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Leveraging Contemporary Technology in Pharmacogenomics Research to Optimize Pharmacotherapy in Substance Abuse

Steven Tung[1*], Joseph Kitzmiller [2,3]

1Department of Anesthesiology, The Ohio State University, College of Medicine, Columbus, OH, USA

2Department of Biological Chemistry and Pharmacology, The Ohio State University, College of Medicine, Columbus, OH, USA

3Center for Pharmacogenomics, The Ohio State University, College of Medicine, Columbus, OH, USA

[*]**Corresponding author:** Steven R. Tung, MD, JD; Department of Anesthesiology, The Ohio State University College of Medicine, 410 West Tenth Avenue, Columbus, Ohio 43210, USA. Tel: +16142938487; Fax: +16142938153; Email: steven.tung@osumc.edu

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Abstract

Substance abuse is a healthcare epidemic with substantial costs to society, both financial and quality of life. Effective pharmacotherapy options for treating substance abuse are critical, and approaches employing precision/personalized medicine hold promise for optimization, not only for improving drug- and dose-selection strategies, but also for addressing reversible, substance abuse-environment-coupled epigenetic changes. By presenting some of the more commonly studied genetic biomarkers relevant to opioid pharmacotherapies, we provide a brief introduction to pharmacogenomics research. Also presented are comprehensive descriptions and examples of contemporary and emerging methodologies and technologies being leveraged to advance pharmacogenomics research.

Although the US comprises only about five percent of the world's population, it consumes nearly seventy percent of the world's opioid production – including ninety-nine percent of the world's hydrocodone [1]. As the uptick in opioid-related deaths continues, the urgency to halt (or at least substantially alleviate) this epidemic becomes increasingly paramount [2]. In 2015 there were 52,404 drug overdose deaths in the United States, and more than sixty percent involved an opioid [3]. In the subsequent year the age-adjusted death rate attributed to drug overdose increased by more than twenty percent to 63,600, representing a 3-fold increase from 1999 to 2016 [1]. Much of the opioid epidemic has been attributed to the consistent rise in use and subsequent abuse of nonmedical pain relievers (NMPR) in past decades. Although NMPR abuse is the most common pathway to abuse of stronger opioids [4], other risk factors include various environmental and genetic factors. Genetic predisposition is thought to be one of the larger contributors to risk (*e.g.*, up to fifty percent in some studies) [5], and both drug abuse and alcoholism share genetic influences including some that are both developmental stage dependent [6].

Pharmacogenomics research involves the study of the effects of genetic variation (*i.e.,* gene mutations or polymorphisms that alter structure, function or expression of gene-encoded proteins) on patient response to pharmaceuticals. As risk of substance abuse has a strong genetic component, pharmacogenomics is poised to provide meaningful guidance for tailoring approaches for risk determination and treatment of substance abuse as well as pain treatment strategies that minimize risk of substance abuse. For most pharmaceuticals genetic variation accounts for twenty-five to fifty percent of interindividual variation in drug response, and contemporary scientific literature suggests its contribution may be even larger for illicit drugs [7]. A few of the more commonly described functional genetic variants pertinent to substance abuse include enzymes, receptors and transporters involving dopamine, glutamate, serotonin and morphine [8-9].

One of the more well-studied genes influencing opioid response is the mu opioid receptor gene (*OPRM1*). Crist *et al*.

