Gene Expression and miRNA Study to Understand Signaling Pathways Inducing Apoptosis in Human Colon Cancer HT29 Cells

Application Note

Authors

Nilanjan Guha[1], Deepak S A[1], Arunkumar Padmanaban[1], Sreekanth Barkeer[2], and Shashikala R Inamdar[2]

[1]Agilent Technologies India Pvt. Ltd, Bangalore India

[2]Karnatak University, Department of Studies in Biochemistry, Dharwad, India

Abstract

This Application Note illustrates a gene expression and miRNA approach for studying signaling pathways that are affected by treatment of lectin, SRL, secreted by *Sclerotium rolfsii*, a soil borne plant pathogenic fungus. The treatment with SRL lectin results in apoptosis of colon cancer cells. The transcriptomics data were acquired using an Agilent Absolutely RNA miRNA Kit, an Agilent 2200 TapeStation, and a SureScan Microarray scanner. Agilent GeneSpring software suite was used to acquire, integrate, and analyze the transcriptomics and miRNA data.

Agilent Technologies

Introduction

The mechanism of apoptosis in colon cancer cell line HT29 upon induction with SRL was studied. *Sclerotium rolfsii*, a soil borne plant pathogenic fungus, secretes a developmental-stage specific lectin (SRL). SRL exhibits strong receptor-mediated antiproliferative activity towards human colon cancer cell lines by inducing apoptosis[1]. In this study, we investigated the signaling pathways that lead to apoptosis by combining transcriptomics data from gene expression and miRNA microarray analysis data, and performed pathway analysis using features of Agilent GeneSpring 13.1 (GS13.1). HT29 cells were treated with lectin, and samples were harvested at early and late time points. The study demonstrates how a cellular response profile can be obtained using the pathway analysis tool of GeneSpring software suite.

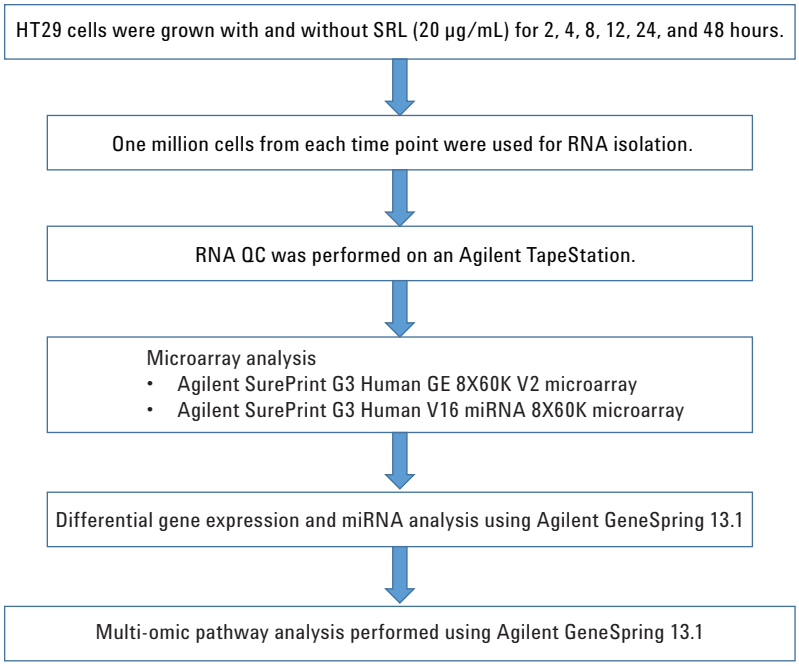

Figure 1. Overview of the experimental design used in this study.

Experimental

Study design

HT29 cells were grown with and without SRL to different time points for isolation of RNA and proteins as follows: cells were seeded at a concentration of 0.8×10^6 cells in a T25 flask (5 mL) and incubated for 48 hours in DMEM containing 10 % FBS (complete media). After incubation, cells were grown without (control) and with SRL (20 µg/mL) (treated) in DMEM media, containing 0.5 % BSA (incomplete media) and were incubated to different time points (2, 4, 8, 12, 24, and 48 hours). Following incubation for the specified time, the spent media was removed, and the cells were washed twice with PBS, then released using a cell scraper. The harvested cells in PBS (2 mL) were transferred to sterile DEPC-treated centrifuge tubes, washed with PBS, then centrifuged at 1,500 rpm at 4 °C. The cell pellet was used to isolate total RNA, or stored at –80 °C for further studies. Cells pooled from three T25 flasks were considered a biological replicate. One million cells were used at each time point for RNA isolation. Figure 1 outlines the workflow.

