Original Article

Pharmacogenomics-Based Detection of Variants Involved in Pain, Anti-inflammatory and Immunomodulating Agents Pathways by Whole Exome Sequencing and Deep *in Silico* Investigations Revealed Novel Chemical Carcinogenesis and Cancer Risks

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What's Known

- Next-generation sequencing enabled new personalized medicine and pharmacogenomics theranostic options. Whole-exome sequencing panels are well-known targeted therapy in the diagnosis of disease pathogenicity and risk alleles in many phenotypes including various cancers.
- The pharmacogenomics impacts on carcinogenesis and cancer incidences in drug consumption, remarkably on pain pathways, are poorly studied.

What's New

- This study investigated the most challenging category of pharmacogenomics known as pain, anti-inflammatory, and immunomodulating agent pathways.
- 54 variants and 128 genes were suggested, a Pharmacogenomics-based Multiplex-Amplification Refractory Mutation System by polymerase chain reaction was established, and risks of chemical carcinogenesis resulting from dysregulation of investigated genes were found via *in silico* analyses.

Abstract

Background: Next-Generation Sequencing (NGS) methods specifically Whole-Exome Sequencing (WES) have demonstrated promising findings with a high accuracy of 91%-99% in Pharmacogenomics (PGx). A PGx-based panel can be utilized to minimize adverse drug reactions (ADRs) and maximize the treatment efficacy. Remarkably, Cancer Pain Management (CPM) is a cutting-edge concept in modern medicine. Thus, this study aimed to investigate the WES results by a PGx-based panel containing genes involved in Pain, Anti-inflammatory, and Immunomodulating agents (PAIma) signaling pathways.

Methods: A total of 200 unrelated Iranians (100 western and 100 northern) were included. 100 WES results were analyzed through the PAIma panel. After DNA extraction, 100 samples were genotyped by Multiplex-Amplification-Refractory Mutation System (ARMS) PCR. A primary *in silico* investigation performed on 128 candidate genes through Protein-Protein Interactions (PPIs) and Gene-miRNA Interactions (GMIs) via the STRING database, and miRTargetLink2, respectively. Additionally, Enrichment Analysis (EA) was applied to find the unknown interplays among these three major pathways by Enrichr.

Results: 55,590 annotations through 21 curated pathways were filtered, 900 variants were found, and 128 genes were refined. Finally, 54 candidate variants (48 non-synonymous single nucleotide variants (nsSNVs), 2 stop-gained, 1 frameshift, and 3 splicing) remained.

Conclusion: Conclusively, six potentially actionable variants including rs1695 (*GSTP1*), rs628031 (*SLC22A1*), rs17863778 (*UGT1A7*), rs16947 (*CYP2D6*), rs2257401 (*CYP3A7*), and rs2515641 (*CYP2E1*) had the most deviations among Iranians, compared with the reference genome, which should be genotyped for drug prescribing. Remarkably, PPIs, GMIs, and EA revealed potential risks of carcinogenesis and cancer phenotypes resulting from PAIma pathways genes.

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Introduction

One subset of patients may respond well to a certain medicine and experience major adverse drug reactions (ADR), whereas other patients may not experience any ADR or therapeutic benefit from it. Growing data suggests that an individual's genetic background plays a remarkable role in observing such different results, accounting for an estimated 20%-95% of diversity in drug administration and consequences.[1] Earlier reports documented that pharmacogenomics testing before drug prescribing leads to disease improvements for about 80 drugs.[2] Pharmacogenomics (PGx) studies the genetic variations in drug-metabolizing enzymes, receptors, transporters, and targets, as well as how these genetic variations link together to create drug-related phenotypes such as drug response or toxicity. Undoubtedly, the advent of next-generation sequencing (NGS) has opened up previously unimaginable capabilities for the analysis of whole genomes and generating a comprehensive portrait of each individual's variome.[3] The important point is that the proteins involved in Absorption, Distribution, Metabolism, and Excretion (ADME) determine the pharmacokinetic characteristics of drugs and their high variability affects drug safety and tolerance in different individuals and various ethnicities. The main factor determining this heterogeneity is the allelic frequency of ADME-associated variants.[4] Previous research has underscored the importance of prescribing the right drug(s) with precise dosage, emphasizing the advantages of personalized medicine and the negative impacts of neglecting PGx, particularly in cancer biology. Precision oncology has recently emerged, challenging its own reliable frameworks to ensure stability and broad implementation.[5, 6] Remarkably, cancer pain management (CPM) has introduced interesting alternatives to opioid prescribing for alleviating cancer-related pains. Therefore, pain-relieving drugs in CPM must be precisely prescribed based on each individual's PGx profile.[7] Notably, no study has reported the possible risks of cancer induction resulting from common drug metabolizing genes involved in pain, anti-inflammation, and immunomodulation through PGx detection.

The identification of variants in actionable pharmacogenes and their clinical application may be beneficial in medication prescription, dosage optimization, and the avoidance of ADRs. The approach utilized in the pharmacogenetic routine at the moment is the study of single nucleotide variations (SNVs) with high frequency in genetic panels.[8] Thus, by saving time in a proactive panel-based strategy, logic and cost-effectiveness will be maximized.[9] Preventive testing by a PGx-based panel can be used to minimize ADRs and improve the treatment efficacy.[2]

Current research and contentious issues have limited the use of pharmacogenomics as a pharmacological guiding tool in clinical trials. Thus, this study aimed to investigate the WES results of unrelated Western Iranians through a PGx-based panel containing variants involved in pain, anti-inflammatory and immunomodulating agents (PAIma) signaling pathways. To test the plausible impacts of population stratifications and to introduce rapid clinical testing of pharmacogenomic variations, the present study additionally set up a Multiplex Multiplex-Amplification-Refractory Mutation System (ARMS) PCR test on the unrelated healthy northern Iranians.

