Aberrations in genes coding for cytochrome P450 family 2 and its putative association with HNSCC.

Apurva Choudhary[1], Anitha P[2], Balachander K[2], Smiline Girija A.S[1], Paramasivam A[2], Vijayshree Priyadharsini J[2*]

[1]Department of Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai 600077, India.

[2]Cellular and Molecular Research Centre, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai 600077, India.

Corresponding author:

Dr. J. Vijayashree Priyadharsini,

Associate Professor, Cellular and Molecular Research Centre, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai - 77, India.

ABSTRACT:

Cytochrome P450s include a large family of enzymes that catalyze the nicotine metabolism with a special emphasis on the metabolism of tobacco specific carcinogens. A few isoforms of this enzyme were found to activate procarcinogens to active carcinogens. One of the important risk factors of head and neck squamous cell carcinoma (HNSCC) is smoking which increases the risk by 5 to upto 25 folds. The present study aims to assess the gene alterations in the CYP2 family of cytochromes so as to derive an association with HNSCC. The analysis follows an observational study design, employing several computational tools to identify and predict the possible outcomes of gene alterations identified in HNSCC patients. cBioportal server was used to identify the gene alterations which was further analysed using tools such as PROVEAN, I-Mutant and gnomAD. A total of 37 genes of the CYP2B family were analyzed, among which 19 genes were identified to harbour gross abnormalities and variations. Polymorphisms in the CYP genes may be associated with diseases and adverse drug reactions. The highest frequency of gene alteration was identified in the gene CYP2AB1P (21%) followed by CYP2R1 (2.8%) and CYP2W1 (2.6%). Gene amplification was a common observation with single nucleotide variations including both synonymous and truncating variants. Several reported polymorphic variants were also identified. The gene alterations identified in the present studied predicted the pathogenicity and protein stability at standard biological conditions. Further experimental studies would provide concrete evidence on the association of observed genetic abnormalities with HNSCC especially in individuals exposed to habitual carcinogens.

Keywords: Cytochrome P450; HNSCC; In silico, mutations, amplification, deletions

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer which is responsible for over 350,000 deaths every year [1]. According to the global cancer observatory, GLOBOCAN, 2018, cancer of lip and oral cavity remains the primary cause of mortality due to cancer in males belonging to the south-Asian countries [2]. It has been demonstrated that more than 90% of the HNSCC arise from the mucosal surface of

the oral cavity, larynx and oropharnyx. The risk for oral cancer is precipitated by several factors such as tobacco chewing, chronic alcoholism, smoking, HPV (Human Papillomavirus) infection, sharp tooth in addition to the genetic makeup. Global Burden of Disease study documents lip and oral cavity cancer as the 15th most common type world-wide and the same occupying the top 10 positions in south-east Asia. Some parts of eastern Europe and in India the incidence of HNSCC is showing an elevation when compared to males. It has been estimated that over 20 males are affected per 100, 000 individuals [3]. Smoking tobacco such as cigarettes and pipes along with smokeless tobacco increased the risk of HNSCC by 2-4 fold [4,5]. The practice of betel nut chewing, a habit most commonly seen in the Asian subcontinent, increased the risk of HNSCC from 2 - 15 fold [6]. Cytochrome P450 or CYPs are enzymes that utilize iron to oxidize substances. They have a prominent role to play in the metabolism of a large variety of drugs and xenobiotic substances. The CYPs are classified based on sequence similarity and clustered into families for example CYP1, CYP2 and a subfamily letter (CYP2B) and isoform number of individual enzymes (CYP2D6) [7]. Polymorphisms in CYPs have been associated with an array of clinical complications including cancers. The association of CYP3A with aflatoxin induced hepatocellular carcinoma, CYP1A2 with heterocyclic arylamine exposure, CYP2E1 with nasopharyngeal cancer related to nitrosamine exposure etc., [8]. Despite the fact that the CYP enzymes are involved in metabolism of nicotine, not much information is available to elucidate the genetic alterations or genetic association with cancer types. In this context, the present study was designed to identify gross aberrations and assess the potential outcomes of the functional variants in HNSCC patients with respect to the cytochrome P450 family 2.

