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Section 1. Clinical Medicine

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NEUROPROTECTIVE THERAPY POSTINSULTNY COGNITIVE FRUSTRATION

Abstract. Stroke remains one of the most important medical and social problems of modern Russian society. Perceptions of etiology, pathogenesis mechanisms, and therapeutic approaches to rational therapy for stroke patients have now expanded. The paper discusses in detail the combined pharmacotherapy of patients with post-insult cognitive impairment, based on the results of their own clinical trials. The effect of the joint use of the preparations "Citicoline" and "Cortexin" compared to monotherapy was analyzed. Positive results of complex pharmacotherapy in recovery of post insult cognitive functions are obtained.

Keywords: neuroprotective therapy, neuroprotection, citicoline, cortexin, ischemic stroke.

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НЕЙРОПРОТЕКТИВНАЯ ТЕРАПИЯ ПОСТИНСУЛЬТНЫХ КОГНИТИВНЫХ РАССТРОЙСТВ

Аннотация. Инсульт остается одним из важнейших медико-социальных проблем современного российского социума. В настоящее время расширились представления об этиологии, патогенетических механизмах и терапевтических подходах к рациональной терапии пациентов

с инсультом. В статье подробно рассмотрена комбинированная фармакотерапия пациентов с постинсультными когнитивными нарушениями на основе результатов собственных клинических исследований. Проанализировано действие совместного применения препаратов «Цитиколин» и «Кортексин» в сравнении с монотерапией. Получены положительные результаты комплексной фармакотерапии в восстановлении постинсультных когнитивных функций.

Ключевые слова: нейропротективная терапия, нейропротекция, цитиколин, кортексин, ишемический инсульт.

Инсульт является чаще всего конечной стадией цереброваскулярных заболеваний (ЦВЗ), представляет собой одну из самых актуальных проблем современной неврологии по частоте встречаемости, смертности и инвалидизации. Универсальным компонентом системной терапии мозгового инфаркта считается нейропротекторная терапия. Это обусловлено тем, что нейропротекторы лишены большинства ограничений, связанных с временным фактором (параметры «терапевтического окна»), тяжестью состояния пациентов, и могут быть использованы на всех сроках заболевания. Нейропротекцию условно разделяют на первичную, направленную на прерывание глутамат-кальциевого биохимического каскада и вторичную, обеспечивающую прерывание отсроченных механизмов клеточной гибели.

В современных клинических исследованиях в последние годы отмечается неуклонный рост числа инвалидизированных пациентов, у 84% пациентов присутствуют когнитивные нарушения после перенесенного инсульта, что является глобальной медико-социальной проблемой [1].

По мнению многих ученых исследователей наиболее проблемным полем являются нарушения когнитивных функций головного мозга. Научные исследования А.Н Бойко, И. В. Дамулина подтверждают, что когнитивный дефицит после перенесенного инсульта достигает приблизительно от 40 до 70% случаев [2; 5].

В современной медицинской науке наиболее известные, распространенные препараты с нейропротективными свойствами, положительно влияющие на восстановление постинсультных когнитивных нарушений: церебролизин, кортексин, актовегин, цитиколин, холинальфоцерат. Однако во многих научных источниках наиболее высокую эффективность по данным метанализов отмечают у цитиколина [9]. Цитиколин оказывает нейропротективное действие при острой и хронической ишемии головного мозга. Известно, что цитиколин улучшает синтез фосфатидилхолина в ишемизированной ткани, в результате чего происходит стабилизация клеточных мембран. Кроме того, в поврежденных нейронах мозга восстанавливается синтез белков, нуклеиновых кислот, ацетилхолина и других нейротрансмиттеров [6; 8]. Особое внимание уделяется свойствам низкомолекулярных нейропептидов, проникающих через гематоэнцефалический барьер и оказывающих многостороннее действие на центральную нервную систему. Одним из нейропептидных нейропротекторов является отечественный препарат кортексин, содержащий комплекс низкомолекулярных пептидов (массой от 1 до 10 кДа). Механизм действия обусловлен активацией нейротрофических факторов мозга нейропептидов; стабилизацией баланса тормозных и возбуждающих аминокислот, серотонина, дофамина; ГАМК–ергическим воздействием; снижением уровня судорожной готовности головного мозга [3; 7; 9].

Сочетание цитиколина с лекарственным средством природного происхождения (нейропептид «Кортексин») может найти применение у больных с сосудистыми когнитивными нарушениями в реабилитационном периоде после перенесенного ишемического инсульта, оба эти

препарата обладают широким спектром фармакологического действия.

Проведено сравнительное исследование фармакотерапевтической эффективности по шкале MMSE лекарственных препаратов «Цитиколин» и «Кортексин» у больных ишемическим инсультом в реабилитационном периоде, сопоставимые по полу, возрасту, длительности заболевания. В исследование были включены пациенты, имеющие когнитивные расстройства в диапазоне от 11 до 27 баллов, у которых длительность заболевания с момента развития инсульта составила более 1 года.

Исключены из группы пациенты с выраженными двигательными (гемиплегия) или речевыми (афазия) расстройствами, причиной которых является инсульт. Для изучения эффективности и переносимости препаратов («Цитиколин» и «Кортексин») отбирались пациенты, прошедшие психологическую диагностику по шкале MMSE. В строгом соответствии с критериями обследовано и пролечено 72 пациента, поступивших на лечение в неврологическое отделение № 3 РКБ имени Н. А. Семашко в период 2016–2019 гг. Методом слепой рандомизации больные были разделены на 2 группы. Первая группа – 36 пациентов, получавших базисную терапию в сочетании с препаратом «Кортексин» 20 мг в/м. Вторая группа – 36 больных, которым проводилась базисная терапия, комбинированная терапия препаратами «Кортексин» 20 мг в/м и «Цитиколин» в дозе 1000 мг в/в кап в сутки.

Для определения различий между группами использовались непараметрические методы: U-критерий Манна-Уитни (U-test Mann-Whitney) для двух независимых выборок и критерий Вилкоксона (Wilcoxonsigned-ranktest) для двух зависимых выборок. Различия между сравниваемыми группами считали статистически значимыми при $p < 0,05$. Статистический анализ произведен в пакете STATISTICA 8.0.

При анализе оценки интеллектуальных нарушений, оцениваемых когнитивных функций шкалой MMSE в день поступления, достоверных различий между группами не было выявлено. Длительность стационарного лечения составила 14 дней.

Средний возраст пациентов в первой группе составил 65,3 года, во 2-й группе – 66,2, что сопоставимо одинаково в обеих группах.

Проведенные клинические исследования в 1-й группе – пациентов, принимавших базисное лечение в сочетании с кортексином, показало, что средние результаты по шкале MMSE, несмотря на повышение значений после лечения статистически значимо, остались ниже нормы в 26 баллов (Z-критерий Вилкоксона = 2,71; $p < 0,006$)

Во второй группе средние показатели достигли нормы (Z-критерий Вилкоксона = 4,52; $p < 0,00001$).

Результаты различий в распределении значений шкал между группами пациентов с разной комбинацией лечебных препаратов демонстрируют, что показатели восстановления когнитивных нарушений после лечения цитиколином и кортексином статистически значимо выше, чем при лечении только кортексином (U-критерий = 194,3; p-уровень = 0,01)

Таким образом, проведенное нами клиническое исследование указывает на эффективность комбинированной терапии цитиколином и кортексином по сравнению с монотерапией кортексином в восстановлении постинсультных когнитивных расстройств. Полученные данные могут быть рекомендованы для лечения постинсультной деменции.

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Section 2. Medbiosciences

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A SYSTEMATIC ANALYSIS OF SLC9A9 SNP SITES AND THEIR ASSOCIATION WITH HUMAN DISEASE

Astract

Attention deficit-hyperactivity disorder (ADHD) is a children's disorder with inattention and hyperactive-impulsive behavior. Recent studies have shown several single-nucleotide polymorphisms (SNPs) of the gene SLC9A9, which encodes for an endosomal sodium/hydrogen ion transporter protein, are genetically associated with ADHD. However, the underlying mechanism and association between these SNP sites and ADHD still remain unknown. In this study, a systematic analysis and annotation of all SLC9A9's SNP sites in human populations were made, then disease-associated SNPs were identified, and finally, ADHD- or ASD-related SNPs were focused on for functional investigation. In total, there are 105,334 SNPs in the SLC9A9 gene locus. The majority of these sites are located in the intron region. There are 725 exonic SNPs including 208 SNPs that encode synonymous amino acids. Among the SNPs that can influence the primary protein sequence of SLC9A9, 41 SNPs were identified as mutant sites in various human cancers. Only two exonic missense variations, rs1248614031 and rs121912597, are associated with ADHD (rs1248614031) and ASD (rs121912597). Both sites are located in the functional cation/H+ exchanger domain of SLC9A9 protein. The former causes a change in the 409th amino acid residue from alanine to proline (A409P), while the latter leads to premature termination of protein translation (R423X). Mechanism study revealed that A409P is not found to be significant in maintaining protein structure, function, or stability. It does not affect active site binding, and likely not stability, but is likely associated with mediating protein-protein interactions that regulate SLC9A9 function.

Keywords: Attention Deficit-Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD), SLC9A9, Single-Nucleotide Polymorphisms (SNPs).

1. Introduction

Attention deficit-hyperactivity disorder (ADHD) is a children's disorder with inattention and hyperactive-impulsive behavior. It is a neurological disorder that affects children, but symptoms may persist into adulthood. Individuals suffering from this disorder exhibit hyperactivity, inattention, impulsivity, and problems in social interaction and academic performance. Recent literature published about ADHD and other neurological diseases relate ADHD-like symptoms in children to autism spectrum disorder (ASD) and reveal a high phenotypical overlap between ASD and ADHD [1]. Studies find that autism spectrum disorder

(ASD) and attention-deficit/hyperactivity disorder (ADHD) are often comorbid. Three distinct pathways between ASD and ADHD were identified: (1) from impulsivity to difficulties with understanding social information, (2) from hyperactivity to stereotypic, repetitive behavior, (3) a pairwise pathway between inattention, difficulties with understanding social information, and verbal IQ. ASD has profound etiological and clinical heterogeneity, which has impeded the identification of risk factors and pathophysiological processes underlying the disorder. A constellation of (i) types of genetic variation, (ii) modes of inheritance and (iii) specific genomic loci and genes have all recently been implicated in ASD risk, and these findings are currently being extended with functional analyses in model organisms and genotype-phenotype correlation studies. The overlap of risk loci between ASD and other NDDs (including ADHD) raises intriguing questions around the mechanisms of risk [2].

ADHD is a neurological disease mainly affecting the prefrontal cortex, a portion of the brain responsible for controlling goal-directed behavior, which involves multiple sensory, motor, and cognitive processes. When engaged in a task, the animal must attend to task-relevant sensory cues, control the initiation and termination of appropriate motor actions, and monitor the outcome of each action in order to adjust future behavioral strategies. ADHD symptoms include a decrease in recognition of sensory cues, inability to control initiation and termination of appropriate motor actions, and incomplete or incorrect adjustment of future behavioral strategies based on outcomes of an action [3]. Other areas of the brain affected are the dorsal striatum and hippocampus.

SLC9A9 (solute carrier family 9, member 9, also known as Na+/H+ exchanger member (NHE9)) is a membrane protein that regulates the luminal pH of the recycling endosome, an essential organelle for synaptic transmission and plasticity. Recent studies have shown several single-nucleotide polymorphisms (SNPs) of the gene are genetically associated with ASD and ADHD. However, the underlying mechanism and association between these SNP sites and ADHD still remain unknown. This study aims to provide a systematic analysis of SLC9A9's SNP sites and their relation to human diseases, specifically ADHD, and find important SNP sites to extensively research and discuss the underlying mechanism between these sites and ASD and ADHD.

2. Materials and methods

Publically available databases and tools were mainly used to complete this study. First, an SNP database was used for SLC9A9 to curate a comprehensive list of SNP sites. Next, these sites were further performed genomic and exonic annotation, and disease association was also analyzed after function annotation to identify those SNP sites associated with ASD and/or ADHD. Once exonic SNP sites were determined to have association with ASD or ADHD, further functional and mechanism analysis was completed including an in-depth examination of effects on protein structure, protein interaction, active site function (small molecule binding, modification, etc.), topology, stability, gene expression level (from GTEX data sets), etc.

Databases and tools used in the study are shown as follows: dbSNP (https://www.ncbi.nlm.nih.gov/snp/), cBioPortal (http://www.cbioportal.org/), wANNOVAR (http://wannovar.wglab.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), InterPro (https://www.ebi.ac.uk/interpro/), UniProt (http://www.uniprot.org/), STRING (https://string-db.org). BioGRID (https://thebiogrid.org), COSMIC (https://cancer.sanger.ac.uk/cosmic/), TCGA (https://portal.gdc.cancer.gov/), GTEx (https://gtexportal.org/), and so on. The general workflow in the current study is shown in (Figure 1).

All SNPs of SLC9A9 were probed mainly through the dbSNP database and annotated using the wANNOVAR tool, such as annotation for disease association. Exonic SNPs with encoded amino acid change were paid more attention for function and disease

mechanism analyses. Serval aspects, including protein structure, protein-protein interaction (PPT), conservative active site, transmembrane topology, protein stability and gene expression, were taken into consideration using their corresponding databases or tools for functional analyses.

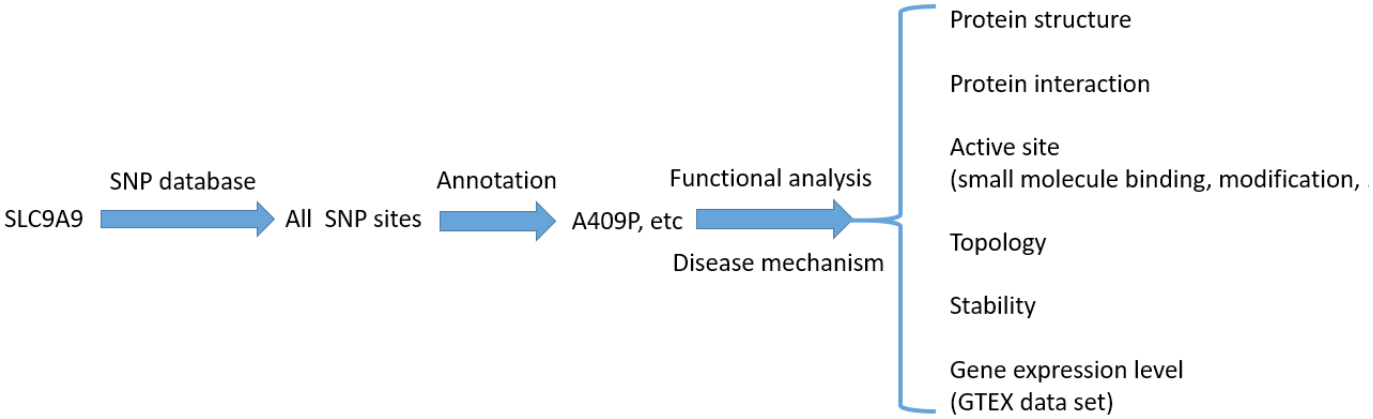


Figure 1. Work flow in the current analysis

3. Results

3.1 Identifying SNP sites of human SLC9A9 gene

Furthermore, an extensive overview of existing literature on the topic of SLC9A9 SNPs/mutations associated with ASD and/or ADHD was conducted, and novel SNPs were considered in the study to be reviewed. SLC9A9 was first identified to be the gene of interest for this study as it is associated with a wide variety of human diseases, including cancer, etc. The human gene *SLC9A9* encodes solute carrier family 9 member A9 (SLC9A9) which is also known as sodium hydrogen exchanger 9 (NHE9). In the past decade, increasing amounts of literature on SLC9A9 and its association with neurological disorders has been noted, indicating it is a relatively new and rapidly growing area of study for genetics associated with neurological health and disease. Within the past two years, three or more studies for SLC9A9 mutations associated with ASD and/or ADHD have been published, a strong indicator that it is a gene worth studying.

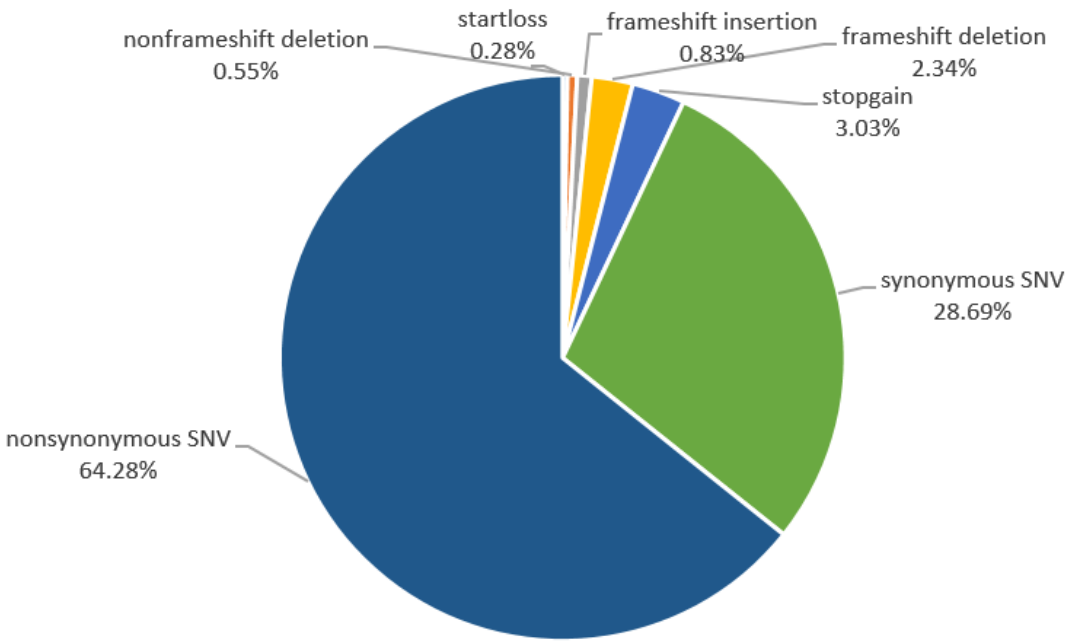


Figure 2. Functional distribution of 725 exonic SNPs

After selecting SLC9A9 as the gene of study, SNP databases were browsed and analyzed for all SNP sites associated with human disease. Altogether, a database of 105,334 SNPs in the *SLC9A9* gene locus was compiled from dbSNP. The result file including all of the SNP sites was converted to VCF (Variant Call Format) format and run through wANNOVAR, which completed a SNP site annotation for all SNP sites. The genome wide SNP site annotation found a majority of SNP sites to be located in the intron region; however, this study focused more on SNPs in exons because of the potential change of amino acid residues. wANNOVAR also provided an exonic annotation of SNP sites and using database analysis methods, ClinVar significant SNP sites were identified.

In total, there were 725 exonic SNPs, and most of the exonic SNPs belong to nonsynonymous SNPs, with only 208 SNPs encoding synonymous amino acids (Table 1, Figure 2), suggesting that potential disease SNP sites could be identified among these sites. For example, there were 49 SNPs that have also been identified as mutant sites in various cancers, suggesting important roles of these sites in human cancer development. About 46% of the exonic SNPs were located in the functional cation/H+ exchanger domain of SLC9A9 protein.

3.2 Disease association analysis for SLC9A9's SNPs

The gene *SLC9A9* is located on chromosome 3 (position 142,984,064–143,567,373 in human hg19 genome version) and is ubiquitously expressed in several tissues. SLC9A9 is responsible for the transport of sodium and hydrogen protons across cell membranes, including endosomal compartments which are where the protein is found most often. In the NCBI Nucleotide Database, SLC9A9 (or Homo sapiens solute carrier family 9 member A9) is found to be heavily associated with cancers in numerous tissues, as well as several different neurological disorders including autism spectrum disorder, epilepsy, and attention-deficit hyperactivity disorder. The NCBI Gene Database reveals SLC9A9 (solute carrier family 9 member A9 [Homo sapiens (human)] gene) is a protein encoding gene that encodes for a sodium/proton exchanger localized to late recycling endosomes in the cell and plays an important role in maintaining cation homeostasis.

A search of TCGA (The Cancer Genome Atlas) database revealed there were a total of 241 somatic mutations in the exons of SLC9A9 among all of 33 human cancers. Interestingly, after mutation annotation analysis using wANNOVAR revealed there were 49 SNPs that potentially could function as mutant sites in human cancers based on COSMIC (Catalogue of Somatic Mutations in Cancer) mutation database (Table 2). Among these sites, 16 missense mutations were also shared in the TCGA data sets. Therefore, these SNPs are suggested to be potentially associated with cancerous development but the cancerous function and detailed mechanism still awaiting further investigation.

Besides cancerous mutation association analysis, GWAS (Genome-wide association study) data were also investigated to find any possible association with other human diseases. The SLC9A9 gene is found in the cytogenetic region 3q24, has 23 associations, 20 studies, and 20 associated traits. Out of the 23 associations (Table 3), one association links SLC9A9 SNP to the attention-deficit hyperactivity disorder trait. This study was reviewed and the SNP site discussed (rs9810857) was found to be phenotypically analyzed only. Since this study was focused on genotypically analyzed SNP sites, this SNP was not discussed and researched further.

