2 MATERIALS AND METHODS

2.1 Laboratory Methods

2.1.1 Cell culture

All cells were routinely maintained in RPMI-1640 (BioWhittaker, Walkersville, MD, USA) containing fetal bovine serum (FBS) and *L*-glutamine (Invitrogen, Carlsbad, CA, USA). Cell lines used in the sequencing experiments were cultured in RPMI 1640 supplemented with 10% FBS and 5mM *L*-glutamine. Cell lines used in the pharmacology experiments were cultured in RPMI 1640 supplemented with 10% FBS and 2mM *L*-glutamine. All cultures were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37^0 C.

2.1.2 DNA sequencing

2.1.2.1 Cell lines

Fifty-nine of the 60 NCI-60 cell lines were provided by the Developmental Therapeutics Program at the NCI (Bethesda, MD, USA). MDA-N was not available at the time of the study because its use was “restricted”. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 5mM *L*-glutamine.

2.1.2.2 Genomic DNA extraction

Genomic DNA was extracted from each of the fifty-nine cell lines using the QIAGEN genomic DNA purification kit (Hilden, Germany).

2.1.2.3 Reagents

Exo / AP (per reaction): *1µl reaction buffer, 1µl dilution buffer, 0.05µl Exonuclease I (20U / µl, New England biolabs), 0.2µl Antartic Phosphatase (5U / µl, New England biolabs), 7.75µl sterile water.*

Exo / AP reaction buffer (stock): *100ml Tris (1M, pH 8.0), 50ml $MgCl_2$ (1M), 350ml sterile water.*

Exo / AP dilution buffer (stock): *25ml Tris (1M, pH 8.0), 475ml sterile water.*

BigDye terminator cocktail (stock): *2.9ml BigDye terminator V3.1 (Applied Biosystems), 17.1ml 5x BigDye reaction buffer (Applied Biosystems), 20ml sterile water.*

Precipitation mix: *500ml Ethanol, 10ml Sodium acetate (3M, pH 5.0), 20ml EDTA (0.1mM).*

2.1.2.4 Primer design

PCR primers were designed to amplify the exons and flanking intronic sequences of twenty-four cancer genes. Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/). The program was configured to design primers for PCR products approximately 500bp in length, with multiple overlapping amplimers for larger exons.

2.1.2.5 PCR

PCR of genomic DNA was carried out in 15µl reaction volumes in 96 well plates. To 1µl genomic DNA (20ng / µl) was added 7.5µl primers (4ng / µl), 1.5µl dNTPs (2mM each), 1.5µl GeneAmp 10x reaction buffer (Applied Biosystems), 0.09µl Thermostart Taq (5U / µl, Abgene) and 3.4µl sterile

water. Cycling was performed on an MJ-Research PTC-225 thermal cycler. Following an initial denaturation step of heating to 95^{o}C for 15 minutes, were 40 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds and a final extension step at 72^{o}C for 10 minutes. PCR products were evaluated by electrophoresis of 4µl aliquots on a 2% agarose gel (containing 0.2µg / ml ethidium bromide). To the remaining 11µl PCR products was added 10µl Exo / AP mix (see above), followed by incubation at 37^{o}C for 30 minutes and 80^{o}C for 15 minutes to remove residual primers and unreacted dNTPs.

2.1.2.6 PCR product sequencing

Sequencing of PCR products was carried out in 8µl reaction volumes in 384 well plates. For each PCR product, forward and reverse sequencing reactions were performed. To 2µl sense or anti-sense primer (15ng / µl) and 4µl BigDye terminator cocktail (see above) was added 2µl Exo / AP treated PCR product. Thermocycling was performed on an MJ-Research PTC-225 thermal cycler. Following an initial activation step of heating to 96^{o}C for 30 seconds, were 44 cycles of denaturation at 92^{o}C for 5 seconds, annealing at 50^{o}C for 5 seconds and extension at 60^{o}C for 2 minutes. DNA was then precipitated by addition of 25µl precipitation mix (see above), and centrifugation (4000rpm, 4^{o}C, 25 minutes). Precipitated DNA was washed twice by addition of 30µl Ethanol (70% v / v in sterile water) followed by centrifugation (4000rpm, 4^{o}C, 4minutes) and removal of the supernatant. The precipitated DNA was allowed to dry and then dissolved in 10µl EDTA

(0.1mM). Sequencing was performed using ABI 3730 DNA analyzer (Applied Biosystems).