Material and Methods

Sampling and Data Collection

The studied population was 200 individuals including 100 healthy people who were referred to the Medical Genetic Laboratory (in the West of Iran, Kermanshah Province) and 100 healthy people from Guilan province (North of Iran). A written informed consent was obtained from each subject. This study was approved by the Research Ethics Committee of Isfahan University and the Biomedical Research Ethics Committee (code: IR.UI.REC.1402.092). The inclusion criteria were as follows: no abnormal clinical characteristics and disease-associated manifestations, normal results of the blood test, age higher than 20, and no consanguinity (relative) status. The exclusion criteria were considered as the healthy subjects with a patient child, subjects with relative parents, subjects who had taken drugs that were not related to the candidate genes, and subjects younger than 20 years old. The cut-off for age range was due to the majority of referred cases to the medical genetic laboratory; this frequently happens because healthy couples (older than 20 years old) request a carrier screening exome test. Finally, 100 WES tests were selected for the NGS analysis. The basic data was extracted from PharmGKB (https://www.pharmgkb.org/). The rationale behind this choice was that a few organizations give explicit guidelines for adjusting drug doses or recommending alternative treatments based on an individual's genetic data. PharmGKB annotates clinical guidelines developed by the Clinical Pharmacogenetic Implementation Consortium (CPIC), the Dutch

Pharmacogenetics Working Group (DPWG), and other professional organizations such as the Canadian Pharmacogenomics Network for Drug Safety (CPNDS) and the French National Network of Pharmacogenetic (RNPGx). The recommendations released by CPIC are developed in partnership with PharmGKB. PAIma category has 37 signaling pathways (21 curated pathways) and is among the most potential evidence-based and challenging pathways classified by PharmGKB. First of all, each of the 21 curated pathways in the PAIma category was investigated, and by filtering the unrepeated, significant, structural, and splicing variants, 55,590 annotations were found among PGx variants and Food and Drug Administration (FDA)-approved drugs. Finally, 128 genes had at least one nsSNV playing a potential role in the PAIma category. Further NGS analyses were performed on this PGx panel.

WES Tests and NGS Analyzing Strategies

All WES tests were done per the following pipeline: A filter-based method was employed for the extraction and purification of genomic deoxyribonucleic acid (gDNA) from the subjects' blood samples, which was then evaluated. For DNA preparation, 1.0 g of gDNA was utilized. The Agilent SureSelect Human All ExonV7 Kit (Agilent Technologies, CA, USA) was applied to generate sequencing datasets, and x-index codes were attached to the sample attribute sequences. Using a hydrodynamic shear procedure (Covaris, Massachusetts, USA), the DNAs were fragmented into 180-280 bp segments. The reactions of exonuclease/polymerase attenuated the residual overhangs, and enzymes were removed from the nest. Adapter oligonucleotides were ligated after adenylation of the 3' ends of the DNA pieces; DNA pieces with adaptor molecules linked at both ends were selectively chosen in a PCR reaction. Collected libraries were enriched with index tags in a PCR reaction to be ready for hybridization. The products were purified via the Beckman Coulter AMPure XP system (USA) and quantified with the Agilent Bioanalyzer 2100 System (USA) and the Agilent High Sensitivity DNA Assay (USA). The Illumina NovaSeq 6000 sequencer was fed with validated libraries. Data quality control, analysis, and interpretation were then carried out on a Unix-based operating system operating on a Generation G9 from an HP server (USA). NGS analyzing was carried out on every FASTQ file by Ubuntu (version 22.04.2) through a filtered-based command-line step and employing genomic packages including fastqc, IlluQC, Cutadapt, Alignment, Post-Alignment, Base Quality Score Recalibration (BQSR), Variant-calling, Variant Quality Score Recalibration (VQSR), Annotation with ANNOVAR, and Filtering. Annotation was performed via three levels including Gene-level (refGene), Region-level (cytoBand), and Frequency-level (cytoBand, exac03, dbnsfp30a, avsnp150, clinvar_20221231, regSNP-intron, and ICGC28) databases (See https://annovar.openbioinformatics.org/en/latest/user-guide/download/).[10] Following merging the candidate panel of 128 PAIma genes on the Variant Call Format (VCF) files, the commands were applied based on the variant (Reference Sequence) RS IDs, and the genotypes were extracted for each individual's variant of interest. Remarkably, to validate the exome results, 10% of the samples were checked by Sanger sequencing.

Rapid Detection by Multiplex-ARMS PCR

Using PAIma high-risk variants, a rapid PGx test was established to provide a time- and cost-saving pharmacogenomics-based test. This set of variants (six actionable variants) was selected from the most important genes of PAIma that had the most differences in minor allele frequency (MAF) compared to the reference genome (available at http://asia.ensembl.org/index.html; Cambridge, UK., and https://www.ncbi.nlm.nih.gov/snp/; NCBI, USA). To be more specific, the selection of these six variants comes from the comparison between the reference genome data (available in public databases) and acquired MAFs of 100 WES tests by calculating the combined frequencies of major and minor alleles according to the genotypic data; the most differed MAFs were selected for designing Multiplex PCR. This study was done to focus more on determining whether there is any plausible population stratification between northern and western Iranians or not by examining a 6-variant set on another Iranian ethnicity -the Guilaks (who reside in northern Iran). A set of primers were designed for six variants including rs1695 (*GSTP1*), rs628031 (*SLC22A1*), rs17863778 (*UGT1A7*), rs16947 (*CYP2D6*), rs2257401 (*CYP3A7*), and rs2515641 (*CYP2E1*). To optimize PCR conditions to achieve uniform amplification across all target variants, we confidently optimized the PCR conditions for Multiplex-ARMS PCR and randomly double-checked some variants in random samples, which indicated the same results.

In Silico Investigations

The primary *in silico* investigations were performed on 128 candidate PAIma genes to uncover the novel interactions in different levels, including Protein-Protein Interactions (PPIs),

Gene-miRNA Interactions (GMIs) by the STRING database version 12 (https://string-db.org/),[11] and miRTargetLink2 version 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2).[12] Moreover, a deep *in silico* analysis was designed through Enrichment Analysis (EA) applying Enrichr (https://maayanlab.cloud/Enrichr/) to investigate the known and unknown interplays among these three pathways.[13] The P values used in this study are calculated through a standard statistical method utilized by most enrichment analysis tools including Fisher's exact test or the hypergeometric test. This is a binomial quantity test assuming a binomial distribution and independence for the likelihood of any gene categorized in any set. The Q value also, is an adjusted P value measured by the Benjamini-Hochberg method for the correction of multiple hypotheses testing.