RNA analysis

RNA was extracted from treated and control HT29 cells using the Agilent Absolutely RNA miRNA Kit (p/n 400814). Both mRNA and miRNA were quantitatively recovered using this kit, allowing gene expression studies of both mRNA and miRNA from the same samples processed as above.

RNA quality control and testing

The RNA integrity number equivalent (RIN^e) values, which indicates the quality of isolated RNA, was determined on an Agilent 2200 TapeStation system (p/n G2964AA) using the RNA ScreenTape assay (p/n 5067-5576). In brief, 1 µL of RNA ScreenTape ladder was aliquoted into the first well of an optical tube strip (8x Strip), followed by the addition of 1 µL of total RNA to the rest of the wells; 5 µL of sample buffer was then added to all wells. The samples were briefly mixed by vortexing, denatured at 72 °C for three minutes, and cooled at 4 °C for two minutes and centrifuged to collect the samples. The samples were then placed in the 2200 TapeStation system, analyzed, and the samples with RIN^e values greater than seven (good quality RNA) were used for microarray analysis.

Preparation of samples for gene expression microarray analysis

Twenty-five nanograms of RNA from control and treated samples were labeled with Cy3 dye using an Agilent Low Input Quick Amp Labeling Kit (p/n 5190-2305). Gene expression microarray analysis was performed using the Agilent SurePrint G3 Human GE 8 × 60K V2 Microarray and an Agilent SureScan Microarray scanner. The gene expression data were extracted using Agilent Feature Extraction Software (11.5.1.1) and analyzed using Agilent GeneSpring GX 13.1. In both mRNA and miRNA analyses, transcripts exhibiting $P \leq 0.05$ and fold changes greater than or equal to two were considered to be differentially expressed.

Preparation of samples for miRNA analysis

One hundred nanograms of miRNA were labeled with Cy3 dye using an Agilent miRNA Complete Labeling and Hyb Kit (p/n 5190-0456). miRNA microarray analysis was performed using the Agilent SurePrint G3 Human v16 miRNA 8X60K Microarray kit and an Agilent SureScan Microarray scanner. The miRNA data were extracted using Agilent Feature Extraction and analyzed using Agilent GeneSpring GX 13.1.

Real-time PCR assay

RNA was extracted from the cell pellets as described earlier. All the primers used in the study were designed using Beacon Designer (Premier Biosoft). One-step qRT-PCR was performed using Brilliant III ultrafast SYBR Green QRT-PCR master mix. The 20 µL reaction contained 10 µL of master mix, 3 µL of forward and reverse primers (150 nM), 1 µL of RT/RNase block, 0.2 µL of DTT, and 16.7 ng of total RNA per reaction. The amplifications were performed in 96-well plates using an Agilent Mx3005P qPCR instrument (p/n 401458).

After performing reverse transcription at 50 °C for five minutes, denaturation was done at 95 °C for three minutes, followed by 40 cycles of 95 °C for 10 seconds, and annealing/extension for 20 seconds (50 to 60 °C depending on the primer pairs). No template controls (NTC) were maintained to ensure the absence of contaminations. The amplicon sizes from the resulting reactions were confirmed by analyzing the products on an Agilent 2100 Bioanalyzer system (pn G2943CA) using the DNA 1000 kit (p/n 5067-1504) according to the manufacturer's protocol.

The RT-qPCR data were analyzed using Agilent MxPro software, and exported for correlation analysis in GS13.1. The Agilent MxPro text output file was modified with column headers **Sample**, **Detector**, **Task**, and **Cq Avg** for uploading into GS13.1 using "RTPCR" as the experiment type. The RT-qPCR entity list was translated into Agilent Single Color technology (GeneSpring Technology 39494). An entity list was created for the microarray data in which only the genes studied by RT-qPCR were included. Comparison of the microarray data, and RT-qPCR data was made using correlation analysis.

Differential and pathway analysis

The microarray data were analyzed for gene expression by mRNA and miRNA using the Gene Expression and miRNA workflow in GeneSpring GX 13.1, respectively. Default flag settings were used to make the detection calls. Signal intensities for each probe were normalized to the 75th percentile without baseline transformation for gene expression analysis. Normalization and baseline transformation was not performed for miRNA analysis. Differential gene and miRNA expression analysis was done using the **Filter on Volcano Plot** option in GeneSpring. The analysis was carried out using a T-test unpaired statistical method with Benjamini Hochberg FDR method; p-values were computed asymptotically.

