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GENE SILENCING;

STUDY THE ROLE OF GENE SILENCING IN CERVICAL CANCER CELL LINE TO CONTROL CELL PROLIFERATION

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ABSTRACT… Objectives: Cancer, the most complex group of genetic disorders results due to over expression or mutation of oncogenes/molecules involved in cell signaling pathways. KRAS is an oncogene that encodes a small GTPase protein with two isoforms KRasA & KRasB and is involved in the regulation of cell division. KRas is frequently found mutated in lung, pancreas, colorectal and many other cancers. Various studies have found that KRasB promotes cell proliferation and inhibits apoptosis whereas KRasA has negligible role in cell proliferation or rather is involved in apoptosis at times. Several experiments have shown tumor growth inhibition by silencing KRas in various tumor models having a differential allelic expression. The goal of our study was to determine the possible differential role of KRas A and B on MAPK Pathway. To examine the disparity in role of various isoforms of KRas on apoptosis, we evaluated the expression of these isoforms through different modalities in HeLa cells before and after silencing KRas through RNA interference. **Study Design:** In vitro study for isolation of protein molecules (Proteomics) and to study various genes (Genomics) through Polymerase chain reaction. **Study Duration:** December, 2011-September, 2014. **Setting:** Center for Research in Molecular Medicine, University of Lahore. **Material & Methods:** In present study, we studied the expression level and behavior of many sets of molecules such as KRasA, KrasB, Bad, Bcl2, BclxL and Mcl-1 through gene quantitation by Real Time PCR. We also analyzed the protein expression through Western blot immune-precipitation. All the tests were done before and after 48-hours of silencing of HeLa cells with shRNA designed for KRas. **Results:** We successfully downregulated KRasB (80%) but found upregulation of KRasA with continued cell proliferation. We also found overexpression of antiapoptotic genes, BclxL and Mcl1 and downregulation of proapoptotic molecule-Bad. Differences were considered significant at $p < 0.01$. Values were expressed as mean ± SEM from six separate experiments. **Conclusion:** We were able to show that in the absence of one proliferative gene, another sister gene upregulates and takes over the role of uncontrolled cell proliferation. This usually leads to failure of most cancer control therapies.

Key words: Apoptosis, GTPase, HeLa Cells, KRas, MAPK Pathway, RNA Interference.

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INTRODUCTION

Human cancer, the most complex genetic disease[1], is one of the major causes of death in the world. Its prevalence rate is estimated as 6 million deaths per year that accounts for 12% deaths worldwide.[2] Cancer is becoming a major cause of death in Asian countries too.[3] Equilibrium in physiological events at cellular level i.e., proliferation and apoptosis are controlled by a complex of network of signaling pathways. Alteration in expression and function of numerous molecules in these intricate pathways, notably in activating components like Ras, PI3K and RTK or in inactive regulators such as NF1, p53, p16, PTEN play the most critical role in the development of cancer.[4] Among these, mutational activation of Ras gene has been observed in 30% of all human cancers.[5]

Ras (Rat sarcoma) family of oncogenes encodes highly related three functional proteins, GTPases with molecular mass of ~21KD (188-189 a. a), first discovered as a transforming oncogene of murine sarcoma viruses. These highly related proteins are H-Ras (Harvey); N- Ras (neuroblastoma); K-Ras (Kristen) alternatively

spliced in two protein isoforms, KRasA & KRasB, that share high homology in the first 165 amino acids and show difference in 25 amino acids at C-terminus.[6] Wild type of Ras proteins control cell growth, proliferation, senescence/cell cycle arrest, survival and differentiation of various cell types.[7]

Synthesis of Ras protein occurs in cytoplasm followed by anchorage at the plasma membrane.[8] Its biological activity is controlled by binding with GTP. Once bound with GTP, Ras becomes active and regulates various downstream effectors, such as Rafs (Rapidly accelerated fibrosarcoma), PLC (Phospholipase C), PI-3K (Phosphatidyl inositide-3Kinase), and Ral (Ras related protein).[9,10] A serine/ threonine kinase Raf, is a direct critical effector of Ras, and one of the members of mitogen activated protein (MAP) kinase signaling cascade.[11,12] Once activated, Raf activates a series of signaling complexes, MEK and then mitogen-activated protein kinase (MAPK/extra cellular signal regulated kinase, ERK) 1/2, MAPK kinase (MAPKK, or MAPK/ERK kinase, MEK) and MAPKK kinase (MAPKKK, or MEK kinase, MEKK).[13,14] Activated ERK1 and ERK2, (44 & 42-KD respectively) phosphorylate a number of cyto-skeletal proteins such as 90 KD ribosomal protein S6 kinases (RSKs), MAPK-interacting kinases (MNK1 & 2), nuclear factor-κB (NF-κB), serum response factor (SRF), cyclic AMP response element-binding protein (CREB), ternary complex factors (TCFs), and c-Myc (Myelocytomatosis).[15,16]

