PGx of Chemotherapy- Induced Peripheral Neuropathy (CIPN): CYP2D6 Genotyping and Validation

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BACKGROUND

Chemotherapy-induced peripheral neuropathy (CIPN) in cancer patients is defined by sensory-motor neuropathy due to chemotherapeutic drug exposure. Prediction of CIPN could be achieved by characterizing patient pharmacogenetic (PGx)-related single-nucleotide polymorphism (SNP) profiles. One common PGx gene- the cytochrome P450 (CYP) 2D6 (*CYP2D6)* is involved in the metabolism of ~21% of currently approved medications, including chemotherapeutic drugs commonly prescribed for cancer treatment. We used Single-Nucleotide Extension (SNE) and a Sanger sequencing-based approach to genotype common clinical variations in CIPN patients to profile PGx loci of CYP2D6 gene.

In a previous study, SNPs in the CYP2D6 gene were analyzed in 23 patients with and without CIPN using Single-Nucleotide Extension (SNE) technology in order to identify markers whose polymorphic profile might be used to predict which patients are at high risk for developing CIPN. Long-range PCR was used to amplify a 4kb section of the CYP2D6 gene. SNE, a multi-locus, multiplexed genotyping method where a single fluorescently-labeled nucleotide incorporated at the primer's 3' end identifies the presence/absence of a specific polymorphism, was then performed on the 4 kb amplicons. Results of the preliminary study are shown in Table 1 below. Twelve primer pairs were split into two multiplexed panels for analysis.

	Primer Assay 1						Primer Assay 2						
Allele	***10R**	***29F**	***9R**	***2R2850**	***3F**	***6F**	***12F**	***17F**	***2R4810**	***41F**	***4F**	***8F**	
Size	25.8	30.76	31.04	35.81	41.86	52.85	23.44	30	31	32	45	58	
Patient ID	100C>T	3183G>A	delAAG	2850C>G	2549delA	1707delT	124G>A	1023C>T	4180G>C	2850C>T	100C>T		**CIPN**
45A	C/G	G	G	G	G	G/T	G	G	G	G	G	NA	**Absent**
47A	G	G	G	G	G	G/T	G	G	G	G	G	G	**Absent**
49A	C/G	G	G	G	G	C/G	G	C/G	G	G	G	G	**Absent**
58A	NA	G	G	C/G	G	G/T	G	C/G	G	G	G	G	**Absent**
62A	G	G	G	G	G	G	G	G	G	G	G	G	**Absent**
63B	G	G	G	G	G	G	G	G	G	G	G	G	**Absent**
66A	G	G	T	G	G	G	G	G	G	G	NA	T	**Absent**
68A	G	G	T	G	G	G	G	G	G	G	NA	NA	**Absent**
69A	G	G	G	G	G	G	G	G	G	G	G	G	**Absent**
78A	G	G	G	G	G	G	G	G	G	G	G	G	**Absent**
79A	G	G/A	G	G	G	G	G	G	G	G	G	G	**Absent**
57A	G	G	G	G	G	G	G	G	G	G	G	G	**Mild**
64A	G	G	G	G	G	G	G	G	G	G	G	G	**Mild**
80A	G	G	G	G	G	G	NA	NA	NA	NA	NA	NA	**Mild**
20A	G	G	G	G	G	G	G	G	G	G	G	T	**Present**
34A	C/G	G	G	G	C/G	G	G	C	G	G	G	T	**Present**
38A	C/G	G	G	G	G	T	G	C/G	G	G	G	T	**Present**
39A	G	G	G	G	G	G	G	G	G	G	G	G	**Present**
42A	G	G	G	G	G	C/G	G	C/G	G	G	G	G	**Present**
70A	G	G	G/T	G	G	G/T	G	G	G	G	G	G	**Present**
71A	G	G	G	G	G	G	G	G	G	G	G	G	**Present**
76A	G	G	G	G/C	G	G	G	G	G	G	G	G	**Present**

Table 1. Results of preliminary study done with SNE.

HYPOTHESIS

Sanger sequencing is an effective methodological approach to validating results of SNE-generated CYP2D6 SNP profiles.

METHODS

Amplification

The process of validation through Sanger sequencing first required optimization for each of the primer pairs. Each primer pair was tested under multiple conditions in order to determine the optimal conditions for patient samples. Patient samples were then selected based off of the presence of a polymorphism according to the previous study. These Patient DNA samples were diluted, then amplified according to their conditions using the thermocycler for each of the primers. An agarose gel was ran to determine which patient DNA was of adequate integrity to generate amplicons for sequencing. Samples used for Sanger sequencing were based on the results of these gels.

CYP2D6*6 - rs5030655
CYP2D6*10- rs1065852
CYP2D6*17- rs28371706

Sanger Sequencing

Patient DNA amplifications resulting in single amplicon bands, as seen in Figure 1, indicated successful and specific amplification. Patient sample amplicons were cleaned up using ExoSAP. Following this enzymatic treatment, cycle sequencing was performed using one of their respective amplification primers using BigDye v3.1 cycle sequencing kit using manufacturer's suggested protocols. Removal of unincorporated fluorescent nucleotides was performed using a Dye-ex spin column. Purified sequencing products were dried down using a SpeedVac and resuspended in 10 ul of HiDi formamide, then loaded on a plate and analyzed on the ABI 3500 capillary electrophoresis system using POP7 polymer and a 50 cm capillary. Sequencing analysis software on the ABI 3500 was used to base call as well as report the quality of the data.

