Chapter III

Materials and methods
3.1. Animal species and acclimation

Male Swiss albino mice (*Mus musculus*) weighing 25-30 g and 10-12 weeks old were procured from National Laboratory Animal Centre (NLAC) of Central Drug Research Institute (CDRI), Lucknow. The animals were housed in plastic cages and acclimated to controlled conditions of light and dark (12 h each), and temperature (23±2°C) for 3 days before drug administration. The animals were randomly assigned to treatment and control groups with a minimum of 4 animals in each group.

3.2. Drug administration and tissue collection

Bulaquine, primaquine (CAS number: 90-34-6) and acetaminophen (CAS number: 103-90-2), and carbon tetrachloride (CAS number: 56-23-5) were procured from Central Drug Research Institute (CDRI), Lucknow, Sigma and Qualigens respectively. All animal procedures were carried out in compliance to institutional animal ethics committee (IAEC) guidelines. Animals belonging to different treatment groups were administered single oral dose of primaquine (40 mg/kg) or bulaquine (equimolar dose to primaquine = 40 mg/kg) dissolved in normal saline whereas carbon tetrachloride (1 ml/kg) and acetaminophen (300 mg/kg) were administered as a single intraperitoneal (ip) injection in olive oil. The animals of respective control groups were administered either 100 µl normal saline or olive oil. Access to food and water was ‘ad libitum’ before and after drug treatment. The animals belonging to control and treatment groups were sacrificed by cervical dislocation at 6, 12 and 24 h. Blood was withdrawn before sacrificing the animals through heart puncture using 1 ml disposable syringe (Dispo Van) and allowed to stand undisturbed for 30 min in 1.5 ml micro centrifuge tube (Axygen). Left lobe of liver was immediately fixed in 10% formalin for histological investigations. Remaining part of liver tissue was washed with normal saline and rapidly snap frozen in liquid nitrogen and subsequently stored at -80°C (Ilshin) until further use.

3.3. Serum biochemistry

The serum was separated from blood cells by centrifugation at 2,000 rpm for 15 min in a minifuge (Sigma) and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were estimated with automated biochemical analyzer (Beckmann). Enzyme activities between the control and drug treated animals were analyzed by one-way ANOVA with Newman-Keuls post analysis test (GraphPad Prism version 3).
3.4. Histopathology

Left lobe of liver was fixed in 10% formalin for at least 48 h. A small piece of tissue was cut and washed overnight under tap water to remove traces of formal saline. It was then dehydrated with acetone (Merck) and acetone: benzene (Merck) (1:1) for 30 min each. Finally, the tissue was cleared thrice in benzene for 30 min. Once the tissue was completely dehydrated it was kept in molten paraffin wax (Merck) for 4 h at 65°C in Histocentre2 workstation (Shandon). Following paraffin embedding, wax blocks containing liver tissue were prepared. Chilled wax blocks were cut into 5 μm sections with a semi-automated microtome (Leica). Ribbons of serial sections were spread on warm water and retrieved on albumin coated glass slides (Blue Star). The sections were incubated over a hot plate for 3-4 h at 37°C to ascertain proper attachment of tissue with slide. Slides were washed twice for 5 min with xylene (Merck) to remove wax before rehydrating with decreasing grades of alcohol. Tissue sections were rehydrated by keeping the section in the following grades of ethanol: 100%, 90%, 70%, 50%, 30% and water, for 5 min in each case. Completely rehydrated sections were stained with hematoxylin for 5 min and washed under running tap water to remove excess stain. The sections were stained with eosin for ~1 min and extra stain removed by 5-6 quick dips in acetone, acetone: xylene (1:1) and xylene (Merck) respectively. The stained slides were mounted in DPX (Fluka) and dried for a week and evaluated to find drug induced histological changes with the help of a light microscope (Leica).