KRAS mutations are prevalent in various malignancies such as pancreatic, colorectal, lung and bladder cancer.[27] Its oncogenecity can be inhibited by inducing apoptosis in cancer cells by prevention of Ras membrane localization through FTase inhibitors[28]; by targeting downstream effectors of Ras protein through small molecule inhibitors[29] or by suppressing its expression through antisense RNAs.[30]

Bcl-2 (B-cell lymphoma 2) family of proteins has a decisive role in apoptosis (a physiological phenomenon of programmed cell death).[31] Pro-apoptotic proteins include Bcl-2 associated X protein (Bax), Bcl-2 associated death promoter (Bad), Noxa, P53 upregulated modulator of apoptosis (Puma) while anti apoptotic proteins are Bcl-2, B-cell lymphoma extra large (Bcl-xl) and Induced myeloid leukemia cell differentiation protein (Mcl-1).[32] Gene silencing through RNA interference, is an experimental tool that not only analyzes gene function but is expected to be an emerging class of gene medicine for targeting cancer. In this technology short strands of double stranded RNAs are introduced into the target tissue that interfere with a particular gene of interest, preventing it from expression of its protein product.[34] To determine the possible differential role of KRas A and B on proliferative pathway we evaluated the expression of different molecules through various modalities in HeLa cells before and after silencing KRas through RNA interference.

MATERIAL AND METHODS

HeLa (Henrietta Lacks) cell line was purchased from American Type Culture Collection (GIBCO, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine 200mM, 10% fetal bovine serum (FBS) and 100U/ml penicillin and 100μg/ml streptomycin (Sigma). Cells were cultured as adherent monolayer at 37° C in humidified atmosphere of 5% CO_2 in six well plates, purchased from Oxygen Life Sciences, California. Versene-EDTA and Phosphate buffer saline (PBS) were obtained from Invitrogen, USA.

When plates were 80-90% confluent, cells were transfected with two doses of a predesigned plasmid of short hairpin RNA of KRas (a generous gift from Dr Aamir Qazi, The University of Toronto, Canada) using effectene reagents from Qiagen, USA and incubated at 37°C for 48 hrs. We used shRNA against KRas having the sequence:
5'CCGGCCTATGGTCCTAGTAGGAAATCTCGA
GATTTCCTACTAGGACCATAGGTTTTTG3'

Clone ID:

NM_033360.2-509s1c1 TRCN0000033262 (Sigma- Aldrich).

Total RNA was isolated from the cells after 48 hours by Trizol (Invitrogen) method and quantified

by Cubbit.

5x RT Buffer (first strand), DTT, M-MLV reverse transcriptase, RNAse inhibitor and Oligo-T, were purchased from Invitrogen, USA. Random Hexamers were the product of Fermentas, Life Technologies USA while the dNTPs were obtained from V Vantis, USA. Conditions used for cDNA synthesis were as follows: 16^0 C for 10 minutes, 25^0 C for 15 min, 40^0 C for 40 min (synthesis time), 42^0 C for 10 min followed by denaturation at 95^0C for 5 min. PCR was then done using the following sequences:
KRasB-F-CCTACgTACCT AT ggTCCTAgTagg R-CTgTCCCACAACTA CTAgC, KRasA-F-GTAGT TGGAGCCTGGTGGCGTAG R-cgacacC CTCTT GACCTGCTGTGTcG, BCl2-F-TTCCcATCTCAT GCCAAGGGGGAA R-GAAAAGCAACGGGGGC CAA, BclxL-F-TGGTcCCTTGCAGTTCAGCACCA R-CCCTGAACCCACCATGAGGA, Mcl1 F-TAC CgAAGAG GAGCTGGACGGGTA R-ATCTC CAGCGACTGCCGGTA AKT3-F-CCCTTTGGT GCTA GGAGCTGA R-gga aACGTCATGCTG TAGTCTTCcG.