Pathway analysis was carried out using the Pathway Analysis Module in GS13.1. The differentially expressed gene entity list for each time point ($p \leq 0.05$ and fold change ≥ 2.0) was selected for pathway analysis. Curated pathways from the KEGG were used for pathway analysis.

Results and Discussion

SRL treatment at early time points

Mitogen-activated protein kinase (MAPK) genes showed marked differential regulation at early time points. The transcription factor c-JUN showed 10-fold up-regulation as early as two and four hours following lectin treatment. The expression level gradually decreased to 3.51 and 2.62-fold, respectively, at eight and 12 hours. Other genes that showed differential regulation at early time points were MAP3K14 and DUSP1, which were up-regulated, and MAP3K4 and ATF2, which were down-regulated at two hours post SRL treatment. The results suggest that the induction of apoptosis by SRL is initiated by an interplay of phosphatases and kinases belonging to the MAP kinase pathway. Figure 4 shows the MAPK pathway generated using the KEGG pathway module of GS13.1. The yellow rectangles show the differentially expressed MAPK genes upon SRL induction, and the histograms near entities indicate the relative levels of the genes.

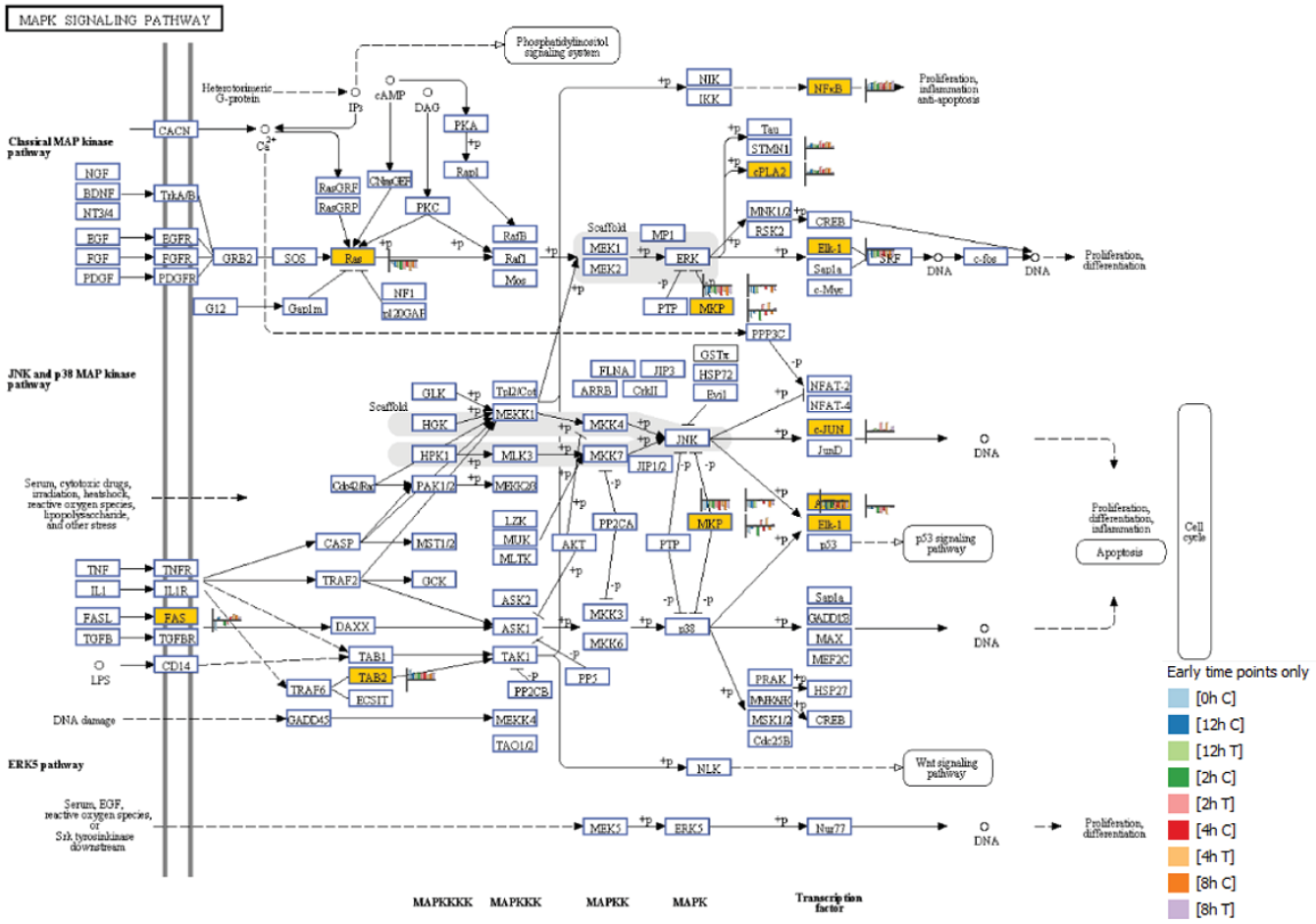

Figure 2. The MAPK pathway at four hours post-SRL treatment. Yellow boxes indicate that transcripts were differentially expressed. The heat strips next to each entity illustrate gene expression levels for each time point.

