Mutation Studies of KRAS Proto oncogene and In Silico Drug Designing against Non-Small Cell Lung Cancer

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Abstract

Based upon the microscopic appearance of the tumor cells, Lung cancers, also known as bronchogenic carcinomas, are broadly classified into two types: small cell lung cancers (SCLC) and non-small cell lung cancers (NSCLC). Lung cancer is the significant contributor of increased cancer deaths. It is evident that the pathway of lung cancer includes both RAS-mediated and non-RAS-mediated mechanisms. Cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes. Mutations in the *K-ras* proto-oncogene are responsible for 10–30% of adenocarcinomas. Many of the mutations of KRAS have been reported worldwide to be involved in Non small cell Lung cancer. The current study involves checking the occurrence of one of these SNPs (rs104894361) in the target population. The work is further extended by investigating the role of natural antioxidants in inhibiting the mutated KRAS thereby reducing the cell proliferation rate. The work involves collection of Blood samples from various lung cancer patients along with their case history and medication details. The blood sample is processed further for DNA isolation, KRAS gene amplification and sequencing. While grouping the samples for processing, emphasis was given on their smoking status and it was concluded that smoking increases the possibility of target SNP hence leading to NSCLC. Further in *in silico* work a comprehensive analysis of KRAS at genomic and Proteomic level was conducted and docking studies were performed on mutated KRAS with screened Antioxidants. A comparative assessment of the docking energies revealed that many antioxidants like 2-Cys peroxiredoxin BAS1, Ascorbate peroxidase, Lectin and Lactoferrin have a better docking interaction with mutated KRAS than Trimetrixate, the conventional drug against NSCLC.

***Keywords*: Non small cell Lung cancer, KRAS, rs104894361, smoking, antioxidants, Lactoferrin**

1. Introduction

Lung cancers can start in the cells lining the bronchi and parts of the lung such as the bronchioles or alveoli. Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and eventually, into other parts of the body. There are two main

types of lung cancer are characterized by the cell size of the tumor when viewed under the microscope. They are called small cell lung carcinoma (SCLC) & Non –small cell lung cancer carcinoma (NSCLC). NSCLC is the most common cause of lung cancer due to long-term exposure to tobacco smoke. About 85% to 90% of lung cancers are due to non-small cell lung cancer (NSCLC). NSCLC can be further divided into different types, each with different treatment options, they are: Squamous cell carcinoma or epidermoid carcinoma, Adenocarcinoma, Bronchioalveolar carcinoma, Large cell undifferentiated carcinoma, adenosquamous carcinoma and sarcomatoid carcinoma. The cells in these types differ in size, shape, and chemical make-up when looked at under a microscope. But they are grouped together because the approach to treatment and prognosis (outlook) are very similar. The survival rates for lung cancers diagnosed at the earliest stage are higher, with approximately 49% surviving for five years or longer. If the cancer started somewhere else in the body and spread to the lungs, it is called metastatic cancer to the lung.

Oncogenic KRAS is found in more than 25% of lung adenocarcinomas, the major histologic subtype of non–small cell lung cancer (NSCLC), and is an important target for drug development. The most important abnormalities detected are mutations involving the ras family of oncogenes. Studies performed on mice suggest the involvement of ras mutations in the molecular pathogenesis of NSCLC. Studies in humans suggest that ras activation contributes to tumor progression in persons with lung cancer.

The KRAS gene belongs to a class of genes known as oncogenes. When mutated, oncogenes have the potential to cause normal cells to become cancerous. The KRAS gene is in the Ras family of oncogenes, which also includes two other genes: HRAS and NRAS. The proteins produced from these three genes are GTPases. These proteins play important roles in cell division, cell differentiation, and the self-destruction of cells (apoptosis).

Oncogenic mutations in the KRas protein, especially in regions specified by codons 12, 13, and 61 of the gene, prevent GTPase dependent down-regulation; therefore, the protein remains permanently active and continuously passes proliferation and survival signals through downstream pathways such as Raf/MAPK (Erk) and PI3K/protein kinase B (Akt). The result of these mutations is constitutive activation of *KRAS* signaling pathways. K-Ras is a membrane-associated GTPase protein that regulates proliferation, differentiation, and cell survival. These mutations lead to forms of RAS with impaired GTPase activity, causing a constitutive activation of RAS signaling pathway. Mutations in K-RAS gene occur frequently in NSCLC.