In the ClinVar analysis summary of the exonic SNP sites related to human disease, only one was identified as related to ASD. This SNP was rs121912597, also known as R423X, and it is a stopgain mutation that leads to premature termination of protein translation. However, in an overview of recent literature published, another exonic missense variation (rs1248614031) was discovered to be associated with ADHD; although not yet reported in ClinVar, the SNP site was chosen for further analysis to determine its potential

effects on the SLC9A9 protein function. We focused on these two SNPs for further study.

PhyreRisk also provided variant analysis and predictions. rs1248614031 (A409P) had a PolyPhen prediction of probably damaging and a SIFT prediction of deleterious (Table 2). rs121912597 (R423X) did not have a Polyphen prediction or SIFT prediction (Table 2).

rs121912597 was associated with ASD in most protein structure and function databases. In NCBI Nucleotide, Protein, and Gene Databases, InterPro, UniProt, Reactome, and Phosphosite, rs121912597 and its related literature are cited as associated with susceptibility with autism. Reactome database provides a pathway analysis of the effect of R423X on the pathway function of SLC9A9. Rs1248614031 was not mentioned in any databases other than NCBI Protein and Gene Databases, in which it is cited in literature as being associated with ADHD. SNP databases were analyzed for more in depth classifications of rs121912597 and rs1248614031. dbSNP reported rs1248614031 as a single nucleotide variant on chr3: 143493743 (GRCh38.p12). It did not have a clinical significance reported in ClinVar, but the reason probably is update not in time.

The associated literature ascertains rs121912597 as a risk factor for autism spectrum disorder in a family of 2 male siblings and a mother. The 2 male siblings had autism spectrum disorder, one with epilepsy, and the mother had speech-language difficulties as a child. Morrow et al. (2008) identified a heterozygous nonsense mutation in the SLC9A9 gene (R423X; 608369.0001) in all three persons of study, indicating a heritable mutation and genetic risk factor for ASD (this information was obtained from OMIM databases). This literature is verified and meets criteria as evidence for R423X being linked to ASD, but there is no verified or updated literature found in dbSNP supporting an experimentally determined linkage between R423X and loss or alteration of function of SLC9A9, which would be linked to ASD and/or ADHD.

3.3 Functional mechanism analysis for disease SNPs of SLC9A9

SLC9A9 is part of the sodium proton exchangers (NHEs) and is an integral membrane protein transporter that counter-transports protons and sodium ions across lipid bilayers. In mammals in general, they are responsible for regulating cell pH, volume, and intracellular sodium concentration, while human NHEs also take part in cell growth of differentiation. The transport of protons out of the cell and sodium ions into the cell eliminates excess acid from actively metabolising cells. In mammals, NHE activity is found in most membranes of cells and cell organelles. There are nine isoforms identified in mammalian cells, and contain 10–12 membrane spanning regions at the N terminus and a large cytoplasmic region at the C terminus. Transmembrane regions M3-M12 are similar in identity with other members of the family, and M6 and M7 regions are greatly conserved in structure across all isoforms. This is considered the region that is involved in transporting sodium and hydrogen ions. Na+/H+ exchanger isoform 9 (SLC9A9) is located in the membranes of late recycling endosomes. SLC9A9 is involved in the effusion of Golgi luminal H+ in exchange for cytosolic cations, and in maintaining organelle ion homeostasis by helping to maintain unique acidic pH values of the Golgi and endosomal compartments of the cell.

SLC9A9 is ubiquitously expressed in all tissues at RNA level that have been tested, but it is expressed at highest levels in heart and skeletal muscle, followed by placenta, kidney, and liver and in the brain, medulla and spinal cord. The Human Protein Atlas provided a summary of SLC9A9 RNA and protein expression in various tissues. In the brain tissue, SLC9A9 RNA expression was highest in the spinal cord and lowest expression in the cerebellum. However, the cerebral cortex, hippocampal formation, and cerebellum had the highest expression of SLC9A9 at protein level, mostly in neuronal, endothelial, and purkinje cells.

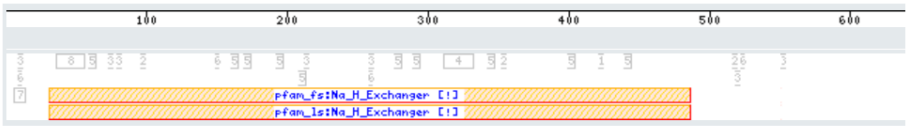
As mentioned above that SLC9A9 has numerous exonic SNPs. To investigate the influence of these SNPs on protein functions, we selected two SNP sites, rs1248614031 and rs121912597, which are associated with ADHD (rs1248614031, A409P) and ASD (rs121912597, R423X). R423X is SLC9A9: NM_173653: exon11: c.C1267T: p.R423X, so it has a heterozygous C—>T transition in Exon 11. The literature provides a diagram of the position and consequences of the C→ T transition. wANNOVAR results confirm that the CGA→TGA change results in arginine residue at position 423 changing to a stop codon. The last diagram the literature provides is comparing the similarity in the nonsense mutations in the last extracellular loop of NHE proteins between SLC9A9 in patients with comorbid autism and epilepsy, and Nhe1 in the slow-wave epilepsy mouse. The SNV is considered rare as it was not found in greater than 3800 control chromosomes within this study.

We considered protein structure including transmembrane topology, protein interaction, active sites, protein stability and gene expression of SLC9A9 itself regulated by SNP to investigate functional mechanisms.

3.3.1 Protein structure

In order to further investigate molecular mechanisms on protein functions by nonsynonymous SNPs, particularly the ADHD and ASD associated SNPs, protein visualization databases and programs (SWISS-MODEL, ModBase, RSCB Protein Data Bank, and PhyreRisk) were used to visualize the loci, structure, and variants of SLC9A9. SWISS-MODEL repository data was searched and Q8IVB4's structure was modeled, including the transmembrane portion of the structure. The full length of SLC9A9 protein includes 645 amino acid residues. SLC9A9 contains 13 transmembrane helices and a conservative sodium/hydrogen exchanger domain that covers the 31–486 aa (amino acids) (Figure 3). For rs1248614031 (A409P) and rs121912597 (R423X), both sites are located in the functional cation/H+ exchanger domain of SLC9A9 protein. The former causes a change in the 409th amino acid residue from alanine to proline (A409P), while the latter leads to premature termination of protein translation (R423X). In addition, both sites are located in the region between the 11th and 12th transmembrane helices.

A



B **Topology**

Feature key	Position(s)	Description	Graphical view	Length
Transmembrane	21 – 41	Helical		21
Transmembrane	46 – 66	Helical		21
Transmembrane	127 – 147	Helical		21
Transmembrane	165 – 185	Helical		21
Transmembrane	204 – 224	Helical		21
Transmembrane	236 – 256	Helical		21
Transmembrane	278 – 298	Helical		21
Transmembrane	302 – 322	Helical		21
Transmembrane	323 – 343	Helical		21
Transmembrane	365 – 385	Helical		21
Transmembrane	387 – 407	Helical		21
Transmembrane	430 – 450	Helical		21
Transmembrane	466 – 486	Helical		21

Figure 3. Domain and topology of SLC9A9. A) SLC9A9 contains a sodium/hydrogen exchanger domain. B) SLC9A9 contains 13 transmembrane helices

RCSB PDB and PhyreRisk were used to visualize the protein sequence and 3D structure of SLC9A9 and the SNPs of interest: R423X and A409P. PhyreRisk databases were used to visualize SNP sites on the protein and predict effect on protein structure and/or function. Both SNP sites were visualized and rs1248614031 (A409P) had a PolyPhen prediction while rs121912597 (R423X) did not. At this stage, existing literature was also reviewed extensively to determine any more experimentally supported effects both SNPs had on protein structure and function. This portion of the study was conducted with caution, as many of the literature was new and relatively unsupported by large amounts of statistical data.

3.3.2 Protein interaction

After protein structure and function analysis, protein interaction was analyzed through data from protein interaction databases (STRING, BioGRID and Protein Data Bank – Europe). BioGrid did not have any updated information on protein interactions not related to cancer, so it was not analyzed extensively. The major data gathered from this portion of the study was STRING and its associated literature. BioGrid and PDB databases are not updated to recent literature publications, but were still analyzed. rs1248614031 (A409P) was identified from existing literature prior to the study began, and STRING databases supported the article's findings. Proteins that interacted with SLC9A9 were identified from STRING databases, most commonly CHP1 and DOCK3 were associated with neurological disease (Figure 4). Further analysis of these proteins was also conducted. Gene co expression data was obtained from STRING as well, supporting high levels of CHP1 and DOCK3 interaction in cells of tissues in the body.

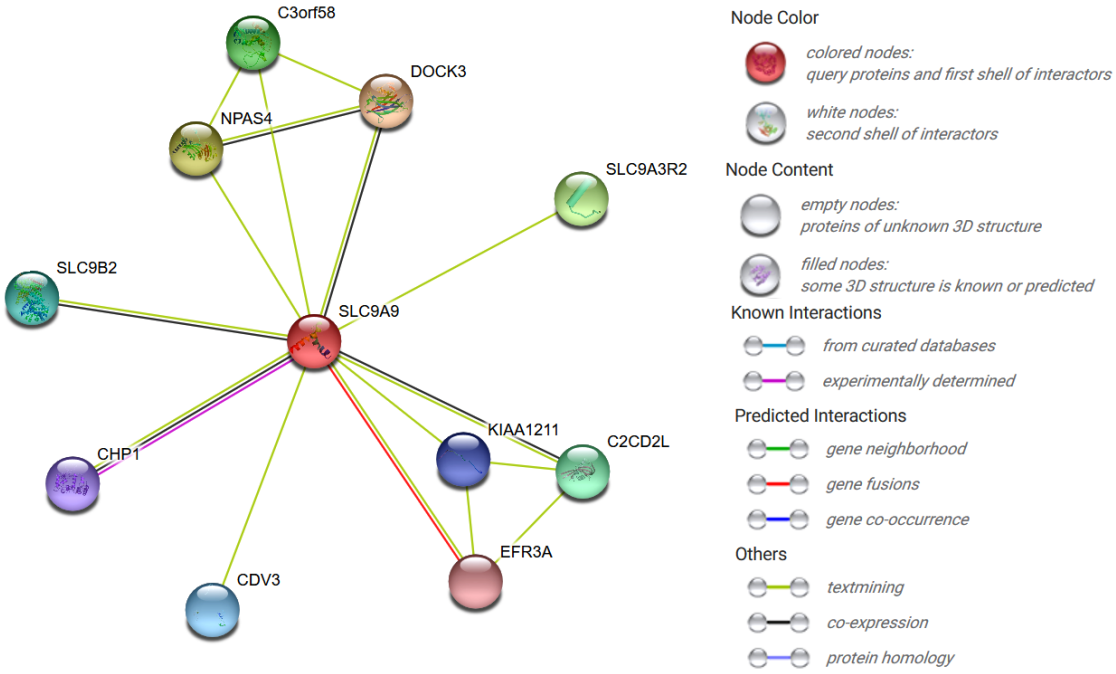


Figure 4. Interacted proteins of SLC9A9

PubMed extracts and articles provided substantial amount of data to support CHP1 and DOCK3 functions being compromised and/or altered in association with ASD and/or ADHD. Experimentally modeled SLC9A9 interactions with CHP1 and DOCK3 were not found in PDB databases, but NHE1 and CHP1 interaction structures were parsed and strongly experimentally supported. Although no parsed structures of SLC9A9 interacting with CHP1 were found in any protein structure databases, parsed structures of NHE1, the isoform 1 of the NHE family, interacting with cofactor CHP1 has been modeled in PDB databases.

According to literature and databases such as InterPro and MatrixDB, CHP1 or calcium-binding protein is involved in many different processes such as regulation of vesicular trafficking, plasma membrane Na(+)/H(+) exchanger and gene transcription. The main role it plays in relation to SLC9A9 is constitutive exocytic membrane traffic and plasma membrane Na(+)/H(+) exchange. It helps in cell pH regulation by controlling plasma membrane-type Na(+)/H(+) exchange activity and affects the pH sensitivity of SLC9A1/NHE1 (and likely its isoforms) by increasing its sensitivity at acidic pH. It is required for the stabilization and localization of SLC9A1/NHE1 at the plasma membrane (and likely its isoforms).

Although many interactors are predicted, whether the amino acids A409 and R423 play a key role to mediate this interaction still need further investigation.

3.3.3 Active site

Next we explored whether the SNP sites of SLC9A9, rs1248614031 (A409P) and rs121912597 (R423X), could represent active sites for the protein's function. Because the reference allele encodes an alanine (A409) or arginine (R423), it means these residues should not be phosphorylation sites that generally happen at serine, threonine and tyrosine. Therefore, phosphorylation prediction tools such as the NetPhos 3.1 server could not obtain any results.

Motif scanning means finding all known motifs that occur in a sequence. We used the tool Motif Scan (https://myhits.isb-sib.ch/cgi-bin/motif_scan) to identify any possible known motifs that could be affected by these two SNPs. Although many motif sites including amidation, glycosylation, phosphorylation, myristoylation and leucine zipper were found in the protein sequence, no sites were truly involved in the above two SNPs, suggesting amino acid alternation could have no affection on post-translational modifications.

In addition, R423X and A409P were analyzed for effects on active site binding and ligand structure and bonding. Active site binding and ligand structure and bonding data came primarily from recent existing literature, so there was not much statistical data to support results.

3.3.4 Protein stability

Protein stability was analyzed mainly based on ubiquitination sites, PEST motifs and protease cleavage sites in proteins. UbPred (http://www.ubpred.org/) is a random forest-based predictor of potential ubiquitination sites in proteins. It was trained on a combined set of 266 non-redundant experimentally verified ubiquitination sites available from large-scale proteomics studies. Because ubiquitination, together with sumoylation, generally occurs in lysine (K), it means both SNPs should not be involved in ubiquitination and sumoylation.

The tool epestfind (http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) was used to identify PEST motifs as potential proteolytic cleavage sites, but no PEST motifs were identified.

3.3.5 Gene expression

Gene expression by SNP was analyzed as well by using GTEx database. Perusing GTEX datasets revealed no significant tissue sQTLs or eQTLs for either SNP site, but the database has not been updated to most recent findings. GTEX datasets did produce expression levels of SLC9A9 in various tissues of the body, confirming high expression levels in brain tissue.

4. Discussion

The findings of the study are supported by data gathered from genomic, proteomic, and other databases, as well as existing literature on the subject. Exonic missense variations of both rs1248614031 and rs121912597 were associated with ADHD (rs1248614031) and ASD (rs121912597), respectively. The former causes a change in the 409th amino acid residue from alanine to proline (A409P), while the latter leads to premature termination of protein translation (R423X). Both SNP sites were functionally annotated and protein mechanism analyzed.

We analyzed protein structure, protein-protein interaction, and protein stability, and so on, to investigate the potential molecular mechanism of these

two SNPs on the function of SLC9A9 (Figure 5). Protein interaction was analyzed through data from protein interaction databases (STRING, BioGRID and Protein Data Bank — Europe). BioGrid did not provide results pertaining to the two variants of interest or diseases of interest. PDB databases were not updated to recent literature findings, so it was only used to visualize SNP sites of interest. The major data gathered from this portion of the study was STRING and its associated literature. rs1248614031 (A409P) was identified from existing literature prior to the study began, and STRING databases supported the article's findings. Proteins that interacted with SLC9A9 were identified from STRING databases, most commonly networks with CHP1 and DOCK3 were associated with neurological disease.

Four recently published studies about genetic mutations associated with ASD and/or ADHD focused on SLC9A9's interaction network with CHP1 (calcineurin-homologous protein 1) and DOCK3 and alterations in these networks that affect cell function and increase pathogenicity, leading to association with ASD and/or ADHD. At least 75% of these articles also ascertain that R423X is a critical SNP in altering the interaction network of SLC9A9, CHP1, DOCK3, and other proteins, which is related to increased pathogenicity of cells in the hippocampus, prefrontal cortex, and other areas of the brain in which pathogenicity relates to neurological disease such as ASD and/or ADHD. The most recently published findings associated with A409P had not been updated into the database, but the article supported ASD and/or ADHD symptoms and disease associated with SLC9A9 and alterations in protein-protein interaction networks with CHP1 and other proteins.

Gene co expression data was obtained from STRING as well, supporting high levels of CHP1 and DOCK3 interaction in cells of tissues in the body. Further analysis of CHP1 and DOCK3 proteins was also conducted. According to literature and databases such as InterPro and MatrixDB, CHP1 or calcium-binding protein is involved in many different processes such as regulation of vesicular trafficking, plasma membrane Na(+)/H(+) exchanger and gene transcription. The main role it plays in relation to SLC9A9 is constitutive exocytic membrane traffic and plasma membrane Na(+)/H(+) exchange. It helps in cell pH regulation by controlling plasma membrane-type Na(+)/H(+) exchange activity and affects the pH sensitivity of SLC9A1/NHE1 (and likely its isoforms) by increasing its sensitivity at acidic pH. It is required for the stabilization and localization of SLC9A1/NHE1 at the plasma membrane (and likely its isoforms).

At this stage, existing literature was also reviewed extensively to determine any more experimentally supported effects both SNPs had on protein structure and function. This portion of the study was conducted with caution, as many of the literature was new and relatively unsupported by large amounts of statistical data. Alterations in protein interaction networks of SLC9A9 is adequately supported by a substantial amount of literature. The main literature cited and verified on R423X does not discuss protein-protein interactions, but it does phenotypically confirm the genetic variant as a risk factor for ASD [4]. They performed a mutational analysis of SLC9A9 in nonconsanguineous pedigrees with comorbid autism and epilepsy. The mutational analysis of SLC9A9 in an AGRE pedigree showed a nonsense variation (also compared to nonsense mutation in Nhe1 in slow-wave epilepsy mice). The 2 sons are included in the pedigree, both with autism but one with comorbid epilepsy; the mother is included but does not have autism and instead has a speech-language symptom often related to ASD. Both sons and the mother are revealed to have the nonsense variation.

Two reports show that mutations of SLC9A9 can affect the interaction of SLC9A9 with CHP1 [5,6]. This particular study used co-immunoprecipitation methods and Western blot to determine that the two mutations found in rats with inattentive ADHD caused CHP and SLC9A9 binding to increase by almost two-fold. Although these two variants are not this study's variants of interest, the data supports the

theory that mutations leading to altered protein interaction networks between SLC9A9 and CHP are associated with ADHD symptoms and pathology in at least mice and possibly humans.

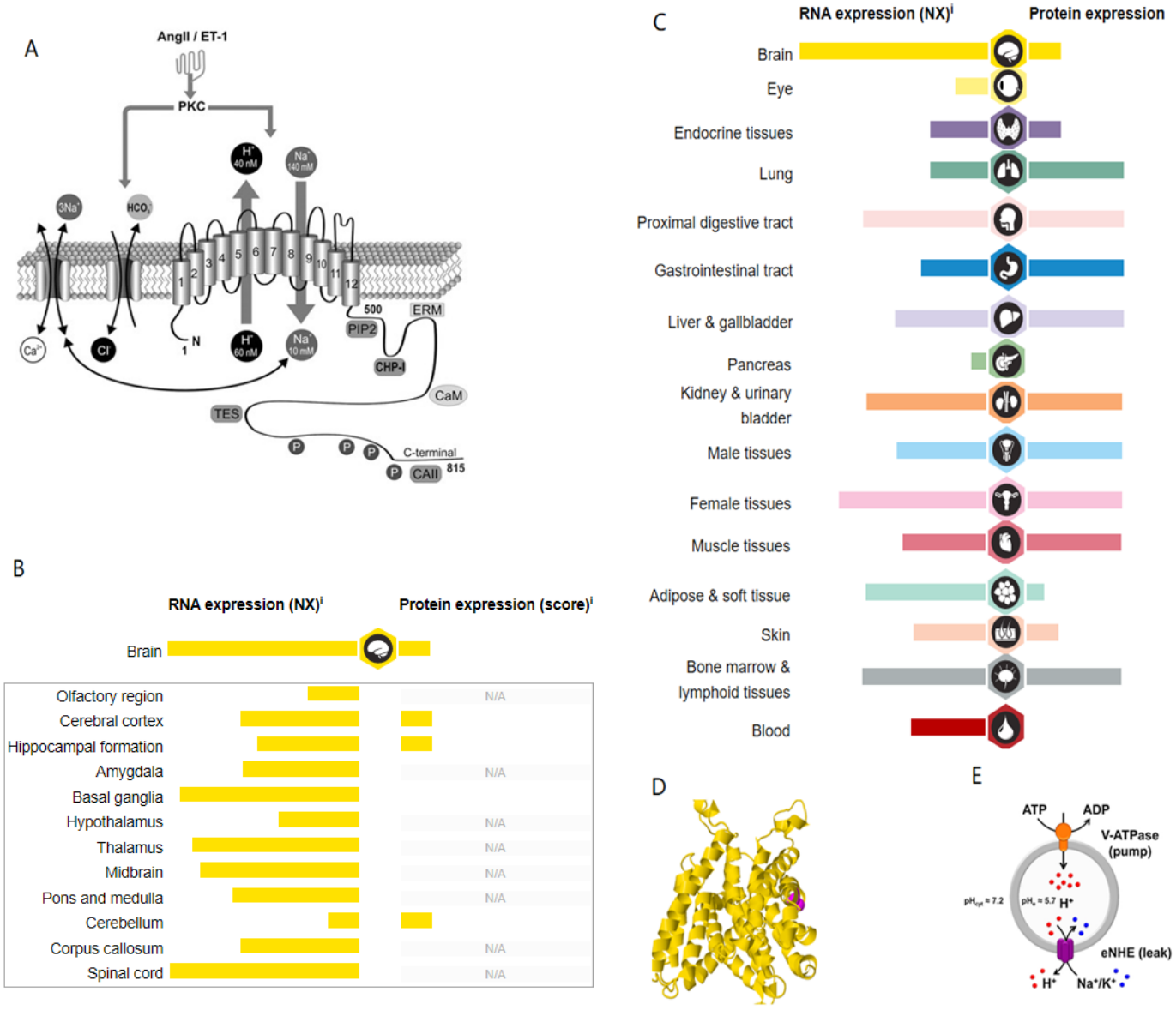


Figure 5. SLC9A9 protein subcellular localization (A), expression (B, C), structure (D) and molecular function (E).