3.5. Chromosomal aberration assay

All the drugs and the doses used in this study were evaluated for their clastogenic effect in vivo. Swiss mice were administered with the drugs as described previously. After 22 h of drug administration, colchicine (Sigma) was administered at a concentration of 4 mg/kg body weight. At 24 h the animals were sacrificed and femur bones removed. The terminal parts of the femur bones were cut carefully with a scissor until a small opening became visible. Bone marrow was flushed out in 5 ml, 1% sodium citrate solution with the help of a hypodermic syringe and aspirated to get a fine suspension of cells, and incubated at room temperature for 15 min before centrifugation at 2,000 rpm for 10 min. The pellet was resuspended in 5 ml, 0.075M potassium chloride (Merck) solution followed by incubation at 37°C for 30 min and then centrifuged at 2,000 rpm for 10 min. The supernatant was discarded and pellet resuspended in 5 ml freshly prepared methanol: acetic acid fixative. The cells were allowed to incubate overnight at 4°C (LG refrigerator) and then centrifuged at 2,000
rpm for 10 min. Finally the cell pellet was resuspended in 500 µl methanol: acetic acid fixative. The cells were dropped on a pre-chilled slide from a distance so as to break swollen cells. Triplicate slides were prepared for each bone marrow sample and stained with Giemsa (Sigma Aldrich) for 15 min. The slides were rinsed thrice with tap water and allowed to dry. The slides were subsequently mounted with DPX (Fluka) and observed under light microscope (Leica).

3.6. 15k and 22.4k mouse cDNA arrays

Pre-spotted microarray slides containing 15,247 duplicate spots (15k) and 22,827 single spots (22.4k) respectively (excluding control spots) were procured from University Health Network, Toronto, Canada. Briefly, the bacterial clone sets from National Institute of Aging (NIA) were PCR amplified and purified before spotting on CMT-GAP II slides (Corning). Post printing quality control tests included a real hybridization using commercially available mouse RNA, vector probe labeling and terminal transferase tailing method to assess spot morphology, background quality and intensity of signal. The slides have both, positive (Chlorophyll synthetase) and negative spots (3X SSC) printed on the slides. More information is available at www.microarrays.ca.

3.7. Preparation of Chlorophyll synthetase of Arabidopsis thaliana as positive control spike

Description of plasmid containing the partial sequence of Chlorophyll synthetase gene and complete methodology involved in in vitro transcription has been described in the following paragraphs.