Results

Data Mining and Analyzing the WES Results

Data mining of 21 curated pathways in the PAIma category from the PharmGKB database represented 55,590 annotations, 900 significant variants impacting the FDA-approved drugs, and 128 genes. Following multiple filtrations, 128 genes remained, which comprised the primary gene list for the WES test analysis. Discarding the non-coding and synonymous variants, 54 candidate variants were found, which showed differences with the reference genome (hg38) based on different MAFs estimated for all 100 WES results. The PAIma panel contained 128 genes including *ABCB1, ABCC2, ABCC3, ABCC4, ABCG2, AKR1B1, AKR1C3, AMACR, ATF2, ATF3, BATF, CES1, CES2, CNR1, CNR2, CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C18, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A, CYP3A4, CYP3A5, CYP3A7, FAAH, FKBP1A, FOS, FOSB, FOSL1, FOSL2, GSTA1, GSTM1, GSTP1, GSTT1, HPGDS, IL2, IMPDH1, IMPDH2, JDP2, JUN, JUNB, JUND, MAF, MAFA, MAFB, MAFF, MAFG, MAFK, MAP2K3, MAP2K4, MAP2K6, MAP2K7, MAP3K1, MAP3K11, MAP3K7, MAPK14, MAPK8, NFATC1, NFATC2, NFATC4, NFKB1, NFKB2, NOS1, NOS2, NOS3, NRL, PLA2G2A, PLA2G4A, PPIA, PPP3CA, PPP3CB, PPP3CC, PPP3R1, PPP3R2, PTGDR, PTGDR2, PTGDS, PTGER1, PTGER2, PTGER3, PTGER4, PTGES, PTGES2, PTGES3, PTGFR, PTGIR, PTGIS, PTGS1, PTGS2, REL, RELA, RELB, S1PR1, S1PR3, S1PR5, SLC22A1, SLC22A11, SLC22A6, SLC22A7, SLC22A8, SLC22A9, SLCO1B1, SLCO1B3, SLCO2B1, SULT1A1, SULT1A3, SULT1A4, SULT1E1, SULT2A1, TBXA2R, TBXAS1, TGFB1, UGT1A1, UGT1A10, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2B15, UGT2B17, UGT2B4,* and *UGT2B7.* Genotype assessments of nsSNVs indicated 54 nsSNVs in the PAIma panel. Additionally, the MAFs of western Iranians revealed that 10 nsSNVs had a MAF of higher than 0.1 and four variants had a MAF of lower than 0.05. According to the function of variants, 48 variants out of 54, were nsSNVs. Furthermore, six variants were either splicing (rs2270860, rs776746, and rs4513095) or highly structure-damaging including two stop-gained (rs17863778 and rs145014075) and one frameshift (rs11572078) variants (table 1). It was found that some nsSNVs had overlapping roles as either functional (missense) or regulatory (promoter, Transcription binding site, enhancer, and CTCF) variants. As mentioned before, rs17863778 (*UGT1A7*) and rs145014075 (*CYP2A6*) are stop-gained mutations, rs11572078 (*CYP2C8*) is a frameshift, and rs2270860 (*SLC22A7*), rs776746 (*CYP3A*; *CYP3A5*), and rs4513095 (*CES1*) are splicing variants.

Multiplex-ARMS PCR as a Rapid PGx Test

To investigate the potentiality of the most different variants compared with the reference genome (GRCh38.p14; from the HapMap project, https://www.genome.gov/10001688/international-hapmap-project), and/or the 1000 Genomes project) and test the possibility of population stratification, the current study designed a Multiplex-ARMS PCR mini-panel as a rapid test on 100 samples from northern Iranians containing the important variants having major pharmacological impacts in the PAIma pathways. Genotyping was set up using ARMS-PCR protocol including 95 °C for 5 min, 95 °C for 40 sec; 59 °C (annealing time) for 40 sec, 72 °C for 40 sec (30 cycles), 72 °C for 5 min, and 4 °C for 5 min as holding time. This rapid test included various variants involved in neoplasm, type 2 diabetes, Human Immunodeficiency Virus (HIV) infection, expression level of *CYP2D6* in the liver, toxic liver disease, and transplantation (and kidney transplantation specifically). Investigating the results of 100 WES tests, six nsSNVs showed the highest differences with reference genomes, which were selected for designing the Multiplex-ARMS PCR. These variants are rs1695 (*GSTP1*), rs628031 (*SLC22A1*), rs17863778 (*UGT1A7*), rs16947 (*CYP2D6*), rs2257401 (*CYP3A7*), and rs2515641 (*CYP2E1*) (figure 1). The MAFs of rs1695 were estimated 0.33 (reference genome), 0.21 (WES tests; western Iranians), and 0.36 (Multiplex-ARMS PCR; northern Iranians). The MAFs of rs628031 were estimated 0.39 (reference genome), 0.66 (western Iranians), and 0.52 (northern Iranians).

Table 1: nsSNVs of PAIma panel with reference genome MAFs compared with Western Iranians MAFs

Variant	Gene(s)	MAF	MAF	Gt	Drugs	Association
rs72551330	UGT1A9	9E-3	1	T>A	Mycophenolate mofetil	Allele C is associated with diminished estimated glomerular filtration rate (eGFR) during the first year after engraftment when treated with mycophenolate mofetil in people with kidney transplantation as compared to allele T.
rs1801030	SULT1A1; SULT1A2	7E-3	9.95E-2	C>T		Genotypes CC+CT are associated with an increased likelihood of Hot Flashes in people with menopause as compared to genotype TT.
rs1751034	ABCC4	0.19	0.87	C>T	Tenofovir	Genotype TT is associated with increased tenofovir renal clearance, and lower AUC when treated with tenofovir as compared to genotypes CC+CT.
rs683369	SLC22A1	0.21	0.86	G>C	Imatinib	Genotype CC is associated with decreased response to imatinib in people with Leukemia, Myelogenous, Chronic, BCR-ABL Positive as compared to genotypes CG+GG.
rs4149117	SLCO1B3	0.16	0.86	T>G	Mycophenolate mofetil	Allele G is associated with decreased dose-normalized Cmax and dose-normalized AUC0to12 h when treated with mycophenolate mofetil in people with kidney transplants as compared to allele T.
rs2257401	CYP3A7	0.13	0.86	C>G	Tacrolimus	Allele C is associated with trough concentration of tacrolimus in people with Kidney Transplantation and Transplantation as compared to allele G.
rs2515641	CYP2E1	0.14	0.8	T>C	Cytarabine; fludarabine; gemtuzumab ozogamicin; idarubicin	Allele T is associated with a decreased likelihood of toxic liver disease when treated with cytarabine, fludarabine, gemtuzumab ozogamicin, and idarubicin in people with leukemia, myeloid, acute as compared to allele C.
rs1799983	NOS3	0.31	0.73	T>C	Hydrochlorothiazide	Genotype GG is associated with an increased reduction in diastolic blood pressure when treated with hydrochlorothiazide in people with Essential hypertension as compared to genotypes GT+TT.
rs628031	SLC22A1	0.39	0.66	A>G	Metformin	Allele A is associated with gastrointestinal toxicity when treated with metformin in people with Diabetes Mellitus, Type 2 as compared to allele G.
rs16947	CYP2D6	0.32	0.6	A>G		Genotypes AA+AG are associated with decreased expression of CYP2D6 in human liver cells as compared to genotype GG.
rs17868323	UGT1A10; UGT1A6; UGT1A7; UGT1A8; UGT1A9	0.4	0.58	T>G	Irinotecan	Allele G is associated with an increased risk of diarrhea within 24 hours and Thrombocytopenia when treated with irinotecan in people with Neoplasms as compared to genotype TT.
rs12529	AKR1C3	0.43	0.56	C>G	Antiandrogens	Genotype CC is associated with an increased risk of prostate cancer-specific mortality when treated with antiandrogens in men with prostatic neoplasms as compared to genotypes CG+GG.
rs7439366	UGT2B7	0.49	0.53	T>C	Lamotrigine	Genotype TT is associated with increased concentrations of lamotrigine in people with Epilepsy as compared to genotypes CC +CT.
rs4292394	UGT2B7	0.49	0.52	C>G	Methadone	Genotype GG is associated with increased severity of opiate withdrawal symptoms when treated with methadone in people with Opioid-Related Disorders as compared to genotypes CC+CG.
rs7438284	UGT2B7	0.49	0.52	A>T	Lorazepam; valproic acid	Allele A is associated with increased response to lorazepam and valproic acid in healthy individuals as compared to allele T.
rs1135840	CYP2D6	0.43	0.49	G>C	Aripiprazole	Genotype GG is associated with increased concentrations of aripiprazole in healthy individuals as compared to genotypes CC+CG.
rs1058164	CYP2D6	0.42	0.48	G>C	Aripiprazole	Genotype GG is associated with increased concentrations of aripiprazole in healthy individuals as compared to genotypes CC+CG.
rs6759892	UGT1A6	0.4	0.42	T>G	Deferiprone	Genotype GG is associated with increased response to deferiprone in people with beta-Thalassemia as compared to genotypes GT+TT.