All primer pairs used were LUX (Light Upon Extention) primers and designed by BioBasic Canada Inc. The levels of all mRNAs were quantitated with a Light Cycler Instrument (Roche Diagnostics, Indianapolis, IN). Amplification reactions and thermal cycling conditions were optimized for each primer-probe set by series of reactions. The total volume of optimized mixture was 20 μl. Platinum Quantitative PCR SuperMix-UDG (cat. No. 11730-017) by Invitrogen, life Technologies was used. Conditions used for PCR were: 56^0 C for 2 minutes, 95^0 C 10 min, 95^0 C for 40 sec, 56-64^0 C for 30 sec followed by extension at 72^0 C for 30 sec.

Cells were harvested as described above and were lysed using Ripalysis cocktail 100μl/well containing 1.5 μl phenylmethylsulphonylfluoride (PMSF), 1.5 μl sodium orthovendate, 1.5 μl protease inhibitor and 150 μl Ripalysis buffer containing 1xTBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 0.004% sodium azide). Twenty μl of protein were loaded per well onto a 12% polyacrylamide gel (SDS-PAGE). Resolution of proteins was carried out by using Mini-PROTEAN® Tetra Cell equipment on 12% sodium dodecyl sulphate- polyacrylamide gel. Proteins were immunoblotted from the gel onto nitrocellulose membrane (BioRad, CA, USA) using mini Rad system. The membrane was blocked with 5% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS) and incubated in specific primary antibody (1:500). Primary antibodies used were KRas (sc-522; Santa Cruz Biotechnology Inc.), BclxL (Z5020199; Biochain Inst. Inc. Ca.); Bcl-2 Z5020197; Biochain Inst. Inc. Ca.); Bad (Z50202000 Biochain Inst. Inc. Ca) and β-actin from Aldrich, Inc. Sigma (St. Louis, MO). Protein markers were obtained from Invitrogen, USA. After incubation with primary antibody, membranes were washed with PBS + 0.1% Tween 20 and incubated with suitable rabbit polyclonal secondary antibody (1:1000 dilutions) horseradish peroxidase-conjugated from Santa Cruze. Blots after washing with PBST (Phosphate Buffer Saline exposed to TMB-Tetramethylbenzidine) substrate purchased from Sigma USA and then visualized in Gel documentation system (BioRad CA, USA).

Statistical Analysis

Each series of experiments were performed in triplicates and results were expressed as mean ± standard deviation of the mean (SE).

RESULTS & DISCUSSION

Before knocking down KRas, we observed high expression level of KRas B with hardly detectable KRas A expression in control HeLa cells on mRNA level (Table-I and Figure-1&2). Hereafter, with shRNA designed for KRas, we successfully knocked down (80%) KRas B variant at mRNA level which was confirmed by protein blotting (Figure-1&2). After getting KRas B suppressed while KRas A upregulated other proteins were blotted. Apoptosis was determined by Trypan blue exclusion test. Anti-apoptotic Bcl2 family members have shown variance in expression after downregulation of KRas B (Figure-2) and concomitant upregulation of KRas A variants. BclxL and Mcl1 were found to be over-expressed on mRNA level (Table-II) and on protein level (Figure-3b,c) confirming our reports of continued cell growth. We can explain that KRas A with its

downstream substrate might be controlling these antiapoptotic genes whereas downregulation of Bcl2 (Figure-3a) along with KRas B show interrelationship with each other (Figure-2). Bad is identified as a convergence point of several antiapoptotic signaling pathways. In order to confirm the results of continuous survival signaling, proapoptotic protein Bad was blotted. It was of great surprise that the level of Bad was negligible in cytosol after KRas silencing and fond to be ubiquitinated in cytosol.

Our findings are in agreement with a previous study documented by Jancik et al.,[35] who showed 88% downregulation of KRasB but intriguingly, KRas 4A variant was found to be elevated, p value <0.001 as shown in data (Figure-4.3) along with continued cell proliferation. This proliferative effect after KRas silencing was in disagreement with previous reports by Morioka and colleagues[36] and Lebedeva and Stein.[37] They reported that KRas RNAi had pronounced anti-proliferative effect with up to 65% inhibition in their studies. Plowman with her colleagues[38] demonstrated that KRas A was dispensable for normal mouse development in the presence of functional KRas B. No such study has been documented so far in this regard where KRasnA takes over the functions of KRas B isoform in HeLa cell or other cell line but Lefloch et al.[39] have shown this behavior of cell survival with reference of silencing another protein isoforms.