2.1.3 Detection of homozygous deletions

Exon deletions in *CDKN2A*, *PTEN*, *RB1* and *SMAD4 (MADH4)* were identified by multiplex PCR. PCR primers were designed to amplify exons 1, 2, and 3 of CDKN2A together with exon 1 of ARF, all 9 exons of PTEN, 27 exons of RB1 and exons 1, 3-13 of MADH4. Control PCR amplimers were designed to beta actin and random intergenic genomic sequences. PCR was carried out as previously described (2.1.2.5), and PCR products were resolved on 2% agarose gels (2.1.2.5). All multiplex PCR experiments were performed in duplicate.

2.1.4 Pharmacology

2.1.4.1 Cell lines

A total of 34 cell lines were used in the pharmacology experiments. Eleven of the 34 cell lines were from the NCI-60 cell lines: SKMEL-28, HT-29, UACC-257, M14, MALME-3M, SKMEL-2, SW620, DU145, A498, MDA-MB-231, and T47D. Twenty-four additional cell lines were provided by the Cancer Genome Project for experimental use: SKMEL-1, SKMEL-3, SKMEL-24, HT144, WM115, IGR-1, SKMEL-30, MEL-JUSO, IPC-298, HMVII, HMCB, MeWo, CHL-1, LS-411N, RKO, COLO-741, NCI-H508, LoVo, SW948, LS174T, LS123, NCI-H716, NCI-H630, HT55.

2.1.4.2 Cell plating

Cell lines were maintained as stated above (2.1.1). All cell lines were grown in T-75cm^2 tissue culture flasks (Costar, USA). Prior to each experiment, an 80-90% confluent flask was aspirated and washed twice with 10ml of phosphate buffered saline. To adherent cells, 2ml of trypsin was added and incubated at 37^0 C for 2 minutes or until cells lifted off the flask. Then the cells were resuspended in 4ml of growth medium. The cell suspension was transferred into a 15ml tube and centrifuged at 2500 rpm for 4 minutes (RT 6000). Suspension cells were directly centrifuged at 2500 rpm for 4 minutes. The resulting supernatant was aspirated off and the cell pellet resuspended in 3ml of medium and gently pipetted to disperse cells. To an Eppendorf tube, 10μl of cells and 10μl of trypan blue were added. From this tube, 10μl of the stained cells was pipetted onto a hemacytometer and cells were counted. Depending on the cell line and its doubling time, 2,500 to 6,000 cells in a total volume of 100μl of medium were plated per well in a 96-well tissue culture plate. Cells were plated in triplicate wells. The 96-well tissue culture plate containing the seeded cells was incubated for 48 hours at 37^0 C.

2.1.4.3 Drug dilutions

Two phenothiazine compounds (NSC 46061, NSC 17474) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD. Stock solution 100mM of each compound

was prepared in 99% DMSO (Sigma, St. Louis, MO) and aliquots stored at –80^0 C.

2.1.4.4 Drug addition

At 48 hours after starting incubation of the plated cells, 1.2-fold and 1.3-fold serial dilutions of NSC 46061 and NSC 17474, respectively, were prepared in medium immediately prior to use. Medium was aspirated from plated cells, and 150μl of drug solution was added to triplicate wells of the 96-well plate. An equal volume of fresh medium was added to triplicate wells of untreated control cells. The plate was then incubated for 48 hours with drug treatment.