Materials and methods

Sample data set

The cBioPortal database (http://cbioportal.org) hosts a large collection of information regarding the molecular profiling information from cancer tissues and cell lines [9-10]. It is user friendly and contains genetic, epigenetic and proteomic information of the cases registered. The dataset used for the present study includes 528 HNSCC cases (530 samples) of which 504 samples harboured information about copy number variations and sequence data. The demographic details of cases in the head and neck squamous cell carcinoma (TCGA, Firehose Legacy) dataset were recorded (Table 1).

Oncoprint data analysis

The oncoprint data is obtained by submitting a user defined query of 36 genes belonging to the family of cytochrome P450. The results demonstrated genetic alterations in 19 crucial genes of the CYP2B family viz., CYP2AB1P, CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2B7P, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2D7, CYP2E1, CYP2F1, CYP2G1P, CYP2J2, CYP2R1, CYP2S1 CYP2W1. The frequency of somatic mutation and the location of mutation in the candidate genes were documented [9,10] (Table 2).

gnomAD analysis

The genome aggregation database (gnomAD) includes data from

approximately 125, 748 exome sequences and 15, 708 whole genome sequences from unrelated individuals. These data were collected and deposited as part of several population genetic and disease specific studies. This source of data was used to ascertain whether the variants identified in the present study are novel or reported elsewhere in other population [11] (Table 2).

Protein stability analysis

Single nucleotide change could have profound effect on the stability of the protein. The I-Mutant server was employed to identify the consequence of mutation and predict stabilization or destabilization conferred due to change in the amino acid composition. The reference protein sequence was retrieved in the FASTA format from the NCBI platform (https://www.ncbi.nlm.nih.gov/protein/). The interpretation was based on the free energy stability change (ΔΔG) value. A value less than and greater than zero implied decrease or increase in protein stability respectively [12] (Table 3).

Pathogenicity analysis

PROVEAN (Protein Variation Effect Analyzer) is yet another server employed to predict the impact of substitution of one amino acid with another (Table 3). The query was run for the missense variants using the reference sequence by setting the default cut-off value as -2.5. The interpretation of the result was based on amino acid substitutions and classified them as either neutral or deleterious depending on the PROVEAN scores [13]. A value less than -2.5 or greater than -2.5 was considered to be deleterious and neutral respectively (Table 3).

Results

Demographic data

The data from The Cancer Gene Atlas (TCGA), Firehose Legacy was included in the present study . The dataset included 528 HNSCC patients (530 samples) with a male:female ratio of 2.7:1. The age group was found to be in the range of 19 - 90 years. The frequency of individuals with the history of smoking and alcohol was roughly around 98% and 67% respectively. The dataset had samples from patients of different ethnic groups including American (85.6%), African (9.1%), Asian (2.1%) and American Indian (0.4%) descent**.** The distribution of patients based on the histologic grade of neoplasm is given in Table 1, of which 59% of patients had grade 2 tumor.

Oncoprint data analysis

The analysis of oncoprint data revealed gene amplification in 17 genes, of which CYP2AB1P (21%) presented with highest frequency of gene amplification, except for CYP2R1. The pattern of amplification as assessed in different groups of smokers showed a greater frequency of gene amplification in smokers with a current reformed smoking history of <15 years when compared to other categories. Interestingly, this gene along with 2 other genes CYP2B7P and CYP2G1P showed the presence of gross abnormality of gene amplifications without any other notable aberrations. The genes CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2E1, CYP2R1 and CYP2W1 demonstrated deep deletions. Although the site of deletion is not available, two patients showed deletion in all four genes CYP2C8, CYP2C9, CYP2C18 and CYP2C19. The CYP2R1 gene harboured the highest number of variations/mutations from among all the

genes identified with alterations (Table 2). Several truncating and mis-sense variants of unknown significance have been documented (Figure 1).

gnomAD analysis

A total of 23 reported variants were identified using gnomAD analysis viz., CYP2A7 (rs768997550), CYP2A13 (rs1048800592), CYP2B6 (rs577681802, rs1405598587, rs766630605), CYP2C8 (rs752621676, rs1253663790), CYP2C9 (rs754487195), CYP2C18 (rs1394512471, rs190647817, rs577595661), CYP2C19 (rs574462231, rs562912432), CYP2D6 (rs967434863), CYP2F1 (rs376080668), CYP2E1 (rs1176734765), CYP2J2 (rs1481873733, rs750655796, rs1189985425, rs146801076), CYP2R1 (11-14899670-A-G), CYP2S1 (rs759878389) and CYP2W1 (rs763439785). All the variants identified in the present study had a minor allele frequency < 0.01, implying the fact that these are rare variants which may be associated with risk of a particular disease.