A) SLC9A9 protein components and interactome within a subcellular membrane [8]. B) SLC9A9 RNA and protein expression in brain tissue, RNA expression and protein expression in various regions of the brain. C) SLC9A9 RNA and protein expression in various body tissues (Human Protein Atlas, https://www.proteinatlas.org/ENSG00000181804-SLC9A9/tissue). D) SLC9A9 3D structure, missense variation rs1248614031 (A409P) is located on 3D structure (purple) (PhyreRisk Sequence Browser and 3D Structure Viewer, http://phyrerisk.bc.ic.ac.uk/variant-detail/Q8IVB4?position=409&alteration=P&original=A&otherInfo=null&variantColour=0xFF00FF&source=User%20defined%20protein%20variant). E) SLC9A9 molecular function in regulating H+ and Na+/K+ ion concentration, maintaining the necessary pH of endosomal subcellular compartment [9]

The third article found in STRING databases supporting protein interaction networks of SLC9A9 and CHP and their implications for ASD and/or ADHD was a continuation of the previous study [7]. In this study, however, they examined SLC9A9 expression and relationship to 9 more genes (including CHP) that could directly or indirectly interact with the protein in the hippocampus of ADHD affected rats. As supported by gene coexpression graphs from STRING, many expression levels of these genes were significantly correlated, including CHP. This study also discussed age-dependent variations of ADHD and differences in SLC9A9 protein networks based on age. Two different types of rat models with different strains of ADHD had similar profiles in adolescence but different ones in adulthood. Both ADHD rat models were distinctly different from the control rat models in adolescence and adulthood.

SLC9A9-CHP interactions were important to intracellular Ca2+ signaling and protein phosphorylation, which are crucial signaling pathways for many cellular functions but especially synaptic transmission and plasticity. So, although SLC9A9 is mainly credited for maintenance of endosomal pH and intracellular pH levels, its interaction network with CHP is crucial for other cell functions that can also affect neurons and other brain cell functions (such as synapse function). Heterozygous stop codon mutation (R423X) is known to truncate the SLC9A9 protein (based on Identifying autism loci and genes by tracing recent shared ancestry. Morrow et al 2008) and remove the C-terminal of SLC9A9 in patients with autism and epilepsy. Although this SNP is referred to, the study is not able to conclude exactly what functions are affected by mutations that affect SLC9A9's C-terminal. From evidence gathered in literature thus far, it seems to be that SLC9A9 protein-protein interaction networks (and especially the C-terminals) is a mechanism that can be affected by changes in SLC9A9 gene sequences and can affect numerous pathways and functions in the cell.

Within these articles, R423X is the only SNP site to have substantial evidence supporting it as a mutation that results in loss of SLC9A9 function, especially in protein-protein networks involving the C-terminus (which it truncates). A recent study quantitatively analyzed the SLC9A9 interactome by co-immunoprecipitation methods and mass spectrometry in order to evaluate the functions of SLC9A9 and effects of disease-associated variants on protein-protein interactions. The study investigated 100 proteins known to interact with SLC9A9, including CHP and others, and enriched them in functional pathways for SLC9A9. They found that A409P (and ADHD-associated mutation) notably altered SLC9A9's interactions with a group of proteins important in caveolae-mediated endocytosis and other signaling pathways. The study also found evidence that R423X (ASD associated nonsense mutation) led to no-detectable amount of SLC9A9 and therefore overall loss of SLC9A9 functional networks.

5. Conclusion

In conclusion, although A409P occurs in the cation/H+ exchanger domain, it is not found to be significant in maintaining protein structure, function, or stability. It does not affect active site binding with ion transport, and likely not stability, although this should be determined by experimentation. A409P is therefore likely associated with mediating protein-protein interactions and pathways, especially with proteins regulating SLC9A9 function in different functional pathways including calcium ion transport and signaling pathways. As for R423X, it is found to be significant in maintaining protein structure and therefore protein function. It is a stop gain variant, so the original amino acid changes to a stop codon and therefore truncates the protein. This truncation results in loss of the C-terminus, a binding site for CHP and other proteins that are crucial for SLC9A9 mediated protein-protein interaction network function. SLC9A9 loses its function and SLC9A9 protein levels in cells decrease. Both A409P and R423X SNPs are clinically associ-

ated with altered protein-protein interactions and function of SLC9A9, which can be positively associated with cell pathogenicity, especially in the brain where SLC9A9 is ubiquitously expressed, and where endosomal functional pathways are especially important to neuronal cell health and function. A summary information is shown in Table 4.

Increased cell pathogenicity in the brain may be associated with certain neurodevelopmental diseases such as ADHD, ASD, etc. rs1248614031 is a rare missense mutation of the SLC9A9 gene that results in a change in amino acid residue at the 409th position from Alanine to Proline and does not have enough evidence to link it to disrupted function, structure, stability, etc. of SLC9A9. This means that while R423X has substantial amounts of evidence in loss of primary function of SLC9A9, its effects on other pathways of SLC9A9 have yet to be studied extensively and may be the reason why it is associated with ASD/ADHD symptoms and disease. Further research must be conducted on A409P to confirm its effects on different pathways of SLC9A9 and investigate why these pathways are related to ASD/ADHD molecular mechanisms and pathophysiology.

Further research should also be conducted on endosomal system pathways and their relationship to neurological disorders, in order to determine important novel pathways that may be implicated in ASD/ADHD and disrupted by SLC9A9 mutations. Thus far, this study's findings support the theory that endosomal system pathways, in particular regulation of luminal pH and primary endosomal functions (transport, signaling reception, etc.) and more specifically protein-protein mediated pathways are highly connected to disruption or damage to neuronal cell development, health, and function and therefore ASD/ADHD cellular mechanisms and pathophysiology. More research should be conducted, experimentally and phenotypically, to confirm these results.

Table 1. – SLC9A9 exonic SNPs and various data

No.	Chr	Start	Ref	Alt	ExonicFunc.ref Gene	AAChange	No.	Chr	Start	Ref	Alt	ExonicFunc.ref Gene	AAChange
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
1	3	142985547	A	G	synonymous	N645N	364	3	143292940	G	A	synonymous	A330A
2	3	142985548	T	A	nonsynonymous	N645I	365	3	143292940	G	T	synonymous	A330A
3	3	142985554	T	A	nonsynonymous	Q643L	366	3	143292941	G	A	nonsynonymous	A330V
4	3	142985555	G	A	stopgain	Q643X	367	3	143292942	C	T	nonsynonymous	A330T
5	3	142985559	T	C	synonymous	Q641Q	368	3	143292946	C	A	nonsynonymous	E328D
6	3	142985559	T	G	nonsynonymous	Q641H	369	3	143292948	C	T	nonsynonymous	E328K
7	3	142985560	T	C	nonsynonymous	Q641R	370	3	143292949	G	A	synonymous	A327A
8	3	142985569	G	A	nonsynonymous	T638I	371	3	143292951	C	T	nonsynonymous	A327T
9	3	142985571	T	C	synonymous	Q637Q	372	3	143292952	A	C	synonymous	S326S
10	3	142985579	G	C	nonsynonymous	L635V	373	3	143292953	G	A	nonsynonymous	S326F
11	3	142985580	C	G	nonsynonymous	K634N	374	3	143292956	A	G	nonsynonymous	L325P
12	3	142985580	C	T	synonymous	K634K	375	3	143292958	G	A	synonymous	F324F
13	3	142985589	A	T	stopgain	Y631X	376	3	143292962	G	A	nonsynonymous	A323V
14	3	142985590	T	C	nonsynonymous	Y631C	377	3	143292963	C	G	nonsynonymous	A323P
15	3	142985591	A	G	nonsynonymous	Y631H	378	3	143292966	T	C	nonsynonymous	S322G
16	3	142985593	C	A	nonsynonymous	G630V	379	3	143292970	A	C	synonymous	S320S
17	3	142985596	C	T	nonsynonymous	G629E	380	3	143292971	G	C	nonsynonymous	S320C
18	3	142985602	C	T	nonsynonymous	G627D	381	3	143292980	A	G	nonsynonymous	F317S
19	3	142985603	C	T	nonsynonymous	G627S	382	3	143292981	A	G	nonsynonymous	F317L
20	3	142985604	G	A	synonymous	L626L	383	3	143292982	A	C	nonsynonymous	F316L
21	3	142985604	G	T	synonymous	L626L	384	3	143292987	G	A	synonymous	L315L
22	3	142985607	G	A	synonymous	D625D	385	3	143292990	C	T	nonsynonymous	G314S

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
23	3	142985607	G	T	nonsynonymous	D625E	386	3	143292991	G	–	frameshift deletion	G314Afs*18
24	3	142985608	T	G	nonsynonymous	D625A	387	3	143292991	G	A	synonymous	T313T
25	3	142985613	C	T	synonymous	E623E	388	3	143292991	G	C	synonymous	T313T
26	3	142985616	A	C	stopgain	Y622X	389	3	143292991	G	T	synonymous	T313T
27	3	142985617	T	C	nonsynonymous	Y622C	390	3	143292998	A	G	nonsynonymous	L311P
28	3	142985630	T	C	nonsynonymous	K618E	391	3	143293000	C	T	nonsynonymous	M310I
29	3	142985633	C	A	nonsynonymous	G617C	392	3	143293002	T	C	nonsynonymous	M310V
30	3	142985633	C	G	nonsynonymous	G617R	393	3	143293003	C	T	synonymous	P309P
31	3	142985634	T	C	synonymous	P616P	394	3	143293004	G	A	nonsynonymous	P309L
32	3	142985635	G	A	nonsynonymous	P616L	395	3	143293004	G	T	nonsynonymous	P309Q
33	3	142985637	C	T	synonymous	T615T	396	3	143293008	A	G	nonsynonymous	F308L
34	3	142985638	G	A	nonsynonymous	T615M	397	3	143293011	C	T	nonsynonymous	E307K
35	3	142985641	–	G	frameshift insertion	Q614Pfs*9	398	3	143293012	A	G	synonymous	C306C
36	3	142985643	G	A	synonymous	P613P	399	3	143293014	A	G	nonsynonymous	C306R
37	3	142985652	T	C	synonymous	K610K	400	3	143293017	G	C	nonsynonymous	L305V
38	3	142985653	T	A	nonsynonymous	K610I	401	3	143293020	T	C	nonsynonymous	K304E
39	3	142985655	C	T	synonymous	Q609Q	402	3	143293021	G	A	synonymous	T303T
40	3	142985657	G	C	nonsynonymous	Q609E	403	3	143293021	G	C	synonymous	T303T
41	3	142985657	G	T	nonsynonymous	Q609K	404	3	143293030	G	T	synonymous	T300T
42	3	142985658	G	A	synonymous	D608D	405	3	143293032	T	G	nonsynonymous	T300P
43	3	142985661	C	T	synonymous	L607L	406	3	143297434	G	A	nonsynonymous	T296I
44	3	142985667	T	-	frameshift deletion	G606Vfs*38	407	3	143297441	T	C	nonsynonymous	I294V
45	3	142985672	T	A	nonsynonymous	R604W	408	3	143297443	G	T	nonsynonymous	A293D
46	3	142985673	T	C	synonymous	A603A	409	3	143297444	C	A	nonsynonymous	A293S
47	3	142985674	G	T	nonsynonymous	A603E	410	3	143297448	C	T	synonymous	A291A
48	3	142985675	C	T	nonsynonymous	A603T	411	3	143297449	G	A	nonsynonymous	A291V
49	3	142985681	G	A	nonsynonymous	P601S	412	3	143297449	G	T	nonsynonymous	A291E
50	3	142985685	G	A	synonymous	C599C	413	3	143297454	C	A	synonymous	G289G
51	3	142985691	T	A	synonymous	S597S	414	3	143297456	C	T	nonsynonymous	G289R
52	3	142985692	G	A	nonsynonymous	S597L	415	3	143297458	A	–	frameshift deletion	M288Rfs*11
53	3	142985693	A	G	nonsynonymous	S597P	416	3	143297459	T	C	nonsynonymous	M288V
54	3	142985700	T	–	frameshift deletion	A595Pfs*10	417	3	143297460	T	G	synonymous	A287A
55	3	142985702	–	C	frameshift insertion	Q594Afs*29	418	3	143297467	G	A	nonsynonymous	S285L
56	3	142985708	G	A	stopgain	Q592X	419	3	143297468	A	T	nonsynonymous	S285T
57	3	142985711	A	C	nonsynonymous	Y591D	420	3	143297469	G	A	synonymous	G284G
58	3	142985716	A	G	nonsynonymous	I589T	421	3	143297470	C	T	nonsynonymous	G284D
59	3	142985717	T	C	nonsynonymous	I589V	422	3	143297471	C	T	nonsynonymous	G284S
60	3	142985718	G	A	synonymous	A588A	423	3	143297472	A	–	frameshift deletion	G284Afs*15
61	3	142985720	C	G	nonsynonymous	A588P	424	3	143297474	C	T	nonsynonymous	A283T
62	3	142985720	C	T	nonsynonymous	A588T	425	3	143297475	G	A	synonymous	F282F
63	3	142985721	T	C	synonymous	L587L	426	3	143297476	A	G	nonsynonymous	F282S
64	3	142985723	G	A	synonymous	L587L	427	3	143297483	C	T	nonsynonymous	G280R
65	3	142985724	T	A	nonsynonymous	E586D	428	3	143297490	A	G	synonymous	N277N
66	3	142985728	T	C	nonsynonymous	D585G	429	3	143297492	T	A	nonsynonymous	N277Y

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
67	3	142985729	C	A	nonsynonymous	D585Y	430	3	143297493	C	T	synonymous	G276G
68	3	142985729	C	T	nonsynonymous	D585N	431	3	143297495	C	T	nonsynonymous	G276R
69	3	142985730	C	A	nonsynonymous	Q584H	432	3	143297497	A	G	nonsynonymous	V275A
70	3	142985734	T	C	nonsynonymous	N583S	433	3	143297499	A	G	synonymous	S274S
71	3	142985735	T	A	nonsynonymous	N583Y	434	3	143297501	A	T	nonsynonymous	S274T
72	3	142985738	C	A	nonsynonymous	V582L	435	3	143297505	G	T	nonsynonymous	F272L
73	3	142985739	A	G	synonymous	I581I	436	3	143297516	C	A	nonsynonymous	A269S
74	3	142985740	A	G	nonsynonymous	I581T	437	3	143297516	C	T	nonsynonymous	A269T
75	3	142985744	A	T	nonsynonymous	C580S	438	3	143297517	G	A	synonymous	A268A
76	3	142985751	A	G	synonymous	D577D	439	3	143297518	G	C	nonsynonymous	A268G
77	3	142985753	C	T	nonsynonymous	D577N	440	3	143297528	C	T	nonsynonymous	A265T
78	3	142985757	CT	-	frameshift deletion	E575Gfs*1	441	3	143297529	A	G	synonymous	N264N
79	3	142985760	T	G	nonsynonymous	K574N	442	3	143297531	T	C	nonsynonymous	N264D
80	3	142985761	T	C	nonsynonymous	K574R	443	3	143297534	G	A	nonsynonymous	P263S
81	3	142985763	T	C	synonymous	L573L	444	3	143297538	C	G	nonsynonymous	E261D
82	3	142985765	G	A	synonymous	L573L	445	3	143297548	C	G	nonsynonymous	S258T
83	3	142985766	C	T	synonymous	Q572Q	446	3	143297550	G	A	synonymous	Y257Y
84	3	142985767	T	G	nonsynonymous	Q572P	447	3	143297555	T	C	nonsynonymous	I256V
85	3	142985771	C	G	nonsynonymous	E571Q	448	3	143297561	T	C	nonsynonymous	I254V
86	3	142987718	C	T	nonsynonymous	G570E	449	3	143297562	A	G	synonymous	S253S
87	3	142987719	C	A	nonsynonymous	G570W	450	3	143371099	T	C	synonymous	T251T
88	3	142987720	A	G	synonymous	Y569Y	451	3	143371107	C	T	nonsynonymous	V249I
89	3	142987721	T	A	nonsynonymous	Y569F	452	3	143371110	T	C	nonsynonymous	I248V
90	3	142987724	G	T	nonsynonymous	A568D	453	3	143371113	C	A	nonsynonymous	A247S
91	3	142987727	T	C	nonsynonymous	Q567R	454	3	143371114	C	T	synonymous	V246V
92	3	142987728	G	A	stopgain	Q567X	455	3	143371115	A	G	nonsynonymous	V246A
93	3	142987729	–	G	frameshift insertion	Q567Sfs*10	456	3	143371119	C	T	nonsynonymous	A245T
94	3	142987736	G	A	nonsynonymous	T564I	457	3	143371129	C	T	synonymous	V241V
95	3	142987740	G	A	nonsynonymous	L563F	458	3	143371133	CT	–	frameshift deletion	S240Cfs*3
96	3	142987750	A	G	synonymous	I559I	459	3	143371138	T	A	synonymous	G238G
97	3	142987751	A	G	nonsynonymous	I559T	460	3	143371141	A	G	synonymous	F237F
98	3	142987753	C	T	synonymous	P558P	461	3	143371145	A	G	nonsynonymous	L236S
99	3	142987754	G	A	nonsynonymous	P558L	462	3	143371150	T	C	synonymous	T234T
100	3	142987755	G	A	nonsynonymous	P558S	463	3	143371150	T	G	synonymous	T234T
101	3	142987757	C	T	nonsynonymous	G557D	464	3	143371153	G	A	synonymous	Y233Y
102	3	142987760	C	T	nonsynonymous	C556Y	465	3	143371156	C	G	synonymous	L232L
103	3	142987761	A	G	nonsynonymous	C556R	466	3	143371164	G	T	nonsynonymous	P230T
104	3	142987770	G	A	nonsynonymous	P553S	467	3	143371167	C	A	nonsynonymous	D229Y
105	3	142987774	TGT	–	nonframeshift deletion	T551del	468	3	143371167	C	T	nonsynonymous	D229N
106	3	142987779	T	C	nonsynonymous	T550A	469	3	143371168	G	A	synonymous	V228V
107	3	142987781	G	C	nonsynonymous	T549S	470	3	143371170	C	T	nonsynonymous	V228I
108	3	142987784	A	T	nonsynonymous	L548Q	471	3	143371171	G	A	synonymous	H227H
109	3	142987786	C	T	synonymous	P547P	472	3	143371174	C	A	synonymous	L226L
110	3	142987787	G	A	nonsynonymous	P547L	473	3	143371176	G	A	synonymous	L226L
111	3	142987790	G	C	nonsynonymous	P546R	474	3	143371176	G	C	nonsynonymous	L226V
112	3	142987793	C	T	nonsynonymous	G545D	475	3	143371182	G	A	nonsynonymous	H224Y
113	3	142987796	G	A	nonsynonymous	S544F	476	3	143371188	T	C	nonsynonymous	I222V

