-chromosome. Consequently, through segregation analysis its X-linked inheritance pattern was validated, with affected members (II-3, II-4, II-5) exhibiting hemizygosity. Similarly, through structural analysis using 3D molecular modeling we have confirmed the potential conformational changes in the protein structure due to mutation c.1207T>A (p.S403T). These findings help us better understand the genetic causes of Intellectual Disabilities (ID) in consanguineous populations, which are crucial for early diagnosis and personalized therapeutic strategies.

Keywords: *Mental retardation, Neurology, Neuromics, Novel Variant, Pakistan*

INTRODUCTION

Intellectual Disability is a neurological condition characterized by certain limitations which further affect cognitive, language, motor, and social skills due to which the patients cannot properly interact with the environment and people, and unable to manage daily tasks. These limitations are comprised of learning, reasoning and problem solving which are mostly evident in teenage. Therefore, standardized intelligence quotient (IQ) test is required to diagnose the patient and an IQ score < 70 along with defective adaptive functioning is considered as the defining criterion for Intellectual disability (1). A range of disorders like developmental delays, speech impairments, behavioral problems, motor dysfunction, and, in severe cases, seizures or other neurological abnormalities are considered as the additional features associated with a person with Intellectual disability (2). Genetics is substantial, hundreds of genes are involved causing different forms of IDs but those present on X-chromosome are called X-lined and less than 50 mutations identified on X-lined genes are involved in causing X-linked Intellectual disability. Similarly, environmental factors such as prenatal exposure to toxins, birth complications, and infections are also involved in the disease etiology of Intellectual Disability (3, 4).

Moreover, several genes are involved in various neurodevelopmental signaling pathways like synaptic function, signal transduction, and chromatin remodeling which further lead to disrupt cognitive and adaptive functioning in Intellectual Disability (ID). Notable examples include *MECP2*, *FMR1*, *SLC9A7*, *SHANK3*, and *IQSEC2 etc* (5). The *SLC9A7* gene resides on X-chromosome is one of the genes associated with IDs as well as autism spectrum disorder (ASD) and epilepsy (6). This X-linked gene, the Solute Carrier Family 9 Member A7 (*SLC9A7*), also known as Sodium/Hydrogen Exchanger 7 (NHE7), which is primarily stationed at trans-Golgi network (TNG). It regulates the internal environment of organelles inside the cell where it maintains pH and ion homeostasis. Furthermore, mutations in *SLC9A7* are most likely cause nonsyndromic intellectual disabilities but some causes also exhibit strabismus along with other neurodevelopmental disorders. Almost, 50 pathogenic variants have been identified as yet, which are clear indication that how much *SLC9A7* is important in motor and cognitive functioning (3).

Genetic disorders and more specifically neurogenetic diseases including autosomal recessive intellectual disability and other neurodevelopmental disorders are prevalent in the regions of South Asia and Middle East due to high rate of consanguineous marriages. This ratio is higher in Pakistan, especially in Khyber Pakhtunkhwa, which is, nearly 60% marriages are between cousins. This high ratio of cousin marriages further intensifies the already higher burden of inherited disorders in Pashtun population (7).

In the current study, we have recruited a consanguineous Pashtun family from Peshawar, Khyber Pakhtunkhwa, Pakistan, exhibiting clinical phenotypes of XLID. Through whole exome sequencing (WES) we have identified a novel hemizygous missense variant c.1207T>A (p.S403T) in the *SLC9A7* gene associated with XLID. Segregation analysis further confirmed the inheritance of this variant within the family, consistent with the observed phenotypes. Moreover, through 3D molecular modeling, the potential structural changes occurred via this novel hemizygous missense variant c.1207T>A (p.S403T) were confirmed. These results expand the mutational spectrum of *SLC9A7* and provide insights into the genetic basis of intellectual disability in consanguineous populations.

MATERIALS AND METHODS

CLINICAL HISTORY AND CONSENT FORM

Ethical approval was obtained from Institutional Bioethical Committee (IBC) of Islamia College University, Peshawar (Ref. No. 602/ORIC/ICP). The consent form for providing the blood samples was signed by legal guardian of those included in this study. Helsinki's declaration was followed to ensure patients' protection and there was no intended or unintended risk involved for the subjects included in this study. No treatment or administered medications were part of this study. Special code was assigned to patient's samples and pedigree, to maintain confidentiality and patient's privacy protection.