Protein stability and pathogenicity analysis

Stability of the protein largely affects the biological function of the protein. Hence, protein stability was assessed for all the non-synonymous variants identified in the study. Majority of missense variants observed were found to decrease the stability of the protein product, thereby giving away a chance for influencing the catalysis process. Although presented with decreased stability all the variants were not found to lead to a deleterious phenotype. Interestingly majority of the variants produced neutral effect with exception in a few gene variants exhibiting deleterious outcomes viz., CYP2A13, CYP2C9, CYP2D6 and CYP2F1.

Discussion

A sustained exposure to environmental or habitual chemicals could impair cellular metabolism with an adverse effect on human health. Cytochrome P450 are a group of enzymes known to protect against toxic compounds. At times the biotransformations mediated by CYPs may result in the activation of procarcinogens into reactive carcinogenic products in a process referred to as “lethal synthesis” [14]. Elevating incidence of oral cancer in Asian countries where the exposure to smoking and smokeless tobacco and other related products underscore the need to understand the molecular mechanisms underlying the process of biotransformation of these chemicals. The present study focused on cytochrome P450 family 2, which is invariably related to the nicotine metabolism, thus imposing the need to study the genes of this family in relation to HNSCC [15].

Single nucleotide variations and copy number alterations in genes encoding drug metabolizing enzymes play a crucial role in the process of carcinogenesis. Loss of function variants in CYP genes were found to affect gene splicing thereby influencing the way the gene is expressed [16]. On the other hand, gain of function variants formed due to gene amplification or duplication, along with promoter and amino acid substitution variants have been found to increase the turnover of enzymes. The selection of substrates and induction of metabolic pathways are greatly affected by these variants [17, 18]. Our study also presents numerous genetic alterations in CYP2 gene family which might exert functional consequence in an individual possessing it.

A narrative literature review by Bandeira et al, identified several members of CYP family and subfamilies such as CYP2A6, CYP2A13, CYP2C19, CYP2E1 gene polymorphisms to be associated with the risk of HNSCC [19]. A meta-analysis based on 21 case control studies on CYP2E1 with HNSCC, with a special emphasis on smoking and alcohol history revealed that homozygous genotypes of PstI/RsaI or DraI polymorphism increased risk of head and neck cancer, in Asian population [20]. The present study also identified missense, truncating and rare variants in the CYP2E1 gene. Although the variants were shown to decrease the stability of the protein encoded, they were not found to be related to deleterious consequences. A similar meta-analysis assessed the risk of harbouring CYP1A1 and CYP2D6 polymorphisms in HNSCC, which revealed numerous studies with positive association between CYP2D6 polymorphisms viz., CYP2D6*4 and CYP2D6*10 in Asian population [21]. A large meta-analysis performed by Zhuo et al., employing data on 43 families of different ethnic groups revealed a significant association on CYP2E1 RsaI/PstI polymorphisms with HNC risk [22]. The text mining strategy employed to discuss the findings of the present study found that CYP2 related variants produced different results based on the ethnic groups and habits, which makes it more interesting and valuable to derive information about the magnitude of risk in certain populations. A PCR-RFLP approach used to screen male patients with HNSCC found significant association with HNSCC, its risk modifiers as well as therapeutic regimen, thus proving the fact that modifications in enzymes could influence the susceptibility to HNSCC and also the chemotherapeutic response to drugs [23]. Supportive evidences on the relatedness of CYP2 polymorphisms in Indian population showed high frequency of CYP2C9*2 and CYP2C9*3 in HNSCC cases, with the risk increasing upon chewing of betel quid, smoking and alcohol intake. In addition, cases with variant alleles were found to have a poor response to radio-chemotherapy [24].

