Screening of Nucleostemin p53-Independent Signal Pathway in NB4 Leukemia Cell

Abdallah Dlykan[1], Sun Xiaoli[2], Atul Dwivedi[3], Wang Ningning[4]

ABSTRACT

Introduction: Nucleostemin is a newly discovered nucleoprotein which is found to be highly expressed in the undifferentiated cells like stem cells and cancer cells. It is an important G1/S checkpoint regulator. The aim of study was to find the specific mechanism of nucleostemin p-53 independent signal pathway. Finding NS p53-independent signal pathway can be an important tool use in gene therapy of cancer patients.

Material and Methods: The present study comprised of sample size of 2719 differentially expressed genes from NB4 experimental group, 847 genes were up-regulated and 1872 genes were down-regulated. RNA quantity and quality were measured by NanoDrop ND-1000. For DNA microarray, the Whole Human Genome Oligo Microarray was used and sequences were compiled from a broad source survey, and then verified and optimized by alignment to the assembled human genome. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v12.0 software package (AgilentTechnologies). Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed genes played in these biological pathways or GO terms. Finally, Hierarchical Clustering was performed to show the distinguishable gene expression profiling among samples.

Results: Inhibition of Nucleostemin mRNA expression succeeded in human leukemia cell lines NB4 by using lentiviral technology, the rate of Inhibition in NB4 cells was 58%, Real-time PCR demonstrated the reliability of the data. These differentially expressed genes may involve in great kinds of functions and signal pathways through GO analysis and Pathway analysis, in which the association with MAPK signaling pathway and NOD-like receptor signaling pathway for up-regulated the most related and significant. RNA transport and mRNA surveillance pathway is the most significant and related pathways for down-regulated. RNA transport from the nucleus to the cytoplasm is fundamental for gene expression.

Conclusion: The data reveals that MAPK signaling pathway is one of the most effected pathway for upregulated genes, thus it is one of the important pathways, that is p53 independent and modifying it can cause NB4 cell apoptosis. Similarly, RNA transport signaling pathway as a most effected pathway of downregulated genes.

Keywords: Nucleostemin; Leukemia Cell; Lentivirus Vector; DNA Microarray; RNA Transport; MAPK Signaling Pathway; Cell Apoptosis

INTRODUCTION

Nucleolar protein called nucleostemin (NS), is a nucleolar GTP binding protein, is considered as a novel regulator of the p53-MDM2 feedback loop initially identified in the nucleolus of rat neural stem cells[1] and subsequently found upregulated in various types of proliferating stem cells and cancer cells[1-4] However, unlike other known nucleolar regulators of this loop, NS surprisingly plays a dual role, as both up and downregulations of its level and could turn on p53 activity. Knockout studies demonstrate that NS is essential for cell proliferation and embryogenesis.[5,6] During stem cell differentiation both in vivo and in vitro experiments, NS levels were rapidly reduced prior to exiting the cell cycle, suggesting the expression of NS is associated with the proliferation potential of cells. Recent work, however, has demonstrated that NS is in fact widely expressed in many types of normal proliferating cells at levels similar to those in malignant cells. For instance, NS is expressed in normal kidney cells and renal carcinoma cells at comparable levels as detected in histological sections.[7] Moreover, p53 is one of the most studied proteins in the whole of contemporary biology, with more than 12,500 papers so far written! Several studies have provided evidence that the p53 signaling pathway is involved in the G1 arrest of the cell cycle induced by the down-regulation of NS. Physical interaction between NS and p53 was initially reported by Tsai and McKay.[1] Later, it was shown that the G1 arrest requires the presence of p53.[8] In the most recent study Dai *et al.*[9] showed that knockdown of NS enhances the interaction between the p53-binding protein MDM2 and the ribosomal protein L5 or L11, preventing MDM2 from inducing ubiquitylation-based p53 degradation.