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
114	3	142987797	A	–	frameshift deletion	S544Lfs*4	477	3	143371189	G	A	synonymous	A221A
115	3	142987797	A	G	nonsynonymous	S544P	478	3	143371194	G	A	synonymous	L220L
116	3	142987801	G	A	synonymous	T542T	479	3	143371196	A	G	nonsynonymous	V219A
117	3	142987801	G	C	synonymous	T542T	480	3	143371198	T	C	synonymous	T218T
118	3	142987802	G	A	nonsynonymous	T542I	481	3	143371199	G	A	nonsynonymous	T218I
119	3	142987803	T	A	nonsynonymous	T542S	482	3	143371201	C	T	synonymous	V217V
120	3	142987807	A	T	synonymous	I540I	483	3	143371202	A	G	nonsynonymous	V217A
121	3	142987809	T	C	nonsynonymous	I540V	484	3	143412035	T	C	synonymous	P216P
122	3	142987812	G	C	nonsynonymous	P539A	485	3	143412036	G	A	nonsynonymous	P216L
123	3	142987819	A	G	synonymous	Y536Y	486	3	143412037	G	T	nonsynonymous	P216T
124	3	142987820	–	A	frameshift insertion	Y536Lfs*18	487	3	143412040	C	G	nonsynonymous	D215H
125	3	142987822	C	T	synonymous	K535K	488	3	143412041	T	C	synonymous	T214T
126	3	143082326	T	C	nonsynonymous	K535R	489	3	143412043	T	C	nonsynonymous	T214A
127	3	143082328	G	A	synonymous	H534H	490	3	143412051	A	C	nonsynonymous	M211R
128	3	143082329	T	G	nonsynonymous	H534P	491	3	143412052	T	A	nonsynonymous	M211L
129	3	143082330	G	A	nonsynonymous	H534Y	492	3	143412054	A	G	nonsynonymous	L210P
130	3	143082331	G	A	synonymous	D533D	493	3	143412061	C	A	nonsynonymous	G208C
131	3	143082336	A	C	nonsynonymous	F532V	494	3	143412066	A	T	nonsynonymous	F206Y
132	3	143082337	G	-	frameshift deletion	F532Lfs*5	495	3	143412067	A	G	nonsynonymous	F206L
133	3	143082337	G	C	nonsynonymous	S531R	496	3	143412068	T	C	synonymous	L205L
134	3	143082337	G	T	nonsynonymous	S531R	497	3	143412078	G	A	nonsynonymous	T202I
135	3	143082338	C	T	nonsynonymous	S531N	498	3	143412085	–	A	frameshift insertion	H200Sfs*3
136	3	143082340	A	G	synonymous	Y530Y	499	3	143412090	T	G	nonsynonymous	D198A
137	3	143082343	C	T	stopgain	W529X	500	3	143412103	G	T	nonsynonymous	L194M
138	3	143082344	C	G	nonsynonymous	W529S	501	3	143412105	T	–	frameshift deletion	Q193Rfs*1
139	3	143082347	A	T	nonsynonymous	M528K	502	3	143412106	G	A	stopgain	Q193X
140	3	143082351	T	G	synonymous	R527R	503	3	143412107	G	T	synonymous	G192G
141	3	143082352	G	C	nonsynonymous	F526L	504	3	143412111	G	A	nonsynonymous	A191V
142	3	143082355	G	A	synonymous	L525L	505	3	143412113	A	G	synonymous	H190H
143	3	143082358	C	G	synonymous	R524R	506	3	143412114	T	G	nonsynonymous	H190P
144	3	143082359	C	T	nonsynonymous	R524Q	507	3	143412118	T	A	nonsynonymous	I189L
145	3	143082360	G	A	nonsynonymous	R524W	508	3	143412120	A	G	nonsynonymous	M188T
146	3	143082360	G	C	nonsynonymous	R524G	509	3	143412121	T	C	nonsynonymous	M188V
147	3	143082364	A	G	synonymous	S522S	510	3	143412124	C	A	nonsynonymous	A187S
148	3	143082365	CT	–	frameshift deletion	S522Cfs*8	511	3	143412124	C	T	nonsynonymous	A187T
149	3	143082367	C	G	nonsynonymous	E521D	512	3	143412125	C	T	synonymous	K186K
150	3	143082371	G	T	nonsynonymous	A520E	513	3	143412129	A	C	nonsynonymous	V185G
151	3	143082373	T	G	nonsynonymous	K519N	514	3	143412134	A	G	synonymous	G183G
152	3	143082374	T	C	nonsynonymous	K519R	515	3	143412137	A	G	synonymous	Y182Y
153	3	143082376	C	T	synonymous	T518T	516	3	143412138	T	A	nonsynonymous	Y182F
154	3	143082377	G	A	nonsynonymous	T518M	517	3	143412138	T	C	nonsynonymous	Y182C
155	3	143082379	C	T	nonsynonymous	M517I	518	3	143412139	A	G	nonsynonymous	Y182H
156	3	143082385	T	C	synonymous	K515K	519	3	143412141	A	G	nonsynonymous	M181T
157	3	143082387	T	A	stopgain	K515X	520	3	143412142	T	C	nonsynonymous	M181V

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
158	3	143082389	T	C	nonsynonymous	D514G	521	3	143412143	A	–	frameshift deletion	M181Cfs*4
159	3	143082392	A	G	nonsynonymous	L513S	522	3	143412144	A	T	nonsynonymous	I180N
160	3	143082395	T	G	nonsynonymous	N512T	523	3	143412148	A	C	nonsynonymous	L179V
161	3	143082401	G	A	nonsynonymous	A510V	524	3	143412149	C	A	synonymous	G178G
162	3	143082402	C	T	nonsynonymous	A510T	525	3	143513843	C	T	nonsynonymous	G178E
163	3	143082404	T	C	nonsynonymous	E509G	526	3	143513848	G	C	synonymous	V176V
164	3	143100902	C	T	synonymous	Q508Q	527	3	143513849	A	T	nonsynonymous	V176D
165	3	143100903	T	C	nonsynonymous	Q508R	528	3	143513850	C	T	nonsynonymous	V176I
166	3	143100908	T	G	nonsynonymous	Q506H	529	3	143513851	G	A	synonymous	I175I
167	3	143100914	G	A	synonymous	S504S	530	3	143513855	C	A	nonsynonymous	C174F
168	3	143100914	G	C	synonymous	S504S	531	3	143513857	G	C	synonymous	S173S
169	3	143100917	G	C	synonymous	P503P	532	3	143513866	A	G	synonymous	T170T
170	3	143100919	G	T	nonsynonymous	P503T	533	3	143513868	T	G	nonsynonymous	T170P
171	3	143100920	G	T	nonsynonymous	D502E	534	3	143513872	C	G	nonsynonymous	L168F
172	3	143100922	C	T	nonsynonymous	D502N	535	3	143513874	A	G	synonymous	L168L
173	3	143100923	C	G	nonsynonymous	E501D	536	3	143513875	G	A	synonymous	F167F
174	3	143100923	C	T	synonymous	E501E	537	3	143513878	G	A	synonymous	A166A
175	3	143100926	C	T	synonymous	K500K	538	3	143513881	A	G	synonymous	Y165Y
176	3	143100927	T	G	nonsynonymous	K500T	539	3	143513882	T	G	nonsynonymous	Y165S
177	3	143100930	A	G	nonsynonymous	L499P	540	3	143513884	C	A	synonymous	T164T
178	3	143100932	A	T	nonsynonymous	N498K	541	3	143513884	C	T	synonymous	T164T
179	3	143100933	T	C	nonsynonymous	N498S	542	3	143513885	G	A	nonsynonymous	T164M
180	3	143100935	T	G	nonsynonymous	E497D	543	3	143513886	T	A	nonsynonymous	T164S
181	3	143100939	T	C	nonsynonymous	D496G	544	3	143513886	–	TA	stopgain	T164fs*0
182	3	143100940	C	T	nonsynonymous	D496N	545	3	143513889	A	G	synonymous	L163L
183	3	143100942	A	G	nonsynonymous	L495P	546	3	143513890	A	G	synonymous	I162I
184	3	143100944	G	A	synonymous	D494D	547	3	143513895	A	T	nonsynonymous	S161T
185	3	143100946	C	T	nonsynonymous	D494N	548	3	143513896	T	A	synonymous	G160G
186	3	143100947	C	T	synonymous	V493V	549	3	143513897	C	A	nonsynonymous	G160V
187	3	143100948	A	G	nonsynonymous	V493A	550	3	143513897	C	G	nonsynonymous	G160A
188	3	143100949	C	T	nonsynonymous	V493M	551	3	143513897	C	T	nonsynonymous	G160E
189	3	143100950	G	A	synonymous	G492G	552	3	143513898	C	T	nonsynonymous	G160R
190	3	143100952	C	T	nonsynonymous	G492S	553	3	143513902	G	A	synonymous	N158N
191	3	143100956	T	C	synonymous	R490R	554	3	143513907	G	A	stopgain	Q157X
192	3	143185879	C	T	nonsynonymous	R490K	555	3	143513908	A	G	synonymous	F156F
193	3	143185882	A	G	nonsynonymous	I489T	556	3	143513914	G	A	synonymous	H154H
194	3	143185885	T	G	nonsynonymous	Q488P	557	3	143513918	C	T	nonsynonymous	R153K
195	3	143185886	G	A	stopgain	Q488X	558	3	143513919	T	C	nonsynonymous	R153G
196	3	143185894	G	A	nonsynonymous	T485I	559	3	143515670	T	G	nonsynonymous	K152Q
197	3	143185894	G	C	nonsynonymous	T485S	560	3	143515674	T	A	synonymous	L150L
198	3	143185902	G	A	synonymous	P482P	561	3	143515678	C	T	nonsynonymous	S149N
199	3	143185903	G	T	nonsynonymous	P482H	562	3	143515683	T	C	synonymous	G147G
200	3	143185904	G	A	nonsynonymous	P482S	563	3	143515687	G	A	nonsynonymous	A146V
201	3	143185904	G	T	nonsynonymous	P482T	564	3	143515688	C	A	nonsynonymous	A146S
202	3	143185906	G	A	nonsynonymous	T481I	565	3	143515689	A	G	synonymous	H145H
203	3	143185906	G	T	nonsynonymous	T481N	566	3	143515692	A	G	synonymous	F144F
204	3	143185907	T	G	nonsynonymous	T481P	567	3	143515697	T	C	nonsynonymous	I143V
205	3	143185910	T	C	nonsynonymous	T480A	568	3	143515705	G	A	nonsynonymous	P140L
206	3	143185911	T	C	synonymous	G479G	569	3	143515706	G	A	nonsynonymous	P140S
207	3	143185912	C	T	nonsynonymous	G479E	570	3	143515706	G	T	nonsynonymous	P140T

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
208	3	143185913	C	T	nonsynonymous	G479R	571	3	143515709	G	A	synonymous	L139L
209	3	143185915	C	T	nonsynonymous	G478E	572	3	143515715	C	T	nonsynonymous	V137I
210	3	143185920	A	G	synonymous	F476F	573	3	143515717	T	C	nonsynonymous	N136S
211	3	143185923	T	A	synonymous	V475V	574	3	143515718	T	G	nonsynonymous	N136H
212	3	143185923	T	G	synonymous	V475V	575	3	143515719	GAA	–	nonframeshift deletion	F135del
213	3	143185926	C	T	stopgain	W474X	576	3	143515719	G	A	synonymous	F135F
214	3	143185929	G	A	synonymous	V473V	577	3	143515724	A	C	nonsynonymous	F134V
215	3	143185938	G	A	synonymous	F470F	578	3	143515731	T	A	synonymous	P131P
216	3	143185943	C	T	nonsynonymous	V469M	579	3	143550860	CCTTTT	–	frameshift deletion	E125Dfs*3
217	3	143185944	G	A	synonymous	L468L	580	3	143550861	CTTTT	–	frameshift deletion	E125Dfs*3
218	3	143185947	G	C	synonymous	L467L	581	3	143550864	T	C	synonymous	E125E
219	3	143185949	G	A	nonsynonymous	L467F	582	3	143550870	T	A	synonymous	I123I
220	3	143185951	A	G	nonsynonymous	L466P	583	3	143550871	A	G	nonsynonymous	I123T
221	3	143185953	C	T	synonymous	T465T	584	3	143550872	T	C	nonsynonymous	I123V
222	3	143185954	G	A	nonsynonymous	T465M	585	3	143550873	A	G	synonymous	A122A
223	3	143185955	T	A	nonsynonymous	T465S	586	3	143550874	G	A	nonsynonymous	A122V
224	3	143185955	T	C	nonsynonymous	T465A	587	3	143550875	C	A	nonsynonymous	A122S
225	3	143185956	A	G	synonymous	T464T	588	3	143550884	G	T	nonsynonymous	Q119K
226	3	143185958	T	C	nonsynonymous	T464A	589	3	143550886	T	C	nonsynonymous	H118R
227	3	143185964	A	G	nonsynonymous	F462L	590	3	143550890	G	T	nonsynonymous	P117T
228	3	143185966	A	G	nonsynonymous	M461T	591	3	143550892	T	C	nonsynonymous	N116S
229	3	143185968	C	A	nonsynonymous	M460I	592	3	143550896	TGT	–	nonframeshift deletion	N114del
230	3	143185968	C	T	nonsynonymous	M460I	593	3	143550900	G	A	synonymous	H113H
231	3	143185970	T	C	nonsynonymous	M460V	594	3	143550903	C	A	nonsynonymous	Q112H
232	3	143185974	T	G	nonsynonymous	K458N	595	3	143550911	T	C	nonsynonymous	I110V
233	3	143185975	T	C	nonsynonymous	K458R	596	3	143550916	C	-	frameshift deletion	R108Kfs*2
234	3	143185979	G	T	nonsynonymous	P457T	597	3	143550916	C	T	nonsynonymous	R108K
235	3	143185980	C	G	nonsynonymous	Q456H	598	3	143550917	T	A	stopgain	R108X
236	3	143185980	C	T	synonymous	Q456Q	599	3	143550920	T	C	nonsynonymous	K107E
237	3	143185984	G	A	nonsynonymous	S455F	600	3	143550921	G	A	synonymous	Y106Y
238	3	143185993	T	C	nonsynonymous	N452S	601	3	143550935	A	G	nonsynonymous	Y102H
239	3	143185995	C	G	synonymous	R451R	602	3	143550941	G	C	nonsynonymous	Q100E
240	3	143185996	C	T	nonsynonymous	R451Q	603	3	143550945	A	G	synonymous	T98T
241	3	143185997	G	A	nonsynonymous	R451W	604	3	143550946	G	C	nonsynonymous	T98S
242	3	143185998	A	C	nonsynonymous	I450M	605	3	143550954	A	–	frameshift deletion	N96Ifs*14
243	3	143186003	C	T	nonsynonymous	A449T	606	3	143550956	C	T	nonsynonymous	V95I
244	3	143186006	A	G	synonymous	L448L	607	3	143550957	C	G	synonymous	L94L
245	3	143186013	T	C	synonymous	A445A	608	3	143550960	C	G	synonymous	L93L
246	3	143186015	C	T	nonsynonymous	A445T	609	3	143550965	T	C	nonsynonymous	T92A
247	3	143186016	G	A	synonymous	I444I	610	3	143550965	T	G	nonsynonymous	T92P
248	3	143186016	G	T	synonymous	I444I	611	3	143550967	G	A	nonsynonymous	S91L
249	3	143186017	A	G	nonsynonymous	I444T	612	3	143550971	G	T	nonsynonymous	P90T
250	3	143186019	C	A	synonymous	A443A	613	3	143550974	T	C	nonsynonymous	S89G
251	3	143186019	C	T	synonymous	A443A	614	3	143550979	G	A	nonsynonymous	T87I
252	3	143186020	G	A	nonsynonymous	A443V	615	3	143550979	G	T	nonsynonymous	T87N

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
253	3	143186021	C	T	nonsynonymous	A443T	616	3	143550982	A	C	nonsynonymous	L86R
254	3	143186022	T	G	synonymous	G442G	617	3	143550982	A	G	nonsynonymous	L86P
255	3	143186026	C	T	nonsynonymous	R441Q	618	3	143550983	G	A	synonymous	L86L
256	3	143186027	G	A	stopgain	R441X	619	3	143550983	G	C	nonsynonymous	L86V
257	3	143186029	A	G	nonsynonymous	L440S	620	3	143550990	A	T	stopgain	C83X
258	3	143212495	C	T	nonsynonymous	G439S	621	3	143550992	A	C	nonsynonymous	C83G
259	3	143212498	A	G	nonsynonymous	S438P	622	3	143550996	A	G	synonymous	Y81Y
260	3	143212506	A	G	nonsynonymous	M435T	623	3	143550996	A	T	stopgain	Y81X
261	3	143212506	A	T	nonsynonymous	M435K	624	3	143551000	A	C	nonsynonymous	V80G
262	3	143212510	T	A	nonsynonymous	M434L	625	3	143551000	A	G	nonsynonymous	V80A
263	3	143212511	G	A	synonymous	H433H	626	3	143551003	G	C	nonsynonymous	T79S
264	3	143212511	G	T	nonsynonymous	H433Q	627	3	143551004	T	A	nonsynonymous	T79S
265	3	143212515	T	C	nonsynonymous	Q432R	628	3	143551004	T	C	nonsynonymous	T79A
266	3	143212517	A	G	synonymous	F431F	629	3	143551009	C	T	nonsynonymous	S77N
267	3	143212519	A	T	nonsynonymous	F431I	630	3	143551011	T	G	nonsynonymous	E76D
268	3	143212521	T	C	nonsynonymous	N430S	631	3	143551021	G	A	nonsynonymous	T73I
269	3	143212522	T	C	nonsynonymous	N430D	632	3	143551021	G	C	nonsynonymous	T73S
270	3	143212530	A	G	nonsynonymous	I427T	633	3	143551022	T	C	nonsynonymous	T73A
271	3	143212531	T	C	nonsynonymous	I427V	634	3	143551023	T	G	synonymous	P72P
272	3	143212542	C	T	nonsynonymous	R423Q	635	3	143551032	A	G	synonymous	A69A
273	3	143212543	G	A	stopgain	R423X	636	3	143551035	A	G	synonymous	Y68Y
274	3	143212546	C	T	nonsynonymous	G422S	637	3	143551037	A	T	nonsynonymous	Y68N
275	3	143212559	G	A	synonymous	F417F	638	3	143551038	T	C	synonymous	R67R
276	3	143212564	A	T	nonsynonymous	S416T	639	3	143551039	C	A	nonsynonymous	R67L
277	3	143212567	G	A	nonsynonymous	L415F	640	3	143551039	C	G	nonsynonymous	R67P
278	3	143212568	G	A	synonymous	P414P	641	3	143551039	C	T	nonsynonymous	R67Q
279	3	143212570	G	T	nonsynonymous	P414T	642	3	143551040	G	A	stopgain	R67X
280	3	143212574	T	C	nonsynonymous	I412M	643	3	143551044	A	T	synonymous	I65I
281	3	143212577	G	A	synonymous	N411N	644	3	143551048	A	G	nonsynonymous	L64P
282	3	143212577	G	T	nonsynonymous	N411K	645	3	143551049	G	A	synonymous	L64L
283	3	143212578	T	C	nonsynonymous	N411S	646	3	143551051	C	A	nonsynonymous	G63V
284	3	143212582	A	T	nonsynonymous	C410S	647	3	143551053	C	G	nonsynonymous	M62I
285	3	143212584	G	A	nonsynonymous	A409V	648	3	143551053	C	T	nonsynonymous	M62I
286	3	143212584	G	C	nonsynonymous	A409G	649	3	143551054	A	G	nonsynonymous	M62T
287	3	143212584	G	T	nonsynonymous	A409D	650	3	143551055	T	C	nonsynonymous	M62V
288	3	143212585	C	G	nonsynonymous	A409P	651	3	143551057	A	G	nonsynonymous	I61T
289	3	143212586	T	C	synonymous	R408R	652	3	143551057	A	T	nonsynonymous	I61K
290	3	143212594	C	T	nonsynonymous	V406I	653	3	143551059	A	T	synonymous	L60L
291	3	143212597	A	G	nonsynonymous	F405L	654	3	143551061	GGCCT	–	nonframeshift deletion	G59_L60delinsV
292	3	143212599	A	G	nonsynonymous	I404T	655	3	143551063	C	T	nonsynonymous	G59D
293	3	143212599	A	T	nonsynonymous	I404N	656	3	143566990	C	T	nonsynonymous	G59S
294	3	143212606	G	A	synonymous	L402L	657	3	143566991	A	G	synonymous	Y58Y
295	3	143214180	G	A	synonymous	A400A	658	3	143566994	C	A	synonymous	V57V
296	3	143214181	G	C	nonsynonymous	A400G	659	3	143567000	T	C	synonymous	A55A
297	3	143214181	G	T	nonsynonymous	A400D	660	3	143567001	G	A	nonsynonymous	A55V
298	3	143214182	C	T	nonsynonymous	A400T	661	3	143567002	C	T	nonsynonymous	A55T
299	3	143214184	C	T	nonsynonymous	G399E	662	3	143567004	C	T	nonsynonymous	G54E
300	3	143214188	G	C	nonsynonymous	L398V	663	3	143567006	T	A	synonymous	G53G
301	3	143214189	T	C	nonsynonymous	I397M	664	3	143567011	T	A	nonsynonymous	T52S
302	3	143214195	A	C	synonymous	L395L	665	3	143567014	C	G	nonsynonymous	E51Q