2.1.4.5 Proliferation assay

At 48 hours after addition of drug, the supernatant was aspirated from plated cells. Phenothiazine derivative compounds' activity was determined by measuring formazan production from MTS (Promega, Madison, WI). A solution containing 100μl of medium and 20μl of MTS (Promega , Madison, WI) per well was prepared. To each well 120μl of this solution was added. The plate was incubated at 37^0 C and absorbance was measured at 490 nm between 1 and 4 hours.

2.1.4.6 Data analysis

Using GraphPad Prism 4.02 (GraphPad Software, Inc., San Diego, CA) drug concentrations were log transformed and nonlinear regression was performed on the A_{490} data using the sigmoidal dose response model with

variable slope. Mean EC_{50} values, SEs, and 95% confidence intervals were determined from the logistic fits. Data are presented as the mean ± SEM of triplicate experiments.

2.2 Bioinformatic Methods

2.2.1 Processing of sequence traces

The sequences generated by the ABI 3730 sequencer were processed using a software program, ASP (http://www.sanger.ac.uk/Software/sequencing/docs/asp) that converts sequence traces into SCF (standard chromatogram format) files (Figure 2-1).

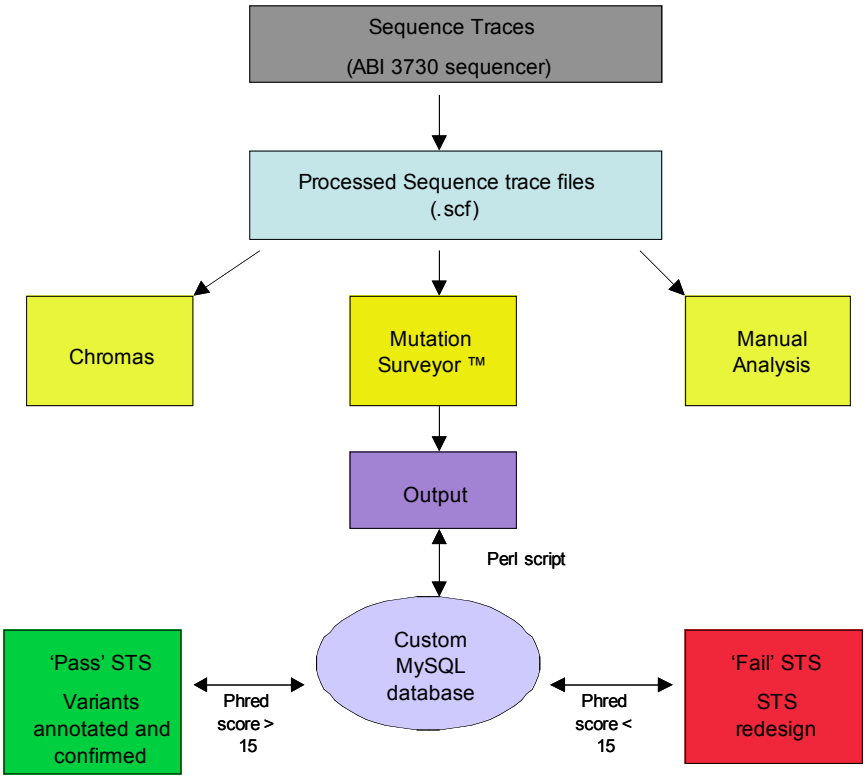

Figure 2-1: Workflow schematic of sequence analysis and sequence trace quality control.

2.2.2 Sequence analysis and confirmation of putative variants

Sequence traces were analysed using a combination of software: Mutation Surveyor™, Chromas, and manual analysis (Figure 2-1). Sequence variants were annotated using NCBI Genome Build 34 (http://www.ncbi.nlm.nih.gov/sites/entrez). Nomenclature for the description of sequence variants was adapted from the Human Genome Variation Society's recommendations (http://www.hgvs.org/mutnomen, Figure 2-2). All putative oncogenic variants and mutations were confirmed by bi-directional sequencing of a second independently amplified PCR product.