Families with consanguineous marriages (first or second cousins) and at least two affected children with genetic neuropathies were given preference to be included in the current study. Before blood samples collection, clinical summary associated with pedigree, videos and photos of the affected subjects were considered complimentary to confirm phenotypes in the patients.

SAMPLE COLLECTION

5ml of blood was collected from all members of the family, including patients, healthy siblings and parents. The samples were stored in freezer at -20 to -50^{o}C, till further process. These samples were used only for the mentioned purpose and nothing else. There was no financial gain for anyone involved in this research. Furthermore, the gDNA from the whole blood samples was extracted using standard phenol-chloroform method of DNA isolation and its concentration was measured via NanoDrop spectrophotometer. Similarly, the DNA isolated was processed for both qualitative and quantitative investigation using agarose gel electrophoresis. Gel documentation was used for visualization of the bands obtained in gel electrophoresis, under the UV light.

SEQUENCING AND DATA ANALYSIS

The DNA isolated from the diseased individual underwent whole exome sequencing following pedigree and phenotypic analysis. Subsequently, the proband DNA was sequenced utilizing the HiSeq-2500

Illumina platform, achieving normal read depth coverage of more than 100X. The resulting raw data was provided having both sequence data and quality scores. Quality assessment of the sequence was conducted using FastQC. Sequence alignment was performed using BWA (Burrows-Wheeler Aligner) with the GRCh38, generating SAM file (8). Sorting of the SAM file resulted into BAM (binary alignment/map) format was accomplished using SAMtools version 1.7(http://samtools.sourceforge.net) (9). Variant calling was executed with GATK, producing a Variant Calling File (VCF) (10). Furthermore, Picard was used for removing the duplicated regions (https://broadinstitute.github.io/picard). The variants obtained in the VCF file was processed for annotation utilizing ANNOVAR, a comprehensive tool accessible at ANNOVAR (http://annovar.openbioinformatics.org) facilitates the functional annotation of genetic variations by leveraging a filter-based approach across diverse genomic resources (11). This process involves scrutinizing variants against several pertinent databases to elucidate their functional implications. Noteworthy databases employed in this analysis include, Mutation Taster (www.mutationtaster.org), SIFT (www.sift.bii.a-star.edu.sg), PolyPhen-2 (www.genetics.bwh.harvard.edu/pph2), the 1KG (www.internationalgenome.org), dbSNP (www.ncbi.nlm.nih.gov/snp), gnomAD (www.gnomad.broadinstitute.org) (12-17).

VALIDATION OF THE TARGET VARIANTS

Sanger sequencing was performed on ABI-3730 genetic analyzer for confirmation and validation of the variant in *SLC9A7* gene. The publicly available tool i.e. Primer3 was used for primer designing. (https://bioinfo.ut.ee/primer3) using the reference sequences of the targeted regions obtained from the Ensemble Genome Browser (www.ensembel.org). Primers designed were tested using in-silico PCR before sending it for synthesis (Table I).

Table I. Set of primers designed for Sanger sequences

Gene	Forward primer	Reverse primer
***SLC9A7*: c.1207T>A**	5′ TGGATATGCTAGGCTTGCCT 3′	5′ CACCTCAAAGAGCTGCACAG 3′

Upon completion of sequencing, the resultant data underwent meticulous scrutiny utilizing BioEdit and ClustalW, sophisticated bioinformatics tools employed for sequence alignment and analysis. This analysis aimed to verify the inheritance pattern of identified variants across familial cohorts, encompassing both affected individuals and unaffected relatives. Furthermore, the sequencing results and validates the presence of hemizygous sequences in affected individuals, as well as heterogeneous sequences in unaffected family members, SnapGene Viewer was used.