It is a protein concentrated in the nucleolus of most stem cells and also in many tumor cells, which has been implicated in cell-cycle progression owing to its ability to modulate p53. It a newly discovered nucleoprotein which found to be highly expressed in the undifferentiated cells like stem cells and cancer cells. It is an important G1/S checkpoint regulator.[10]

NS is gaining popularity as an important protein and may be helpful in down regulating cancer cell gene. This may be an important tool in gene therapy. The present study was conducted with aim to find the specific mechanism of nucleostemin p-53 independent signal pathway.

[1]PhD Student, Department of Hematology, Department of Laboratory Medicine, [3]PhD Student, Department of Orthopedics, [4]MD Doctor, The First Affiliated Hospital of Zhengzhou University, [2]MD Doctor, First Affiliated Hospital of Henan TCM University, China

Corresponding author: Abdallah Dlykan, Foreign Hostel, Building, 40 Daxue Road, Yixue Yuan, Zhengzhou University, Zhengzhou, 450052, Henan, China

How to cite this article: Abdallah Dlykan, Sun Xiaoli, Atul Dwivedi, Wang Ningning. Screening of nucleostemin p53-independent signal pathway in NB4 leukemia cell. International Journal of Contemporary Medical Research 2017;4(4):914-919.

MATERIAL AND METHODS

The present study comprised sample size of 2719 differentially expressed genes, from 2631 group NB4 experimental group 847 genes were up-regulated and 1872 genes were down-regulated. RNA quantity and quality were measured by NanoDrop ND-1000. RNA integrity was assessed by standard denaturing agarose gel electrophoresis. Figure 1 shows experiment workflow. For DNA microarray, the Whole Human Genome Oligo Microarray was a broad view that represents all known genes and transcripts in the human genome. Sequences were compiled from a broad source survey, and then verified and optimized by alignment to the assembled human genome. For RNA labeling and array hybridization, sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Briefly, total RNA from each sample was linearly amplified and labeled with Cy3-UTP. The Labeled cRNAs were purified by RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/µg cRNA) were measured by NanoDrop ND-1000. 1 µg of each labeled cRNA was fragmented by adding 11 µl 10 × Blocking Agent and 2.2 µl of 25×Fragmentation Buffer, then heated at 60 °C for 30 min, and finally 55 µl 2 × GE Hybridization buffer was added to dilute the labeled cRNA. 100µl of hybridization solution was dispensed into the gasket slide and assembled to the gene expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (part number G2505C).

STATISTICAL ANALYSIS

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed with using the GeneSpring GX v12.0 software package (Agilent Technologies). After quantile normalization of the raw data, genes that at least 6 out of 6 samples have flags in Detected ("All Targets Value") were chosen for further data analysis. Differentially expressed genes with statistical significance were identified through Fold Change filtering. Hierarchical Clustering was performed using the Agilent GeneSpring GX software (version 12.0). GO analysis and Pathway analysis were performed in the standard enrichment computation method.

We have completed the human expression microarray analysis of the samples. Total RNA from each sample was quantified by the NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. For microarray analysis, Agilent Array platform was employed. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, total RNA from each sample was amplified and transcribed into fluorescent cRNA with using the manufacturer's Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the Whole Human Genome Oligo Microarray (4 x 44K, Agilent Technologies). After having washed the slides, the arrays were scanned by the Agilent Scanner G2505C.