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
303	3	143214197	G	C	nonsynonymous	L395V	666	3	143567017	G	C	nonsynonymous	H50D
304	3	143214198	A	G	synonymous	A394A	667	3	143567018	C	T	synonymous	L49L
305	3	143214199	G	A	nonsynonymous	A394V	668	3	143567025	C	A	nonsynonymous	R47L
306	3	143214209	T	C	nonsynonymous	I391V	669	3	143567025	C	T	nonsynonymous	R47H
307	3	143214212	G	T	nonsynonymous	H390N	670	3	143567026	G	A	nonsynonymous	R47C
308	3	143214213	A	T	nonsynonymous	N389K	671	3	143567031	C	A	nonsynonymous	R45L
309	3	143214222	C	T	synonymous	T386T	672	3	143567032	G	T	synonymous	R45R
310	3	143214223	G	A	nonsynonymous	T386M	673	3	143567033	A	G	synonymous	H44H
311	3	143214226	A	T	nonsynonymous	F385Y	674	3	143567036	A	T	nonsynonymous	N43K
312	3	143214227	A	G	nonsynonymous	F385L	675	3	143567038	T	C	nonsynonymous	N43D
313	3	143214231	T	C	synonymous	A383A	676	3	143567043	A	G	nonsynonymous	F41S
314	3	143214237	G	A	synonymous	G381G	677	3	143567047	A	G	synonymous	L40L
315	3	143214242	T	C	nonsynonymous	M380V	678	3	143567048	C	T	stopgain	W39X
316	3	143214244	T	G	nonsynonymous	Y379S	679	3	143567049	C	T	stopgain	W39X
317	3	143214248	A	G	nonsynonymous	C378R	680	3	143567051	G	A	synonymous	I38I
318	3	143214249	G	A	synonymous	F377F	681	3	143567052	A	G	nonsynonymous	I38T
319	3	143214251	A	G	nonsynonymous	F377L	682	3	143567053	T	C	nonsynonymous	I38V
320	3	143214257	C	G	nonsynonymous	V375L	683	3	143567055	G	T	nonsynonymous	T37K
321	3	143214257	C	T	nonsynonymous	V375I	684	3	143567059	A	G	synonymous	L36L
322	3	143214258	G	A	synonymous	N374N	685	3	143567061	A	G	nonsynonymous	I35T
323	3	143214259	T	C	nonsynonymous	N374S	686	3	143567067	A	C	nonsynonymous	L33R
324	3	143214262	T	C	nonsynonymous	E373G	687	3	143567073	A	T	nonsynonymous	L31H
325	3	143214264	C	T	synonymous	A372A	688	3	143567074	G	A	nonsynonymous	L31F
326	3	143214265	G	A	nonsynonymous	A372V	689	3	143567075	–	A	frameshift insertion	L30Ffs*12
327	3	143214266	C	A	nonsynonymous	A372S	690	3	143567080	A	C	nonsynonymous	F29V
328	3	143214276	C	T	nonsynonymous	M368I	691	3	143567081	A	G	synonymous	N28N
329	3	143214286	A	G	nonsynonymous	F365S	692	3	143567087	G	A	synonymous	V26V
330	3	143271204	C	T	synonymous	Q363Q	693	3	143567095	G	A	synonymous	L24L
331	3	143271205	T	C	nonsynonymous	Q363R	694	3	143567096	C	G	nonsynonymous	E23D
332	3	143271206	G	T	nonsynonymous	Q363K	695	3	143567100	A	C	nonsynonymous	V22G
333	3	143271207	T	C	synonymous	K362K	696	3	143567100	A	T	nonsynonymous	V22E
334	3	143271211	G	C	nonsynonymous	T361S	697	3	143567103	G	A	nonsynonymous	A21V
335	3	143271218	T	C	nonsynonymous	I359V	698	3	143567110	G	C	nonsynonymous	Q19E
336	3	143271220	T	G	nonsynonymous	K358T	699	3	143567111	A	T	nonsynonymous	H18Q
337	3	143271222	G	A	synonymous	S357S	700	3	143567112	T	C	nonsynonymous	H18R
338	3	143271227	C	G	nonsynonymous	D356H	701	3	143567125	A	G	nonsynonymous	Y14H
339	3	143271228	C	T	synonymous	S355S	702	3	143567126	C	G	nonsynonymous	E13D
340	3	143271229	G	A	nonsynonymous	S355L	703	3	143567126	C	T	synonymous	E13E
341	3	143271238	T	C	nonsynonymous	N352S	704	3	143567129	A	C	nonsynonymous	D12E
342	3	143271240	G	A	synonymous	N351N	705	3	143567129	A	G	synonymous	D12D
343	3	143271240	G	C	nonsynonymous	N351K	706	3	143567131	C	T	nonsynonymous	D12N
344	3	143271241	T	C	nonsynonymous	N351S	707	3	143567132	C	T	synonymous	K11K
345	3	143271242	T	C	nonsynonymous	N351D	708	3	143567133	T	C	nonsynonymous	K11R
346	3	143271243	G	C	stopgain	Y350X	709	3	143567133	T	G	nonsynonymous	K11T
347	3	143271249	A	G	synonymous	Y348Y	710	3	143567137	C	G	nonsynonymous	E10Q
348	3	143271250	T	C	nonsynonymous	Y348C	711	3	143567138	T	C	synonymous	S9S
349	3	143271254	G	A	nonsynonymous	H347Y	712	3	143567140	A	C	nonsynonymous	S9A
350	3	143271257	C	T	nonsynonymous	A346T	713	3	143567141	C	T	nonsynonymous	M8I
351	3	143271263	T	A	nonsynonymous	T344S	714	3	143567146	C	A	nonsynonymous	V7F
352	3	143271268	C	T	nonsynonymous	G342E	715	3	143567146	C	T	nonsynonymous	V7I

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
353	3	143271281	C	G	nonsynonymous	V338L	716	3	143567148	C	G	nonsynonymous	R6T
354	3	143271283	G	A	nonsynonymous	A337V	717	3	143567148	C	T	nonsynonymous	R6K
355	3	143271284	C	A	nonsynonymous	A337S	718	3	143567150	T	C	synonymous	S5S
356	3	143271286	A	T	nonsynonymous	V336D	719	3	143567151	G	T	stopgain	S5X
357	3	143271290	T	A	nonsynonymous	I335L	720	3	143567154	T	G	nonsynonymous	Q4P
358	3	143271292	C	T	nonsynonymous	G334E	721	3	143567156	TC	-	frameshift deletion	R3Tfs*9
359	3	143292930	C	T	nonsynonymous	G334R	722	3	143567157	C	T	nonsynonymous	R3K
360	3	143292931	T	C	synonymous	T333T	723	3	143567160	T	G	nonsynonymous	E2A
361	3	143292932	G	A	nonsynonymous	T333I	724	3	143567163	A	C	startloss	E2_M8del
362	3	143292938	C	T	nonsynonymous	G331D	725	3	143567164	T	A	startloss	E2_M8del
363	3	143292939	C		nonsynonymous	T							G331S

Table 2. – SNPs associated with cancer

Chr	**Position**	**Ref**	**Alt**	**dbSNP**	**COSMIC_ID**	**Cancer type**	**Exonic Function**	**AAChange**
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
3	142985638	G	A	rs767114012	COSM5987135	salivary_gland	nonsynonymous	T615M
3	142985708	G	A	rs752770927	COSM1039503	endometrium	stopgain	Q592X
3	142985753	C	T	rs757449602	COSM3125514	upper_aerodigestive_tract	nonsynonymous	D577N
3	142987786	C	T	rs61750363	COSM1039505	endometrium	synonymous	P547P
3	143082359	C	T	rs769786135	COSM5664679	lung	nonsynonymous	R524Q
3	143082360	G	A	rs574582502	COSM1226613	large_intestine	nonsynonymous	R524W
3	143082373	T	G	rs771266539	COSM3125518	stomach	nonsynonymous	K519N
3	143082376	C	T	rs141051651	COSM3427182	large_intestine	synonymous	T518T
3	143082377	G	A	rs759634686	COSM728924	lung; large_intestine	nonsynonymous	T518M
3	143100923	C	T	rs747034373	COSM3915071	skin	synonymous	E501E
3	143100949	C	T	rs775249012	COSM3783668	bone; oesophagus; prostate; large_intestine	nonsynonymous	V493M
3	143100950	G	A	rs201448381	COSM383360	lung; endometrium	synonymous	G492G
3	143185912	C	T	rs143224544	COSM107028	skin	nonsynonymous	G479E
3	143185920	A	G	rs61734421	COSM4114204	stomach	synonymous	F476F
3	143185943	C	T	rs768229420	COSM4584016	bone	nonsynonymous	V469M
3	143185954	G	A	rs771359670	COSM1419701	large_intestine	nonsynonymous	T465M
3	143185968	C	T	rs761372851	COSM3588352	skin	nonsynonymous	M460I

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
3	143185970	T	C	rs61734409	COSM3846440	breast	nonsynonymous	M460V
3	143185984	G	A	rs759376193	COSM3846441	breast; NS	nonsynonymous	S455F
3	143185997	G	A	rs752878648	COSM205616	skin	nonsynonymous	R451W
3	143186015	C	T	rs761337323	COSM5549901	prostate	nonsynonymous	A445T
3	143186016	G	A	rs141105678	COSM3588354	oesophagus; skin	synonymous	I444I
3	143186019	C	T	rs144930120	COSM5604940	skin	synonymous	A443A
3	143212542	C	T	rs368254745	COSM3125528	large_intestine	nonsynonymous	R423Q
3	143212543	G	A	rs121912597	COSM5455144	large_intestine	stopgain	R423X
3	143214209	T	C	rs138653730	COSM4114206	stomach	nonsynonymous	I391V
3	143214222	C	T	rs140335350	COSM5007475	large_intestine	synonymous	T386T
3	143214258	G	A	rs137994789	COSM1266084	stomach	synonymous	N374N
3	143214264	C	T	rs533183607	COSM1039507	liver; endometrium	synonymous	A372A
3	143271229	G	A	rs552019939	COSM1039509	stomach; endometrium	nonsynonymous	S355L
3	143292940	G	T	rs140574815	COSM4319719	bone	synonymous	A330A
3	143293003	C	T	rs144088456	COSM4727884	bone; large_intestine	synonymous	P309P
3	143293004	G	A	rs775325802	COSM3846442	breast	nonsynonymous	P309L
3	143293012	A	G	rs6763202	COSM4406945	haematopoietic_and_lymphoid_tissue; liver	synonymous	C306C
3	143297448	C	T	rs140901762	COSM1039511	endometrium	synonymous	A291A
3	143297516	C	T	rs143468139	COSM3001742	small_intestine	nonsynonymous	A269T
3	143297517	G	A	rs759374253	COSM5026058	breast	synonymous	A268A
3	143371182	G	A	rs778413733	COSM4901315	skin	nonsynonymous	H224Y
3	143371201	C	T	rs1242069	COSM3759808	large_intestine	synonymous	V217V
3	143513851	G	A	rs377682237	COSM1536649	skin; lung	synonymous	I175I
3	143513878	G	A	rs752509231	COSM4497507	skin	synonymous	A166A
3	143513885	G	A	rs201948779	COSM4781526	pancreas	nonsynonymous	T164M
3	143513907	G	A	rs866080325	COSM3588362	skin	stopgain	Q157X
3	143550892	T	C	rs762785258	COSM4998907	pancreas	nonsynonymous	N116S
3	143550916	C	T	rs767428360	COSM6096454	lung	nonsynonymous	R108K

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
3	143551032	A	G	rs112237207	COSM728916	lung	synonymous	A69A
3	143551039	C	T	rs372558008	COSM1039515	endometrium	nonsynonymous	R67Q
3	143567075	–	A	rs765712953	COSM1180943	large_intestine	frameshift insertion	L30Ffs*12
3	143567080	A	C	rs534139654	COSM1419714	large_intestine	nonsynonymous	F29V

Table 3. – GWAS Catalog SLC9A9 associations with disease

Variant and risk allele	**P–value**	**P–value annotation**	**RAF**	**OR**	**Beta**	**CI**	**Trait(s)**
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
rs7632299–A	4×10^{-6}		0.17	–	0.45 unit increase	[NR]	non–alcoholic fatty liver disease, cirrhosis of liver
rs7645841–?	5×10^{-6}		NR	–	–	–	QT interval, Trypanosoma cruzi seropositivity
rs201023017–?	6×10^{-7}		NR	–	–	–	response to platinum based chemotherapy, ovarian carcinoma, cytotoxicity measurement, response to paclitaxel
rs185347221–A	4×10^{-6}		0.0011	–	1.7791 unit increase	[1.02–2.54]	BMI–adjusted hip circumference
rs10935496–A	3×10^{-8}	(GO:0015238 |MF|03|drug transmembrane transporter activity)	NR	–	5.527629 Z–score decrease	NR	gut microbiome measurement
rs35312258–G	4×10^{-6}		0.17	–	–	–	forced expiratory volume, allergen exposure measurement, asthma
rs1875463–?	2×10^{-6}		NR	1.111605	–	–	rheumatoid arthritis
rs1135438–?	9×10^{-6}		NR	–	–	–	radius bone mineral density
rs1868175–A	7×10^{-17}		0.748	–	0.22 unit increase	[0.17–0.27]	HIV–1 infection, viral load
rs12637224–G, rs16853354–G, rs1501626–G, rs7641708–G, rs2197063–A, rs17636071–T, rs7617034–A, rs7639109–C, rs16853335–A	1×10^{-7}		0.009	–	–	–	acute myeloid leukemia

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
rs35312258–G	4×10^{-6}		0.17	–	–	–	forced expiratory volume, allergen exposure measurement, asthma
rs1875463–?	2×10^{-6}		NR	1.111605	–	–	rheumatoid arthritis
rs1135438–?	9×10^{-6}		NR	–	–	–	radius bone mineral density
rs1868175–A	7×10^{-17}		0.748	–	0.22 unit increase	[0.17–0.27]	HIV–1 infection, viral load
rs12637224–G, rs16853354–G, rs1501626–G, rs7641708–G, rs2197063–A, rs17636071–T, rs7617034–A, rs7639109–C, rs16853335–A	1×10^{-7}		0.009	–	–	–	acute myeloid leukemia
rs1372288–C, rs294470–G	7×10^{-9}		0.201	–	–	–	acute myeloid leukemia
rs9810857–T	6×10^{-6}		0.49	1.41	–	[NR]	attention deficit hyperactivity disorder
rs294468–?	6×10^{-9}		NR	–	–	–	mean corpuscular hemoglobin
rs2800–G	3×10^{-6}	(ALT)	0.34	–	0.61 unit increase	[NR]	serum alanine aminotransferase measurement, non–alcoholic fatty liver disease
rs1371924–?	4×10^{-6}	(RWLTA)	NR	–	–	–	blood pressure
rs2166775–G	3×10^{-6}	(EA)	NR	–	0.99 unit increase	[0.54–1.44]	response to candesartan, hypertension
rs17636071–G	2×10^{-6}	(MMSE)	0.09	–	4.74 unit decrease	[NR]	response to cholinesterase inhibitor, Alzheimer's disease
rs1404568–G	2×10^{-6}		0.18	–	–	–	forced expiratory volume, allergen exposure measurement, asthma
rs4839603–C	4×10^{-6}	(additive model)	NR	–	0.03503 unit decrease	–	response to opioid, analgesia requirement measurement
rs115134572–?	6×10^{-7}		–	–	0.4727 unit decrease	[NR]	sporadic amyotrophic lateral sclerosis, survival time
rs17635531–A	7×10^{-7}	(sleep duration)	NR	–	0.403 unit decrease	[0.24–0.56]	sleep duration
rs4839604–A	4×10^{-13}	(Tetrasialylated)	0.23	–	0.22375393 (zscore) increase	[0.16–0.28]	N–glycan measurement

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
rs1372288–G	3×10^{-8}	(Tetrasialylated, men)	0.25	–	0.24889249 (zscore) increase	[0.16–0.34]	N–glycan measurement

Table 4. – A summary information of two disease SNPs related to ADHD or ASD

	rs1248614031	**rs121912597**
Organism	Homo sapiens	Homo sapiens
Position	chr3:143493743 (GRCh38.p12)	chr3:143493701 (GRCh38.p12)
Alleles	C>G	G>A
Variation Type	SNV Single Nucleotide Variation	SNV Single Nucleotide Variation
Frequency	G=0.00000 (1/251382, GnomAD_exome)	A=0.00005 (12/251342, GnomAD_exome) A=0.00002 (3/121390, ExAC) A=0.0000 (1/78696, PAGE_STUDY) (- 2 less) A=0.0000 (1/31388, GnomAD) A=0.000 (1/5008, 1000G)
Gene: Consequence	SLC9A9 : Missense Variant	SLC9A9 : Stop Gained
Code	GCC to ACC	CGA to TGA
Amino acid change	A409P	R423X
Protein domain	cation/H+ exchanger domain	cation/H+ exchanger domain
Protein interaction	Yes	Yes
Topology	not in transmembrane helix	not in transmembrane helix
Active site	No	No
Stability	No	No
Expression	No	No

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MODERN VIEWS ON LIVER STEM CELLS AND THEIR OCCUPIED NICHES

Abstract. In human liver and laboratory animals, two populations of resident progenitor cells have been described: hepatic stem cells and biliary tree stem cells. An analysis of the scientific literature showed that in the future they will become a promising source for the development of methods for cell therapy of diseases of the liver and pancreas.

Keywords: hepatic stem cells, biliary tree stem cells, liver.

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СОВРЕМЕННЫЕ ВЗГЛЯДЫ НА СТВОЛОВЫЕ КЛЕТКИ ПЕЧЕНИ И ЗАНИМАЕМЫЕ ИМИ НИШИ

Аннотация. В печени человека и лабораторных животных описаны две популяции резидентных прогениторных клеток: печеночные стволовые клетки и стволовые клетки билиарного дерева. Анализ научной литературы показал, что в будущем они станут перспективным источником для разработки методов клеточной терапии заболеваний печени и поджелудочной железы.

Ключевые слова: печеночные стволовые клетки, стволовые клетки билиарного дерева, печень.

В настоящее время в печени взрослого человека и лабораторных животных описаны две популяции резидентных прогениторных клеток: печеночные стволовые клетки (HpSCs) и стволовые клетки билиарного дерева (BTSCs). Вопросом о роли данных популяций клеток в процессе регенерации

печени при ее повреждении остается малоизученным. Проведенные исследования демонстрируют вариабельность данных, что затрудняет сопоставления полученных результатов [1; 2].

Целью статьи является обобщение современных научных данных в области изучения стволовых клеток печени и определение перспектив их использования в регенеративной медицине.

Печеночные стволовые клетки (HpSCs) и занимаемая ими ниша

В здоровой печени человека и животных HpSCs представляют собой маленькие покоящиеся клетки с овальным ядром, небольшим количеством цитоплазмы и низкой скоростью пролиферации. При обычных гистологических методах окраски данные клетки трудно верифицировать. Общепринятыми маркерами идентификации HpSCs являются иммуногистохимические маркеры CK-7 и CK-19. Согласно литературным данным печеночные клетки-предшественники способны так же экспрессировать маркеры стволовых клеток (Sox9, Lgr5, CD44, CD133), молекулу адгезии эпителиальных клеток EpCAM, молекулу адгезии нервных клеток NCAM и маркер гепатоцитов CK18. Вероятно, при идентификации HpSCs в печени необходимо учитывать их локализацию и иммунофенотип [1; 3].

Печеночные стволовые клетки расположены в каналах Геринга – специализированной анатомической и функциональной нише. Каналы представляют собой наименьшие и наиболее периферические ветви желчного дерева. Экспансия, пролиферация и дифференцировка HpSCs зависит от клеток ниши, состава внеклеточного матрикса и сигнальных путей. В настоящее время среди исследователей роль ниши в пролиферации и дифференцировке HpSCs находится в центре активных дискуссий. Нишу печеночных клеток-предшественников ученые описывают как клеточное и внеклеточное микроокружение. Взаимодействие HpSCs с клетками микроокружения является ключевым механизмом, регулирующим процессы состояние покоя и дифференцировки стволовых клеток. При этом необходимо учитывать, что сигнальные и адгезивные молекулы внеклеточного матрикса ниши способны влиять на состояние покоя стволовых клеток, их самообновление, дифференцировку и функцию. В костном мозге и кишечнике молекулярные каскады сигнальных путей Wnt, Notch, Hedgehog поддерживают покой стволовых клеток, контролируют пролиферацию и управляют их дифференцировкой [4].

Установлено, что заболевания печени, связанные с повреждением желчного эпителия, характеризуются активацией в нише стволовых клеток сигнального пути Notch. Звездчатые клетки печени и портальные миофибробласты секретируют лиганды пути сигнального Notch (например, Jagged1), стимулируя дифференцировку HpSCs в билиарном направлении. В нише печеночных клеток-предшественников локализуются портальные миофибробласты, звездчатые клетки, гепатоциты, холангиоциты, эндотелиальные клетки, резидентные макрофаги (клетки Купфера). Вероятно, все эти клетки могут взаимодействовать с HpSCs, влияя на их пролиферацию и дифференцировку. При поражениях печени клетки воспалительного инфильтрата секретируют ряд цитокинов и хемокинов, так же оказывая влияния на HpSCs. Например, Т-клетки экспрессируют TNF-подобный слабый индуктор апоптоза (TWEAK), стимулирующий пролиферацию HpSCs. Другие молекулы воспалительного ответа (лимфотоксин-β, интерферон-γ, фактор некроза опухоли-α и гистамин) стимулируют пролиферацию HpSCs [1; 5].

При вирусном гепатите, стеатогепатите и первичном билиарном циррозе печени выявлена корреляция между степенью повреждения, пролиферацией HpSCs и фиброзом. Предполагают, что HpSCs влияют на активацию звездчатых клеток и наоборот, активированные звездчатые клетки способны стимулировать пролиферацию HpSCs и управлять их дифференцировкой. Вероятно, активация HpSCs и звездчатых клеток происходит независимо друг

от друга, а возможно совместно с помощью схожих медиаторов или стимулов. Это является предметом дальнейших исследований [6; 7].