Antioxidants are abundant in fruits and vegetables, including other foods like nuts, grains, meat, poultry and fish. Antioxidants are phytochemicals, vitamins and other nutrients that protect our cells from damage caused by free radicals. In vitro and in vivo studies have shown that antioxidants help prevent the free radical damage that is associated with cancer and heart disease. Antioxidants can be found in most fruits and vegetables but also culinary herbs and medicinal herbs can contain high levels of antioxidants. Numerous studies with plant

phytochemicals show that phytochemicals with antioxidant activity may reduce risk of cancer and improve heart health.

The current work emphasize on two approaches, (i) The wet lab approach wherein 85 samples were tested for the presence of targeted SNP and: (ii) Computational biology approach wherein the scrutinized specific natural antioxidants available in the daily diet were docked with the targeted mutated Site of kras receptor to check their probable anti cancerous activity.

Materials and Methods

The work involves the collection of 3ml blood samples in K3 EDTA tubes from 60 NSCLC patients and 25 controls along with their case history and medication details. The samples were grouped into diseased smokers- 35, diseased non smokers-25, healthy smokers-15 and healthy non smokers-10. All the samples were stored at –20°C. After DNA extraction, KRAS amplification, and sequencing, the occurrence of target SNP was checked in all the samples.

Genomic DNA Isolation, purification and qualitative estimation

Genomic DNA was isolated by the Bunce's method in which the anticoagulated blood was treated with a solution of Tris HCl, Sucrose and $MgCl_2$. Further EDTA and NaCl solution was added to the pellet followed by the subsequent treatment with Sodium acetate and ice cold chloroform. To the supernatant obtained an equal volume of ice cold iso propanol was added for proper DNA precipitation. For purification the precipitated DNA was incubated at -20^{o}C overnight and Centrifuged at 6000rpm/10min.The pellet was washed with 1 ml of 70% ethanol and recentrifuged. The air dried DNA pellet was dissolved in100µl of 1xTE buffer.

The agarose gel electrophoresis was carried out to check the integrity and quality of the DNA yield. All the samples were run on 0.8% Agarose at 100V for 30 minutes. Furthermore the spectrophotometric readings were taken to obtain the ratio at wavelengths of 260 and 280nm. Samples were then diluted to 25 ng/µl each for using them in PCR.

Primer designing and amplification

Specific primer was designed using Primer BLAST for the **target region of kras consisting of the SNP Site .**The mixture of the PCR reaction had a final volume of 25 µl and contained 50 ng of genomic DNA (2µl), 5 µl of Taq buffer, 1 µl of 1.5mM $MgCl_2$, 2 µl of 0.2 mM dNTP, 2 µl of 20 pmol of each primer, 2 µl of 1.00 Unit Taq DNA polymerase ("Fermentas") and 9 µl of nuclease free water. The amplification was performed in 'Bio-Rad Thermo cycler' using the following cycling parameters:

- pre denaturation (94°C) 5 minutes,
- 30 cycles of denaturation (95°C) 45 second, annealing (55°C) 30 second, extension (72°C) 1minute,
- final extension (72°C) 7 minutes.
- The amplicons were subjected for sequencing and the obtained sequences were analysed for the presence of SNP.

Results

gDNA extraction and Qualitative & Quantitative estimation: Genomic DNA was successfully extracted from the blood

samples of all the 96 patients. A clear sharp band indicative of contaminant free DNA was obtained in the gel as viewed under gel documentation system. The range of A260/A280 for all the samples was found to be from 1.4 to 1.8 indicative of pure DNA content. The concentration of DNA in the samples was in the range of 25-45µg/ml which were further diluted to 25µg/ml for amplification.

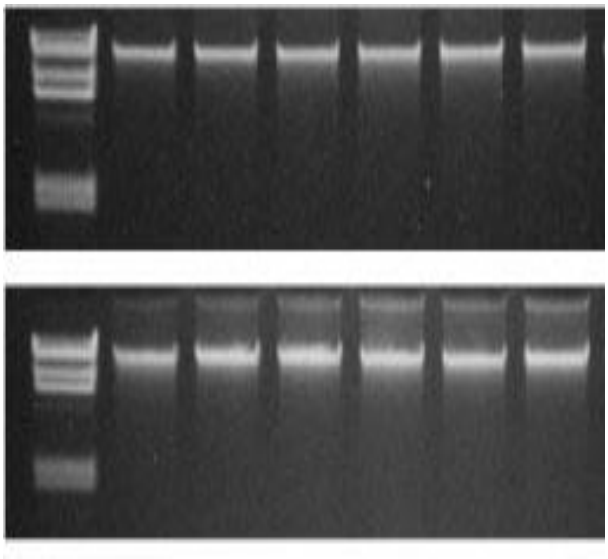

Fig 1: gDNA bands as obtained on 0.8% Agarose gel

SNP Selection, Primer Designing and Amplification

rs104894361 *[Homo sapiens]*
GCCTGCTGAAAATGACTGAATATAA
[A/T]CTTGTGGTAGTTGGAGCTGGTG
GCG
Chromosome: 12:25245370