recently reported that a single nucleotide polymorphism (SNP pronounce "snip") in the mu opioid receptor gene (*OPRM1*) was associated with clinical outcomes in a 582-patient cohort of European-Americans of a 24-week, randomized, open-label trial of methadone or buprenorphine/naloxone (Suboxone) for the treatment of opioid dependence [10]. The authors report the SNP (rs10485058), characterized by a guanin to adenine substitution resulting in variant mRNA and reduced expression of mu-opioid receptors, was associated with decreased risk (relative risk=0.76, 95% confidence intervals=0.73–0.80, *P*=0.0064) of opioid abuse relapse as determined by urine screen. They also reported supportive findings from analysis of self-reported data in the Comorbidity and Trauma Study (CATS) of 1215 Australian opioid dependent individuals of European descent. In the CATS patient cohort, rs10485058 predicted abstinence of relapse (measured during the final 30 days of the 24-week study) after achieving abstinence (p=0.003) [10]. Importantly, Oslin *et al.* had previously reported rs10485058 was associated with efficacy of naltrexone for the treatment of alcohol dependence. Homozygous rs10485058-Carriers had lower rates of relapse (0.26 vs. 0.47, OR=2.27, p=0.044) compared to homozygous wild-type (normal) and heterozygous carriers of rs10485058 [11]. Polymorphisms in *OPRM1*, including rs10485058 and others (*e.g.*, rs1799971, rs2075572, rs558025, rs9384179 and rs62638690), demonstrates promise potential for aiding prescribers in opioid and dose selection as well as opioid-abuse treatment strategies to achieve optimal clinical outcomes.

Another important gene influencing opioid response is *CYP2D6*, encoding the metabolism enzyme cytochrome P450 family 2 subfamily D member 6 cytochrome. Evidence demonstrating its utility in opioid and dose selection is sufficient that official recommendations have been established by the Clinical Pharmacogenetics Implementation Consortium (CPIC) [12] and in FDA-approved drug labeling for certain opioids [13]. Comprehensive information including references to additional clinical and translational studies as well as population-specific polymorphism frequencies for polymorphisms in a myriad of genes affecting the pharmacology of opioids and/or a variety of other pharmaceuticals is maintained by the NIH-sponsored website, www.pharmgkb.org [14]. Recent technologic developments improving our capacity to examine the human genome have greatly improved our understanding of the interplay between genetic structure, regulation and the subsequent downstream effects on clinical response to pharmacotherapies. The clinical relevance of gene-medication associations continues to expand as does the list of FDA-approved drug labels containing pharmacogenomic guidance. In fact, more than 150 medications are now included in the FDA's Table of Pharmacogenomic Biomarkers in Drug Labeling table. [13].

Contemporary Technology in Pharmacogenomic Research

Bioinformatics

Bioinformatics applications include computational tools to organize, analyze, visualize and store information associated with biological macromolecules. Advances in bioinformatics allow for efficient processing and storage of large multivariate data sets of molecular structures, genetic interactions, high-throughput genotyping data and differential gene expression. With the aid of contemporary bioinformatics methodologies, gene expression studies can now be conducted without necessitating implementation of *in vitro* experiments. In addition, *in silico* gene expression analysis allows for quantitative analysis of the genes comprising the entire genome (rather than only a subset of genes fitted to a microchip array) [15].

Paramount to the recent transition of national health policy towards the principles of precision medicine, bioinformatics has merged with genomic medicine by (1) linking biobanks with electronic health records (EHR) for genomics analysis, (2) initiating patient genomics testing, (3) assimilating pharmacogenomics into routine medical care, and (4) utilizing genomics in drug development. [16].

Electronic Health Records and Biobanks

Electronic health records (EHR) are routinely utilized for storage and retrieval of patient health data, and inclusion of patient genomic data is becoming more common. Optimal EHR systems are user-friendly, scalable, and capable of storing large amounts of data for annotation, search and retrieval. As most patients are likely to change healthcare systems several times during their life, efficient, and accurate, transfer of data across various EHR platforms is another essential attribute. Common limitations in utilizing EHR for pharmacogenomics research include (1) inaccurate, incomplete or incompatible data, (2) inability to accurately identify and define phenotypes from available clinical data, (3) biased health records, and (4) difficulty in generating phenotype algorithms from complex data [17]. Biobanks are repositories of patient tissue specimens, often including genetic material. They are commonly linked to EHRs, creating a vast resource of genomic and health related data, enabling researchers to reclassify diseases based on molecular pathways. Schemes

linking EHRs to biobanks provide a powerful, unprecedented opportunity for investigating the relationship between heritability and drug response. Compared to traditional clinical trials, these methods involving the linkage of longitudinal phenotype data (captured via EHR queries of prescriptions, procedures and socioeconomic status) [18] to large patient repositories of either genetic data or access to biological samples for genomic analysis are superior for cost savings, efficiency and cohort size [19-20].