Variant	Gene(s)	MAF	MAF	Gt	Drugs	Association
rs1105879	UGT1A10; UGT1A6; UGT1A7; UGT1A8; UGT1A9	0.35	0.4	A>C	Valproic Acid	Allele C is associated with increased glucuronidation of valproic acid.; Genotype AC is associated with an increased dose of valproic acid in people with Epilepsy as compared to genotype AA.
rs7586110	UGT1A9	0.35	0.39	T>G	Irinotecan	Allele G is associated with an increased risk of Anemia when treated with irinotecan in people with Neoplasms.
rs11692021	UGT1A10; UGT1A6; UGT1A7; UGT1A8; UGT1A9	0.35	0.38	T>C	Irinotecan	Genotypes CT+TT are associated with decreased likelihood of severe myelosuppression when treated with irinotecan in people with Neoplasms as compared to genotype CC.
rs2306283	SLCO1B1	0.43	0.37	A>G	Pitavastatin	Genotypes AG+GG are associated with increased pitavastatin plasma concentrations (AUC) and Cmax when exposed to pitavastatin in healthy individuals as compared to genotype AA.
rs3740066	ABCC2	0.33	0.36	C>T	Antiepileptics	Genotypes CT+TT are associated with increased resistance to antiepileptics in people with Epilepsy as compared to genotype CC.
rs1042597	UGT1A8	0.21	0.33	C>G	Cyclosporine; mycophenolate mofetil	Genotype CC is associated with increased risk of Diarrhea when treated with cyclosporine and mycophenolate mofetil in people with Kidney Transplantation as compared to genotypes CG+GG.
rs2070959	UGT1A10; UGT1A6; UGT1A7; UGT1A8; UGT1A9	0.32	0.31	A>G	Valproic acid	Genotype AG is associated with increased dose of valproic acid in people with Epilepsy as compared to genotype AA.
rs3745274	CYP2B6	0.26	0.29	G>T	Efavirenz	Allele A is not associated with decreased clearance of nevirapine in people with HIV Infections as compared to allele T.
rs2273697	ABCC2	0.19	0.29	G>A	Deferasirox	Genotype AG is associated with increased exposure to deferasirox in children with beta-thalassemia as compared to genotype GG.
rs2279343	CYP2B6	0.25	0.27	A>G	Efavirenz	Genotype GG is associated with increased concentrations of efavirenz in people with HIV Infections as compared to genotypes AA+AG.
rs28365063	UGT2B7	0.17	0.23	A>G	Carvedilol	Allele G is associated with decreased clearance of carvedilol in people with heart diseases as compared to allele A.
rs1695	GSTP1	0.33	0.21	C>T	Fluorouracil; irinotecan; oxaliplatin	Genotype AA is associated with an increased risk of neurotoxicity Syndromes and Neutropenia when treated with fluorouracil, irinotecan, or oxaliplatin in people with colorectal neoplasms as compared to genotypes AG+GG.
rs324420	FAAH	0.21	0.19	C>A	Methamphetamine	Allele A is associated with an increased risk of methamphetamine dependence due to methamphetamine as compared to allele C.
rs60140950	SLCO1B3	0.13	0.19	G>C	Telmisartan	Allele C is associated with increased concentrations of telmisartan in healthy individuals as compared to allele G.
rs1042028	SULT1A1	0.32	0.185	C>G		Genotypes CT+TT are associated with a decreased likelihood of Breast Neoplasms in people with at least three pregnancies and premenopausal as compared to genotype CC.
rs1126545	CYP2C18	0.15	0.18	C>T	Clozapine	Allele G is associated with an increased risk of Death when treated with clopidogrel in people with Acute coronary syndrome as compared to allele A.
rs4244285	CYP2C19	0.15	0.16	G>A	Cyclophosphamide	Genotype GG is associated with an increased risk of toxicity when treated with cyclophosphamide in women with Lupus Erythematosus, Systemic as compared to genotypes AA+AG.