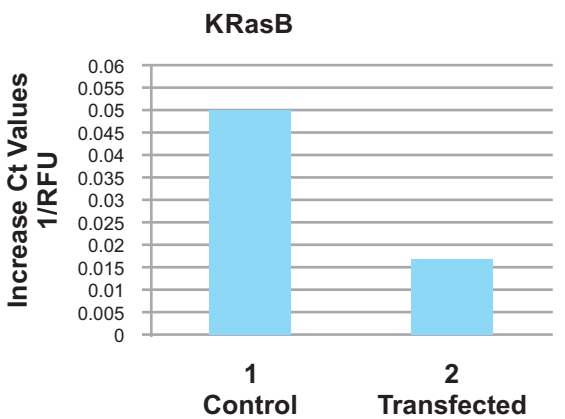

Figure-1a. Graphic representation of relative concentration of mRNA of KRas B in control and transfected HeLa cells.

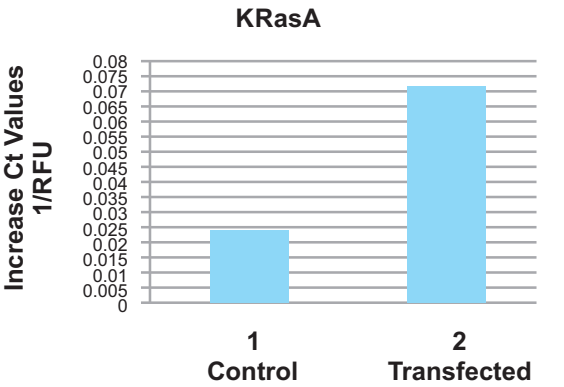

Figure-1b. Graphic representation of relative concentration of mRNA of KRas A in control and transfected HeLa cells.

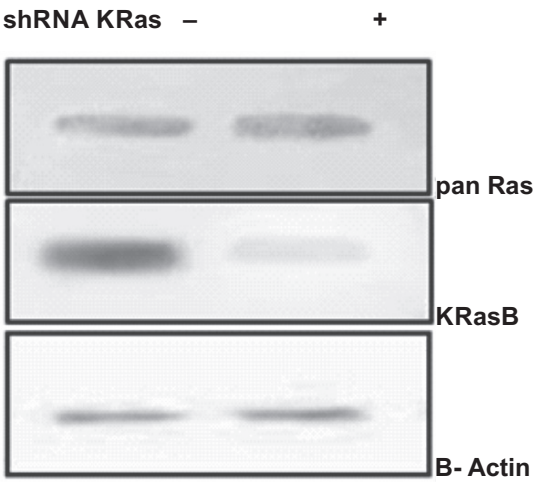

Figure-2. Determination of panRas, KRas B and β- Actin protein expression separated by 12% SDS-PAGE in control and transfected HeLa cells.

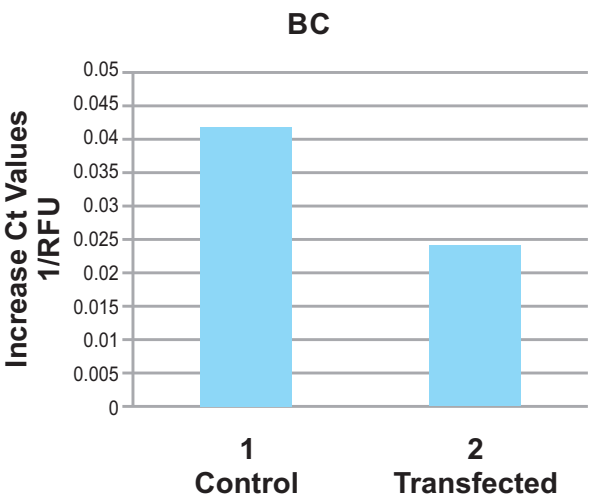

Figure-3a. Graphic Representation of relative concentration of mRNA of Bcl2 in control and transfected HeLa cells.