IN-SILICO SITE DIRECTED MUTAGENESIS OF *SLC9A7* PROTEIN

Three-dimensional AlphaFold model of *SLC9A7* was retrieved with PLDDT score of >90. Through Insilico site directed mutagenesis mutant models of SLC9A7 (Ser403Thr) were built and evaluated for model quality (18). UCSF Chimera tool was used for structure minimization, refinement and visualization (19).

EFFECT OF IDENTIFIED MUTATIONS ON *SLC9A7* PROTEIN STRUCTURE AND FUNCTION

The online HOPE tool was utilized to analyze the impact of the identified mutations ($SLC9A7^{Ser403Thr}$) on the physicochemical characteristics of the proteins. By using the UniProt database and predictions from the Reprof software, HOPE evaluates structural alterations caused by the mutant amino acid on the protein's structure.

RESULTS

CLINICAL FEATURES OF THE FAMILY

There are three individuals affected in this family (II-3, II-4, II-5). Upon their developmental and behavioral assessment, they were having similar symptoms of mental retardation. They had started crawling after 2 years of their birth and were unable to sit for 2.5 years, and become able to stand vertically after 3 years. Moreover, they were assisted while walking and normally dependent on wheel-chair, delay speech,

strabismus, motor stereotypies, aggression, shyness, self-harm behavior, behavioral issues were all characteristics of their appearance (Fig. 1).

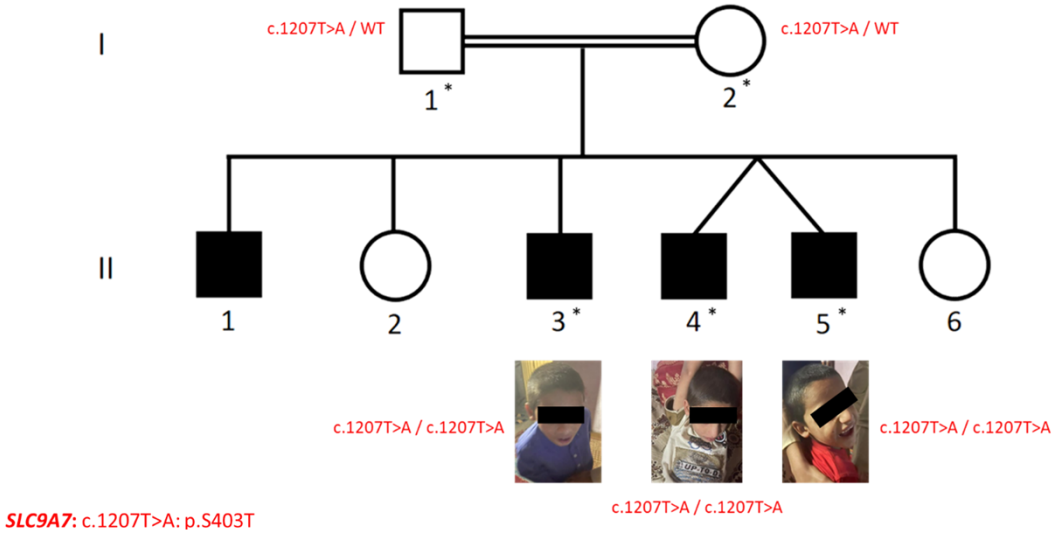

Fig. 1. Pedigree of the proband affected with intellectual disability

At the time of the last clinical assessment, the patients exhibited growth delays. Heights ranged from 102 cm to 122 cm, and weight was 24-30 kg. Head circumference measurements were smaller than age-appropriate standards, ranging from 43 cm to 48 cm. Motor assessment of these patients has previously shown neonatal or infantile hypotonia, infantile hypotonia progressing to spasticity, weakness, muscles wasting, in both their fore and hind limbs. Increased deep tendon reflexes in their upper limbs and ankle jerk, upgoing toes, drooling, jaw jerk, frequent urination, urinary incontinence, scoliosis, contractures, foot abnormality. Similarly, extrapyramidal movement such as rigidity was also found in all the three patients.