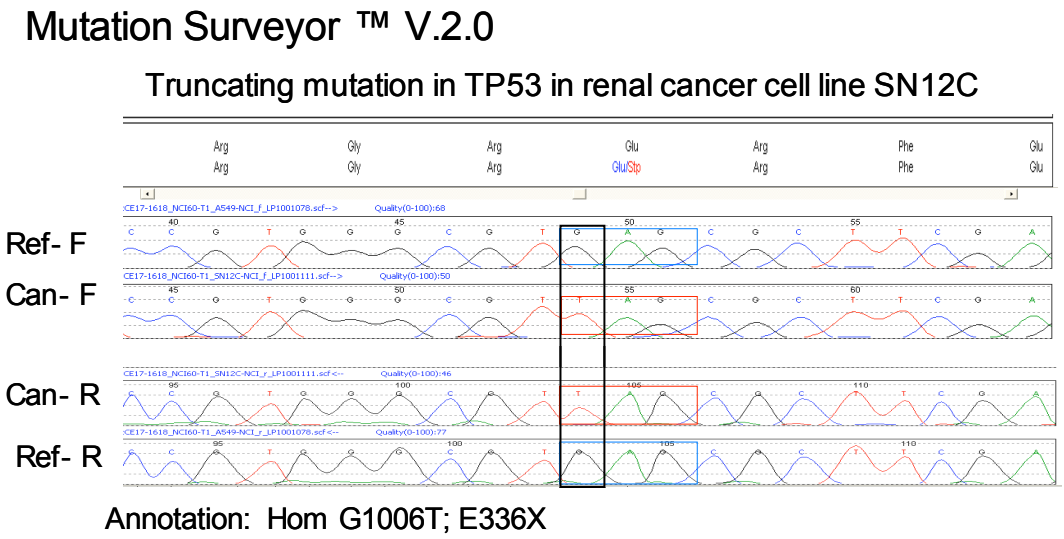

Figure 2-2: An example of sequence analysis in Mutation Surveyor™ and annotation of a nonsense mutation in TP53 gene in renal cancer cell line, SN12C, of the NCI-60 cell line set. Legend: Ref –F (Reference DNA Forward strand), Can-F (Cancer Sample DNA Forward strand), Can-R (Cancer Sample DNA Reverse strand), Ref-R (Reference DNA Reverse strand), Hom (Homozygous), G (Guanine), T (Thymine) 1006 (nucleotide position within

TP53 gene); E (Glutamic acid), X(Termination codon), 336 (Amino acid position within TP53 gene).

2.2.3 Storage of sequence variants

A custom MySQL relational database, version 4.0.21 (http://dev.mysql.com/), named ***nci60***, was created. The database was used to store all annotated sequence variants identified, to maintain quality control of sequencing markers, and to store the drug response data. The database consists of five tables: Sts, Mutation, Experiment, Celline, and Drugdata (Figure 2-3). To interface between the output from Mutation Surveyor™ and the MySQL database, a perl script, ***passfail.pl***, was written. The perl script, ***passfail.pl***, parses sequence quality scores for each sequence marker and cell line pair from the output of Mutation Surveyor™ as a designation of 'pass' or 'fail' into the Experiment table of the MySQL database. The designation of 'pass' was attributed to a sequence marker with a sequence quality score greater than 15 and a 'fail' was attributed to a sequence marker with a sequence quality score less than or equal to 15. Failed sequence markers were redesigned and re-sequenced in the cell line(s) of interest.