В соответствии с современными научными данными HpSCs окружены внеклеточным матриксом обогащенным фибриллярным белком ламинином. В здоровой печени наблюдается минимальная экспрессия галектина-3 (Gal-3, CBP-35, Mac-2, L29). Галектин-3 принадлежит к семейству бета-галактозид-связывающих протеинов. В зависимости от типа клеток и состава внеклеточного матрикса ниши Gal-3 способен как ингибировать, так и индуцировать рост и дифференциацию стволовых клеток. Макрофаги, фибробласты и нейтрофилы экспрессируют Gal-3. При повреждении печени количество Gal-3 увеличивается. Его С-терминальный участок связывается с ламинином и запускает процесс пролиферации HpSCs. Заболевания печени с высоким содержанием ламинина во внеклеточном матриксе (например, алкогольный гепатит), характеризуются более низким уровнем пролиферации и дифференцировки HpSCs. У мышей Gal-3$^{-/-}$ значительно снижена пролиферация HpSCs [1; 3; 7].

При острых и хронических повреждениях печени активация HpSCs характеризуется появлением реактивных протоков (дуктулярная/протоковая реакция, DR). При морфологическом исследовании реактивные протоки представляют собой цепочки пролиферирующих клеток, напоминают извилистые структуры без отчетливого просвета и протоки с четко оформленным просветом. Исследования выявили, что дуктулярная реакция представлена клетками с вариабельным маркерным профилем. Вероятно, гетерогенность клеток здесь обусловлена стадией дифференцировки протоков и разной этиологией заболеваний печени [2; 8; 9].

Результаты последних научных исследований показали, что при определенных условиях HpSCs дифференцируются в гепатоциты и холангиоциты. В процессе дифференцировки HpSCs приобретают промежуточный фенотип, характеризующийся модификацией иммуногистохимических маркеров. Однако это процесс описан только у грызунов. Печеночные стволовые клетки являются остатками протоковой пластинки печени плода и новорожденного, сохраняющейся во взрослой печени как популяция Sox9+ клеток. Schmelzer et al. и Weiss et al. продемонстрировали, что клетки EpCAM+ и Thy-1+, выделенные из эмбриональной или постнатальной печени, способны давать начало зрелым паренхиматозным клеткам печени человека [2; 9].

Следует отметить, что ни один из описанных маркеров не является полностью специфическим для HpSCs. Использование HpSCs в клинической практике нуждается в дальнейших исследованиях. Необходима разработка протоколов стандартизации процессов от фундаментальных исследований до разработки клинических испытаний [1; 3; 9].

Стволовые клетки билиарного дерева (BTSCs) и занимаемая ими ниша

В стенке внепеченочных и крупных внутрипеченочных желчных протоков локализованы железистые элементы. В современной научной литературе их называют трубчато-альвеолярные перибилиарные железы (peribiliary glands, PBG). Перибилиарные железы описаны у человека и большинства млекопитающих. До 2011 года их функцией считали секрецию муцина, защищающего эпителий от агрессивного действия желчи. В настоящее время появились данные свидетельствующие о том, что PBG являются нишей для мультипотентных стволовых клеток. В зарубежной литературе данные клетки называют стволовые клетки билиарного дерева (BTSCs). В настоящее время данные, свидетельствующие об функции PBG остается дискутабельным [2; 10].

Стволовые клетки билиарного дерева обладают уникальной способностью дифференцироваться в зрелые холангиоциты, гепатоциты и клетки островков поджелудочной железы. Вероятно, это связано с тем, что эпителиальные клетки пече-

ни и поджелудочной железы имеют общий источник развития. Клетки BTSCs идентифицируют по экспрессии поверхностных маркеров Sox17, Pdx1, Sox9, EpCAM, Sall4 и Lgr5 [10; 11].

Ряд исследований продемонстрировали, что выделенные из печени взрослого человека BTSCs способны сохраняться в недифференцированном состоянии в среде Кубота в течение восьми недель, экспрессируя поверхностный маркер EpCAM. При изучении процесса дифференцировки, выделенные BTSCs пересевали в различные питательные среды. Для получения популяции гепатоцитов их пересевали в питательную среду HDM–L, холангиоцитов – в среду HDM–C, а клеток островков поджелудочной железы – в среду HDM-P. Результаты исследований показали, что в питательной среде HDM–L по периферии образовавшихся колоний располагались клетки, экспрессирующие маркеры CK18 и альбумин (маркеры гепатоцитов), а в центре колоний локализовались клетки, несущие на поверхности маркер EpCAM. При культивировании стволовых клеток билиарного дерева в среде HDM–C по периферии колоний выявлены клетки с поверхностными маркерами CK19 и CK7 (маркеры холангиоцитов). В среде HDM-P культивируемые клетки экспрессировали инсулин, глюкагон, PDX1, C-peptide и соматостатин (маркеры клеток островков поджелудочной железы) [2; 12; 13; 14].

Стволовые клетки билиарного дерева представляют собой резервный отсек стволовых клеток, который можно стимулировать к дифференцировке посредством фармакологической модуляции. Молекулярные сигнальные молекулы, которые управляют пролиферацией и дифференцировкой данных клеток, а также роль ниши в этих процессах до конца не изучены.

Заключение

Анализ литературы показал, что резидентные прогениторные клетки печени являются перспективным источником для разработки методов клеточной терапии заболеваний печени и поджелудочной железы и требует дальнейших исследований. По-прежнему остаются не изученными способы выделения и методы дифференцировки клеток и их фенотипические характеристики. Несмотря на накопленные новейшие результаты исследований из-за отсутствия в научных работах описания их детального протокола проведения не представляется возможным их полное воспроизведения.

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Section 3. General biology

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PREDICTING CHILDHOOD ADHD USING INDIVIDUAL CHARACTERISTICS AND ENVIRONMENTAL RISK FACTORS

Abstract. Attention-deficit/hyperactivity disorder (ADHD) is frequently found in children these days, but its risk factors remain uncertain. The main aim of this study is to identify and indicate the main risk factors for ADHD. Family income, household and family sizes, demographic information about the household reference person, and other demographic information such as gender, age, race, education and country of birth were assessed in 1576 youths examined in the NHANES National Youth Fitness Survey (NNYFS). Logistic regression analysis was performed to analyze the relationship between a set of potential risk factors and the outcome (ADHD). In addition, Kolmogorov-Smirnov (KS) statistic and Receiver operating characteristic curve (ROC curve) were used to evaluate the predictive model. The linear regression models indicate factors such as male, younger age and maternal smoking will largely increase the risk of developing ADHD while significant influence was not found in other factors like race, family income, education and birth weight.

Keywords: ADHD, mental disorder, methylphenidate, dextroamphetamine, maternal smoking.

1. Background

Attention-deficit/hyperactivity disorder (ADHD) is a disorder marked by an ongoing pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development [1]. Inattention and hyperactivity/impulsivity are the key behaviors of ADHD. Some people with ADHD only have problems with one of the behaviors, while others have both inattention and hyperactivity-impulsivity [1]. Most children have the combined type of ADHD. Researchers are not sure what causes ADHD. Like many other illnesses, several factors can contribute to ADHD, such as genes, cigarette smoking, alcohol use, drug use during pregnancy, exposure to environmental toxins during pregnancy such as high levels of lead, at a young age, low birth weight, and brain injuries [1]. ADHD is more common in males than females, and females with ADHD are more likely to have problems primarily with inattention. Based on the current uncertainty of risk factors that may cause ADHD, data analysis is used in this study in order to more accurately confirm the risk factor that cause ADHD [1].

2. Study methods:

2.1 Data source

The NHANES National Youth Fitness Survey (NNYFS, website: https://www.cdc.gov/nchs/nnyfs/index.htm) was conducted in 2012 as a one-time

survey by the Division of Health and Nutrition Examination Surveys, National Center for Health Statistics, part of the Centers for Disease Control and Prevention. It collected data on physical activity and fitness levels of children and teens in the U.S. ages 3 to 15 years. The NNYFS collected data through interviews and fitness tests. The fitness tests included standardized measurements of core upper and lower body muscle strength, as well as a measurement of cardiovascular fitness by walking and running on a treadmill. The family and sample person demographics questionnaires were asked, in the home, by trained interviewers. An adult family member, aged 18 years or older, was interviewed as a proxy for the survey participant.

The demographics file provides individual, family, and household level information on the following topics:

- family income;
- Household and family sizes;
- Demographic information about the household reference person;
- Other selected demographic information, such as gender, age, race/Hispanic origin, education, and country of birth.

NNYFS2012included a total of 1,640 youths' data from the interview, and among them, 1,576 youth were examined.

2.2 Study variables

ADHD

Children on common ADHD medications were identified by medication use information. The most commonly used stimulant medications include methylphenidate (Ritalin, Concerta), mixed amphetamine salts (Adderall), dextroamphetamine (Dexedrine), and lisdexamfetamine (Vyvanse). Non-stimulant medications with a specific indication for ADHD include atomoxetine (Strattera), √guanfacine (Intuniv).

Variables of potential predictors

We tried to include variables from literature that are suggested as related to ADHD risk and meanwhile with available data from the survey. The following variables of participants are included:

- Gender;
- Age;
- race;
- birth weight;
- maternal smoking;
- Ratio of family income to poverty.

Other information of the participant's mother was not directly available. Therefore, we used the reference person's information as a proxy, including the following:

- gender;
- education;
- marital status.

2.3 Analysis

To formally train and then test a predictive model, data was split randomly into two datasets, with one half for model development ("training" data), and the other half for model validation ("testing" data).

Using the training data, we performed logistic regression analysis to build the predictive model. Logistic regression is a type of generalized linear regression for analyzing relationship between a set of explanatory variables and a binary outcome variable (i.e., with yes/no value). In this study, the outcome is if a student has above average grades. The model is:

$$\ln(\text{odds of outcome event}) = \ln(\text{Prob}/\text{Prob-1}) = b_0 + b_1 * X_1 + b_2 * X_2 + \ldots . + b_n * X_n$$

"Prob" is the probability of an event, and is convertible with odds. $X_1, X_2, \ldots X_n$ are explanatory variables. b is regression coefficient for a specific X.

- If the coefficient of a variable X is above 0, it means that X is related to a higher odds/probability of the event. The corresponding Odds Ratio will be above 1.
- If the coefficient of a variable X is equal to 0, it means that X is NOT related to the event. The corresponding Odds Ratio equals to 1.
- If the coefficient of a variable X is below 0, it means that X is related to a lower odds/prob-

ability of the event. The corresponding Odds Ratio will be below 1.

Lastly, the prediction model was tested using the testing data to examine if the model provides good prediction of the outcome. The following measures are used to evaluate if the model is a good fit:

- Kolmogorov-Smirnov (KS) statistic. KS is the most commonly used model evaluation metric for models predicting binary outcomes. It reflects the distance between distributions of positive outcome and negative outcome. A higher KS means more separation of positive vs negative. Therefore, a higher value indicates better model fit. KS ranges from 0% to100%. A rule of thumb is that a KS of 0.4 suggest good discrimination of the outcome [1].
- Receiver operating characteristic curve (ROC curve) and the Area under the ROC Curve (AUC). ROC curve is a graphical plot that illustrates the diagnostic ability of a model [2]. For AUC, the higher the better.

3. Results

The study sample included 50% boys and 50% girls. Average age was 9 years.

4% of the study sample were found with ADHD. this is similar with the reported national average.

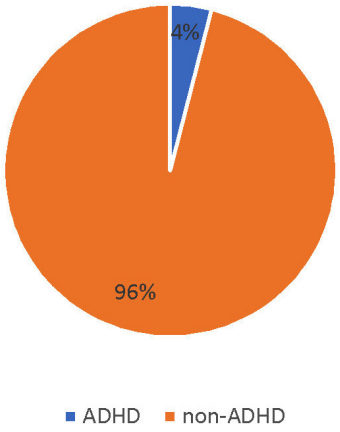


Figure 1. Prevalence of ADHD in study sample

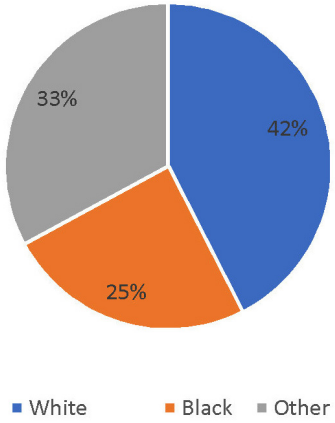


Figure 2. Racial composition of study sample

3.1 Development of the prediction model

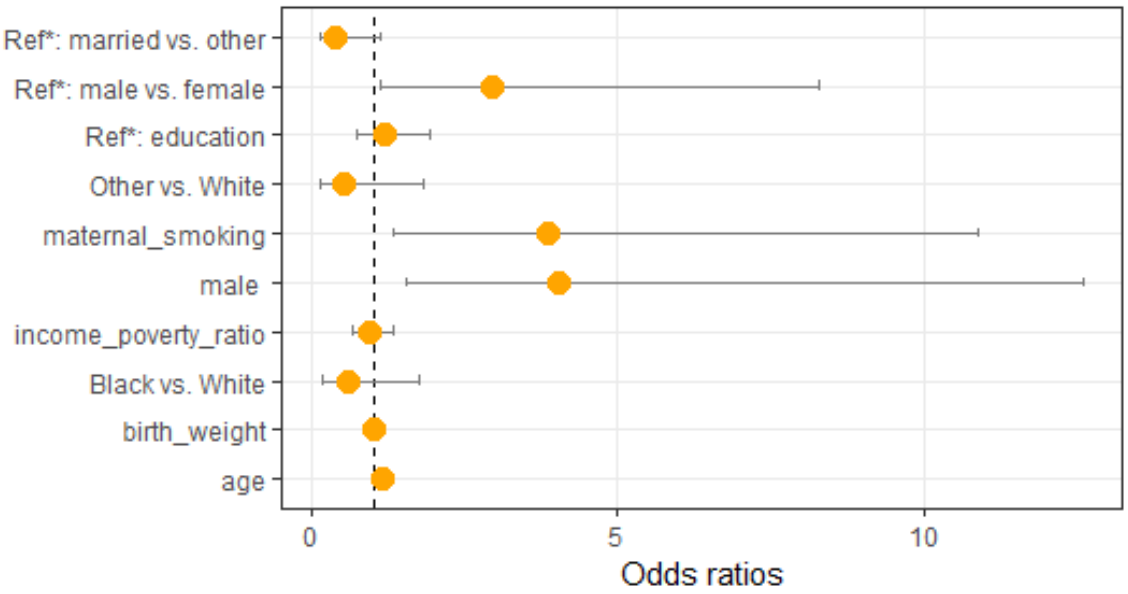
Table of Coefficients:

Table 1.– Of coefficients from training data

	Estimate	standard error	z value	p-value
	–6.867	1.784	–3.848	<0.001
male	1.39	0.527	2.638	0.008
age	0.129	0.059	2.178	0.029
Race				
Black vs. White	–0.548	0.587	–0.934	0.35
Other vs. White	–0.653	0.671	–0.973	0.33
birth_weight	0	0	0.809	0.418
income_poverty_ratio	–0.059	0.18	–0.333	0.739
maternal_smoking	1.354	0.533	2.54	0.011
reference person's information				
male vs. female	1.086	0.504	2.151	0.031
education	0.158	0.239	0.662	0.507
married vs. other	–0.939	0.529	–1.774	0.076

Table 2.– Of odds ratios from training data

	Odds Ratio	Lower CI	Upper CI
male	4.018	1.536	12.633
age	1.138	1.016	1.285
Race			
Black vs. White	0.577	0.167	1.739
Other vs. White	0.52	0.124	1.817
birth_weight	1	0.999	1.001
income_poverty_ratio	0.941	0.656	1.337
maternal_smoking	3.876	1.317	10.896
reference person's information			
male vs. female	2.962	1.129	8.294
education	1.171	0.743	1.909
married vs. other	0.39	0.137	1.112



*Ref: reference person

Figure 1. Factor predicting childhood ADHD

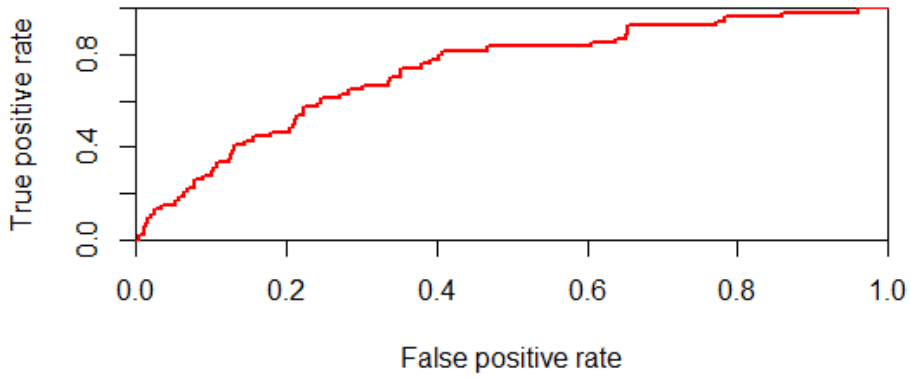


Figure 2. ROC curve

As introduced in the Methods section, an Odds Ratio above 1 means that the variable is related to a higher risk of the outcome, while an Odds Ratio below 1 means that the variable is related to a lower risk of the outcome. From the tables, we can see that three variables are related to a *higher* risk of ADHD: male gender, older age, and maternal smoking during pregnancy. Reference person's gender also seems to related to ADHD but practically this finding is not very meaningful.

3.2 Validation of the prediction model

When applying the above model to the testing data, the KS statistic was 0.41. Meanwhile, the following ROC curve was generated. AUC was 73%.

4. Discussion

After the data of study samples were analyzed, the prediction model was developed. Based on the table of coefficients, as those coefficients that are greater than 0 mean the risk factor is highly related to ADHD, conditions including gender (male), age, maternal smoking, and education fulfill the requirements we are looking for. Another standard that has to be met is the p-value, which if it is under 0.05, it shows the risk factor is related. Therefore, education can be eliminated from the list.

In the table of odds ratios from training data, the table shows that all the potential risk factors that can be shown related in the last table are also shown relatable to ADHD. By simply looking at the table, risk factors including gender, age, and maternal smoking all have a odds ratio that are greater than 1, which shows a great likelihood of those risk factors related to ADHD.

In addition, the prediction model was tested by KS statistic and ROC curve. In this study, as the testing data was applied, the KS statistic turned out to be 0.41, which indicates a relatively data-fitting model. Also, after the ROC graph was produced, the AUC was calculated to be 73%, showing a good fit of the data and the relativity.

Contrary to the former researches [3; 4], some results from this study predicting risk factors like prenatal factors appear to be contradictory, while genetic factors like gender seem to be mutual. The uncertainty of whether maternal smoking being a risk factor might be due to the difficulty surveying mothers' smoking history, including the length and frequency of their smoking history.

Based on the fact that ADHD has already become a prevalent disorder, studying its risk factor has a great significance reduce the rate from people getting it, and that is the major objective of this study.

5. Conclusion

Male sex and younger age are significant risk factors of ADHD. Smoking during maternal presidency will also largely increase the risk developing the disorder.

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Section 4. Professional medicine

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ANALYSIS OF PRENOSOLOGICAL MONITORING OF THE ORAL CAVITY (ON THE EXAMPLE OF KRMU APPLICANTS)

A**bstract.** the article provides data from a preventive examination and questioning among applicants. The high intensity and prevalence of dental caries and dentoalveolar anomalies was established; 85% of applicants need oral cavity sanitation.

Keywords: analysis, examination, oral cavity, need, sanitation, questioning.

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АНАЛИЗ ДОНОЗОЛОГИЧЕСКОГО КОНТРОЛЯ СОСТОЯНИЯ ПОЛОСТИ РТА (НА ПРИМЕРЕ АБИТУРИЕНТОВ КРМУ)

Аннотация. В статье предоставлены данные профилактического осмотра и анкетирования среди абитуриентов. Была установлена высокая интенсивность и распространенность

кариеса зубов и зубочелюстных аномалий; 85% абитуриентов нуждаются в санации полости рта.

Ключевые слова: анализ, осмотр, полость рта, нуждаемость, санация, анкетирование.

Стоматологическое здоровье у лиц молодого возраста является одним из важнейших показателей благосостояния общества. Реформирование ведущих отраслей народного хозяйства и, в первую очередь, здравоохранения, повлияли на основные показатели общественного здоровья и в первую очередь, на наиболее уязвимую категорию – молодое население (Баранов А. А.,2003; Вельтищев Ю. Е., Балева Л. С., 2004, Герасименко Н. Ф.,2004; Ваганов Н. Н., 2005). Новые социальные ориентиры требуют фундаментальных и прикладных исследований. При этом важным является оценка донозологического контроля состояния полости рта, которое позволяет предупредить ряд стоматологических заболеваний и устранить факторы, негативно влияющих на здоровье.