MOLECULAR FINDINGS

The whole exome sequencing (WES) of the proband was conducted to detect the gene responsible for the intellectual disability phenotype in this family. Over 95000 variants discovered through whole exome sequencing in the proband. Various filter options were applied, as a result we identified one novel hemizygous missense variant c.1207T>A; p. S403T) in the *SLC9A7* gene on the X-chromosome. Moreover, the pathogenicity was predicted using datasets from SIFT, PolyPhen, MutationTaster, PROVEAN and others, which determined that the variant in *SLC9A7* gene are disease causing. This variant was identified within the three affected members (II-3, II-4, II-5) of the family. The variant was proceeded for further analysis and five members of the family were sequenced by Sanger sequencing. The Sanger sequencing further validated that the parents have heterozygous variants while the other three patients are hemizygous confirming the X-linked inheritance pattern of variant (Fig. 2).

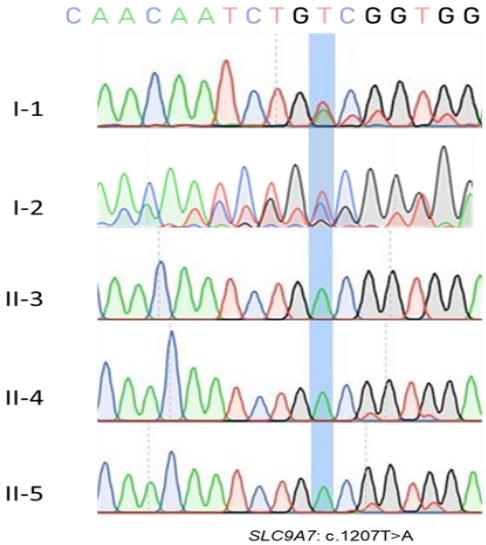

Fig. 2. Segregation analysis of the identified variant in the proband's family through

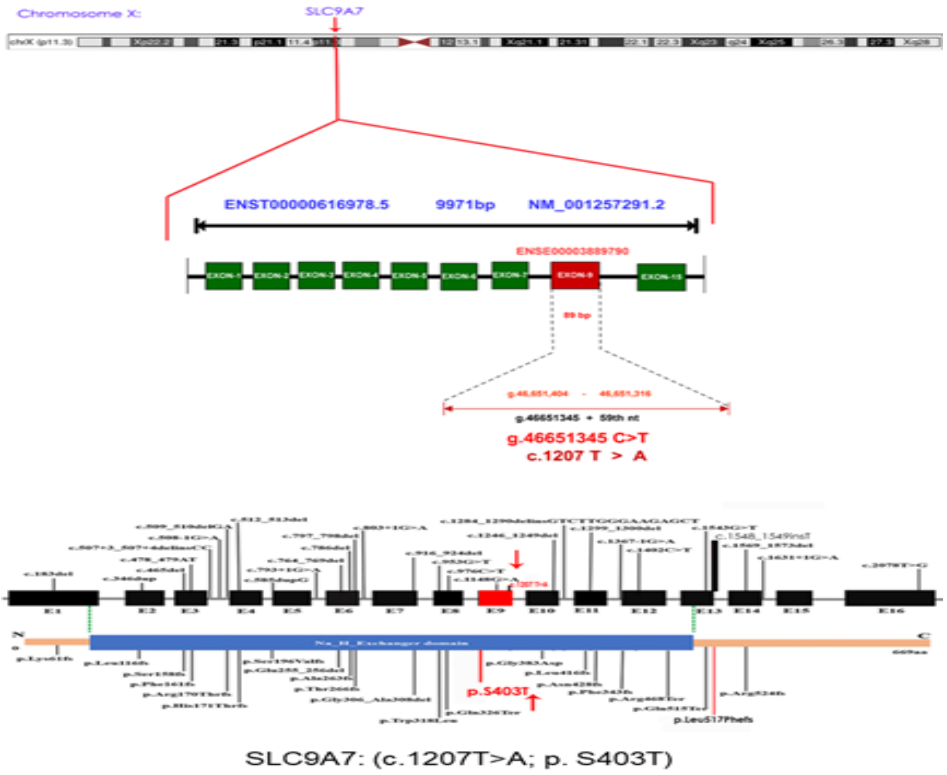

Fig. 3. Map of the human X chromosome. The centromeric region flanked by *SLC9A7* is shown below the chromosome along with the published polymorphism, with all known genes in this region

INSILICO MUTAGENESIS AND STRUCTURE ANALYSIS

The wild type of model of *SLC9A7* and its predicted mutant model was superimposed and RMSD value was calculated to measure the structural changes. Comparison of wild type and mutant *SLC9A7* protein indicated the change protein structure with RMSD score of 1.02 Aº (Fig. 4).