Genome-Wide and Phenome-Wide Association Studies

A Genome-wide Association Study (GWAS) is a statistical analysis that simultaneously measures association between hundreds of thousands of genetic polymorphisms and select phenotypes (traits). Without bias regarding location or plausibility of the genes and polymorphisms investigated, GWAS are particularly useful for ***discovery*** of association(s). A primary disadvantage of GWAS is that the p-value thresholds must account for multiple hypothesis testing (*e.g.,* p-values often need be $< 5x10^{-6}$ to be considered statistically significant rather than the customary p-value cut-off of $5x10^{-2}$), resulting in potentially important associations (those with biological plausibility) going undetected. Additional limitations include the inability to determine causality and linkage-dependent under- or over-estimation of association(s).

Coverage of most gene loci is commonly adequate to allow reliable utilization of proxies in GWAS. When the polymorphism of interest is not specifically included on a genetic assay, a proxy with known linkage to the polymorphism of interest can be substituted. The use of a proxy, however, introduces additional probability estimate(s) when linkage is not absolute. Haplotype maps (HapMap) are constructs of several proxy polymorphisms that can substitute (accurately predict) large genomic segments when several genotypes occur with near perfect linkage [21-23]. While GWAS have collectively made profound contributions to genetic discovery, GWAS findings alone are insufficient for properly informing clinical decision strategies. Replication studies utilizing a candidate-gene approach, limited to fewer select genes and polymorphisms, are superior for better characterizing association(s) and potential clinical importance. The utility of GWAS may likely be enhanced when coupled with emerging methodologies and technologies, providing greater clinical relevance and deeper understanding of the complex relationships constituting the heritability of response [24].

Measuring association(s) between genetic polymorphism(s) and a variety of phenotypes, Phenome-Wide Association Studies (PheWAS) are another complementary approach to GWAS. Phenotypic data, often retrieved from EHR(s), can be aggregated (*e.g.,* similarly-defined phenotypes can be combined assuming reasonable scale homogeneity) to drastically increase statistical power. As PheWAS-GWAS replication rates near seventy percent were reported as earlier as 2013 (near the infancy of PheWAS) [25], the potential for improved replication and PheWAS-GWAS symbiosis remains promising as PheWAS continue to integrate emerging methodologies and technologies [26].

Sequencing

Traditional DNAsequencing (Sanger sequencing) utilizes dideoxy nucleotide triphosphates (DDNTPs) as DNA-chain terminators, fragments large DNA polynucleotides into smaller components and utilizes computer-based assembly of the contiguous sequences. Considered the gold standard of DNAsequencing for the past 30 years, this method has been greatly successful in detecting monogenetic pathologic traits in well-recognized phenotypes. Primary limitations of Sanger sequencing include inability to identify copy number variants and limited success for studies involving complex, heterogeneous genetic diseases. As most diseases are not monogenic (i.e., their etiology can include hundreds of genes and mutations), Sanger sequencing has substantial limitations [27].

Compared with traditional sequencing, Next Generation Sequencing (NGS) provides superior (time, cost, accuracy and reproducibility) genetic sequencing. Introduced in 2007, NGS has led to great enhancements in our abilities to understand and interpret genetic data in disease and clinical decision models. As early as 2014, readily available services were able to offer genetic sequencing with nearly 100-fold improvement (financial and time cost per nucleotide) compared to traditional sequencing. For example, Illumina introduced the HiSeq X Platform in 2014 with reported capability to sequence 16 entire human genomes in 3 days at a depth of 30-fold at a cost of only 1,000 USD per genome [28]. NGS involves the direct sequencing of DNA molecules and does not rely on amplification *a priori* to sequencing. By eliminating the for DNA cloning and nucleotide-library construction steps, NGS has emerged as a cost-effective alternative to real-time Polymerase Chain Reaction (PCR) and DNA microarray methods for the evaluation of global gene expression [29].