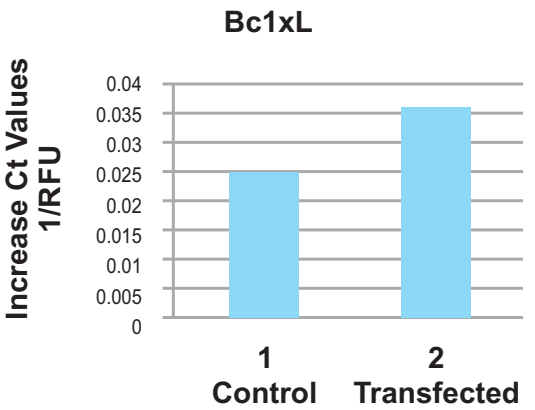

Figure-3b. Graphic Representation of relative concentration of mRNA of BclxL in control and transfected HeLa cells.

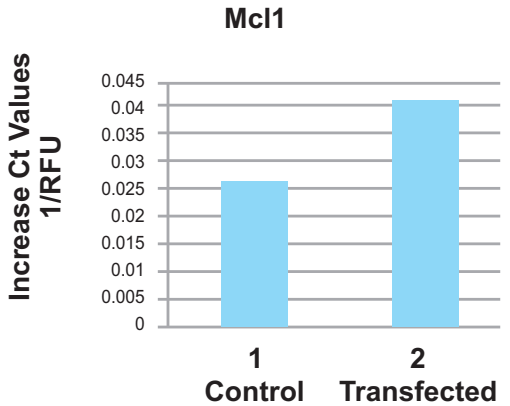

Figure-3c. Graphic Representation of relative concentration of mRNA of Mcl1in control and transfected HeLa cells.

The sustained proliferative signal through sister allele explains that some unknown splicing factor or formation of complex transcriptomes[40] might be involved in selective/inductive cum adaptive mean. Taken together our results, the notion placed earlier is confirmed that KRas A acts as key players in cancer progression.

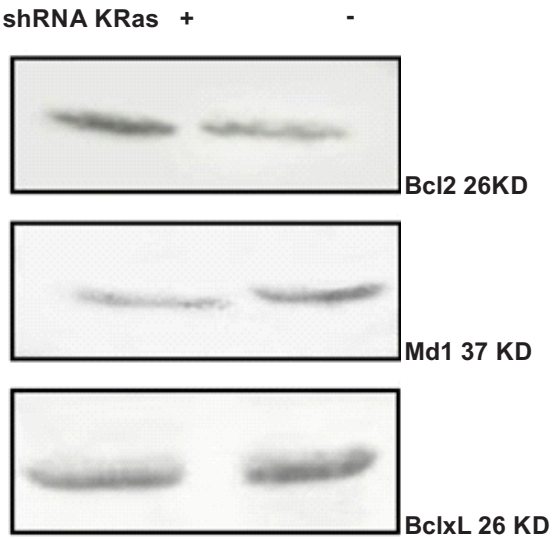

Figure-4. Determination of antiapoptotic protein Bcl2, BclxL and Mcl1expression separated by 12% SDS-PAGE in control and transfected HeLa cells.

Conclusion

Highly consistent results in present study, explain that expression of KRas A isoform escapes the silencing effect of shRNA constructed for both isoforms KRas A &B and restores the proliferative effects through MAPK pathways. In future, it will be of great interest to determine exactly how the activation of KRas A took place. But it can be suggested that some unknown/ mutation in known splicing factors and proteins making spliceosome, are involved or some other phenomenon like splitting of gene might be involved. This could be one of the reasons why single therapy against cancer control usually fails as one gene and protein in the signaling pathway is controlled whereas some other gene from same or other pathway gets activated.

		KRas A		KRas B		p-Value
Group	N	Mean	SEM	Mean	SEM	
Control Sample	6	19.8000	0.88428	19.8000	1.19205	<0.001
Transfected Sample	6	59.4733	2.88345	59.4733	1.20396	

Table-I. Mean of Ct values of KRasA and KRasB with standard errors of means P value =< 0.01

Group	N	Bcl2			BclxL			Mcl 1		
		Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value
Control	6	24.02	2.09	<0.013	40.01	0.36	<0.002	30.09	0.56	<0.014
Transfected	6	41.26	0.49		27.71	0.30		24.68	1.22	

Table-II. Mean of Ct values of Bcl 2, BclxL and Mcl 1 with standard errors of means P value =< 0.01

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Don't let yesterday take up to much of today.

– Will Rogers –

AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Farah Deeba Khan	Original thesis work and compilation of article.	
2	Ghazala Irshad	Editing and manuscript writing.	
3	Samra Hafiz	Statistical work.	