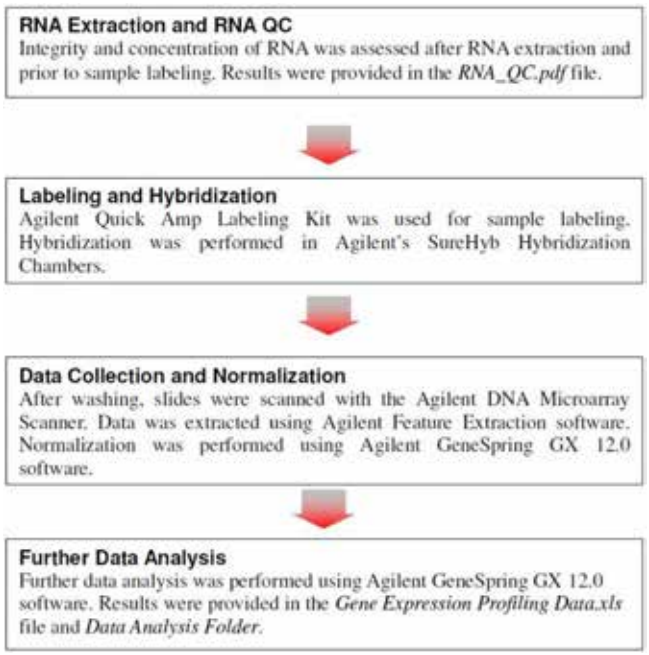


Figure-1: Experiment Workflow

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies). After quantile normalization of the raw data, genes that at least 6 out of samples have flags in Detected ("All Targets Value") were chosen for further data analysis. Differentially expressed genes with statistical significance were identified through Fold Change filtering for two compared samples. Pathway analysis and GO analysis were applied to determine the roles of these differentially expressed genes played in these biological pathways or GO terms. Finally, Hierarchical Clustering was performed to show the distinguishable gene expression profiling among samples.

RESULTS

Inhibition of Nucleostemin mRNA expression succeeded in human leukemia cell lines,NB4 by using lentiviral technology, the rate of Inhibition in NB4 cells was 58%,. The purity, integrity and quality of RNA were met the requirements for microarray hybridization. 2719 differentially expressed genes were identified (Figures 2,3) through Fold Change≥2 or ≤0.5, in which 847 genes were up-regulated and 1872 genes were down-regulated. Real-time PCR demonstrated the reliability of the data. These differentially expressed genes (Figure 4) may involve in great kinds of functions and signal pathways through GO analysis and Pathway analysis, in which the association with MAPK signaling pathway (Pathway map 2) and NOD-like receptor signaling pathway for up-regulated the most related and significant (Figure 5b).

RNA transport and mRNA surveillance pathway is the most significant and related pathways for down-regulated (Figure 5a). RNA transport (Pathway map 2) from the nucleus to the cytoplasm is fundamental for gene expression.

The mRNA surveillance pathway (Table 1) is a quality control mechanism that detects and degrades abnormal mRNAs.

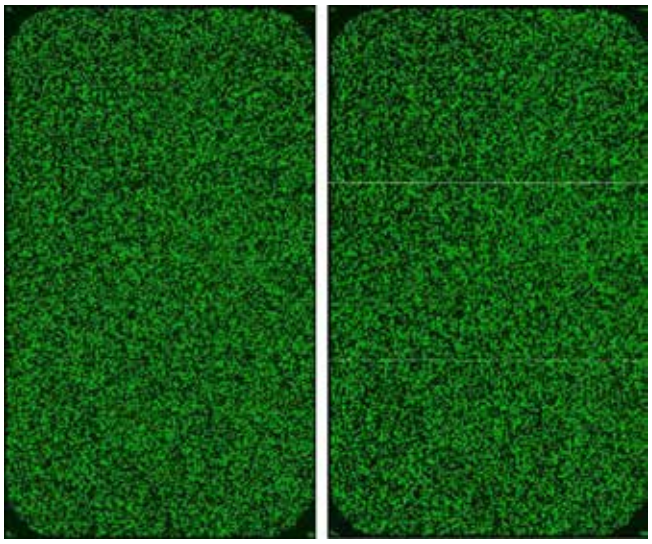


Figure-2: Graphs of Gene Chip, left controller right experimental of NB4 cell DNA microarray