Analysis of Sanger Sequencing Data

Analyzing sanger sequencing data may be done in a variety of ways. The preferred method of analyzing this data is using a tertiary software with an AB1 file to analyze the chromatograms. The other method and the one used in comparing the CYP2D6 data to the human genome involved the use of a text file of that data. This procedure is done by using the best guess of the chromatogram in a text file. The text file (.txt) is compared to the Human Reference Genome through BLAST. This software provides data on pseudogenes, bands, transcript variants, and identifies polymorphisms by showing gaps. The limitations to this method include the possibility of low quality and the inability to identify heterozygotes. Though even with these limitations, this software was used to identify a polymorphism found in a patient's sample amplified with the *6 primer amplicon. Within most patients as well as the Human Reference Genome, at position 297, a CC is found. The polymorphism found in the particular patient's sample indicated this with a gap at this position. This patient's "C" was changed to a "G". The BLAST technique is useful in determining information as such.

RESULTS

Patient DNA samples were used to generate amplicons containing the *6, *10 and *17 polymorphisms (See example amplification results in Figure 1). Those patients generating the appropriately-sized amplicons (*6= 505 bp; *10= 499 bp; *17= 505 bp) were subjected to Sanger sequencing. Positive control genomic DNA confirmed the expected amplicon sizes. NTC, no template controls indicated no contamination

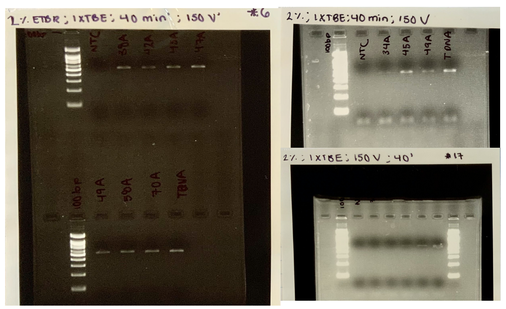

Figure 1. Prior to sanger sequencing, amplification of the polymorphic regions of CYP2D6 were performed for *6 (left), *10 (top right), and *17 (bottom right).

Amplicons for *6 were sequenced using the reverse primer while amplicons for both *10 and *17 were sequenced using the forward primer. Sanger sequencing data for *6 and *17 showed acceptable quality values (QV) and peak threshold. *10 sequencing data failed the QV threshold and showed high signal to noise ratio. Example sequencing electropherograms are shown in Figure 2. Sequencing identified additional SNPs in *6 and *17 amplicons. The *6 region showed synonymous rs1058164 polymorphisms while the *17 region had two intronic SNPs, namely rs28371702 and rs28371701. To examine whether the synonymous rs1058164 *6 region SNP segregated with CIPN, *6 amplicons from all 23 patients are currently being sequenced.

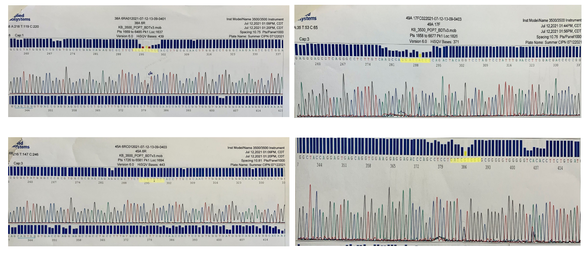

Figure 2. Sanger sequencing data portraying polymorphisms in certain patient samples for *6 *rs1058164* (top and bottom left), *17 *rs28371702* (top right), *17 *rs28371701* (bottom right).

Preliminary validation results indicated a low concordance between Sanger sequencing results and the SNE assay findings in the original CIPN study. All loci from the original CIPN study/SNE method were monomorphic by Sanger sequencing (see Table 2).

Amplicon	**6R rs5030655*	**6 rs1058164*	**17 rs28371706*	**17 rs28371702*	**17 rs28371701*
Patient ID					
20A	M	M	N/A	N/A	N/A
34A	N/A	N/A	M	M	M
38A	M	P	N/A	N/A	N/A
42A	M	M	M	M	M
45A	M	P	N/A	N/A	N/A
47A	M	M	N/A	N/A	N/A
49A	M	M	M	P	P
57A	M	M	N/A	N/A	N/A
58A	M	M	M	P	P
64A	M	M	N/A	N/A	N/A
70A	M	M	M	M	M
71A	M	M	N/A	N/A	N/A
80A	M	M	N/A	N/A	N/A

Table 2. Results of the Patient samples is highlighted within this table based on Sanger Sequencing results. **M- Monomorphic // Polymorphic**

CONCLUSION

In the current study, we used a Sanger sequencing-based approach to validate the previously generated SNE results for CYP2D6 polymorphisms in CIPN patients. Polymorphic patient samples for *6, *10 and *17 SNPs were selected for locus-specific amplification. Results from this analysis revealed that the original SNE assay resulted in a high degree of false positive base calls. This approach is known to show design and optimization-related variations and limitations but calibration issues on the GeXP capillary electrophoresis system (Beckman Coulter) used most likely also contributed to these anomalies.

Although, Sanger sequencing of amplicons containing the interrogated CYP2D6 polymorphisms was a better system for evaluating SNPs for risk assessment studies, this approach is also limited due to low sensitivity, scalability, limit of detection, and thus discovery power. Future efforts to assess CIPN risk using SNP profiles are currently utilizing a PGx panel (ThermoFisher) run on an Ion Torrent platform, to more broadly detect low and moderate frequency variants at higher coverage and perform association analyses of these variants with CIPN.

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