Variant	Gene(s)	MAF	MAF	Gt	Drugs	Association
rs717620	ABCC2	0.19	0.13	C>T	Antiepileptics	Genotypes CT+TT are associated with increased resistance to antiepileptics in people with epilepsy as compared to genotype CC.
rs11045819	SLCO1B1	0.15	0.11	C>A	Fluvastatin	Genotype CC is associated with decreased LDL-C reduction when treated with fluvastatin in people with hypercholesterolemia as compared to genotypes AA+AC.
rs12208357	SLC22A1	0.07	0.1	C>T	Morphine	Genotype TT is associated with decreased clearance of morphine in children with adenotonsillectomy as compared to genotypes CC+CT.
rs1799853	CYP2C9	0.11	0.09	C>T	Celecoxib	Genotype CT is associated with higher plasma concentrations of celecoxib when exposed to celecoxib in healthy individuals as compared to genotype CC.
rs8187710	ABCC2	0.06	0.08	G>A	Rosuvastatin	Allele A is associated with decreased exposure to rosuvastatin in healthy individuals as compared to allele G.
rs1042008	SULT1A1	0	0.08	G>A	Desmethylnaproxen	Allele A is associated with decreased sulfation of desmethylnaproxen as compared to allele G.
rs3211371	CYP2B6	0	0.08	C>T	Cyclophosphamide; doxorubicin	Allele C is not associated with response to efavirenz or non-nucleoside reverse transcriptase inhibitors in women with HIV infections as compared to genotype TT.
rs1138272	GSTP1	0.08	0.07	C>T	Thiotepa	Allele T is associated with increased non-inducible thiotepa clearance and decreased tepa clearance when treated with thiotepa.
rs2306168	SLCO2B1	0.04	0.06	C>T	Rosuvastatin	Warfarin
rs12248560	CYP2C19	0.22	0.04	C>T	Clopidogrel	Allele T is associated with decreased cardiovascular events when treated with clopidogrel in people with acute coronary syndrome.
rs3842787	PTGS1	0.07	0.03	C>T	Rofecoxib	Allele T is associated with a reduction in COX-1 inhibition and depression of the urinary thromboxane metabolite when exposed to rofecoxib in healthy individuals as compared to allele C.
rs11568681	ABCC4	1.8E-2	0.02	G>T	17beta-estradiol glucuronide	Allele T is associated with increased catalytic activity of ABCC4 when assayed with 17beta-estradiol glucuronide as compared to allele G.
rs138417770	CYP2D6	0	5E-3	G>C	Bufuralol; dextromethorphan	Allele T is associated with decreased clearance of bufuralol or dextromethorphan in insect microsomes as compared to allele C.
rs17863778	UGT1A7	0.4	0.56	C>A	Atazanavir; Ritonavir	Genotype AA is associated with an increased likelihood of hyperbilirubinemia when treated with atazanavir and ritonavir in people with HIV infections.
rs145014075	CYP2A6	0.04	0.06	G>T	Nicotine	Genotype GT is associated with increased concentrations of nicotine as compared to genotype GG.
rs11572078	CYP2C8	0.17	0.31	T>TA	Carboplatin; Gemcitabine	Allele A is associated with decreased severity of thrombocytopenia when treated with carboplatin and gemcitabine in people with carcinoma and non-small cell lung cancer as compared to allele del.
rs2270860	SLC22A7	0.33	0.39	C>T	Capecitabine	Genotype TT is associated with an increased likelihood of drug toxicity when exposed to capecitabine in people with colorectal neoplasms as compared to genotypes CC+CT.
rs776746	CYP3A; CYP3A5	0.11	0.06	C>T	Tacrolimus	Genotype CC is associated with a decreased likelihood of recurrence when treated with tacrolimus in children with nephrotic syndrome as compared to genotype CT.
rs4513095	CES1	0.02	0.05	C>A	Sofosbuvir	Allele A is associated with a decreased response to sofosbuvir in people with chronic hepatitis C as compared to allele C.

MAF and Gt mean Minor Allele Frequency and Genotype, respectively. Note that rs17863778 and rs145014075 are stop-gained, rs11572078 is frameshift, and rs2270860, rs776746, and rs4513095 are splicing variants.

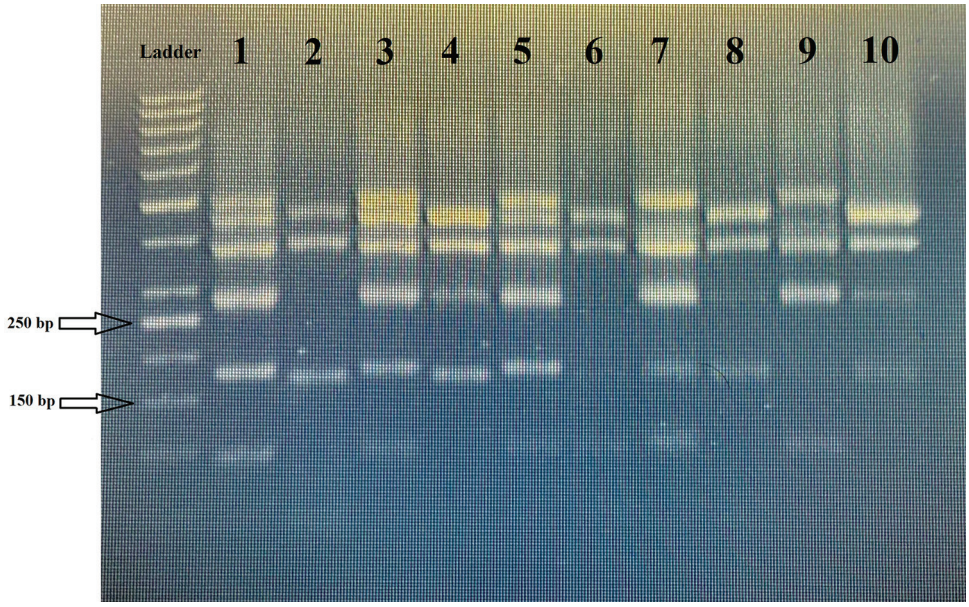

Figure 1: The Gel Electrophoresis image of Multiplex-ARMS PCR products for five samples including six pharmacogenomics-based variants, including rs1695 [*GSTP1*; 92 base pairs (bp)], rs628031 [*SLC22A1*; 180 bp], rs17863778 [*UGT1A7*; 288 bp], rs16947 [*CYP2D6*; 387 bp], rs2257401 [*CYP3A7*; 459 bp], and rs2515641 [*CYP2E1*; 508 bp]. Numbers 1 and 2 represent the major allele and minor for the 1st sample, and the other numbers following this major-minor order to the last numbers 9 and 10 for the 5th sample.

The MAFs of rs17863778 were estimated 0.4 (reference genome), 0.56 (western Iranians), and 0.38 (northern Iranians). The MAFs of rs16947 were estimated 0.32 (reference genome), 0.6 (western Iranians), and 0.48 (northern Iranians). For rs2257401, the reference, western, and northern Iranians MAFs were 0.48, 0.14, and 0.28, respectively. Finally, for rs2515641, the reference, western, and northern Iranians MAFs were 0.14, 0.2, and 0.24, respectively (table 2).

Table 2: Variants included in Multiplex-ARMS PCR as a rapid PAIma test

Variant	Genes	PCR Product (bp)	Gn	Drug(s)	MAF [ref]	Maf (WI)	MAF (NI)	Association
rs1695 [mis+ Promoter]	GSTP1	93	G>A	Fluorouracil; irinotecan; oxaliplatin	0.33	0.21	0.36	Genotype AA is associated with an increased risk of neurotoxicity syndromes and neutropenia when treated with fluorouracil, irinotecan, or oxaliplatin in people with colorectal neoplasms as compared to genotypes AG+GG.
rs628031 [mis= Enhancer]	SLC22A1	180	A>G	metformin	0.39	0.66	0.52	Allele A is associated with gastrointestinal toxicity when treated with metformin in people with diabetes mellitus, type 2 as compared to allele G.
rs17863778 [stop-gained]	UGT1A7	288	C>A	Atazanavir; ritonavir	0.4	0.56	0.38	Genotype AA is associated with an increased likelihood of Hyperbilirubinemia when treated with atazanavir and ritonavir in people with HIV Infections.
rs16947	CYP2D6	388	G>A		0.32	0.6	0.48	Genotypes AA+AG are associated with decreased expression of CYP2D6 in human liver cells as compared to genotype GG.
rs2257401	CYP3A7	459	G>C	Tacrolimus	0.48	0.14	0.28	Allele C is associated with trough concentration of tacrolimus in people with kidney transplantation and transplantation as compared to allele G.
rs2515641	CYP2E1	511	C>T	Cytarabine; fludarabine; gemtuzumab ozogamicin; idarubicin	0.14	0.2	0.24	Allele T is associated with a decreased likelihood of toxic liver disease when treated with cytarabine, fludarabine, gemtuzumab ozogamicin, and idarubicin in people with leukemia, myeloid, acute as compared to allele C.