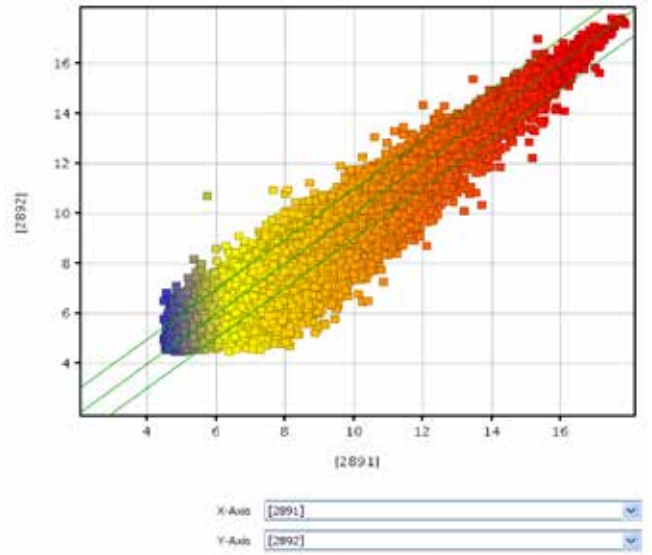


Figure-3: The scatterplot is a visualization that is useful for assessing the variation (or reproducibility) between chips.

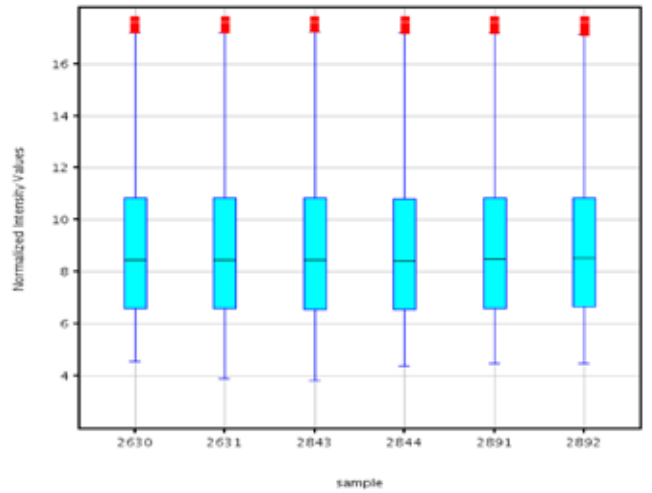


Figure-4: Boxplot view is used to look at, and compare, the distributions of expression values for the samples or conditions in an experiment after normalization.

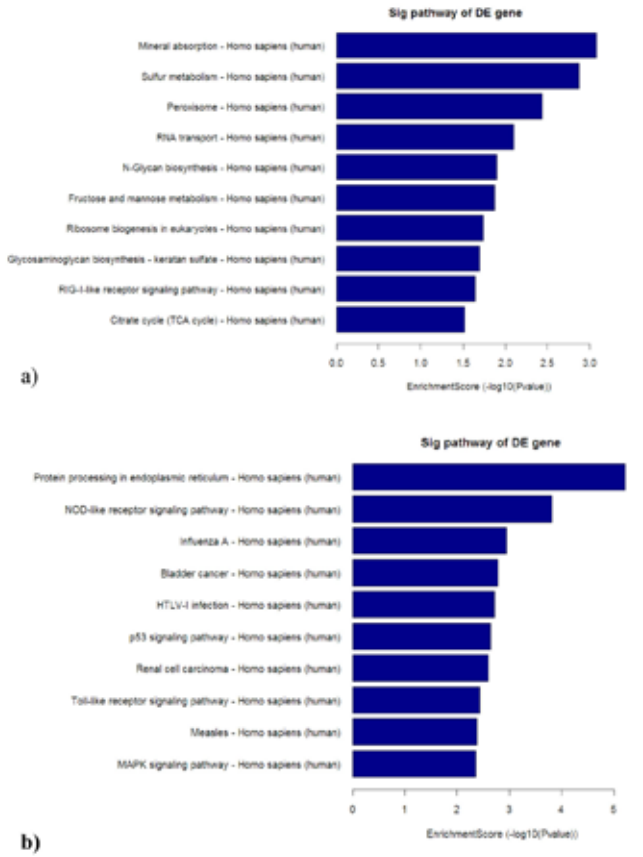


Figure-5: a) down-regulated pathways. b) up-regulated pathways.in NB4 cell.