mRNA/miRNA correlation analysis

miRNA microarrays on SRL-treated and untreated HT29 cells at various time points were performed to identify miRNA candidates affecting gene expression. At two and four hours, eight and seven miRNAs were differentially regulated respectively with the number of miRNAs increasing significantly after four hours of lectin treatment. At eight and 12 hours, 85 and 144 miRNAs were differentially expressed, respectively, between the treated and control samples ($p \leq 0.05$). GS13.1 enables users to perform correlation analysis among the entities of two experiments performed by two different technologies including mRNA/miRNA pairing. Figure 3 shows correlation of differentially expressed miRNAs and differentially expressed MAPK genes at four hours. The correlation coefficient scatter plot shown in Figure 3B shows the dependency of the expression of JUN on hsa-miR-487b, suggesting that JUN can be a potential target of that miRNA.

Validation of microarray gene expression data with qRT-PCR

The gene expression microarray data were compared with qRT-PCR data. Both up- and down-regulated genes were chosen for the purpose. Overall, the gene expression data were in good concordance with qRT-PCR data, with most of the genes showing similar trends.

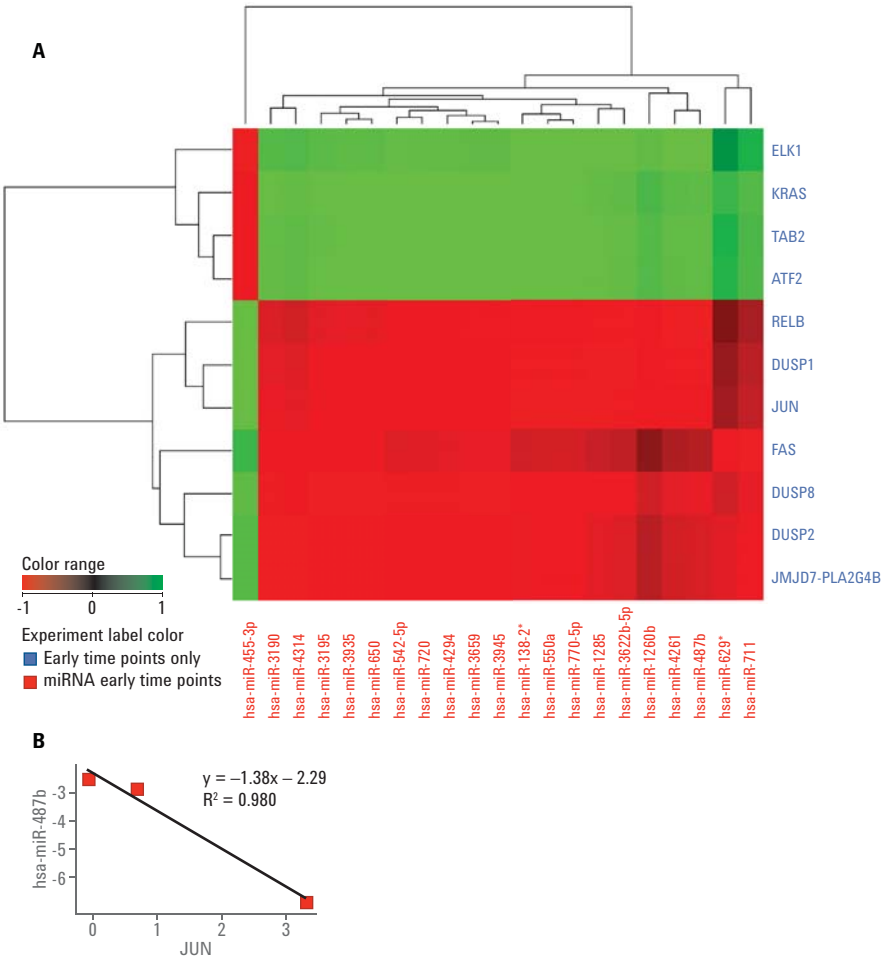

Figure 3. A) Correlation analysis showing the correlation pattern between differentially expressed MAPK mRNAs and miRNAs differentially expressed at four hours. B) Scatter plot showing the relationship between JUN and hsa-miR-487b.