Gn, bp, MAF, ref, WI, and NI refer to Genotype, base-pair, Minor Allele Frequency, reference, western Iranians, and northern Iranians, respectively.

To test the reliability of Multiplex-ARMS test genotypes, ten samples were confirmed by Sanger sequencing. The sequences of the Multiplex-ARMS PCR will be available upon request.

In Silico Findings

Protein-Protein Interactions (PPIs)

STRING database represents a unique network with completely linked 128 proteins of coding genes. This finding is a strong clue for including three pathways in one categorization as well as showing certain genes that have various variants in all these three pathways. PPI enrichment P value was lower than 1.0E-16 (figure 2).

Gene-miRNA Interactions (GMIs)

For investigating the regulatory interactions among the PAIma genes, miRTargetLink2 (version 2) was employed to find the potential miRNAs associations. By adjusting the strong validated evidence only, the output indicated a two-circled concentric model of GMIs with the inner circle having the most interactions. In the inner circle of concentric model, three miRNAs had the greatest interactions including hsa-miR-146a-5p associated with the *PTGS2, TGFB1, PTGES2*, and *NOS1* genes; hsa-miR-155-5p correlated with the *MAFB, FOS, JUN, NFKB1, MAPK14*, and *NOS3* genes; and hsa-miR199a-5p linked with the *MAFB, PTGES2, NFKB1, MAP3K11, JUNB,* and *SULT1E1* genes (figure 3).

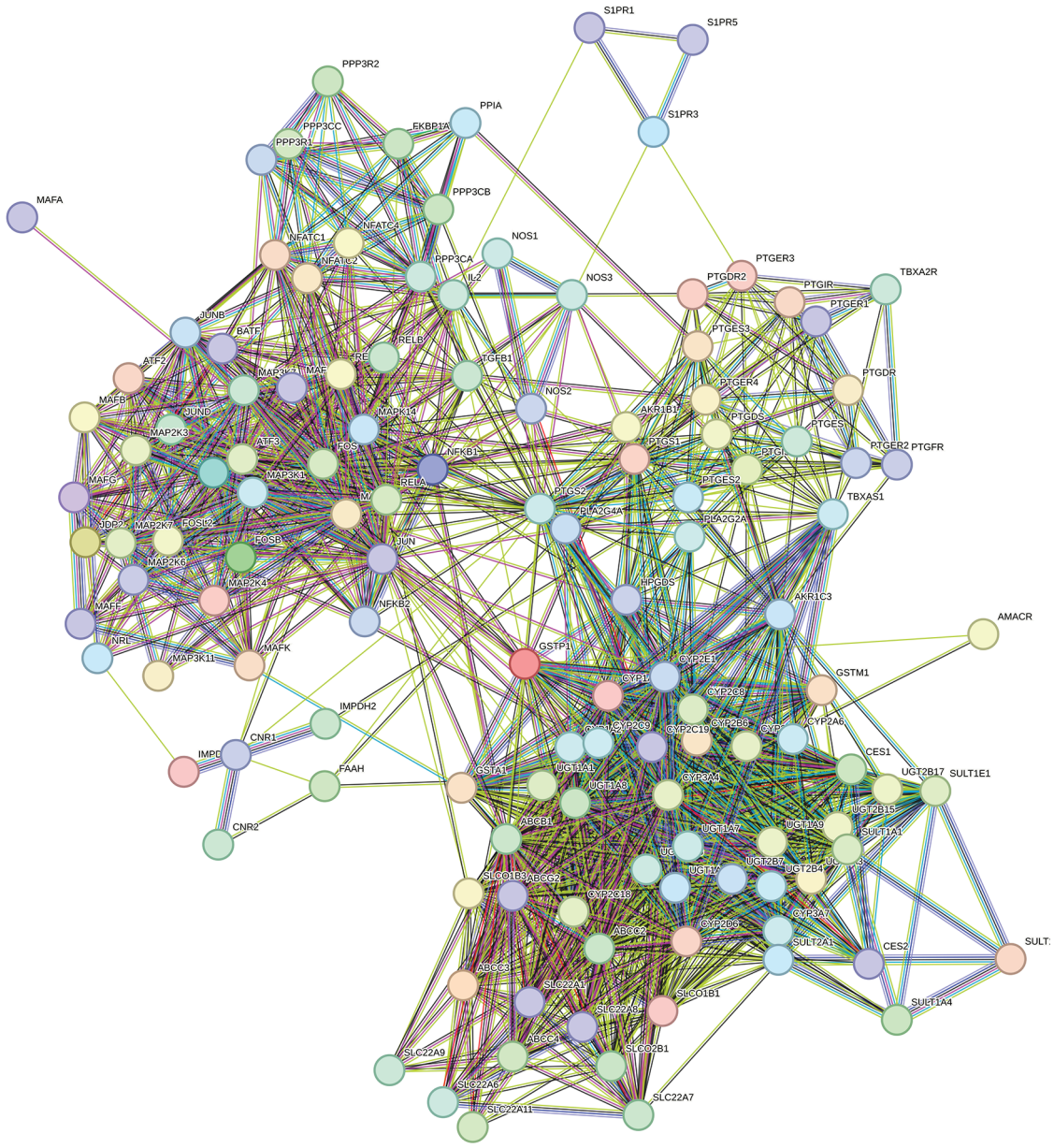

Figure 2: The STRING model of 128 genes involved in the PAIma signaling pathways.