DISCUSSION

Nucleostemin before was supposed to be P53 dependent signal pathway. Many new studies conducted found that NS has independent P53 signal pathway. Nucleostemin is a recently discovered nucleolar protein predominantly associated with proliferating rat neural and embryonic stem cells, and some human cancer cell lines. A comprehensive study of nucleostemin in human adult bone marrow stem cells is lacking.[11]

It was all started from Tsai and MC Kay, when they identified a novel protein, nucleostemin, found in the nucleoli of CNS. Stem cells, embryonic stem cells, and several cancer cell lines and preferentially expressed by other stem cell-enriched populations.[1]

NS through the p53 pathway plays a biological role. It is a newly discovered nucleoprotein which is found to be highly expressed in the undifferentiated cells like stem cells and cancer cells. It is an important G1/S checkpoint regulator.[12]

The mitogen-activated protein kinase (MAPK) cascade is a highly-conserved module that is involved in various cellular functions, including cell proliferation, differentiation and migration. Mammals express at least four distinctly regulated groups of MAPKs, extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38alpha/beta/gamma/delta) and ERK5, that are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK3/6 for the p38, MKK4/7 (JNKK1/2) for the JNKs, and MEK5 for ERK5. Each MAPKK, however, can be activated by more than one

#Column "PathwayID" stands for Pathway identifiers used in KEGG
#Column "Definition" stands for the definition of the PathwayID
#Column "OriginalWebSite"stands for The map's website,the nodes with red frame and red symbol are associated with the DE genes,
#Column "p-value"
"EASE-score" stands for the enrichment p-value of the PathwayID used EASE method, EASE Score is modified Fisher's exact pro "Fisher-Pvalue" stands for the enrichment p-value of the PathwayID used Fisher's exact test
"Hypergeometric-Pvalue" stands for the enrichment p-value of the PathwayID used Hypergeometric test
#Column "SelectionCounts" stands for the Count of the DE genes' entities directly associated with the listed PathwayID

PathwayI	Definition	O	Fisher-Pvalue	Sele	Sele			FDR	Enrichme	Genes
hsa03040	Spliceosome - Homo sapiens	h	3.71589E-	34	59	12	608	7.1345E-	7.4299	ACIN1//BUD31//
hsa03013	RNA transport - Homo sapiens	h	2.51665E-	32	59	15	608	0.002415	4.5991	AAAS//ACIN1//
hsa03410	Base excision repair - Homo	h	0.0002630	11	59	34	608	0.016836	3.5799	APEX1//FEN1//H
hsa00520	Amino sugar and nucleotide sugar	h	0.0005313	13	59	48	608	0.025502	3.2746	GALE//GALK1//
hsa05221	Acute myeloid leukemia - Homo	h	0.0011510	14	59	58	608	0.043422	2.9389	AKT
hsa04142	Lysosome - Homo sapiens	h	0.0013923	23	59	12	608	0.043422	2.8562	ABCA2//AP1M1
hsa04141	Protein processing in endoplasmic	h	0.0015831	29	59	16	608	0.043422	2.8004	AMFR//BAK1//C
hsa03050	Proteasome - Homo sapiens	h	0.0034298	11	59	45	608	0.081213	2.4647	PSMA7//PSMB9/
hsa05213	Endometrial cancer - Homo	h	0.0038068	12	59	52	608	0.081213	2.4194	AKT
hsa04666	Fc gamma R-mediated	h	0.0046109	18	59	95	608	0.088529	2.3362	AKT
hsa04662	B cell receptor signaling pathway -	h	0.0055621	15	59	75	608	0.097084	2.2547	AKT
hsa00310	Lysine degradation - Homo sapiens	h	0.0068585	11	59	49	608	0.109737	2.1637	DOT1L//EHMT
hsa04910	Insulin signaling pathway - Homo	h	0.0077578	23	59	13	608	0.113615	2.1102	AKT
hsa05130	Pathogenic Escherichia coli	h	0.0082844	12	59	57	608	0.113615	2.0817	ABL1//ARHGEF
hsa04150	mTOR signaling pathway - Homo	h	0.0108534	11	59	52	608	0.138923	1.9644	AKT2//DDIT
hsa04722	Neurotrophin signaling pathway -	h	0.0116563	21	59	12	608	0.139875	1.9334	ABL1//AKT
hsa00511	Other glycan degradation - Homo	h	0.0205715	5	59	17	608	0.218776	1.6867	GBA//HEXA//M
hsa03030	DNA replication - Homo sapiens	h	0.0211445	8	59	36	608	0.218776	1.6748	FEN1//LIG1//MC
hsa00480	Glutathione metabolism - Homo	h	0.0216497	10	59	50	608	0.218776	1.6645	G6PD//GGT
hsa05140	Leishmaniasis - Homo sapiens	h	0.0242224	13	59	73	608	0.221462	1.6157	C3//CYBA//ELK1
hsa05220	Chronic myeloid leukemia - Homo	h	0.0242224	13	59	73	608	0.221462	1.6157	ABL1//AKT
hsa03015	mRNA surveillance pathway -	h	0.0333813	14	59	84	608	0.291327	1.4765	ACIN1//CPSF1//
hsa03420	Nucleotide excision repair - Homo	h	0.0416868	9	59	48	608	0.347994	1.38	DDB2//ERCC1//