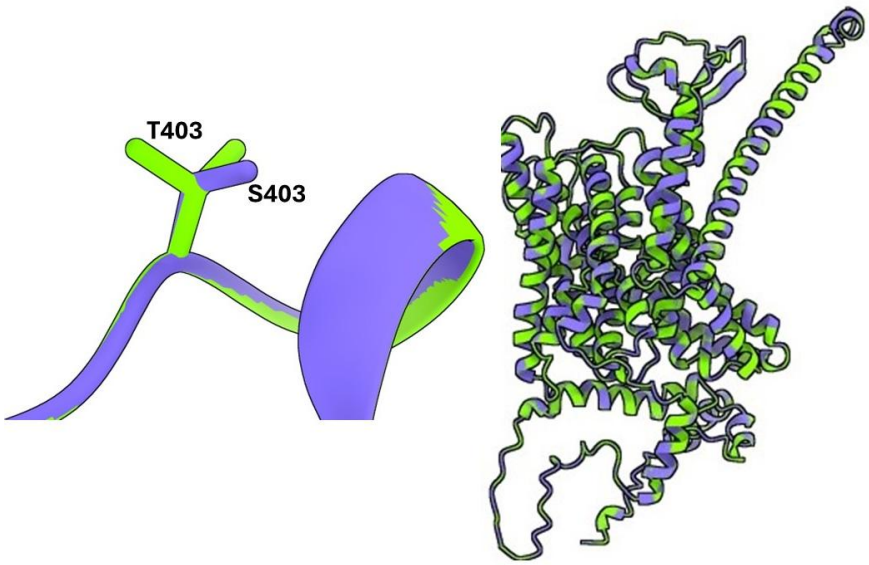

Fig. 4. Structure comparison of wild type and mutant *SLC9A7* protein. Superimposition of wild type (Purple ribbon) and mutant (green ribbon) *SLC9A7* protein. Wild type and mutant residues are shown in stick representation in zoom view

STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF THE MUTATION

The mutation in *SLC9A7* protein is in the transmembrane region of protein which is highly conserved. Substitution of serine, a small polar residue, with threonine, which is bulkier due to its additional methyl group might change protein's conformation by altering intrahelical interactions. Wild type residue Ser at position 403 form hydrogen bond and hydrophobic interactions with His355, Glu361, Ser406, Leu405 and Arg407 (Fig. 5). All these interactions are necessary for protein stable conformation and change of wild type

residue Serine to mutant residue Threonine abolish these interactions with ΔΔG energy of -0.66 kcal/mol indicating the decrease in protein stability.

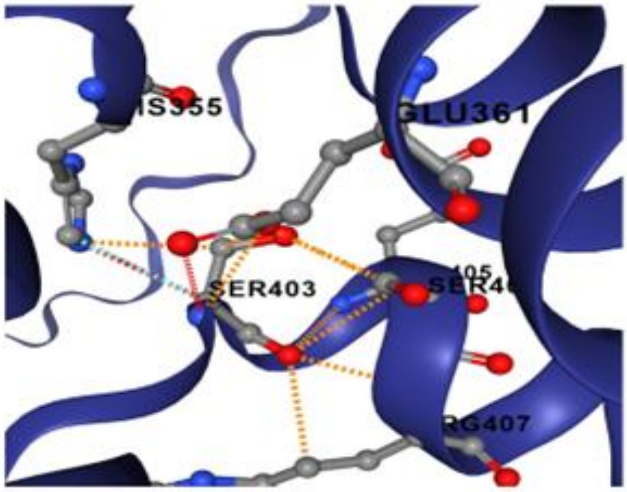

Fig. 5. Intramolecular interactions of SLC9A7^{Ser403Thr}**.** Hydrophobic interactions are shown in green dotted lines and hydrogen bond and polar interactions are shown in red dotted lines

These findings elucidate the structural consequences of this novel missense variant, c.1207T>A (p.S403T) which provide a biophysical approach to its potential pathogenesis. By correlating these alterations with clinical phenotypes, the study underscores the importance of integrating protein modeling into the functional annotation of novel genetic variants.