pARAB: This plasmid contains a fragment of Chlorophyll synthetase gene of Arabadopsis thaliana (as found by sequencing/BLAST search for partial sequence of the insert) and is not homologous to any human, yeast, or mouse sequences. It was provided by the University Health Network, Toronto, along with the microarray slides. The partial sequence of the Chlorophyll synthetase gene is as follows:

```
GAATTTGGTACCGGCCCCCCTCGAGGTGCCCAGGCTGTGGGTAATTTAGGGATGTGATCTTCCCGGATGATGATGTGCT
GTCGGGCTCTGTCCTTACTGGGCTATACACAGACAATCAACGACTGGTATGATAGAGATATCGACGCAATTAATGAGCAT
CATATATGTCACATATCTGAGAATATCAATTACAGACAGAGGTTATTACACAGTCTGGGTGCTATTATTGGGAGGT
CTTGGTATTGCTGGAATATTAGTGTGCTGGGATACCGATACCCGCTCTACCTCTTGGGGAGT
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The size of this insert is approximately 1.3-1.4 kb and is identical to the one that has been spotted on mouse cDNA microarray slides used in this study. The plasmid has a SacI (Ec136II) restriction site that can be used to linearize the plasmid and a T7 promoter (located at the end of the plasmid opposite to the restriction site). Furthermore, the plasmid contains a sequence which introduces polyA tail during T7 run off transcription reaction. This polyA tail allows the RNA to be converted into labeled cDNA when added to cDNA labeling reaction as a positive control. The plasmid also encodes ampicillin resistance to allow for selection during transformation. Detailed description of steps involved in the preparation of positive spike RNA is as follows:

3.7.1. Preparation of competent cells

Single isolated colony of *E. coli* DH5α (Central Drug research Institute, Lucknow) were incubated overnight in 5 ml Leuria Bertini (LB) medium at 37°C and 225 rpm. Of the overnight grown culture, 150 μl sample was reinoculated in 25 ml LB and incubated at 37°C for 1\(\frac{1}{2}\) h so as to harvest the bacteria at an optical density (OD) of 0.3-0.5 at A600. Approximately 10 ml of freshly grown DH5α cells were transferred to a polypropylene tube (Tarsons) and centrifuged at 5,000 rpm for 5 min at 4°C in refrigerated centrifuge (Sigma). The supernatant was discarded and pellet was incubated for 1 h in 5 ml pre-chilled solution of 0.1M CaCl₂ (Glaxo) after proper mixing. It was then centrifuged at 5,000 g for 5 min at 4°C. The supernatant was discarded and pellet resuspended in 2 ml, 0.1M CaCl₂+15% glycerol (Merck). Aliquots of 200 μl were stored in -80°C for further use.

3.7.2. Transformation with pARAB and efficiency check

200 μl competent cells prepared in the previous step was mixed with 1 μl pARAB (0.5 μg/μl) in micro centrifuge tube and incubated on ice for 15 min for efficient attachment of plasmid with competent cells. It was then transferred to a pre-heated water bath (Julabo) at 42°C for 90 sec for efficient translocation of plasmid into the competent cells. Further incubation was carried out on ice for 5 min to facilitate constriction of pores in cell wall and proper retention of plasmid inside the cells. The transformed competent cells (200 μl) containing pARAB were inoculated in 800 μl LB medium and incubated at 37°C at 225 rpm for 1 h. 100 μl of fresh culture was spread on petri plates containing LB with ampicillin (100 μg/ml final conc, Sigma) as selection
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marker. The plates were incubated for 12-16 h at 37°C and screened to estimate the efficiency of competent cells by calculating the number of transformed colonies.

Efficiency = No. of colonies / 1μg plasmid DNA

3.7.3. Plasmid DNA Isolation

A single transformed colony of DH5α was carefully picked and inoculated in 5 ml of LB medium in a culture tube and incubated at 37°C overnight at 225 rpm. Qiaprep spin miniprep kit (Qiagen) was used to isolate plasmid DNA from overnight grown culture following manufacturer’s protocol. Briefly, 3 μl of the overnight grown culture was centrifuged at 5,000 g and pellet resuspended in 250 μl buffer P1. It was ensured that RNase A has been added to buffer P1 before use. Further, 250 μl buffer P2 was added and mixed gently by inverting the tube 4-5 times followed by incubation at room temperature for 5 min. The lysis reaction was not allowed to proceed for more than 5 min. To this lysate 350 μl buffer N3 was added immediately and mixed gently till the lysate became less viscous. At this stage, the precipitated material contains genomic DNA, protein, cell debris and SDS. The lysate was centrifuged at 12,000 g for 1 min at 25°C and clear supernatant containing the plasmid promptly separated. The clear supernatant was readily transferred to a pre-equilibrated column and centrifuged at 12,000 g for 1 min. (The column equilibration was carried out by applying 200 μl buffer PE and centrifuged at 12,000 g for 2 min). The column was washed with 0.5 ml of buffer PB and centrifugation at 12,000 g for 1 min to remove the contaminants. A second wash was given to remove traces of contaminants for achieving better results. Finally the plasmid DNA was washed with 0.75 ml of buffer PE and centrifuged at 12,000 g for 1 min. Pure plasmid DNA was eluted in 50 μl molecular grade water and evaluated for quality on 1.2% agarose gel and quantity with GeneQuant Pro (Amersham).