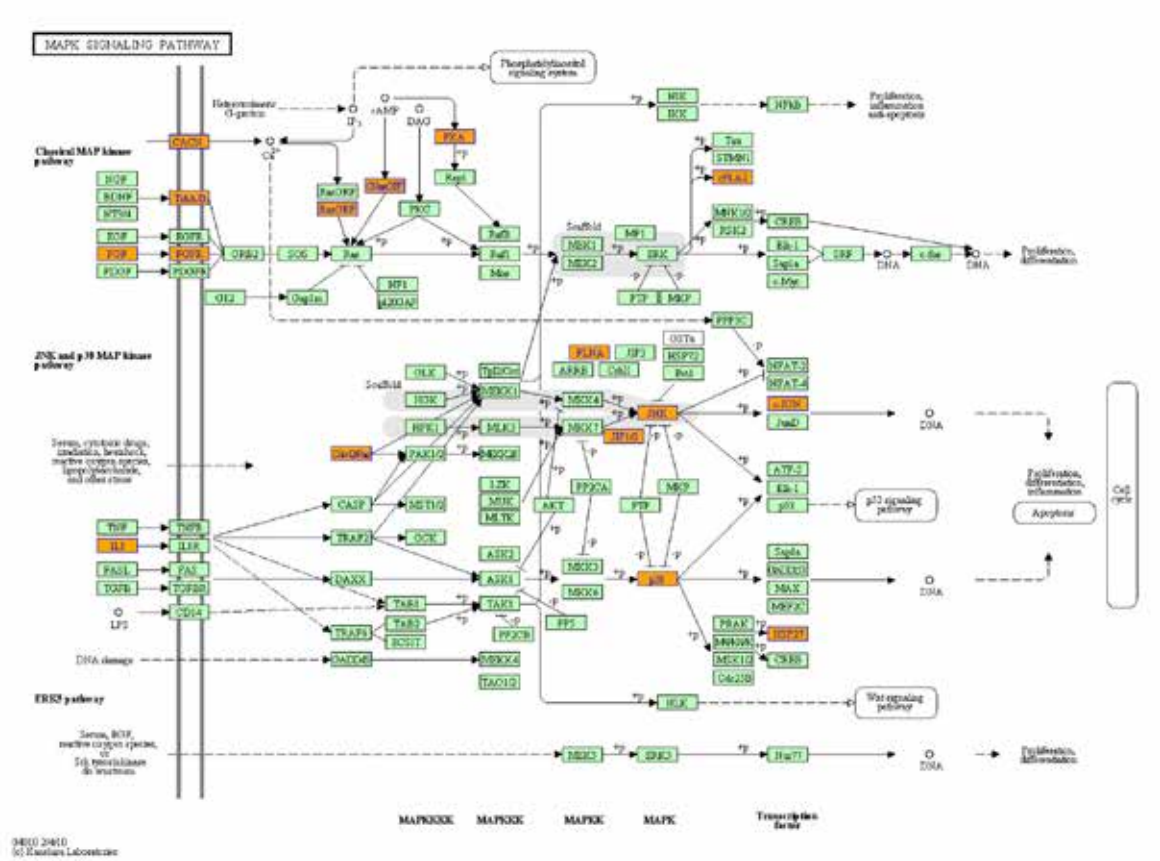
Table-1: Down-regulated pathways and associated DE genes