Next Generation Sequencing has also advanced the understanding of human diseases through epigenetics, genomics and transcriptomics. This family of technologies provides the high-throughput computing power needed to run Large-scale Unbiased Sequencing (LUS), which includes DNA-Seq (genomics), RNA-Seq (Transcriptomics), ChIP-Seq and Methyl-Seq (epigenetics). DNA-Seq is applied to Whole Genome Sequencing and Whole Exome Sequencing.. Exomes are the coding portions of the genome (*i.e.,* the portion of sense that code for the amino acid sequence of the protein product). A newer technology, RNA-Seq, allows sequencing of the entire transcriptome, measuring environment-associated changes in gene expression

including altered transcription secondary to coding and noncoding genetic modifications. ChIP-Seq allows for sequencing of the epigenetic architecture of the genome, detecting genome-wide transcription factors, binding sites and chromatin-associated modifications. Methyl-Seq profiles DNA methylation at the single-nucleotide level. Collectively, these LUS methods provide genetic data that is far deeper, richer and more flexible compared with that from SNP-based genotyping, transcript profiling, or multigene panel sequencing [30].

Omics Bioscience

Epigenomics

Epigenetics is the study of heritable changes in gene expression that result from factors other than polymorphism in DNA sequence. Epigenetic modifications can have significant influence on both disease and patient response to pharmaceuticals. Importantly, epigenetic changes are potentially reversible and vary with age and tissue. Histone modification and DNA methylation are commonly studied epigenetic molecular mechanisms. A major epigenetic pathway for gene expression modification involves methylation of cytosine at the 5' position of CpG dinucleotide sites. Methylation at the promotor region of a gene leads to decreased gene expression, a signal for those genes to be silent. DNA methylation is essential for normal organismal development and for tissue-specific gene expression. Variation in DNAmethylation is influenced more by environment than genetics. Environmental influence is about 90% and genetic influence is about 10% [7]. Another pathway of epigenetic regulation chromatin remodeling (via methylation, acetylation and phosphorylation of the four histone proteins surrounding DNA).

Modifications of chromatin structure (chromatin includes DNA- and RNA-wrapped proteins) are an additional transcription regulatory mechanism. In short, DNA is wrapped around the nucleosome (consisting of a segment of DNAwound in sequence around a histone octamer consisting of 2 copies each of the core histones H2A, H2B, H3, and H4) with histone tails extruding from tightly packed histone protein cores. Exposed protein cores are subject to modification (methylation, acetylation and phosphorylation) of the histone tails to form specific combinations that establish open/closed transcription states regulating protein transcription. Although DNA methylation and chromatin remodeling regularly vary across different tissues and tissue regions, both acute and chronic administration of drugs of abuse result in specific transcription-modifying changes in the nucleus accumbent and other brain areas comprising the reward center paramount in addiction behaviors [31]. Epigenetic alterations occur over time or immediate and can be secondary to drugs of abuse, pharmacotherapy for drug addiction, prior stressful life experiences, or through genomic imprinting.

As epigenomic modifications occur in real-time, newer technologies allowing characterization of epigenetic markers across the genome need to account for epigenomic vulnerability, environmental stressors, nutritional assessments and cell-specific epigenetic patterns [32]. The DNA methylation state can be accessed via the Chromatin Immunoprecipitation Procedure (ChIP) consisting of the following: proteins are crosslinked with DNA to shear off DNA; fragments with methylated cytosines are extracted by immunoprecipitation utilizing antibodies specific for the 5'-methyl-cytosine; and the resulting DNA precipitate is purified, amplified, tagged with a fluorescent label, and subsequently subjected to DNA microarray assay. Modification of histones, another epigenetic modification, is studied via mass spectrometry. Recently reported modifications in histone H4 isoforms that regulate cell differentiation of human stem cells were detected using mass spectrometry [33].