3.7.4. pARAB linearization and gel extraction

10 μl (~4 μg) purified pARAB was digested with 2 μl (10U/μl) Sacl (Fermentas). The reaction was carried out in the presence of 2 μl; 10X Sacl buffer containing 10mM Tris-HCl (pH 7.4), 100mM KCl, 1mM DTT, 1mM EDTA, 0.2 mg/ml BSA in 50% (v/v) glycerol. The reaction volume was reconstituted to 20 μl and incubated at 37°C for 1 h and linearized pARAB DNA observed for a band of 1.2 kb on 0.7% agarose gel. Once the exact size of linearized plasmid was confirmed on the agarose gel under UV light, it was excised with a clean scalpel blade (Ghia sergi blade)
to retrieve from the gel. Care was taken to excise the part of gel having pARAB only and any extra agarose was discarded. The piece of excised gel was weighed in a clear tube and 3 volumes of buffer QG was added to the weight of the gel slice. The gel was dissolved completely by incubating at 50°C for nearly 10 min. (After the gel slice is dissolved completely, the resulting solution should appear yellow. Anyhow, if the color of the mixture was either orange or violet, 10 μl, 3M sodium acetate (pH 5) was mixed in order to ensure efficient adsorption of DNA to QIAquick membrane, which is maximum at pH ≤7.5. Conveniently, buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of optimal pH for DNA binding). After this an equal volume of isopropyl alcohol was added and placed on QIAquick column. The column was centrifuged at 12,000 g for 1 min at 25°C and flow through was discarded. Again 0.5 ml of buffer QG was added to QIAquick column and centrifuged at 12,000 g for 1 min at 25°C. To the column 0.75 ml of buffer PE was added and allowed to stand for 2-5 min before centrifugation at 12,000 g for 1 min at 25°C. An additional spin was given for complete removal of traces of buffer PE from the column. Finally the DNA was eluted by placing 50 μl of molecular grade water to the centre of the QIAquick column and centrifugation at 12,000 g for 1 min at 25°C. The quality of the DNA was confirmed on 1% agarose gel and quantified with the help of GeneQuant Pro.

3.7.5. In vitro transcription and purification of positive spike RNA

The linearized pARAB DNA containing T7 promoter site (TAATACGACTCATAATAGGGAGA) was used as a template for in vitro transcription reaction using MEGAscript in vitro transcription kit (Ambion). The in vitro transcription reaction consisted of 2 μl each of ATP, CTP, GTP, UTP, 10X buffer, enzyme and 8 μl template DNA. To this was added 1 μl DNase I and incubated at 37°C for 15 min. All the components were mixed properly in a nuclease free tube and incubated for 6 h at 37°C. The in vitro transcribed RNA was cleaned with the help of Megaclear purification kit (Ambion). The RNA sample from in vitro transcription reaction was reconstituted to 100 μl with elution buffer provided with the kit and mixed gently. To this, 350 μl of binding solution was added followed by 250 μl of absolute alcohol and centrifugation at 13,000 g for 1 min. The flow through was discarded and collection tube reused for subsequent washing steps. The column was incubated with
500 μl wash solution and centrifuged at 13,000 g for 1 min. A band of ~1kb RNA was confirmed on denaturing, formaldehyde gel and RNA sample was quantified with GeneQuant Pro. This stock was diluted to get a concentration of 3 ng/μl RNA for further use in labeling reaction.

3.8. RNA isolation and quality assessment

Approximately 50 mg of frozen liver tissue was crushed manually in liquid nitrogen and immediately homogenized (Heidolph) in 1 ml, TRI reagent (Sigma). The homogenized tissue was incubated for 10 min at room temperature followed by the addition of 200 μl chloroform (Sigma) per ml of TRI reagent used and mixed vigorously for 10-15 sec. The sample was allowed to stand undisturbed for 15 min at room temperature and then centrifuged at 12,500 g for 20 min at 4°C. The aqueous phase was carefully transferred to a fresh tube and RNA was precipitated with 500 μl isopropyl alcohol (Merck). Further centrifugation at 12,000 g for 15 min at 4°C and washing (twice) with 1 ml, 75% ethanol was done to recover pure RNA pellet. Finally, the RNA pellet was air dried and resuspended in molecular grade water (Sigma). The quality of RNA was assessed electrophoretically on denaturing formaldehyde agarose gel (Genei) and spectrophotometrically with GeneQuant Pro. Briefly, 3-4 μl of RNA sample was mixed with 10 μl formamide (Sigma), 2 μl; 5X formaldehyde gel running buffer and 3.5 μl formaldehyde. It was heated at 65°C for 15 min and immediately placed on ice. After 5 min, 2 μl formaldehyde gel loading dye was added followed by 0.5 μl EtBr (10 mg/ml stock). The contents were mixed properly and loaded on to gel for electrophoretic separation. RNA samples with approximately 2:1 ratio of 28S: 18S rRNA and 260/280 values >1.7 were used for cDNA labeling reaction. Potential inter-individual variability was removed by pooling equal amounts of RNA from individual mice belonging to each group for gene expression studies.