MAPKKK, increasing the complexity and diversity of MAPK signaling. Presumably each MAPKKK confers responsiveness to distinct stimuli. For example, activation of ERK1/2 by growth factors depend on the MAPKKK c-Raf, but other MAPKKKs may activate ERK1/2 in response to pro- inflammatory stimuli.[13-16]

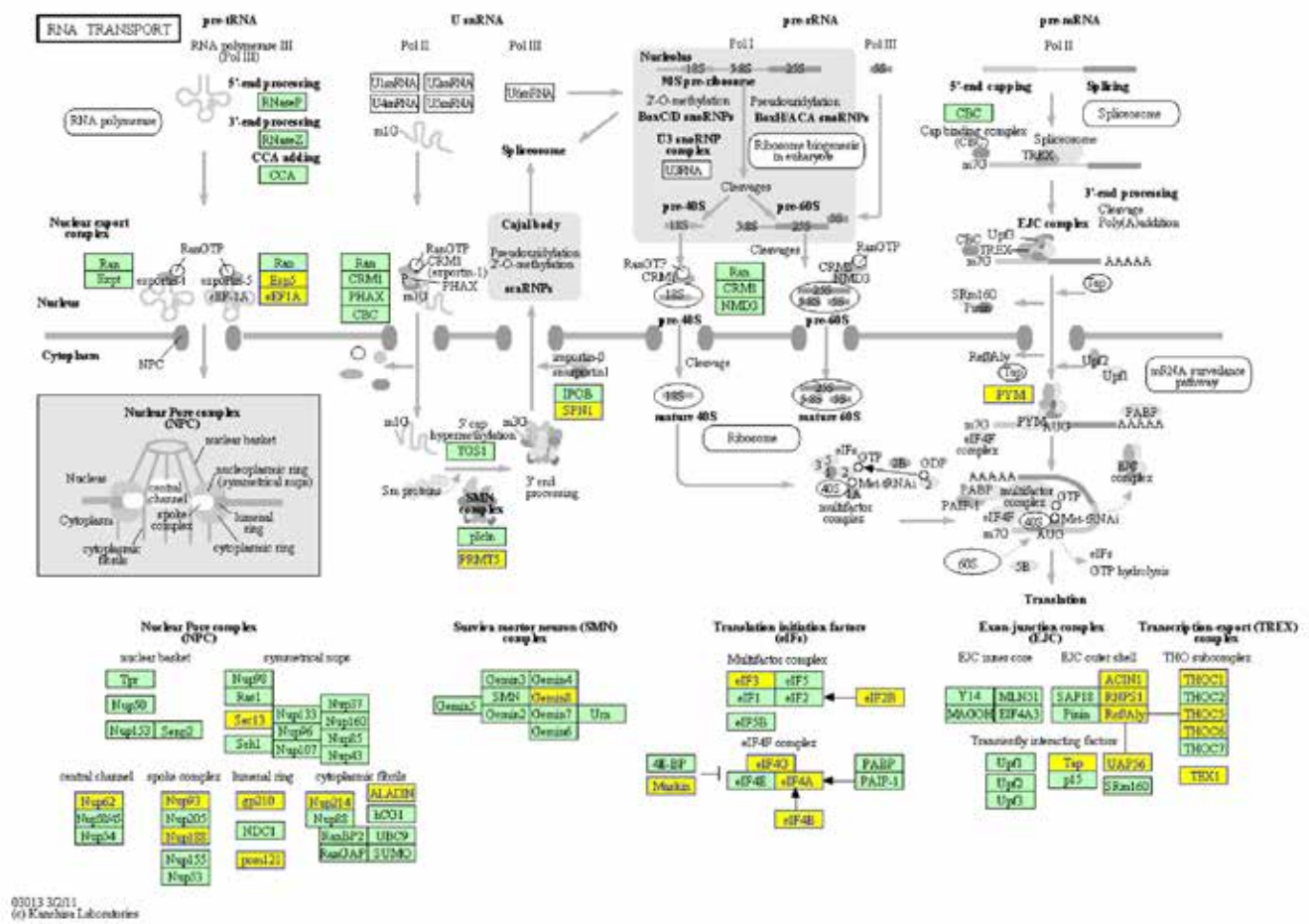
NOD-like receptor signaling pathway specific families of pattern recognition receptors are responsible for detecting various pathogens and generating innate immune responses. The intracellular NOD-like receptor (NLR) family contains more than 20 members in mammals and plays a pivotal role in the recognition of intracellular ligands. NOD1 and NOD2, two prototypic NLRs, sense the cytosolic presence of the bacterial peptidoglycan fragments that escaped from endosomal compartments, driving the activation of NF-{kappa}B and MAPK, cytokine production and apoptosis. On the other hand, a different set of NLRs induces caspase-1 activation through the assembly of multiprotein complexes called inflammasomes. These NLRs include NALP1, NALP3 and Ipaf. The inflammasomes are critical for generating mature proinflammatory cytokines in concert with Toll-like receptor signaling pathway.[17-21]