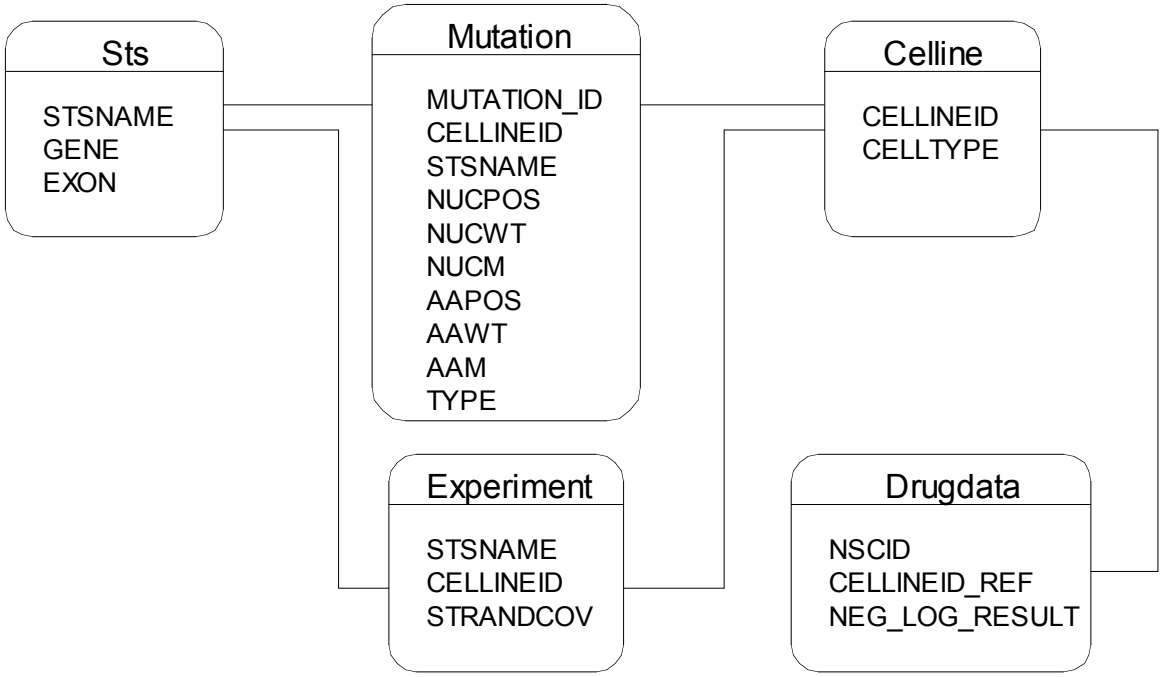

*Figure2-3: Schema of the structure and content of the **nci60** custom MySQL database. The Mutation table is linked to the Sts table by the STSNAME and linked to the Celline table by CELLINEID. Similarly, the Experiment table is linked to the Sts and Celline tables by STSNAME and CELLINEID, respectively. The Drugdata table is linked to the Celline table by CELLINEID_REF.*

2.3 Statistical analysis

2.3.1 Relationship between cancer gene mutations and drug activity

We compiled pharmacological data ($-\log_{10}$ (GI50)) for 7794 compounds from the Developmental Therapeutics Program (DTP) 60-cell line screen

(http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). A matrix of the ($-\log_{10}$ (GI50)) measures of the NCI-60 cell lines was imported into the R statistical programming environment (http://www.r-project.org). Another matrix of the mutation status of cancer genes, as determined by sequence analysis in the NCI-60 cell lines, was imported into R. A Wilcoxon rank sum test was used to assess differential drug sensitivity between mutant and wild type cell lines in the NCI-60. A False Discovery Rate (FDR) of 0.25 was applied to the nominal p-values to correct for testing multiple hypotheses. Compounds with the most significant p-values (FDR adjusted p-value < 0.05) following correction for multiple testing were selected for follow-up analysis.

2.4 Cheminformatic screen of phenothiazine compounds

A structural similarity search for phenothiazine compounds tested in the DTP 60 cell line screen, with NSC 46061 as the query compound, was performed using LeadMiner, a software package developed by Leadscope Inc. (Columbus, OH). The structural similarity search compared the global collection of structural features between the query compound and every compound tested in the DTP 60 cell line screen database. For every compound in the database, a ratio of common features to total features in the two compounds, known as a similarity value, was calculated. A user-defined structural similarity threshold of 0.5 was applied and all compounds with a similarity value greater than 0.5 were returned. A total of ninety-one additional chemical compounds containing the phenothiazine ring system were retrieved.