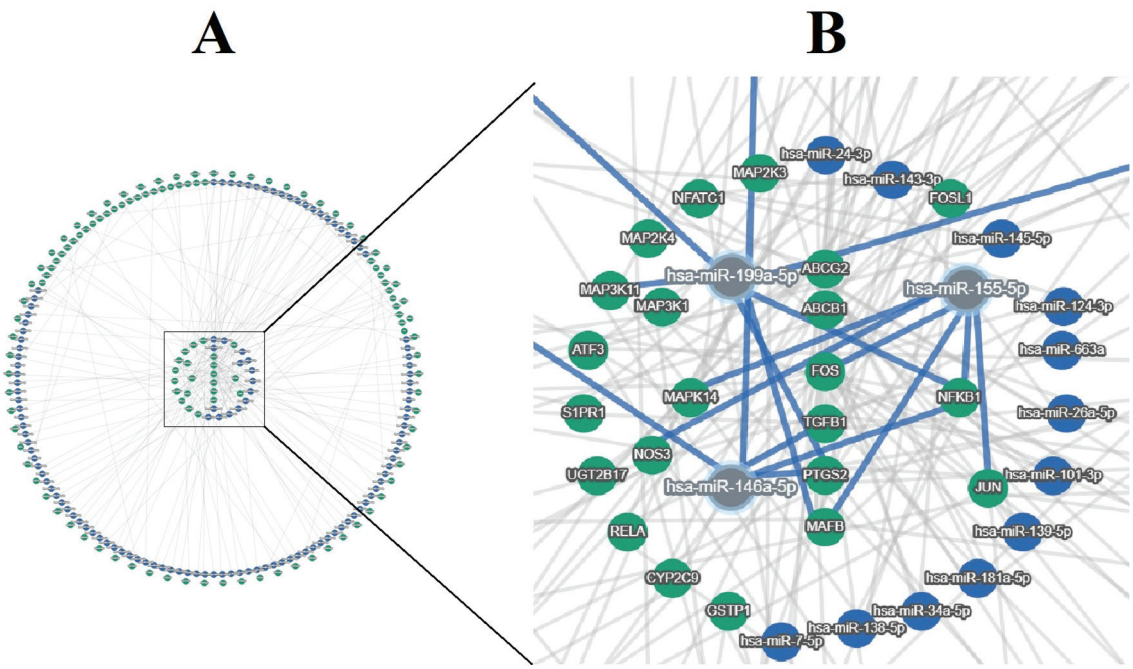

Figure 3: The concentric model of 128 PAIma genes, visualized by miRTargetLink2 and indicating A) The complete concentric model, and B) the inner circle of the concentric model.

Table 3: Enrichment Analysis of pharmacogenomics-associated genes involved in pathways by KEGG and Reactome

Index	Name	P value	Q value	OR
Reactome	Drug ADME R-HSA-9748784	5.25E-58	2.13E-55	138.9
KEGG	Chemical carcinogenesis	1.93E-44	3.64E-42	43.1
Reactome	Biological oxidations R-HSA-211859	9.01E-43	1.83E-40	44.23
KEGG	Drug metabolism	1.18E-41	1.11E-39	77.68
KEGG	Metabolism of xenobiotics by cytochrome P450	7.27E-39	4.58E-37	101.05
Reactome	Aspirin ADME R-HSA-9749641	2.70E-35	3.65E-33	169.37
KEGG	Bile secretion	4.82E-31	2.28E-29	64.75
Reactome	Paracetamol ADME R-HSA-9753281	3.40E-31	3.44E-29	276.52
KEGG	Retinol metabolism	2.23E-30	8.43E-29	82.78
KEGG	Osteoclast differentiation	6.60E-29	2.08E-27	44.29
KEGG	Steroid hormone biosynthesis	1.22E-27	3.31E-26	82.3
Reactome	Phase II- conjugation of compounds R-HSA-156580	9.64E-26	7.81E-24	45.15
KEGG	Arachidonic acid metabolism	7.15E-24	1.69E-22	69.02
KEGG	Lipid and atherosclerosis	3.50E-23	7.34E-22	23.78
Reactome	Arachidonic acid metabolism R-HSA-2142753	2.62E-22	1.77E-20	65.88
KEGG	MAPK signaling pathway	3.54E-21	6.69E-20	17.69
Reactome	Prostanoid ligand receptors R-HSA-391908	1.35E-20	7.82E-19	178848
Reactome	Metabolism R-HSA-1430728	3.05E-20	1.55E-18	6.33
Reactome	Phase I- functionalization of compounds R-HSA-211945	1.46E-19	6.56E-18	34.83
Reactome	Synthesis of prostaglandins (pg) and thromboxanes (TX) R-HSA-2162123	2.35E-19	9.53E-18	336.73

Q value refers to Adjusted P value and OR: means Odd ratio; Fisher's exact test, the hypergeometric test, and the Benjamini-Hochberg method were utilized for the P value, Q value, and OR calculations, repectively.

Enrichment Analysis (EA)

Enrichr was applied to test the EA of 128 PGx-associated genes. Results were extracted from three layers including Pathway analysis, Gene Ontology (GO), and Disease/Drugs analysis (DDA). Reactome and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases are considered for pathway analysis. According to Reactome and KEGG, the most significant pathways were Drug ADME (R-HSA-9748784) (Q value=2.13E-55) and Chemical carcinogenesis (Q value=3.64E-42), respectively. Notably, biological oxidations R-HSA-211859 (Q value=1.83E-40) and drug metabolism (Q value=1.11E-39) were in the third and fourth places (table 3). The most significant processes in GO analyses were as follows: Arachidonic Acid Metabolic Process

Table 4: Enrichment analysis according to the disease drugs analysis databases including DisGeNET and GeDiPNET for candidate genes

Index	Name	P value	Q value	OR
DisGeNET	Mammary neoplasms	1.27E-34	4.32E-31	10.07
DisGeNET	Asthma	1.92E-31	3.26E-28	11.15
DisGeNET	Lung neoplasms	1.21E-30	1.36E-27	11.4
DisGeNET	Malignant neoplasm of breast	2.88E-30	2.44E-27	8.31
DisGeNET	Breast carcinoma	5.24E-30	3.55E-27	8.19
DisGeNET	Liver carcinoma	2.73E-27	1.54E-24	7.26
DisGeNET	Malignant neoplasm of prostate	7.79E-27	3.39E-24	7.23
DisGeNET	Cholestasis	8E-27	3.39E-24	21.04
DisGeNET	Liver diseases	3.49E-26	1.31E-23	13.79
DisGeNET	Colorectal carcinoma	3.99E-26	1.35E-23	7.18
GeDiPNet	Lung Neoplasms	8.95E-18	5.44E-15	15.42
GeDiPNet	Prostatic neoplasms	2.15E-16	6.53E-14	9.15
GeDiPNet	Lung Cancer	1.69E-15	3.42E-13	12.68
GeDiPNet	Lucey-Driscoll syndrome	1.33E-14	1.61E-12	574.75
GeDiPNet	Crigler-Najjar syndrome	1.33E-14	1.61E-12	574.75
GeDiPNet	Gilbert disease	4.40E-14	4.39E-12	383.15
GeDiPNet	Kidney failure	5.05E-14	4.39E-12	13.5
GeDiPNet	Prostate cancer	2.10E-13	1.60E-11	6.8
GeDiPNet	Nervous system disorder	1.25E-12	8.42E-11	39.08
GeDiPNet	Acute kidney insufficiency	4.66E-11	2.83E-09	20.66

Q value and OR refer to adjusted P value and odd ratios, respectively. Fisher's exact test, the hypergeometric test, and the Benjamini-Hochberg method were utilized for the P value, Q value, and OR calculations.