Furthermore, RNA transport and mRNA surveillance pathway is the most significant and related pathways for down-regulated (Figure 5a). RNA transport (Pathway map 2) from the nucleus to the cytoplasm is fundamental for gene expression. The different RNA species that are produced in the nucleus are exported through the nuclear pore complexes (NPCs) via mobile export receptors. The majority of RNAs, such as tRNAs, rRNAs, and U snRNAs, are transported by specific export receptors, which belong to the karyopherin-beta family proteins. A feature of karyopherins is their regulation by the small GTPase Ran. However, general mRNA export is mechanistically different. Nuclear export of mRNAs is functionally coupled to different steps in gene expression processes, such as transcription, splicing, 3'-end formation and even translation.[22-26]

The mRNA surveillance pathway (Table 1) is a quality control mechanism that detects and degrades abnormal mRNAs. These pathways include nonsense-mediated mRNA decay (NMD), nonstop mRNA decay (NSD), and no-go decay (NGD). NMD is a mechanism that eliminates mRNAs containing premature translation-termination codons (PTCs). In vertebrates, PTCs



Pathway map-1: MAPK transport



Pathway map-2: RNA transport

trigger efficient NMD when located upstream of an exon junction complex (EJC). Upf3, together with Upf1 and Upf2, may signal the presence of the PTC to the 5'end of the transcript, resulting in decamping and rapid exonucleolytically digestion of the mRNA. In the NSD pathway, which targets mRNAs lacking termination codons, the ribosome is believed to translate through the 3' untranslated region and stall at the end of the poly(A) tail. NSD involves a n eRF3-like protein, Ski7p, which is hypothesized to bind the empty A site of the ribosome and recruit the exosome to degrade the mRNA from the 3' end. NGD targets mRNAs with stalls in translation elongation for endonucleolytic cleavage in a process involving the Dom34 and Hbs1 proteins.[27-30]

Therefore, we nominated those four pathways MAPK signaling pathway and NOD-like receptor signaling pathway for up-regulated and RNA transport and mRNA surveillance pathway for down-regulated furthermore we suggest that indicate that

MAPK signaling pathway and RNA transport they are most probably responsible for NB4 leukemia cells apoptosis, and the weak proliferation capacity tumorigenic respectively

CONCLUSION

Inhibition of Nucleostemin mRNA expression succeeded in three human leukemia cell lines HL-60, NB4 and K652 by using lentiviral technology, the rate of Inhibition in NB4 cells was 58%, that's increase the probability of presence of Nucleostemin p53-independent signal pathway, and push for more follow-up studies.

This research is a forward step to explore the exact specific mechanism of Nucleostemin p53-independent signal pathway in NB4 leukemia cells which will help to improve more effective treatment for cancer patients. Especially with more follow up studies.

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Source of Support: Nil; **Conflict of Interest:** None

Submitted: 18-03-2017; **Accepted:** 12-04-2017; **Published:** 09-05-2017