Gene: KRAS (GeneView)

Functional Consequence:missense

Allele Origin:T(germline)/A(germline)

Clinical significance: Pathogenic

Primer designed

Sequence (5'->3')	Template Strand	Length	Tm	GC%
Forward primer ACCCTCTCACGAAACTCTGAA	Plus	21	58.68	47.62
Reverse primer CGTAGGCAAGAGTGCCTTGA	Minus	20	60.04	55.00

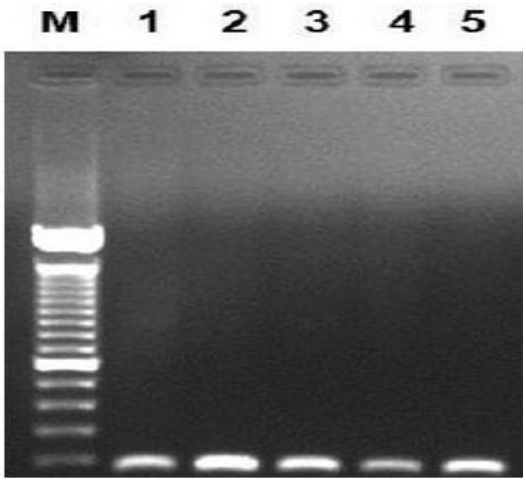

Fig 2: Bands of amplicons as obtained on 1.2% agarose gel with novel primers

Sequencing and SNP Identification

Upon amplification and further purification the consensus sequences were obtained for all the 85 samples and aligned to identify the SNP. Following is the partial image of the alignment showing SNP in Sample numbers 51, 52, 53, 54, 55, 56, 58, 59 and 60 while sample number 57 was found to have the normal allele A.

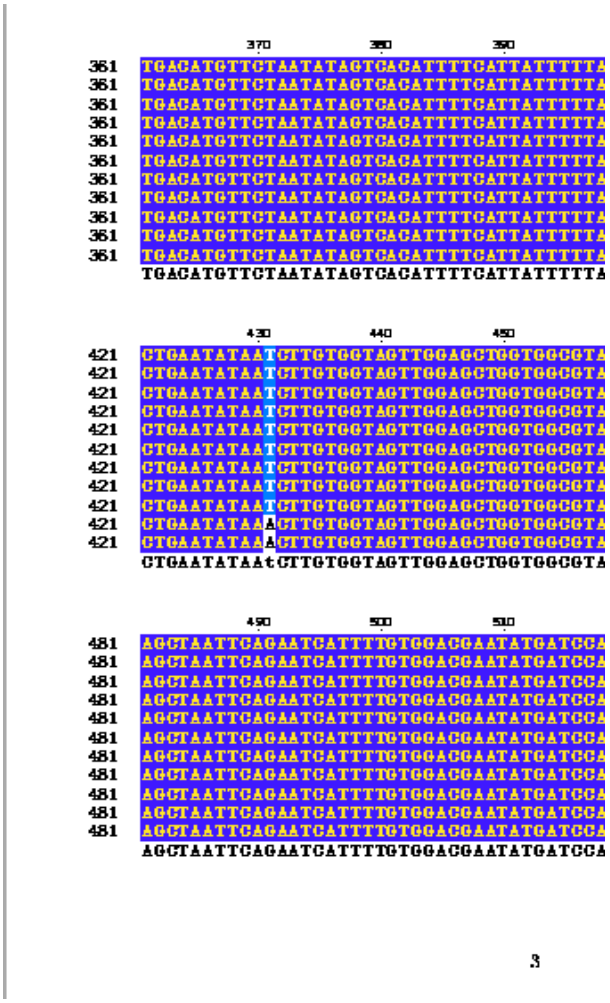

Fig 3: Tex Shade obtained upon Multiple sequence Alignment

SNP Analysis

Following samples were found to have T instead of wild type A at position 25245370 of the 12th Chromosome

diseased smokers- 21

diseased non smokers-09

healthy smokers-07

healthy non smokers-00

From the above result it is evident that the A to T transition may play a significant role in causing lung cancer. The chance of disease increases in smokers wherein there is a high probability of this SNP. Out of 50 smokers 28 (56%) and out of 60 diseased 30 (50%) were found to have this SNP.