DISCUSSION

In this study, the phenotypes of all the patients were comprised of intellectual disability, spasticity, developmental delays, and structural abnormalities like contractures and scoliosis, combined with X-lined inheritance explains the genetic etiology of the disease. Previous reports have documented similar multisystem involvement in X-linked intellectual disability syndromes, underscoring the relevance of phenotypic expansions in this group of disorders (20, 21). Due to the scarce of available genetic as well as clinical literature about the SLC9A7 gene, its phenotypic spectrum cannot be explained properly. In this particular research, we have reported a consanguineous Pashtun family from Peshawar, Khyber Pakhtunkhwa, Pakistan. There were three patients in this family all were having the same phenotypes of intellectual disability and related symptoms. For further analysis whole exome sequencing was performed and a novel hemizygous missense variant: c.1207T>A (p.S403T) in the *SLC9A7* gene was identified. Variants in *SLC9A7* have been previously implicated in neurodevelopmental disorders through independent studies, highlighting its functional relevance in neuronal processes (22). Similarly, its segregation was confirmed through Sanger sequencing and its potential structural disruption was studied via performing 3D modeling.

This novel hemizygous missense variant: c.1207T>A (p.S403T) in the *SLC9A7* gene, is associated with XLID and strabismus as well other neurodevelopmental disorders. Consistent with this, an earlier case series described missense mutations in *SLC9A7* leading to overlapping clinical features including intellectual impairment and ocular abnormalities (23). Through this study, we have further expanded the mutational spectrum of *SLC9A7* gene along with its structural and functional annotation. There are less than 50 mutations so far reported in *SLC9A7* gene and their association with a number of neurogenetic disorders such as neurodevelopmental disorders and X-linked Intellectual Disability. The novel missense variant, c.1207T>A (p.S403T) further broadens the genotypic and known phenotypic variability of *SLC9A7* gene. Furthermore, it highlights the need for further research to clearly emphasize the potential role in disease pathogenesis. Integrating clinical, genetic, and structural data has been proposed as a key strategy for characterizing pathogenic mechanisms in rare X-linked neurodevelopmental disorders (24).

Furthermore, to study the complete molecular mechanisms, and the associated signaling pathways and related synaptic transmission channels, functional analysis is crucial to understand the pathogenic pattern of c.1207T>A (p.S403T) variant. As there were significant potential structural changes identified

during 3D modeling which signifies potential disruption in the functional domains. Therefore, advance molecular modeling, molecular dynamics techniques, and functional analysis are required to further explain the broader implications of these findings to contribute targeted therapeutic and early diagnostic approaches. Functional studies including electrophysiology and in vitro modeling have been effective in other rare neurogenetic disorders for clarifying pathogenic effects of novel variants and identifying therapeutic targets (25).

CONCLUSION

In conclusion, we identified a novel hemizygous missense variant, c.1207T>A (p.S403T), in the SLC9A7 gene, expanding the genotypic and phenotypic spectrum of X-linked intellectual disability. The findings underscore the importance of structural and functional analyses to understand variant pathogenicity. Further research is warranted to elucidate molecular mechanisms and support early diagnosis and targeted therapeutic strategies.

Conflict of interest:

The authors declare that they have no competing interests.

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Availability of data and materials:

The datasets used and analyzed supporting our findings are included in the main manuscript. The raw data during the current study is available to researchers on request from the corresponding author.

Consent for publication:

Informed written consent for publication of medical data and images was obtained from the legal guardian of family.

Authors' contribution:

MI &SA Performed the experiments; WA, ZA & MH helped in sample collection; MI, MA, IK & VK Provided technical outputs and wrote the manuscript; DN Did clinical study; NB, MA & WA did the bioinformatic analysis; HG & IK Provided relevant literature; MI, MA, IK & VK Proof reading.

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