Proteomics

Complementary to genomics approaches, proteomics involves the analysis of the complete set of proteins in an organism and can uncover protein regulation not dependent on changes in genetic code (*e.g.,* post-translational modifications secondary to environmental or multigenic influences). Clinical proteomic schemes generate protein profiles of various chemical compounds found in tissue-specific fluids to serve as biomarkers. Cryptic biomarker patterns in complex mass spectra require specialized bioinformatics algorithms [34], and validation in clinical studies requires a delicate balance of protective measures to reinforce study design and statistical analysis [35]. Proteomics also depends heavily on bioinformatics and technology. Computer algorithms for database query and retrieval, mass spectrometry for protein identification, ultra-performance liquid chromatography and nucleotide sequence databases must be carefully designed to ensure accuracy and reproducibility of studies.

Metabolomics

Metabolomics is the study of the complete set of metabolites in the organism. The metabolome is composed of a large diversity of small molecules that are molecular intermediates and end-products of different cellular and physiological processes. These small molecules are present during health, disease and during treatment and may function as biomarkers. Proteomics and metabolomics are highly linked with proteomics focusing on large molecules (proteins) and metabolomics focusing on small molecules (metabolites). Underlying this symbiotic relationship, proteins are designed to act on the metabolome and metabolites are designed to act on the proteome [36]. The downstream product of the genome are

endogenous metabolites resulting from transcription and translation. The environment acts on the organism and gives rise to the upstream product - exogenous metabolites (including metabolites of drugs of abuse). Metabolomics is an ideal tool for studying gene-environment interaction [37].

Metabolomics involves analytical chemistry coupled with computational methodologies to characterize complex biochemical mixtures. Separation methods typically include high-resolution liquid chromatography or gas chromatography, and metabolites are subsequently characterized by mass spectroscopy or nuclear magnetic resonance.

Transcriptomics

Gene expression is a dynamic process influenced by cellular, genetic and environmental influences. The transcriptome is the complete set of mRNA transcripts in a cell at a specific developmental stage or physiological condition. Transcriptome analysis reveals the molecular constituents of cells and functional elements of the genome, both essential for understanding mechanism of disease. Transcriptomics, also termed gene-expression profiling, is the direct analysis of mRNA as a representation of gene-expression patterns. While microarray technology has traditionally been used, analysis of the entire transcriptome can now be accomplished using RNA-seq. Sequencing of full length mRNA within a single cell allowing results in single-cell transcriptomic analysis, and utilization of bioinformatics-coupled high-throughput sequencing allows for *in situ* multi-omic detection analyses [38].

Gene expression can be regulated by small non-coding RNA species including small interfering RNA (siRNA) and microRNA (miRNA). They silence gene expression by blocking mRNA translation and their range of regulation (physical coverage) is about 30% of exons (gene segments that code for proteins) [39]. Derived from longer double-stranded RNAs processed to siRNA and miRNA, their discovery occurred while observing mammalian cell culture introduced to short double-stranded RNA oligonucleotides (siRNA) resulted in RNA interference (RNAi) without inducing interferon response [40]. Identification of siRNA species can be obtained via siRNA high-throughput screening experiments using > 5000 siRNA libraries assuming appropriate biostatistics methodologies [41].

While most pharmacogenomics studies have focused on polymorphisms in DNA exons (*i.e.,* DNA segments that code for the amino-acid sequence of the protein product), variants in DNA introns (*i.e,* regulatory segments of DNA including promotor, enhancers and miRNAs) can significantly influence drug response. Variants in miRNA impacts the protein product, and variation in enhancer or promotor regions impact mRNA. Human disease and variability in patients' drug response are associated with genetic variation in both intronic and exonic DNA segments. More than ninety-five percent of impactful SNPs reported have been within noncoding regions (*i.e.,* introns) [42].

With expression scores similar to that of microarrays, RNA-seq is an accurate and cost-effective method to analyze mRNA transcripts. RNA-seq can survey the entire transcriptome without prior knowledge of the transcribed regions and can identify novel and alternative transcripts of protein-coding genes not possible with microarrays [43]. RNA-seq datasets are generated by extracting, purifying and segmenting mRNA; subsequently producing cDNA by subjecting segmented mRNA to *reverse transcriptase*; attaching adapters to fragments and sizing selection via sequencing of the cDNA [29].