In Silico gene Analysis of KRAS

S.N0	SOFT WARE/ TOOLS	RESULTS
1.	**FEX** To find Exonic regions	Total potential exons: 7. Positions: 182-751, 3141-3201, 2701-2716, 2445-2451, 813-856, and 2839-2966.
2.	**POLY- AH** To find Poly-A tail sites	6 potential poly-A sites at positions 527, 2279, 2285, 3710, 3887 and 4215.
3.	**F-SPLICE** To find Splice sites	Direct chain - 41 Splice sites [Acceptor (AG) sites=30 &Donor (GT) sites=11], Reverse chain – 36 Splice sites. [AG sites=18 & GT sites=18]
4.	**PROMOTER SCAN** To find the promoter regions	Promoter regions : PEA1(1435), AP-1(1441), TFIID (1474), TFIID (1557)
5.	**COMET** To find Cis elements	8 Motif regions: TATA 640 to 654, NF-1 2128 to 2145, LSF-5209 to 5223, ERE-5239 to 5252 & 1443 to 1456, Mef-2 5276 to 5287 & 1633 to 1644, Myf-4695 to 4706 & 4056 to 4067, Ap-1 4389 to 4399, Tef-5123 to 5134.
6.	**TIS MINER** Translation Initiation site prediction	Five positions at 182, 380,395, 512, and 276.
7.	**HCTATA** To find TATA regulatory elements	Total TATA signal prediction is 57

8.	**CPG ISLAND** To find total GC content.	The maximum GC% was present at the region 1 to 200 with 72.50%.

Table 1: Showing Results of various Tools used for gene analysis of KRAS

Docking studies and Drug Library Creation:

Docking associations were performed on mutated KRAS and short listed Antioxidants and docking energies were analyzed

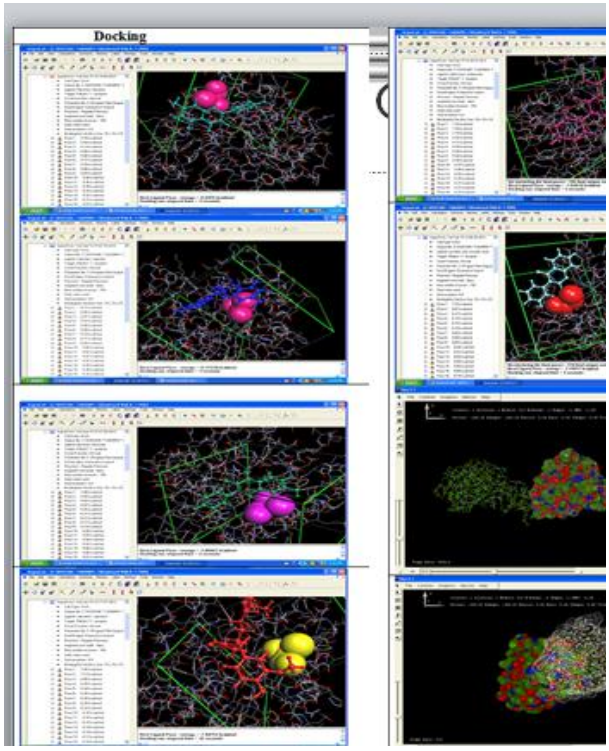

Fig 4: Docking of RKAS with Lactoferrin

S.NO	Antioxidant name	Docking energy
1.	Luteolin (Bell peppers)	-6.70kcal/mol
2.	Catechins (Tea plant)	-6.72kcal/mol
3.	3,7-dihydroxyflavone (Blue berries)	-7.00kcal/mol
4.	Capsaicin (Bellpeppers)	-7.45kcal/mol
5.	Isoflavones (soya bean)	-7.62kcal/mol
6.	Ascorbic acid (sweet orange)	-7.75 kcal/mol
7.	2-Cys peroxiredoxin BAS1 (spinach)	-260.26 kcal/mol
8.	Ascorbate peroxidase/ APX (spinach)	-286.92 kcal/mol
9.	Catalase (Honey)	-390.01 kcal/mol
10.	Lectin (Cicerarietinum)	-595.05 kcal/mol
11.	Lactoferrin (milk)	-736.73 kcal/mol
12	Trimetrixate	-216.46 kcal/mol

4. Conclusion

The SNP rs 104894361 reported in NSCLC Indian patient looks to be promising and may be used as a predictive tool or Genetic Predisposition testing tool for Lung cancer if found positive in a wider sample size.