3.9. cDNA labeling and purification

Two parallel reactions containing 25 μg total RNA, from pooled treated and control samples, was converted into labeled cDNA using CyScribe First-Strand cDNA Labeling Kit (Amersham) following manufacturer’s protocol in a thermal cycler (MJ Research). Briefly, a reaction mixture of 25 μl contained 1 μl oligo(dT) primers, 1 μl dCTP mix, 1 μl labeled CTPs, 2 μl; 0.1M DTT, 4 μl; 5X RT buffer and 1 μl CyScribe reverse transcriptase. Primer annealing was carried out at 70°C for 10 min followed by incubation at room temperature for 15 min. Primer extension was carried out at 42°C
for 2 h. Template RNA was degraded by 2 μl, 2.5M NaOH at 37°C for 15 min. The reaction mixture was neutralized by adding 10 μl of 2M HEPES (N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid), Sigma) buffer before purifying it from unincorporated nucleotides through GFX columns (Amersham). Purified labeled cDNA was concentrated by evaporation under vacuum in a speedvac after calculating the percent incorporation of the dyes with spectrophotometer (Thermo). Dye swap technical replicate experiments were performed with aliquots of pooled RNA preparations to address inconsistencies regarding dye incorporation and other technical means of variance.

3.10. Hybridization, scanning and image analysis

The Cy5 and Cy3 labeled cDNA samples were mixed in CyScribe Hyb buffer (Amersham) containing sheared salmon sperm DNA (10 μg/ml), and yeast tRNA (10 μg/ml, Ambion) as blocking agents. The samples were heated to 95°C for 2 min and immediately chilled to avoid secondary structures. The labeled samples were manually hybridized to mouse cDNA arrays and incubated for 18 h at 42°C. The arrays were washed with 1X SSC + 0.2% SDS (Sigma) and 0.1X SSC + 0.2% SDS at 42°C C and 0.1X SSC at room temperature for 15 min each. The arrays were dried immediately at 1000 rpm for 2 min and scanned to collect raw data for both Cy3 and Cy5 with Array scanner generation III (Molecular Dynamics) supported with ImageQuant version 5. Scanned images were analyzed to extract feature intensity values with ArrayVision version 8.

3.11. Microarray data analysis

Raw intensity data was analyzed with Avadis Express version 4.3 (Strand life Sciences) and the background corrected intensity data was LOWESS normalized (Cy5 against Cy3) to obtain log (base 2) ratios. Technical replicate data belonging to all the three time points were transformed to account for dye swap discrepancies. Two-sample t-test was applied between the control and the treatment groups on mean log transformed data belonging to all the three time points to find probes with statistically significant p values (uncorrected) and fold-changes. Finally the data was filtered to identify probes that satisfied p<0.01 and 2 fold criteria at all time points. Furthermore, log2 values of duplicate spots were averaged in order to get a single mean value. Mean log data of each time point was analyzed to infer the value of k to perform k-means clustering with MeV version 3.1 (TM4, The Institute of Genomic Research, Saeed et al., 2003). Each expression cluster was further clustered hierarchically with Euclidean
distance matrix and average linkage to identify gene with similar expression patterns. Raw and log transformed data has been submitted to Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/) having series accession nos. GSE 4874, GSE 5979 and GSE 5980) and conforms to MIAME guidelines developed by microarray gene expression data society (MGED).

3.12. Pathway analysis, functional characterization and in-silico validation