The consequences of human genetic variations in the CYP2 family genes in connection with HNSCC has been reported in several studies. These variants were shown to exert their effect in metabolising anti-cancer drugs and demonstrated that certain genotypes were considered to be poor metabolizers leading to drug inactivity or toxic accumulation of drugs. A study conducted by Yadhav et al., identified that a greater frequency of drug non-responders had CYP2C19*2 (74%) and CYP2C19*3 (50%) [25]. Furthermore, a combination of poor metabolizing genotypes of CYP2D6 with CYP2C9 or CYP2C19 were found to interact synergistically in regulating the treatment response. Despite the fact that the role of these enzymes are not clearly established, their indirect association with disease risk is noteworthy. CYP2A13, the enzyme expressed in the extrahepatic tissues such as the respiratory tract, induces metabolic activation of nitrosamine present in nicotine, thereby enhancing smoking induced lung cancer. The variant allele, CYP2A13(*)2 was found to reduce the expression which is in part linked to the decreased incidence of lung adenocarcinoma in smokers. The role of genetic variants of CYP2A13 involved in the detoxification of tobacco components with risk of head and neck cancer was examined by Sharma et al [26]. The group identified two novel polymorphisms of

which one was found to be a protective genotype (T478C/T494C) and the other risk allele "T" of C578T was found to be exclusively present in cancer patients**.**

Another important finding of the present study is the high frequency of amplification observed in the CYP2AB1P gene, which is a pseudogene. The pseudogenes are inactive form of gene originated during the evolution process. They were once thought to be non-functional and non-protein encoding, but now it has been established that pseudogenes can also be transcribed to long non-coding RNAs (lnRNAs). These lnRNAs can exert its effect by acting as gene expression regulators or modifiers. The future scope of the study lies in screening the genetic alterations observed in the South Asian population, so as to prepare a panel of genetic markers which can be used to identify the at risk group who are exposed to direct or indirect carcinogens [27]. The study opens new avenues to develop cost effective methodologies to curate gene markers associated with a malignant phenotype. Such studies employing computational approach has aided in the identification of candidate genes and potentially deleterious variants which could have an effect on the HNSCC phenotype [28-34]. Extensive studies in various domains of research [35-44] have encouraged us to initate the persent study on cancer informatics.

The study presents some limitations which are discussed here (a) several different groups of individuals were included in the study, which made the study population more diverse than specific. Since each individual belonging to an ethnic group or population have been exposed to different chemical carcinogens, environmental pollutants or habits, a presice and strong conclusion about the relatedeness of the variants with the disease phenotype could not be derived, (b) epigenetic factors also play an influential role on the disease phenotype, hence, investigations on the methylation, histone modification, microRNA interference should be carried out to delineate the role of genetic factors upon HNSCC phenotype. The present study has taken a step forward to address the genetic abnormalities in the "hub genes" which could invariably convert compounds to its carcinogenic form eventually leading to malignant transformation of the cells. Advancements in computational approaches in biology have led to the development of methods which can be used for identification of genetic abnormalities within a short span of time in a cost effective manner. The present study is one such attempt to accumulate information related to the genetic abnormalities in the cytochrome P450 family B and HNSCC. Findings of the study might aid in solving the puzzle underlying the molecular process involved in the metabolism of xenobiotic chemicals and their role in HNSCC.

Conflicts of interest – All authors declare that they have no poten- tial conflict of interest for this work.

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Table 1: Demographic details of patients analyzed in the present study (as obtained from the cBioportal site)

Gender	Male (n = 386) Female (n = 142)
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515 Data not available: 12 Unknown: 1
Alcohol history	Yes – 352 No – 165 Data not available: 11
Neoplasm Histologic grade	Grade 1: 63 Grade 2: 311 Grade 3: 125 Grade 4: 7 Grade GX: 18 Data not available: 4
Race category	White: 452 African: 48 Asian: 11 American Indian or Alaska native: 2 Data not available: 15

Table 2: Genetic alterations in genes associated with Cytochrome P450 family 2

Gene	Protein	Alteration	Cytogenetic Loci	% of alteration	Variant allele frequency in tumor sample	gnomAD Frequency data
CYP2AB1P	Cytochrome P450 family 2 subfamily AB member 1, pseudogene	Gene amplification	3q27.1	21	-	-