Precision medicine may be an effective tool towards cancer therapy. Our results are indicative of several drugs with better docking energies than the conventional drug trimetrixate. The antioxidant Lactoferrin shows promising efficiency for inhibition of mutated KRAS protein. Hence it can be targeted as a novel drug against Non Small Cell Lung Cancer Patients. Lactoferrin can be further studied for formulation, Dosage designing followed by animal trials as Lung cancer drug as an efficient substitute of conventional drug trimetrixate. Potential Drug library against KRAS Mutation which includes the successful and unsuccessful lead molecules (Lactoferrin being the most efficient drug) has also been created as a deliverable.

Acknowledgements and Conflict of interest

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References

1.Akiko Hisamoto, Eisei Kondo, Katsuyuki Kiura, Toshiaki Okada, Shinobu Hosokawa, Junko Mimoto, Nagio Takigawa, Masahiro Tabata, Mitsune Tanimoto. Point mutation of K-ras gene in cisplatin-induced lung tumours in A/J mice, Lung cancer Volume 58, Issue 1, Pages 15–20

2. Gregory J. Riely, Jenifer Marks and William Pao1. KRAS Mutations in Non–Small Cell Lung Cancer: Proc Am Thorac Soc. 2009 Apr 15;6(2):201-5. doi: 10.1513/pats.200809-107LC.

3. Koji Okudela, Tetsukan Woo, Hitoshi Kitamura. KRAS gene mutations in lung cancer**:** Particulars established and issues unresolved: Article first published online: 12 JUL 2010,DOI: 10.1111/j.1440-1827.2010.02580.

4. Li M, Liu L, Liu Z, Yue S, Zhou L, Zhang Q, Cheng S, Li RW, Smith PN, Lu S.The status of KRAS mutations in patients with non-small cell lung cancers from mainland China: Oncol Rep. 2009 Nov; 22(5):1013-20.

5. McKeage, Mark J., et al. Phase II Study of ASA404 (vadimezan, 5,6-dimethylxanthenone-4-acetic acid/DMXAA) 1800 mg/m2 Combined With Carboplatin and Paclitaxel in Previously Untreated Advanced Non-Small Cell Lung Cancer. Lung Cancer 65.2 Aug.

2009: 192-197.

6. Noriaki Sunaga, David S. Shames, Luc Girard, Michael Peyton, Jill E. Larsen, Hisao Imai, Junichi Soh, Mitsuo Sato, Noriko Yanagitani ,Kyoichi Kaira, Yang Xie, Adi FGazdar, Masatomo Mori and John D. Minna. Knockdown of Oncogenic KRAS in Non–Small Cell Lung Cancers Suppresses Tumor Growth and Sensitizes Tumor Cells to Targeted Therapy:
American Association for Cancer Research **DOI:** 10.1158/1535-7163.MCT-10-0750 Published February 2011

7. Okudela K, Hayashi H, Ito T et al. K-ras gene mutation enhances motility of immortalized airway cells and lung adenocarcinoma cells via Akt activation: Possible contribution to non-invasive expansion of lung adenocarcinoma. Am J Pathol 2004; 164: 91–100.

8. Okudela K, Yazawa T, Woo T et al. Down-regulation of DUSP6 expression in lung cancer: Its mechanism and potential role in carcinogenesis. CrossRef,PubMed,CAS,Web of Science Am J Pathol 2009; 175: 867–81.

9. Ruano-Ravina A, Figueiras A, Freire-Garabal M, Barros-Dios JM. Antioxidant vitamins and risk of lung cancer: Curr Pharm Des. 2006;12(5):599-613.

10. United States. National Cancer Institute (NCI). "Lung Cancer." July 26, 2007.
http://www.cancer.gov/cancertopics/types/lung.

11. "What Are the Key Statistics About Lung Cancer?" American Cancer Society. Oct. 20, 2009. http://www.cancer.org/docroot/CRI/content/CRI_2_4_1x_What_Are_the_Key_Statistics_About _Lung_Cancer_15.asp?

12."What You Need to Know About Lung Cancer." National Cancer Institute, U.S. National Institutes of Health. July 26, 2007.
http://www.cancer.gov/cancertopics/types/lung.