Individual gene expression data of different time points were condensed to make gene-expression dataset using GenMAPP version2 (Dahlquist et al., 2002) and subsequently analysed to find the pathways affected by different drugs used in this study. Furthermore, the gene expression dataset file (.gex) was exported to MAPPFinder to calculate percentage of genes with significant expression change, statistical score for each Gene Ontology (GO) term and respective z scores (Doniger et al., 2003). Functional annotation for the identified probes was inferred from the information available on NIA (http://lgsun.grc.nia.nlm.gov), NCBI (www.ncbi.nlm.nih.gov), MGI (www.informatics.jax.org), SwissProt (www.expasy.org/sprot), SOURCE (www.smd.stanford.edu/cgi-bin/source/sourceBatchSearch) and other related databases. The final list of genes with p<0.01 and 2 fold differential expression was validated with extensive data available on GEO profiles (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo&cmd=search&term=), Comparative Toxicogenomics Database (CTD, http://ctd.mdibl.org), Pubmed (www.ncbi.nlm.nih.gov/literature) and related databases regarding information on direction and extent of deregulation in similar studies conducted on various mammalian species.

3.13. Real Time PCR analysis

Aliquots of pooled samples of total RNA used in microarray gene expression studies were utilized for real time PCR analysis using iCycleriQ real time PCR (Biorad) and Quantitect SYBR Green real time PCR kit (Qiagen). Genes selected for validations were based on the microarray results and included only those that passed p<0.01 and 2 fold selection criteria (Table 1). PCR primers were synthesized with the help of PrimerSelect (DNA Star version 6) and procured from Sigma. Initial optimization of cycling conditions of primer pairs was performed with the help of one step RT-PCR kit (Qiagen) following manufacture’s protocol. Briefly, 50 μl reaction mixture consisted of 25 μl; 2X Quantitect SYBR green RT-PCR mix, 0.5 μM forward and reverse primers, 0.5 μl Quantitect RT mix and 500 ng template RNA. Final volume was adjusted with
RNase free water. The PCR cycling protocol include reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 sec, gene specific annealing for 30 sec at 56-67.5°C (Table 1) and extension at 72°C for 30 sec. A gradual increase in temperature from 67°C to 95°C was carried out to calculate melt curves. Following amplification the inbuilt software was used to set the baseline and threshold for each reaction. A cycle threshold (Ct) was assigned at the beginning of logarithmic phase of PCR amplification and difference in Ct value of control and treated samples were used to determine the relative expression (fold changes) of the gene using a Microsoft excel macro.

**Materials Used**

**Primaquine, bulaquine, acetaminophen, and Carbon tetrachloride stock solutions**

10 mg primaquine (Sigma) and 8 mg bulaquine (Central Drug Research Institute) was dissolved separately in 1 ml normal saline. A stock solution of acetaminophen was prepared by dissolving 100 mg acetaminophen (Sigma) in 2 ml olive oil (Bertolli Lucca) to achieve 50 mg/ml final concentration. Carbon tetrachloride (Qualigens) and olive oil were mixed in the ratio of 3:7 and proper mixing was done with the help of a few drops of alcohol followed by vigorous homogenization. **Normal saline**

0.9 g NaCl (Sigma) was dissolved in 100 ml triple distilled water (TDW).

**10% formalin**

Mixed 10 ml formaldehyde (37-41%, Qualigens) with 90 ml normal saline.

**1% sodium acetate solution**

Dissolved 1 g sodium acetate (Glaxo) in 100 ml TDW

**0.075 M potassium chloride solution**

Dissolved 0.5592 g potassium chloride (Merck) in 800 ml TDW and adjusted the pH to 7.2 with 1N NaOH (Qualigens). Final volume of solution was adjusted to 1 liter with TDW.

**Methanol: acetic acid fixative**

Mixed methanol and glacial acetic acid (Merck and Qualigens respectively) in the ratio of 3:1
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Diethyl pyrocarbonate treated water
0.1% diethyl pyrocarbonate (DEPC) treated water was prepared by mixing 1 ml DEPC (Sigma) with 999 ml TDW. The solution was continuously stirred for 6 to 7 h on a magnetic stirrer (Remi) and autoclaved for 25 min at 15 lb/sq. in. on liquid cycle.

Ampicillin stock solution
Dissolved 100 mg ampicillin (Sigma) in 1 ml TDW, filtered through 0.22 μm syringe filter and stored at 4°C.

Luria-Bertani medium
Dissolved, 10 g tryptone (Sigma), 10 g NaCl (Sigma) and 5 g Yeast extract (Himedia) in 950 ml TDW. The pH of the solution was adjusted to 7 with 5N NaOH and volume to 1 liter with TDW. The solution was sterilized by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

0.5M ethylenediaminetetraacetic acid (EDTA)
Dissolved 186.1 g of disodium ethylenediaminetetraacetic acid (Sigma) in 800 ml TDW and mixed vigorously. Adjusted the pH to 8 with 10N NaOH (Qualigens) and reconstituted the final volume to 1 liter with TDW.

50X Tris-acetate (TAE) buffer
One liter concentrate stock solution of TAE was prepared by mixing 242 g of Tris base (SRL) with 57.1 ml glacial acetic acid (Qualigens) and 100 ml, 0.5M EDTA (pH 8, Sigma) in TDW. It was autoclaved for 20 min at 15 lb/sq. in. on liquid cycle.

50 mM sodium acetate solution
Dissolved 4.1 g sodium acetate (Glaxo) in 800 ml TDW and final volume was adjusted to 1 liter.

Ethidium bromide stock