CYP2A6	Cytochrome P450 family 2 subfamily A member 6	Gene amplification E152K E390K	19q13.2	1.2	- 0.15 0.35	- Novel Novel
CYP2A7	Cytochrome P450 family 2 subfamily A member 7	Gene amplification E63V D108H R446K	19q13.2	1.4	- 0.17 0.07 0.10	 Novel Novel rs768997550
CYP2A13	Cytochrome P450 family 2 subfamily A member 13	Gene amplification G5R R190C F155S K425N	19q13.2	1.6	- 0.25 0.29 0.29 0.03	- Novel rs1048800592 Novel Novel
CYP2B6	Cytochrome P450 family 2 subfamily B member 6	Gene amplification G71* Q286L L470V L88P A176T	19q13.2	1.8	- 0.11 0.41 0.23 0.02 0.45	- rs577681802 Novel Novel rs1405598587 rs766630605
CYP2B7P	Cytochrome P450 family 2 subfamily B member 7, pseudogene	Gene amplification	19q13.2	0.8	-	-
CYP2C8	Cytochrome P450 family 2 subfamily C member 8	Gene amplification Deep deletion P427H K474Q R144H E326D D265H	10q23.33	1.8	- - 0.14 0.35 0.12 0.06 0.06	- - rs752621676 Novel rs1253663790 Novel Novel

		S482P				
CYP2C9	Cytochrome P450 family 2 subfamily C member 9	Gene amplification Deep deletion E93K A149T E253K K138N *491Rext*42	10q23.33	1.8	- - 0.03 0.45 0.12 0.19 0.64	- - Novel rs754487195 Novel Novel Novel
CYP2C18	Cytochrome P450 family 2 subfamily C member 18	Deep deletion W212* E199* E122D R478S	10q23.33	1	- 0.11 0.12 0.12 0.21	- Novel rs1394512471 rs190647817 rs577595661
CYP2C19	Cytochrome P450 family 2 subfamily C member 19	Deep deletion E154D E400Q F239L L287F R261G I387T	10q23.33	1.6	- 0.24 0.21 0.15 0.18 0.32 0.29	- Novel Novel Novel Novel rs574462231 rs562912432
CYP2D6	Cytochrome P450 family 2 subfamily D member 6	Amplification P102S Y124C A305V G471V	22q13.2	1.2	- 0.09 0.37 0.10 0.45	- Novel Novel rs967434863 Novel

CYP2D7	Cytochrome P450 family 2 subfamily D member 7	Amplification	22q13.2	0.4	-	-
CYP2F1	Cytochrome P450 family 2 subfamily F member 1	Amplification R336W G92E	19q13.2	1.2	- 0.63 0.24	- rs376080668 Novel
CYP2E1	Cytochrome P450 family 2 subfamily E member 1	Amplification Deep deletion R149S R233* I476V I289M R374L T266S	19q13.2	1.8	- - 0.45 0.13 0.25 0.18 0.43	- - Novel Novel rs1176734765 Novel Novel Novel
CYP2G1P	Cytochrome P450 family 2 subfamily G member 1, pseudogene	Amplification	19q13.2	0.8	-	-
CYP2J2	Cytochrome P450 family 2 subfamily J member 2	Amplification S80* R117* T143A A10V R19G F201L	1p32.1	1.8	- 0.24 0.13 0.08 0.31 0.11 0.19	- rs1481873733 rs750655796 rs1189985425 rs146801076 Novel Novel
CYP2R1	Cytochrome P450 family 2 subfamily R member 1	Amplification Deep deletion K3N E160Q E203K A17T W414C	11p15.2	2.8	- - 0.29 0.15 0.18 0.37 0.09 0.27 0.30	- - Novel Novel Novel Novel Novel Novel

		502Lext ? G236S I76F				11-14899670-A-G Novel Novel
CYP2S1	Cytochrome P450 family 2 subfamily S member 1	T501R	19q13.2	0.8	0.08	rs759878389
CYP2W1	Cytochrome P450 family 2 subfamily W member 1	P277S V261M	7q22.3	2.6	0.42 0.20	Novel rs763439785

Table 3: Protein stability and pathogenicity analysis of functional variants identified in the study