Systems Biology

A multidisciplinary, holistic approach, systems biology integrates all interacting networks of genes, proteins and biochemical reactions of the organism. The techniques and technologies of Systems Biology are those that support Epigenomics, Proteomics, Metabolomics and Transcriptomics. Although not focused on a drug of abuse nor on a pharmacologic treatment for substance abuse, Folkersen's *et al.* recent report of the COMBINE biobank, one of the largest collection of patient multi-omics data, nicely demonstrates the high potential for discovery and replication achievable via Systems Biology. The overall hypothesis, response to medication can be predicted by a precision-medicine Systems-Biology approach coupled with a multi-omics patient biobank, was tested with DNA, RNA and protein measurements in 451 blood samples (61 controls and 185 cases collected pre-treatment, baseline and 3-months post-treatment). Impressively, more than fifty percent of the variation in patient 3-month response was accounted with a sensitivity of 0.73 and specificity of 0.78 [44]. With the inclusion of other contemporary and emerging modalities (*e.g.,* epigenetics), Systems-Biology approaches will provide opportunity for discovery and integration of genetic biomarkers with real clinical significance.

The range of epigenetic changes characterized in substance abuse patients continues to expand as researchers increasing harness the investigative power of these emerging technologies and methodologies. The following provides some examples of recently reported and promising findings. **Nicotine** Epigenetic modification of DNA occurs in hematopoietic stem and progenitor cells during smoking and persists in peripheral blood many years after cessation, [45] is partially reversible, [46] and is transmitted to the fetus *in utero* by maternal smoking [47]. Maternal smoking is associated with variable DNA methylation in fetal lung and placental tissues that suggests a fetal origin for chronic pulmonary and immune-related diseases [48]. **Ethanol** Ethanol-induced up-regulation of the NMDA receptor of the

NR2B gene was found after chronic administration of ethanol and was associated with demethylation [49] and histone acetylation [50]. Fetal Alcohol Spectrum Disorders (FASD), characterized by irreversible cognitive and behavioral disability, results from significant ethanol exposure *in utero*. Demethylation of normally hypermethylated imprinted regions in sperm DNA is associated with chronic alcohol use, suggesting a potential molecular mechanism for paternal transgenerational transmission of FASD [51]. **Opioids** Epigenetic activation and silencing of mu opioid receptor (MOR) expression can be achieved through coordination at both the histone and DNA levels. DNA methylation and histone deacetylation at the *OPRM1* promoter decrease/silence *MOR* expression. The *in vivo* interaction between the histones and MeCP2, a methyl-CpG binding protein, which binds preferentially to methylated DNA and directly represses transcription, was reduced in the *MOR* promoter region upon differentiation, and *MOR* expression increased. When siRNA was used to disrupt the *MeCP2*, *MOR* expression increased [52]. The *MOR* gene in blood and sperm DNA was significantly increased in opioid addicts, suggesting evidence of a mechanism for transgenerational continuation of the opioid dependence phenotype [53]. **Cocaine** Acute and chronic administration of cocaine produces immediate and lasting gene expression changes through epigenetic modifications mirroring the behavior adaptations seen in human cocaine addiction. Acute cocaine treatment induced hypermethylation of the promoter region of the DNA methyltransferase gene (*DNMT*) and resulted in transcriptional downregulation (transcriptional silencing) in the nucleus accumbens. Repeat cocaine administration resulted in hypomethylation and upregulation of *fosB* (immediate early transcription factor) in the nucleus accumbens [54].

Conclusion

Substance dependency and addiction result largely from environment-gene interactions occurring in specific areas of the brain. Pharmacogenomics focuses on heritable variations in the genome leading to individual differences in drug response and interfaces with bioinformatics, molecular genetics, neuroscience, clinical pharmacology, EHR/Biobanking, genome and phenome-wide association studies, proteomics, transcriptomics, and metabolomics. Interdisciplinary systems-biology approaches are increasingly needed to harness these contemporary and emerging technologies and methodologies to adequately characterize this complex disease at the molecular level. The linking of EHR and biobank systems and the application of genomic science and technologies to substance abuse are highlighting the potential of personalized-medicine approaches related to substance abuse.

Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content of this article.

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