(GO:0019369) with a Q value of 3.58E-26 in GO Biological Process, Endoplasmic Reticulum Membrane (GO:0005789) with a Q value of 7.33E-11 in GO Cellular Component, and Heme Binding (GO:0020037) with a q-value of 1.62E-15 and Prostaglandin Receptor Activity (GO:0004955) (Q value=1.72E-15) in GO Molecular Function were found for candidate genes. Lastly, Disease Drugs Analysis (DDA) indicated a high risk of various cancers based on DisGeNET and GeDiPNet. As shown in table 4, the most significant phenotype result from the interplays among candidate genes is Mammary Neoplasm (Q value=4.32E-31) according to the DisGeNET database and also the top-scored disease risk. Additionally, based on GeDiPNET database, Lung Neoplasms were scored as the high-risk phenotype (Q value=5.44E-15).

Discussion

This study conducted a multiplex-ARMS PCR as a PGx rapid test by genotyping six important PGx variants. Deep Enrichment Analysis predicted a high risk of chemical carcinogenesis and various cancer types, remarkably mammary and lung neoplasms, which may result from the drug metabolism processes of the 128 refined candidate genes.

To have a comprehensive overview of PGx literature, the PubMed search engine was filtered in the title for the two main keywords including "pharmacogenomics" and "exome". Results revealed 68 publications (2012-2023). Among them, 51 publications were related. Remarkably, nine of them had a panel-based strategy, but none of them mentioned a pathway-based strategy of analysis; thus, the present study might be the first PGx report to employ a pathway-based strategy of analysis on exome results. Several studies examined exome results and identified pharmacogenes, which mostly focused on the dispersed introduction of pharmacogenes and pharmacogenetic variants in various populations such as Spanish, Colombian, Thai, Dutch, American, Korean, and Canadian.[14-19]

Recent reports represented increasing attention to testing the population stratifications by investigating PGx panels. PGx-based panels according to these studies will be more powerful than other investigations introducing a set of pharmacogenes. Using the WES datasets of the Qatari population, Sivadas and others studied the PGx impacts of commonly prescribed antiplatelet drugs, warfarin, and clopidogrel.[20] Nagar and colleagues hypothesized that genetic ancestry would give better precision for stratifying PGx risks because it is a more reliable factor for genetic heterogeneity than self-identified race/ethnicity (SIRE), which has an important social component.[21] The current study is inconsistent with these reports.

We selected the PAIma pathways because this was the most challenging categorization in PharmGKB (which in turn is based on CPIC, DPWG, and other aforementioned well-known

guidelines). Another challenging issue was that this categorization includes 21 curated pathways such as the Diclofenac pathway, which in turn has various genes involved in different phenotypes; for instance, *ABCC2* (associated with epilepsy), *CYP2B6* (associated with HIV infections), *SLC22A7* (associated with colorectal neoplasms), and *SLCO1B1* (associated with hypercholesterolemia). Western and northern Iranian ethnicities are among the most important gene pools of Iran. By comparing their MAFs for the same variants, possible population stratifications were checked. As the most important gene in the heart of pharmacogenomics, CYP2D6 is included in this rapid test, which shows individuals carrying the genotypes AA+AG are associated with decreased expression of CYP2D6 in human liver cells compared to genotype GG. The other five variants of this test are associated with colorectal neoplasms (rs1695), type 2 diabetes (rs628031), HIV Infections (rs17863778), kidney transplantation (specifically) and transplantation (generally) (rs2257401), and Acute Myeloid Leukemia (AML) risk (rs2515641). An additional important response is that all cancers are along with various kinds of pain including chronic or acute pains during cancer metastasis, cancer therapies, or post-operative cancer pains. Therefore, there are increasing numbers of research and review articles concentrated on CPM. Opioids such as morphine and methadone are widely used in alleviating the pain of cancers and due to their addictive consequences, personalized medicine procedures are suggested for minimizing the side effects of opioid consumption.[7, 22, 23] Despite the experimental and clinical reports indicating critical associations between pain and cancer,[24-26] there is no study concentrated on the pharmacogenomics impacts of pain, inflammation, and immunomodulation pathways on cancer incidence and chemical carcinogenesis; thus, the deep *in silico* findings of this study for the first time uncovered the potential cancer risks resulting from PGx-associated variants, which need clinical follow-up and future studies on this new predictive application of PGx in cancer biology and CPM.

Remarkably, there were some limitations, which are highly recommended to be considered in future studies such as larger sample size, genotyping of the suggested panel, and included variants, specifically six important variants in the other Iranian ethnicities (central, southern, and eastern Iranians) and other ancestries including East Asians (Japanese, Chinese, Indians) European, Hispanic, and Americans. Additionally, to cover and investigate other variants in the intronic locations and non-exonic regions, future researchers can utilize the WGS technique or SNP-array technologies. Furthermore, incorporating experimental validation alongside computational analysis would enhance the robustness of current results and will improve the *in-silico* suggestions. Finally, while WES offers advantages in studying protein-coding regions, it's important to note that comprehensive detection of structural variants (SVs) and copy number variations (CNVs), particularly within pharmacogenes, is better suited for techniques such as WGS and long-read sequencing due to their broader coverage of the entire genome.

Conclusion

This study suggested a PGx panel containing 128 genes involved in PAIma pathways and also a time and cost-saving Multiplex-ARMS PCR test for genotyping six important PGx variants as a rapid test conducted on northern Iranians. Interestingly, primary and deep *in silico* findings proposed the chemical carcinogenesis and cancer risks resulting from dysregulations in drug metabolisms of pain, inflammation, and immunomodulation pathways, which play roles in novel unknown mechanisms that need to be studied soon. Notably, the PAIma genes and their included variants are presented as the first signaling-based PGx panel, which can be considered by working groups and utilized by NGS analyzers.

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Authors' Contribution

A.S: acquisition, analysis, and interpretation of data for the work, drafting the work; M.M: design of the work, reviewing the work critically for important intellectual content; P.K: interpretation of data for the work, reviewing the work critically for important intellectual content. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the

accuracy or integrity of any part of the work are appropriately investigated and resolved.
Conflict of Interest: None declared.

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