A stock solution of ethidium bromide (EtBr) was prepared by dissolving 10 mg of EtBr (Sigma) in 1 ml TDW. The tube was wrapped with aluminum foil to protect from light.

6X gel loading dye
Dissolved 2.5 mg bromophenol blue (Sigma) and xylene cynol (Sigma) each in 1 ml, 30% glycerol (Qualigens) prepared in TDW.

RNA loading dye
Dissolved 2.5 mg bromophenol blue and xylene cynol (Sigma) each in 1 ml, 50% glycerol (Qualigens) prepared in TDW. To this was added 2 μl, 0.5M EDTA solution (1mM final concentration). The contents were mixed and stored at 4°C.
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5X formaldehyde gel running buffer

Dissolved 20.9 g of 3-(N-morpholino)propane-sulfonic acids (Sigma) in 800 ml, 50 mM sodium acetate (Glaxo) solution prepared in DEPC treated water. Adjusted the pH to 7 with 2N NaOH (Qualigens) and added 10 ml, 0.5M EDTA (pH 8) prepared in DEPC treated water. Reconstituted the final volume to 1 liter with DEPC treated water and sterilized the solution by filtration through 0.22 μm syringe filter (Millipore).

20X SSC

Dissolved 175.3 g of NaCl and 88.2 g of sodium citrate (Glaxo) in 800 ml of TDW and adjusted the pH to 7 by adding a few drops of 10N NaOH (Qualigens). The final volume was adjusted to 1 liter with TDW and sterilized by autoclaving.

Hematoxylin stain

For preparing 1 liter of hematoxylin stain, 5 g of hematoxylin (Merck) was dissolved in 100 ml of absolute alcohol. In a separate flask 100 g of potassium alum (Merck) was dissolved in 900 ml TDW and boiled. Hematoxylin solution was mixed with potassium alum while the latter is still hot. To this solution was added 2.5 g mercuric oxide (Merck) and rapidly cooled by plunging the flask in to cold water. The solution was filtered and stored in dark colored bottles.

Eosin stain

Dissolved 5 g of eosin (Merck) in 500 ml of TDW and mixed.

1% agarose gel for DNA electrophoresis (35 ml)

0.35 g agarose (Genei) was melt in 35 ml, 1X TAE buffer and allowed to cool for a few min at room temperature. To this was added 2 μl EtBr (10 mg/ml stock) solution to get 0.5 μg/ml final concentration. The molten agarose was poured in the gel casting tray (Genei) guarded on sides by clear tape. It was allowed to set for some time and DNA was run at constant voltage.

1% denaturing formaldehyde gel (35 ml)

Melted 0.35 g of agarose in 22 ml DEPC treated water and immediately added 7 ml of 5X formaldehyde gel running buffer (1X final conc.) followed by 6 ml of formaldehyde (2.2 mM final conc.). The molten agarose was poured in gel casting tray and allowed to set at room temperature.

Primer designing

Primers used for real time PCR analysis were designed with PrimerSelect module of Lasergene version 6 (DNA STAR) and procured from Sigma. The nucleotide sequences of selected genes were downloaded from reference sequence...
(RefSeq) database and saved as .seq files using EditSeq module of Lasergene. These files served as input data for PrimerSelect. Default settings of primer length ranged between 17 and 24 nucleotide having Tm values ranging between 55-60°C and product length ranging between 150-200 bp. Primer sequences were checked for their self complementary and avoided if they did not fulfill default parameters.

List of forward and reverse primers used for quantitative real-time PCR (qRT-PCR) analysis.

<table>
<thead>
<tr>
<th>S No.</th>
<th>Gene Name</th>
<th>Primer Sequence (5'............3')</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arhgap</td>
<td>FP: GATGATTGTTCCTCAGTGCCTACCC</td>
<td>67°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: GACCTGCGCTCTGCACACATCTCCCT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>H2-Eb1</td>
<td>FP: GCTACATGAACTGGGCTCTGAAA</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: CCGCGCGCTTTAGGTTACAGATTCAA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Zfp592</td>
<td>FP: TGCTCCTGTGGGATGGGAAAATTGG</td>
<td>66°C</td>
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<tr>
<td></td>
<td></td>
<td>RP: GCAGGACAGACAGCAGAAGGAAAC</td>
<td></td>
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<tr>
<td>4</td>
<td>S1c44</td>
<td>FP: GTGATGACACAGCCTGCACCTT</td>
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<tr>
<td></td>
<td></td>
<td>RP: CAGCGGGGAGGCTGTGGTATG</td>
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</tr>
<tr>
<td>5</td>
<td>Pcsk6</td>
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<td>RP: TTTCGGAAGGACGTGACCTGAGA</td>
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<td>Arih1</td>
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<td>RP: TGCCCTGTGACCTATCCGCTCT</td>
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<td>Polr2h</td>
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<td>8</td>
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<td>9</td>
<td>Hsd3b1</td>
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