Актуальность изучения влияния социально-гигиенических факторов наряду с клиническими аспектами на показатели стоматологической заболеваемости населения, обусловлена их чрезвычайно широкой распространенностью, как среди взрослого, так и детского населения. Роль социально-гигиенических факторов подтверждается своеобразной динамикой заболеваемости населения в различных социальных условиях. Не меньшее значение имеют медико-биологические и клинические факторы, а также поведенческие факторы: несоблюдение здорового образа жизни, нерациональное питание, отсутствие гигиены полости рта, пренебрежительное отношение к своему здоровью. Не исключается влияние миграции населения, климатогеографических особенностей местности и др. Социально-гигиеническое значение распространенных стоматологических заболеваний связано не только с весьма большими затратами на их лечение, но и с большой зависимостью возникновения этих заболеваний от условий жизни человека. Поэтому стоматологическая заболеваемость и, в первую очередь, кариес зубов, как наиболее часто встречающаяся нозологическая форма, рассматривается, как социальное заболевание (А. В. Алимский с соавт., 2005; Э. М. Кузьмина, 2005; А. Н. Галлиулин, 2007).Заболевания кариесом зубов является одним из существенных факторов риска возникновения инфекционно-аллергической и хронической патологии: бронхолегочной, гастроэнтерологической патологии и т.д. (В. Г. Васильев с соавт., 2005; В. М. Гринин с соавт, 2006; И. В. Кузнецова, 2007). При этом следует уделить особое внимание предупреждению заболеваний, проведению оздоровительных мероприятий, устранению факторов, негативно влияющих на здоровье. К числу проблем, разработка которых имеет большое социальное значение, относятся вопросы предупреждения и лечения стоматологических заболеваний. Очевидным является то, что здоровье закладывается в детском возрасте, а стоматологические заболевания могут отрицательно воздействовать и воздействуют на организм людей любого возраста, в том числе среди студентов, подвергавшие большой нагрузки и стрессовым ситуациям в период сессии.

Вопросы организации и совершенствования стоматологической помощи населению до недавнего времени, в основном, решались в отрыве от всестороннего анализа медико-демографических и социально-гигиенических факторов. Всемирной Организацией Здравоохранения предложены Европейские цели стоматологического здоровья к 2010 году и 2020 году, одной из которых является функционирование эффективной информационной системы на основе статистически достоверных данных, сопоставимых на межгосударственном уровне. Формирование банка данных стоматологического здоровья различных медико-социальных групп, а также комплексный

подход к организации стоматологической помощи студентам, т.е. социально-уязвимой группе является приоритетным направлением.

Цель: провести донозологический контроль состояния полости рта и разработать профилактические мероприятия основных стоматологических заболеваний (на примере студентов КРМУ).

Задачи:

1. Изучить распространенность стоматологической заболеваемости среди студентов КРМУ, в т.ч. абитуриентов, и выявить частовстречающие формы стоматологической патологии по обращаемости и по данным профилактического осмотра.

2. Выявить зависимость уровня стоматологической заболеваемости у студентов КРМУ от медико-биологических, социально-гигиенических и медико-организационных факторов развития заболевания;

Актуальность изучения влияния социально-гигиенических факторов наряду с клиническими аспектами на показатели стоматологической заболеваемости населения, обусловлена их чрезвычайно широкой распространенностью как среди взрослого, так и детского населения.

Роль социально-гигиенических факторов подтверждается своеобразной динамикой заболеваемости населения в различных социальных условиях. Также имеют значения медико-биологические и клинические факторы, а также поведенческие факторы: несоблюдение здорового образа жизни, нерациональное питание, отсутствие гигиены полости рта, пренебрежительное отношение к своему здоровью. Не исключается влияние миграции населения, климатогеографических особенностей местности, употребление нездоровой пищи и др. Социально-гигиеническое значение распространенных стоматологических заболеваний связано не только с весьма большими затратами на их лечение, но и с большой зависимостью возникновения этих заболеваний от условий жизни человека. Поэтому стоматологическая заболеваемость и, в первую очередь, кариес зубов, как наиболее часто встречающаяся нозологическая форма, рассматривается, как социальное заболевание (А. В. Алимский с соавт., 2005; Э. М. Кузьмина, 2005; А. Н. Галлиулин, 2007).

Заболевания кариесом зубов является одним из существенных факторов риска возникновения инфекционно-аллергической и хронической патологии: бронхолегочной, гастроэнтерологической патологии, сердечно-сосудистой и т.д. (В. Г. Васильев с соавт.,2005; В. М. Гринин с соавт, 2006; И. В. Кузнецова, 2007).

Широкая распространенность и высокая частота заболеваний тканей пародонта и частота рецидивов приобрела масштаб общемедицинской, а так же общесоциальной проблемы. Причина в том, что очаги инфекции в пародонтальных карманах оказывают негативное влияние на организм в целом, а пародонтит, в частности, способствует потере зубов [1]. По распространенности в мире заболевания пародонта среди стоматологических заболеваний занимают 2-е место, и имеют тенденцию к повсеместному распространению вне зависимости от пола, возраста и среды проживания [2; 3; 4; 5; 6].

Согласно обобщенным данным Всемирной Организации Здраво-охранения от 2000 г., интактный пародонт наблюдается не более чем в 2–10% наблюдений, пародонтит средней степени тяжести – в 25–45%, тяжелой степени – в 5–20%. Соответственно, для возрастной группы 35–44 года распространенность заболеваний пародонта в мире соответствует 94,3%. У взрослого населения воспалительные заболевания пародонта, приводящие к патологическим изменениям в зубочелюстной системе, и связанные с потерей зубов, выявляются примерно у 90–95%, что в 5 раз чаще, чем при осложнениях кариеса [7; 8; 9; 10].

Здоровье полости рта является неотъемлемой составляющей здоровья человека в целом. В 1988 году ВОЗ предложил Европейские цели достижения стоматологического здоровья, как ориентир для разработки и внедрения мероприятий

по профилактике наиболее распространённых заболеваний твёрдых тканей зубов, тканей пародонта, слизистой оболочки полости рта, зубочелюстных аномалий и деформаций, воспалительных и неопластических процессов мягких тканей лица и челюстно-лицевой области. Предполагается, что 80% 6-летних детей будут свободны от кариеса; интенсивность кариеса зубов у 12-летних детей не будет превышать КПУ 1,5. Регулярное посещение стоматолога (минимум два раза в год) позволяет выявить возникшие изменения в твёрдых тканях зубов и тканях пародонта на ранних стадиях, а рекомендации врача позволяют сохранить зубы. Исследования, проводимые в г. Алматы, выявили наибольшую распространенность апикального периодонтита среди лиц молодого возраста, что свидетельствует о риске ранней потери зубов [11].

Изучение уровня стоматологического здоровья среди населения – важный этап работы стоматолога с целью предотвращения таких проблем как кариес, пульпит, периодонтит, зубной налет, камни, воспаление десны, неприятный запах изо рта, заболевания слизистой оболочки полости рта неинфекционного происхождения. Поэтому необходимость проведения профилактических осмотров, особенно среди лиц молодого возраста является важным и необходимым этапом работы врача.

Гиппократ завещал: «Болезнь легче предупредить, чем лечить». В настоящее время, наряду с развитием малоинвазивных методов, внедрением последних достижений медицины, новых методов диагностики и лечения в практической деятельности врача имеет значение развитие профилактического направления. Любой врач, а также в подготовке будущих врачей профилактическое направление должно иметь приоритет. В частности, включать в учебные программы студентов вопросы гигиенического обучения и воспитания населения, формирования здорового образа жизни. Эффективность проводимых профилактических мероприятий оценить довольно сложно, так как нужны годы наблюдений. Однако, профилактика заболеваний, в т.ч. стоматологических и укрепление здоровья населения отражены в Государственной программе развития здравоохранения Республики Казахстан «Денсаулық» на 2016–2019 годы. Целью этой Программы является укрепление здоровья населения для обеспечения устойчивого социально-экономического развития страны. Одной из действующих задач – развитие системы общественного здравоохранения, совершенствование профилактики и управления заболеваниями.

Материалы и методы исследования:

Донозологический контроль состояния полости рта проводился среди абитуриентов, в период с июля по август 2019 года, в стоматологическом кабинете, по адресу: пр. Абылай хана 51/53, учебный корпус 1, кабинет № 105. Абитуриентам КРМУ проводился профилактический стоматологический осмотр с использованием одноразовых стерильных инструментов. Перед проведением осмотра абитуриенты заполняли информированное согласие (утверждено на заседание ЛЭК КРМУ, 10.06.2019 г.).

После осмотра абитуриенты отвечали на предложенную и разработанную нами анкету «Оценка состояния гигиены полости рта» (утверждена на заседание ЛЭК КРМУ, 10.06.2019 г.).

Для фиксации результатов использовалась электронная карта стоматологического осмотра, в которой были зафиксированы паспортные данные и зубная формула, по которой была установлена нуждаемость в санации и дана оценка профилактической работе.

Результаты исследования и обсуждение:

В рамках данного исследования участвовали студенты 3 и 5 курсов по специальности “Стоматология”, которыми были осмотрены 114 абитуриентов КРМУ.

В результате проекта было установлено, что основной проблемой полости рта остаются заболеваний твердых тканей зубов: кариес зубов

(85%), встречались различные формы хронического пульпита и периодонтита (17%) и у более 15% абитуриентов, ранее запломбированные зубы нуждались в перелечивании, т.е. основная часть абитуриентов нуждается в санации полости рта (рисунок 11).

Рисунок 1. Количество случаев заболеваний

Рисунок 2. Частота поражения зубов кариесом

Рисунок 3. Частота поражения кариеса зубов в зависимости от групповой принадлежности

Среди абитуриентов наиболее проблемными зубами, по частоте поражения кариесом, были жевательные зубы: моляры нижней челюсти – 36, 37, 46, 47 и моляры верхней челюсти – 16, 17, 26 и 27, что подтверждает данные различных исследований (рисунки 2, 3).

В ходе анкетирования абитуриентов были установлены следующие факты: в анкетировании приняли участие 114 абитуриентов, их них 55,3% – абитуриенты мужского пола и 44,7% – женского пола (рисунок 4).

Рисунок 4. Половозрастной состав абитуриентов

На вопросы: «Как часто вы чистите зубы?» был получен наибольший ответ – утром и вечером – 77,2%, что свидетельствует о том, что абитуриенты знают правила чистки зубов. Один раз в день чистят- 21,9% абитуриентов и 0,9% ответили, что редко чистят зубы (рисунок 5).

На вопрос: «Вас беспокоит кровоточивость дёсен при чистке зубов?» были получены результаты: да – среди 16,7% опрошенных; нет – 62,3%; иногда – 21,1%. Эти данные позволили нам сделать вывод о том, что состояние тканей пародонта у большинства абитуриентов–благоприятное, но тем не менее 24,5% абитуриентов нуждаются в лечении заболеваний тканей пародонта (рисунок 6).

На вопрос: «Как часто вы обращаетесь к врачу-стоматологу?» наиболее распространенными ответами среди абитуриентов были «иногда» – 49,1%; «в случае острой зубной боли» – 23,7%; «два раза в год» – 11,4%; «один раз в год» – 7,9%; «никогда» – 7,9% (рисунок 7).

Рисунок 5. Правила чистки зубов (процентное соотношение)

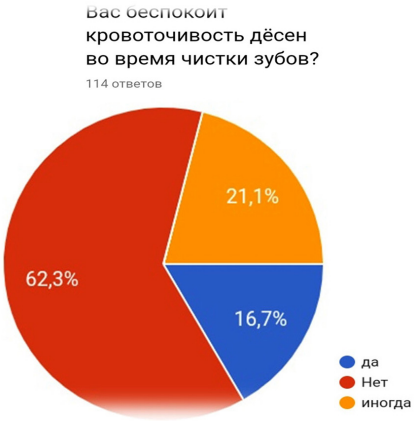

Рисунок 6. Субъективные данные состояния тканей пародонта

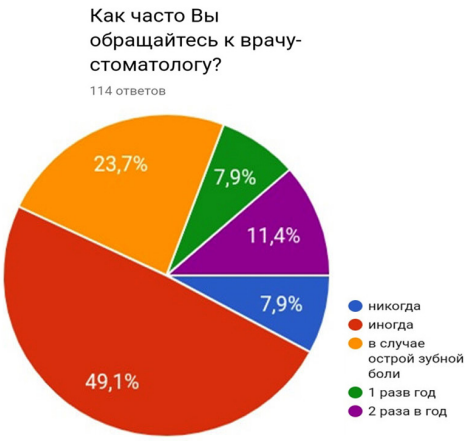

Рисунок 7. Обращаемость, в процентном соотношении, к врачу-стоматологу

Из 113 абитуриентов на вопрос «Как часто вы меняете зубную щётку?» были получены следующие ответы: «один раз в 3 месяца» – 71,7% опрошенных абитуриентов; «раз в полгода» – 23,9%; ряд таких вопросов «раз в год», «раз в месяц», «раз в вечность … » – в незначительном процентном соотношении (рисунок 8).

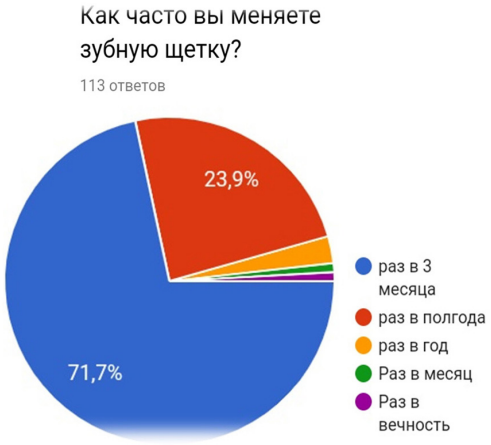

Рисунок 8. Частота смены зубной щетки

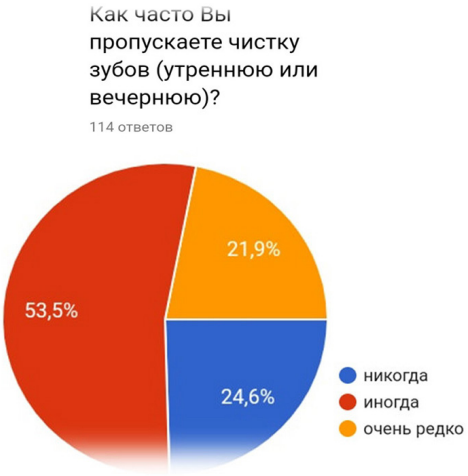

Рисунок 9. Показатели частоты чистки зубов

На вопрос «Как часто вы пропускаете чистку зубов (утреннюю или вечернюю)» респонденты ответили следующим образом: наибольшее количество – 53,5% – иногда; 24,6% – никогда и 21,9 – очень редко (рисунок 9).

На вопрос «Как вы компенсируете этот пропуск?» были получены следующие варианты ответов: 52,2% абитуриентов используют различные ополаскиватели для полости рта; 28,3% абитуриентов чистят зубы в течение дня; 19,5% -никак не компенсируют пропуск чистки зубов (рисунок 10).

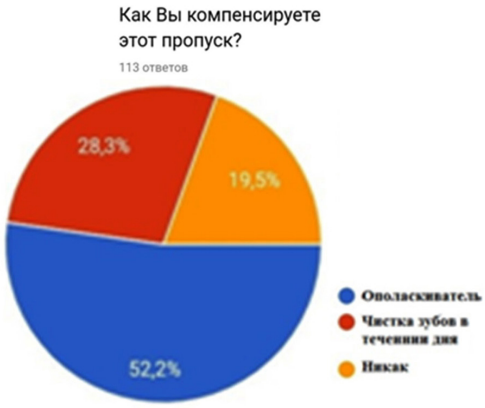

Рисунок 10. Результаты соблюдения правил чистки зубов

Таким образом, проведенный стоматологический осмотр 114 абитуриентов позволил нам установить высокую интенсивность и распространенность кариеса зубов и зубочелюстных аномалий; 85% абитуриентов нуждаются в санации полости рта. Данные результатов анкетирования показали, что многие респонденты не владеют навыками гигиены полости рта, отмечается низкий уровень обращаемости к врачу-стоматологу. Эти результаты свидетельствуют о том, что не достаточный уровень проведения санитарно-просветительной работы среди населения, и в том числе среди молодёжи остаётся актуальной проблемой.

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Section 5. Pharmacalogues

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SYNTHETIC LETHAL SCREENING OF ATP-LIKE PROTEIN INHIBITOR TO TERMINATE MITOSIS

Abstract

Background: In attempt to create a molecular drug that inhibits mitosis in cancerous cells (Myc gene overexpressed cancer cells), a drug – HA-75 is designed and made in resemblance with ATP molecules. Primary testing showed HA-75 to be effective in inducing cancer cell death.

Objective: this study aims to 1) determine the regulatory protein inhibited by the drug HA-75 during mitosis, and 2) investigate the effectiveness of HA-75 as a synthetic lethal screening approach to treating cancer, and 3) seek the possibility of future drugs/mechanism similar to that of HA-75.

Data and Methods: Data is mainly collected using time lapse photography(1) and immunofluorescent staining(2). Pictures are taken after applying HA-75 for 24 and 48 hours and then analyzed. Staining of different regulatory proteins are used to indicate whether each of those proteins have been inhibited. Positive and Negative control groups are assigned to be photographed as comparison to the experimental groups. Specific cell photographs are analyzed to determine the inhibitions made.

Results: specifically, 2 proteins regulators are associated with mitotic arrest and cell death – Mklp2 and Aurora A. These two proteins have shown consistent inhibition when HA-75 was introduced and the mitotic process was affected severely as a result of these inhibitions. Time lapse photography further shows that after metaphase cell was not able to fully divide and experienced mitotic arrest followed by apoptosis. Over 81.2% of cells under 20um concentration of HA-75 was unable to survive mitosis.

Conclusion and Discussion: The direct conclusion from the study is that, the proteins that are affected by HA-75 are Aurora B and Mklp2. It can also be concluded that a drug (like HA-75) has a dual target synthetic lethal effect on cancerous cells. Further research may be conducted on how best to develop HA-75 so that it is better suited to inhibit Mklp2 and Aurora B.

Keywords: Immunofluorescent staining, Time lapse photography, polyploidy, multi-target drug.

Introduction

Myc oncogenes is one of the best targets when creating a synthetic lethal drug, it is common to over 70% of cancer types ranging from solid tumors to hematological tumors. A successful drug that has a lethal effect when combined with overexpressed Myc oncogenes will provide a solution to most cancers. The concept of a synthetic lethal drug is appealing

because it does not target non-cancerous, thereby minimizing the side effects cells. However, a drug that direct inhibits Myc oncogenes is almost impossible as Myc genes has important functionality in normal cells. Thus, a different approach must be taken to create a synthetic lethal drug.

Cytokinesis is the final step in mitosis, and it ensures the correct separation of the cell's nucleus, many regulators regulates cytokinesis. Such a regulator – Mklp2 helps the correct positioning of the Aurora B protein in the spindle mid-zone. The spindle mid-zone guides the cleavage to form between the two daughter cells. The dislocation of the spindle mid-zone will lead to uneven division of the cell's genetic material and trigger the termination of mitosis. But, by this point, the cell's nucleus has already divided, and thus a polyploidy cell forms.

Other regulators of mitosis include Aurora A, CREST, and Histone-3. All of these regulators are located on major components involved in mitosis: Aurora A being on the centrosome, CREST on the chromosomal passenger complex (CPC), and Histone-3 being on histone proteins in the chromosome. Centrosome regulates the formation of spindles that direct chromosomes to opposite ends of the cell, CPC also help orchestrate spindle direction, and histone proteins holds the chromosome compact.

Another kinase protein that is being investigated ins the MKlp2 kinase. MKlp2 kinase forms a conjugate complex with the Aurora B protein during anaphase of the cell cycle (1), helping it to correctly move away from the centromeres during anaphase.

HA-75, though its targets are unknown, is a feasible synthetic lethal drug that is directed to Myc overexpressing genes. HA-75 combines the structure of the recognition site of ATP molecules and primary inhibition structures. The drug causes polyploidy to form in cancer cell lines and kills cancerous cells. HA-75 also matches the requirements of a synthetic lethal drug of Myc oncogenes.

Targeted to investigate the specific inhibitions caused by HA75, immunofluorescence staining of different regulatory proteins in mitosis can be of aid in identifying the inhibitions. One such protein complex is the chromosomal passenger complex (CPC) involved in the anaphase of mitosis. Specifically, two members of the CPC is involved in the separation of the sister chromosomes into singular chromosomes – Aurora B protein and CREST protein. Aurora B is located at the kinetochore of the separating chromosomes and is left behind in the spindle midzone after anaphase, and CREST is located in the NUMA transport proteins that congregates around the spindle. Aurora B in particular is of importance for the completion of mitosis, hence is a determining factor in the formation of polyploidy cells which results in mitotic catastrophe (2). Correct location of the spindle mid-zone is crucial for cytokinesis to be carried out, without the correct localization cytokinesis will stop and the cell will not divide.

Other methods such as transfection can be an alternative to provide insight to the target of a specific drug. Transfection allows the ability to do time laps, a technique that allows a long-term photography of sample cells. Time laps provides how the cells became polyploidy during mitosis and indicate the phase that mitosis stopped in.

Effectiveness of HA75

Through staining the nucleus of cancerous cells and comparing the experimental groups and the control groups, the effectiveness of the drug HA-75 to induce polyploidy is proven. The following graph demonstrates the effectiveness of the drug HA-75 under different concentrations by recording the. number of normal and polyploidy cells in the cell culture. A baseline culture that is used as a negative control is established using DMSO medium. A positive control is established by applying excess ADZ inhibitors, this is positive control group specifically inhibits the Aurora A protein complex. Another positive control is established by applying ADZ, this positive control specifically inhibits the Histone H3 protein. The number of cells are estimated by using a digital cell counter.