Gene	**Alteration**	**I-Mutant prediction**	**I-Mutant Score**	**PROVEAN prediction**	**PROVEAN Score**
CYP2A6	E152K E390K	DECREASE DECREASE	-2.6 -1.17	DELETERIOUS NEUTRAL	-3.594 -1.649
CYP2A7	E63V D108H R446K	DECREASE DECREASE DECREASE	-0.67 -1.02 -1.76	DELETERIOUS DELETERIOUS NEUTRAL	-4.834 -3.293 -2.447
CYP2A13	G5R R190C F155S K425N	DECREASE DECREASE DECREASE INCREASE	-0.8 -1.71 -1.48 0.08	DELETERIOUS DELETERIOUS DELETERIOUS DELETERIOUS	-2.962 -6.900 -3.628 -4.396
CYP2B6	Q286L L470V L88P A176T	INCREASE DECREASE DECREASE DECREASE	0.07 -1.50 -2.31 -1.02	DELETERIOUS NEUTRAL DELETERIOUS NEUTRAL	-3.789 -1.012 -6.179 -1.712
CYP2C8	P427H K474Q R144H E326D D265H S482P	DECREASE DECREASE DECREASE DECREASE DECREASE DECREASE	-1.13 -1.12 -1.86 -0.19 -1.47 -1.29	DELETERIOUS NEUTRAL DELETERIOUS DELETERIOUS DELETERIOUS NEUTRAL	-6.652 -0.344 -4.402 -2.751 -6.101 -1.142

CYP2C9	E93K	DECREASE	-0.75	DELETERIOUS	-2.864
	A149T	DECREASE	-0.66	DELETERIOUS	-3.812
	E253K	DECREASE	-0.80	DELETERIOUS	-2.502
	K138N	DECREASE	-0.51	DELETERIOUS	-4.830
CYP2C18	E122D	INCREASE	0.31	NEUTRAL	-2.383
	R478S	DECREASE	-2.76	NEUTRAL	2.109
CYP2C19	E154D	DECREASE	-0.91	DELETERIOUS	-2.603
	E400Q	DECREASE	-1.10	DELETERIOUS	-2.584
	F239L	DECREASE	-2.52	NEUTRAL	-1.408
	L287F	DECREASE	-0.76	DELETERIOUS	-3.712
	R261G	DECREASE	-0.86	DELETERIOUS	-5.763
	I387T	DECREASE	-0.99	DELETERIOUS	-3.571
CYP2D6	P102S	DECREASE	-1.31	DELETERIOUS	-6.495
	Y124C	INCREASE	1.02	DELETERIOUS	-5.470
	A305V	INCREASE	0.41	DELETERIOUS	-3.846
	G471V	INCREASE	0.57	DELETERIOUS	-6.901
CYP2F1	R336W	DECREASE	-0.50	DELETERIOUS	-6.728
	G92E	DECREASE	-0.59	DELETERIOUS	-4.489
CYP2E1	R149S	DECREASE	-1.44	NEUTRAL	-0.734
	I476V	DECREASE	-1.19	NEUTRAL	0.128
	I289M	DECREASE	-1.22	NEUTRAL	-0.182
	R374L	DECREASE	-1.02	NEUTRAL	-2.210
	T266S	DECREASE	-0.40	NEUTRAL	-1.351
CYP2J2	T143A	DECREASE	-1.04	NEUTRAL	-1.384
	A10V	INCREASE	1.07	NEUTRAL	-1.333
	R19G	DECREASE	-0.77	NEUTRAL	-2.413
	F201L	DECREASE	-0.85	DELETERIOUS	-5.674
CYP2R1	K3N	DECREASE	-0.16	NEUTRAL	-0.365
	E160Q	DECREASE	-0.14	NEUTRAL	-1.833
	E203K	DECREASE	-0.80	NEUTRAL	-1.562
	A17T	DECREASE	-1.51	NEUTRAL	-0.715
	W414C	DECREASE	-1.54	DELETERIOUS	-11.695
	G236S	DECREASE	-1.00	NEUTRAL	0.149
	I76F	DECREASE	-1.97	DELETERIOUS	-3.518
CYP2S1	T501R	INCREASE	0.23	NEUTRAL	-0.299
CYP2W1	P277S	DECREASE	-0.58	NEUTRAL	-0.681
	V261M	DECREASE	-1.6	NEUTRAL	0.179

Figure 1: Oncoprint data showing genetic alterations in the cytochrome P450 family 2 genes