Conclusions

In this study, we investigated the signaling pathways that are affected by SRL using Agilent GeneSpring 13.1 to identify the signature genes and miRNAs that lead to apoptosis of HT29 cells. The results suggest that SRL induces apoptosis in HT29 cells by affecting multiple signaling pathways leading to cellular apoptosis. These findings demonstrate the signature profiles of SRL-induced gene expression, which helps in understanding the mechanism of action.

GeneSpring offers an interactive computing environment that enables mapping of differential entities (genes, proteins, or metabolites) to biological pathways. Multi-omics data can be visualized in a pathway context using the Pathway Architect tool of GS 13.1 to facilitate further exploration, and suggest follow-up experimentation. In addition, correlation analysis can be used to see the relationship among expression levels of various entities from within an experiment or cross-experiment and different omics.

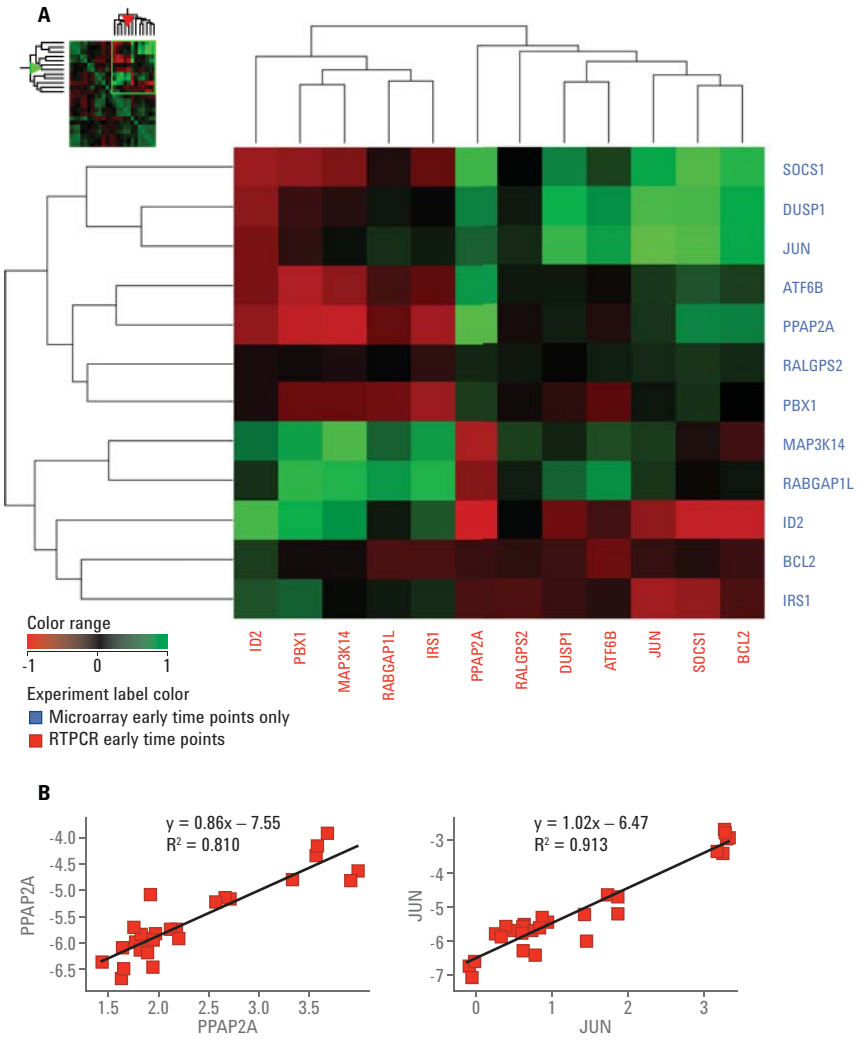

Figure 4. A) Correlation analysis showing the correlation of microarray gene expression data with qRT-PCR data. B) Scatter plots showing the correlation of expression of PPAP2A and JUN between microarray and qRT-PCR.

Reference

1. Barkeer, S.; *et al.* Molecular mechanism of anti-cancer effect of Sclerotium rolfsii lectin in HT29 cells involves differential expression of genes associated with multiple signalling pathways; A microarray analysis. *Glycobiology*. **2015** Sep 7. [Epub ahead of print]

www.agilent.com/chem

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

Published in the USA, March 1, 2016
5991-6377EN

Agilent Technologies