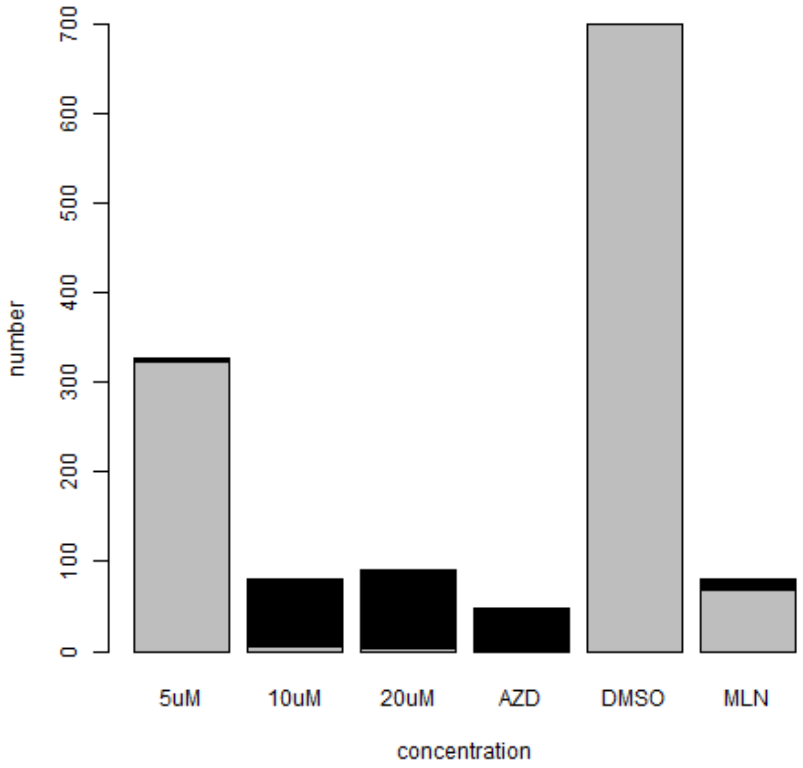


Figure 1.Number of polyploidy cells

The graph demonstrates extremely high number of polyploidy cells in cultures that are introduced with 10uM+ of HA-75. Over 95% of cells that in the culture are polyploid, this demonstrates high effectiveness of HA-75 when compared to the negative control. The graph also demonstrates a difference in cell number between the experimental groups and the baseline (DMSO group). This indicates that the drug effectively killed mitotic cells during the incubation period before the staining process thus further demonstrating the effectiveness of the drug.

Localizing protein inhibition through Immunofluorescent staining (IS)

After determining that the drug HA-75 is effective in treating cancer cell lines, investigation into the localization of proteins that are inhibited by HA-75 can begin. 4 motor proteins involved in the mitotic process is being examined. These four proteins all play essential roles in the cell cycle and the inhibition (one/several) of these proteins may be the cause of the formation of polyploidy cells. The four proteins that are under examination in this case are: Aurora A, Aurora B, CREST, and Histone H3 ser10.

The method of Immunofluorescent staining is used to determine the specific inhibitions that are caused by HA-75. Immunofluorescent staining is a technique that can identify target proteins in a cell. Target cells must first be transferred from a petri dish onto disks. The process of IS kills the cells on the disk, fixing them so that antibiotics can be applied. Two antibiotics are applied to the target disk, the first to identify the protein by binding to the surface, and the second to illuminate the first via a fluorescent stain. Antibiotics varies according to the protein that they target, for example Aurora B is stained using a Rabbit antibiotic – Biotin. Before that staining the disks are soaked in P+T that fix them to the disks, thereby providing a clearer image.

Staining that indicates a normal localization of Aurora A protein indicates a normal functioning of the Aurora A protein in mitosis (1). Staining that indicates a normal localization of Histone H3 ser10 protein

indicates the normal functioning of the Aurora B motor protein complex (1). Staining that indicates the normal localization of CREST and Aurora B proteins indicate the correct functioning of MKLP2 in mitosis.

Procedure

Samples used in the immunofluorescent staining procedure are cultured on disks and the corresponding amount of concentrated drug is added prior to the staining of the cells. The specific culture technique is shown below:

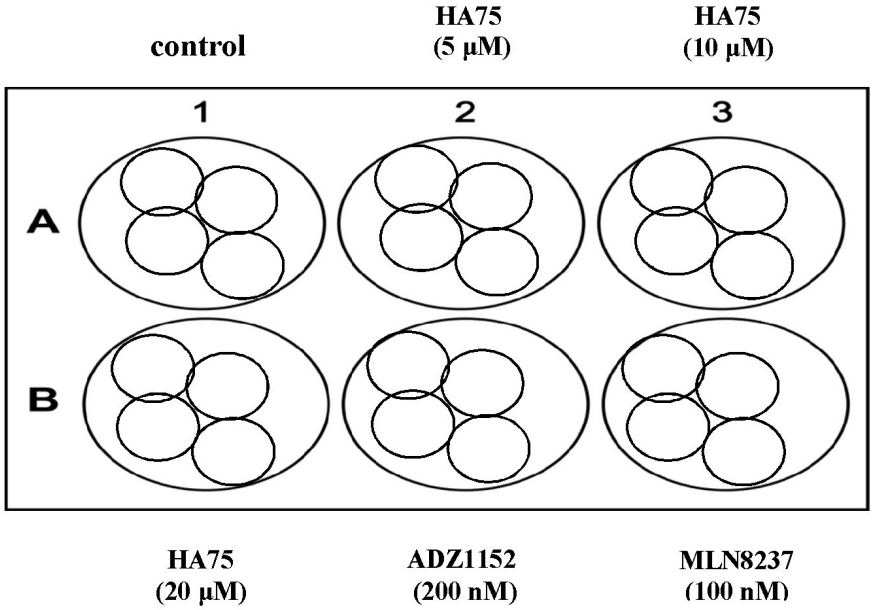


Figure 1. The layout of a 6 well plate with all of the test disks in corresponding wells

A six well plate is used for the culturing of the cell samples. As shown above, the disks are removed from a growth medium after 48 hours of culturing (or after a usable cell culture is established) and placed into a six well plate. The disks are kept in the well in groups of four (the number of staining being done) for 24 hours to achieve a sustainable sample of cell. Each well is labeled with the solution added and the corresponding solution is added. A negative control group is established with DMSO growth medium to examine cells that are in their natural state. Two positive control groups are established with 200nM ADZ1152 solution and 100nM MLN8237 solution.

Aurora A p-Thr288 protein is located in the centrioles during mitosis. Under staining the normal localization of Aurora A protein should display distinct "dots" on either side of the anaphase nucleolus. incorrect localization of the protein Aurora A may induce the inability of forming spindle and thereby inhibiting mitosis. Histone 3 phosphor ser-10 is the third phosphorylated protein involved in the formation of histone structure. The localization of this protein demonstrates the viability of Aurora B. The breaking of Histone structures also plays an important role in mitosis. Aurora B protein, when correctly located, is located at the spindle midzone of early telophase. CREST protein is located around the central protein complex that surrounds the centrioles. The location of CREST is similar to that of Aurora A but CREST localization covers a much larger area.

After the 24-hour incubation in the six well plate, cell disks are rinsed with DMSO and then fixed onto the disk and a immunofluorescent staining photography machine is used to take pictures of the fixed cells.

Using transfection technique to achieve a time lapse of mitotic cells

Transfection is a technique where plasmid is used to introduce targeted DNA information into E. coli bacteria to create specific proteins or add specific functionality to the bacteria. In this experiment, time

lapse is used in hope to achieve a 48-hour time lapse of polyploidy cells decaying and deteriorating. Since immunofluorescent staining requires the cells to be fixed, a live time lapse cannot be achieved.

The transfection consists of two different DNA segments – a first segment that identifies the nucleus and binds to it, and a second that targets the first segment and provide a florescent glow under ultraviolet lighting. Both these segments are infused into a premade plasmid segment through altering the temperature of the mixture of the plasmid and the segments. After the engineering of the plasmid is complete, the plasmids are injected into E. coli bacteria and the bacteria is cultured on a petri dish until a usable colony is established.

After the bacteria colony is established, the colony is harvested. Transfection is then performed and the plasmids are inserted into MBC cells and the cells are cultured. The cell, after leaving time for growth, are then moved to a observational petri dish and left under the time lapse photography machine overnight. Interval of photography is set to 1/1 hour. The results can indicate how/when the cell develops into polyploidy and experiences apoptosis.

Results

Immunofluorescent staining results shows a strong, positive correlation between the concentration of ATP-like drug HA-75 applied and the inability of locating motor proteins correctly. The concentration of HA-75 applied in this experiment are in ascending order: 5uM, 10uM, and 20uM. The more concentrated the drug is, the more significant the effect:

Staining of Aurora A protein that demonstrates the activity of the motor protein Aurora A during mitosis of a cell:

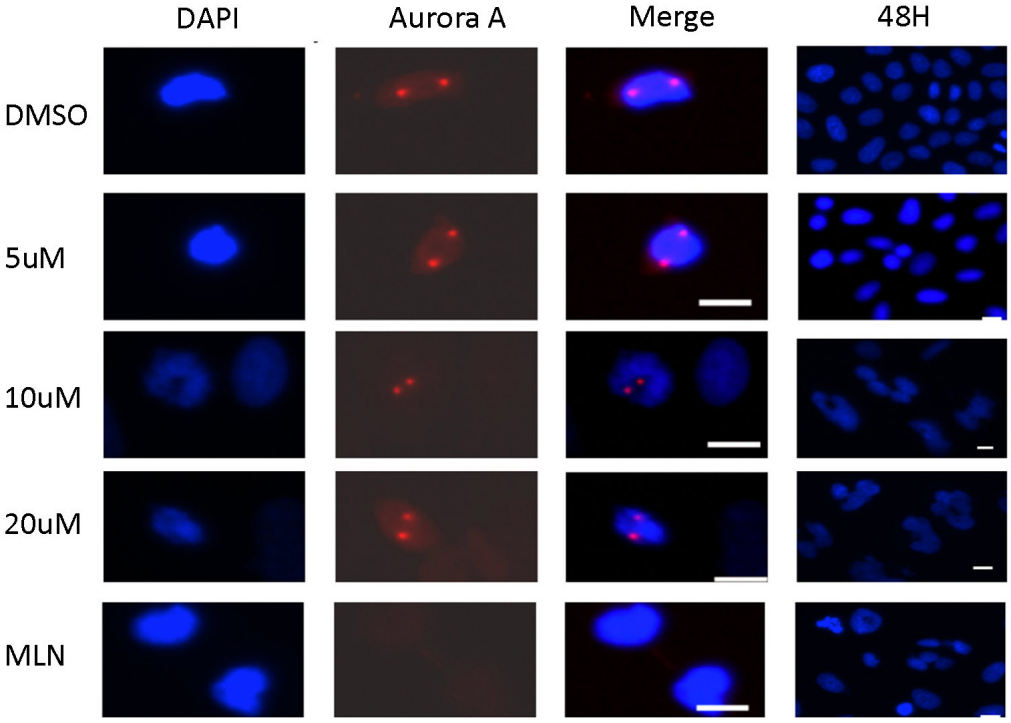


Figure 2.

The staining of Aurora A shows that the function of Aurora A motor protein is not affected by ATP-like drug. The positive control of MLN shows that no localization of Aurora A protein is visible when the function of Aurora A is suppressed.

Staining of Histone H3 ser10 protein in mitotic cell that demonstrates the localization of Aurora B protein activity in mitotic cell:

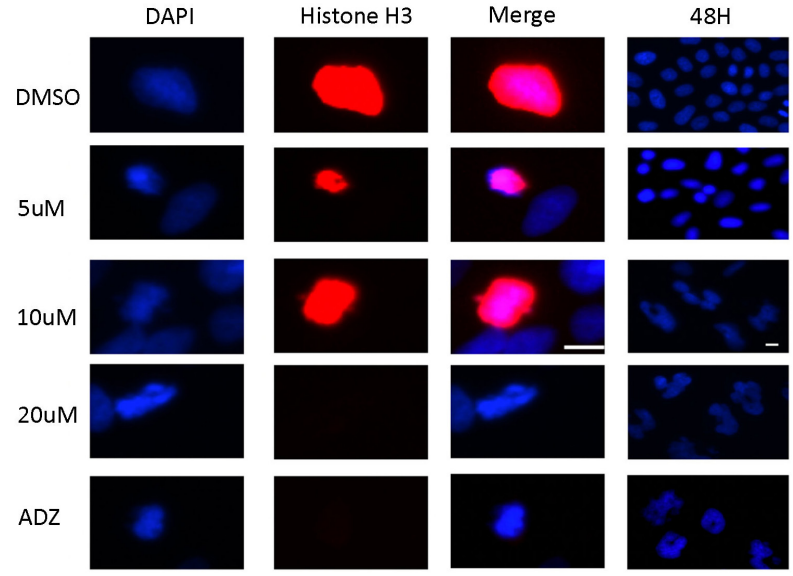


Figure 3.

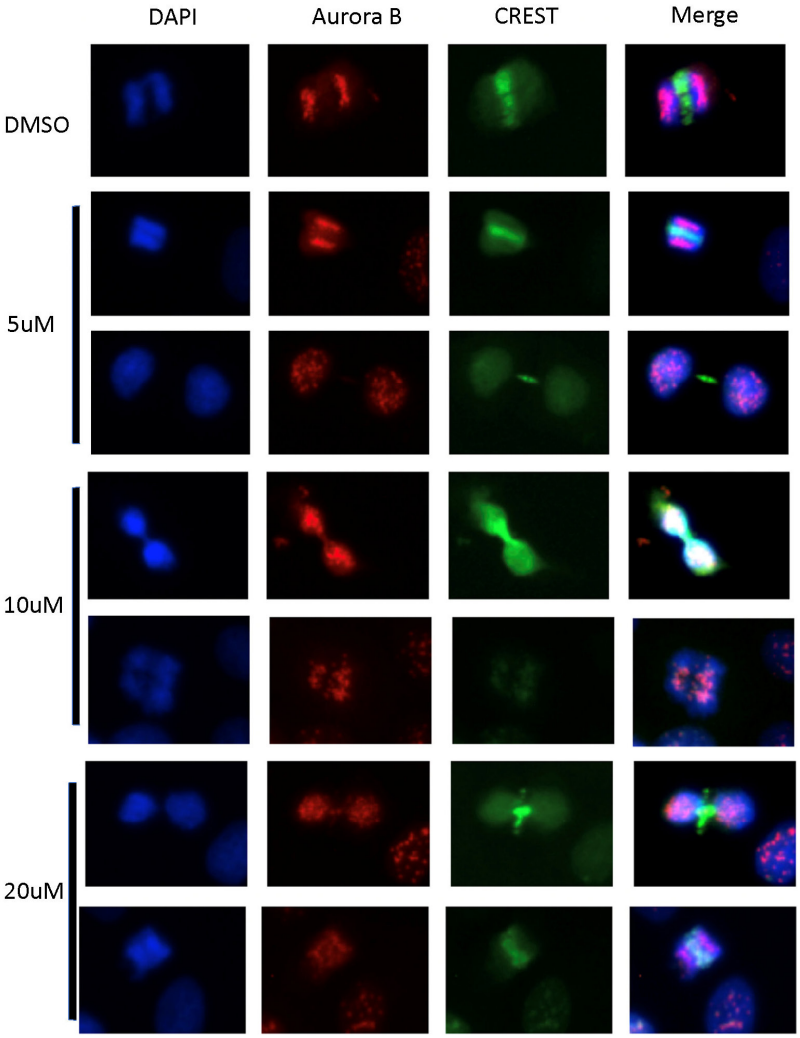


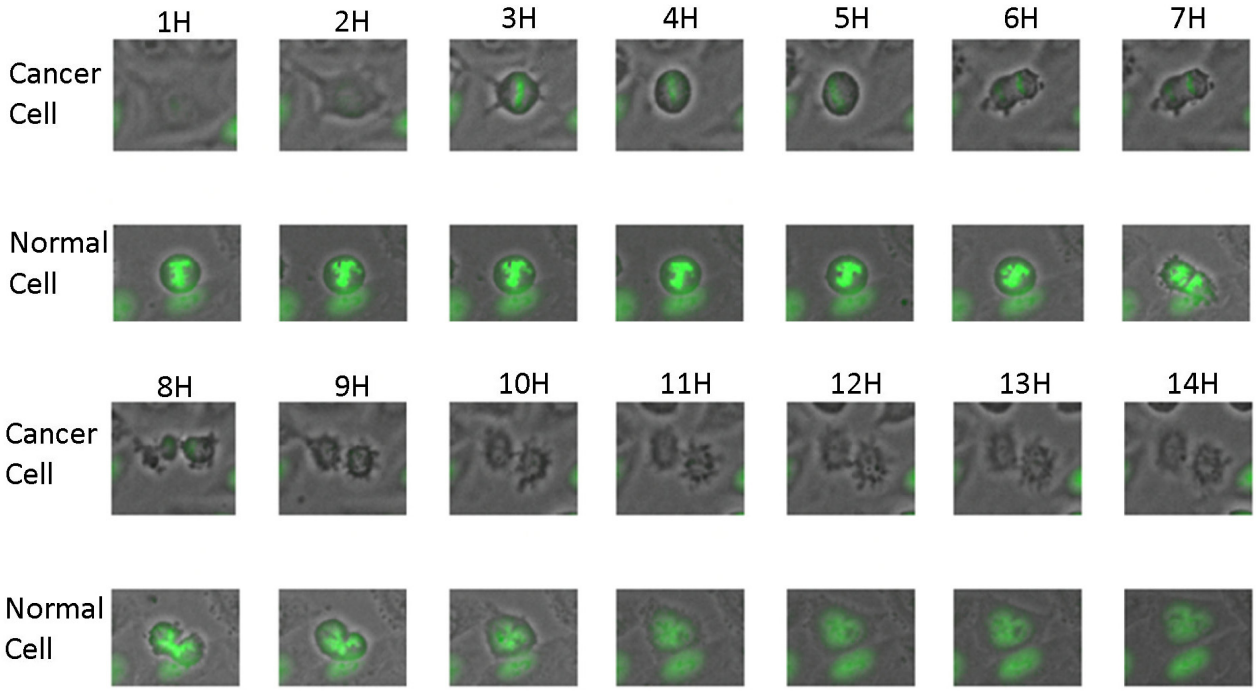
Figure 4.

The staining of Histone H3 ser10 protein showed that drug that are made in homologous to ATP does have effect on suppressing the localization of Aurora B proteins. The imaging at 20uM of HA-75 indicates that the Histone H3 had completely disappeared, similar to that of the positive control ADZ. This indicates that at 20uM the drug HA-75 suppressed the localization of Aurora B protein.

Staining of Aurora B and CREST protein in a mitotic cell that demonstrates the activity of MKLP2 protein in a mitotic cell:

The staining of CREST and Aurora B proteins shows that the drug HA-75 does have effect on the location of MKLP2 proteins. The imaging altered dramatically between the DMSO negative control group and the experimental groups. Imaging of both Aurora B and CREST proteins showed dislocation of the proteins at 10uM. this implies that MKLP2 is a key factor that is altered when ATP like inhibitors are introduced to a cancerous cell.

Time lapse results:



Time lapse photography clearly indicates difference between normal, untreated cells mitosis process and cancerous, treated cells mitosis process. The green region indicated on the photography is the DNA of the cell. In the cancer cell, the DNA is separated during anaphase, but the cell could not complete telophase correctly thus the cell develops into a polyploidy cell and apoptosis is triggered leaving the cell unable to divide. The normal cell successfully developed into two separate cells that both have viable DNA information (shown in green). This demonstrates the apoptosis process of cancer cell that had been treated by HA-75 drug.

Conclusion

From the study, many implications may be drawn. First the target of the drug simulating ATP is discovered, and the first stages of the experiments suggests that it is plausible that this is a revenue for inhibiting mitosis and causing mitotic catastrophe. The study also shows that taking Myc genes as a target is one of the most effective ways, this is shown both in Myc gene's pervasiveness in cancer and HA75's effects at treating Myc abnormalities.

This study also shows that HA-75 uses both Histone H3, MKLP2 protein, and other protein as targets to interfere with Mitosis. This indicates a multi-

target drug is a good way to attack cancer cell mitosis and to terminate the multiplication of cancer cells.

This study also suggests that with further structural change to increase the effectiveness, the drug HA-75 is a possible drug that can be used in reality. The effects of HA-75 on the cancer cell lines were effective and the concentration that was needed to cause a response in the cancer cells is low enough that the drug could be viable to be manufactured as a cancer-suppressant.

This study is also a prove that Synthetic Lethal treatment using biomolecular engineering is an extremely effective way of defeating cancer. This study determined a specific chemical to use as a template (ATP) because of its special uses in Mitosis and added molecules that will inhibit active sites to produce a drug that will effectively suppress cancer development. A similar approach could be applied to the recent Nobel award winning discovery of cellular oxygen regulation. A drug could be developed that will specifically inhibit the oxygen regulating protein, causing the cell to suffer hypoxia and die.

This study is a step towards the possibility of a general assay for cancer drug production: a targetable protein is identified that is unique to a type of cancer; an inhibitor is created specifically to inhibit that protein(s); and the inhibition occurs, ending mitosis of the cancer cell.

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