

Analysis of RAS Subfamily involved in Cancer via in silico Approach and Designing of Potent Drug against Cancer

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Abstract:

RAS subfamily belongs to a class of proteins called small GTPase, which are a group of hydrolase enzymes that can bind ('on state') and hydrolyze ('off state') guanosine triphosphate (GTP). Hence, *RAS* proteins act as binary molecular switches and play a consequential role in intracellular signal transduction which regulates processes such as actin cytoskeletal integrity, proliferation, differentiation, cell adhesion, apoptosis, and cell migration. Upon mutation oncogenic forms of *RAS* proteins beget overactive signaling inside the cell, which ultimately leads to cancer. Therefore, discerning the functionality of *RAS* proteins, and the identification of inhibitors that block the mutated *RAS* proteins, may lead a successful step towards cancer therapy. The present bio-computational analysis was performed using web-based tools and servers. Multiple sequence alignment of selected human *RAS* subfamily proteins with other homologous sequences revealed highly conserved regions. The present work determined the physico-chemical properties of selected *RAS* proteins such as their hydrophilic nature; alpha-helical structure; close evolutionary relationship with higher vertebrates. Functional sites and domains within the protein were analyzed and the Protein-protein interaction study was made that revealed the functional association of *RAS* proteins with several effector proteins. The target site on the receptor protein was predicted based on the mutation analysis and the best binding site was selected. Comparative analysis of the binding affinity between the receptor protein and final screened chemical ligands was performed using Docking analysis, which determined Trimetrexate as the best ligand acting against mutation in *HRAS*, *NRAS*, *KRAS* and *DIRAS1* proteins due to its lowest docking energy. The present work supports Trimetrexate as potential chemical compound to block oncogenic *RAS* activity.

Keywords: Cancer, Drug-designing, Proteomic analysis, *RAS* subfamily proteins.

Introduction:

Cancer is abnormal growth of cells caused by multiple changes in gene expression leading to deregulated balance of cell proliferation and cell death, ultimately evolving into population of cells that can invade tissues and metastasize to distant sites causing significant morbidity. The characteristic that delineates the malignant cancer from a benign tumor are the abilities to invade locally to spread to regional lymph nodes and

to metastasize to distant organs in the body. The kind of genes that are involved are oncogenes, which when activated lead to deregulated cell proliferation and the tumor suppressor genes would become inactivated or deleted, producing a loss of cell's check and balance in controlling cell proliferation and differentiation. The single most common, if not universal, trait that occurs in all cancers is genetic drift, or the ability of cells to lose the stringent requirement for precise DNA replication and to acquire the ability to undergo sequential progressive changes in their genomes, through mutations, gene rearrangements and gene deletions. This has sometimes been called the acquisition of "mutator phenotype" [1]

RAS Proteins and Cancer: *RAS* protein family members belong to a class of protein called small GTPase, and are involved in cellular signal

transduction and in cell growth, differentiation, and survival. Mutations in *RAS* genes can cause unintended and overactive signaling inside the cell; as these signals result in cell growth and division, overactive *RAS* signaling can ultimately lead to cancer [2] *RAS* oncogenes can be activated by point mutations so that the GTPase reaction can no longer be stimulated by GAP – this increases the half life of active *RAS*-GTP mutants [3]

Constitutively active *RAS* (*RAS*^D) is one which contains mutations that prevent GTP hydrolysis, thus locking *RAS* in a permanently 'On' state. Mutations in the *RAS* family of proto-oncogenes are very common, being found in 20% to 30% of all human tumors [4]. High rates of *KRAS*-activating missense mutations have been detected in non-small cell lung cancer (15 to 20% of tumors) [5], colon adenomas (40%)

[6] and pancreatic adenocarcinomas (95%) [7], making it the single most common mutationally activated human oncoprotein. In some tumors, *HRAS*- or *NRAS*-activating mutations are also seen. More than half of the most malignant thyroid tumors, characterized as poorly differentiated or undifferentiated, harbor a mutation in *KRAS*, *HRAS*, or *NRAS* [8]. In addition to mutational activation, *RAS* genes are amplified or over expressed in some tumors [9]. Other mechanisms leading to *RAS* over activation in tumor cells include the deletion of genes encoding negative regulators (for example NF1, a GAP for *RAS*, in neurological tumors) [10-13] and over expression of positive regulators (such as SOS1, a GEF for *RAS*, in renal cancer cells) [14]. Analysis of *RASSF2* protein expression in a series of human lung tumor cell lines shows that the protein is frequently down-regulated. *RASSF2* inhibits the growth

of tumor cells, and its growth-inhibitory properties are enhanced by activated *KRAS*. *RASSF2* promotes both cell cycle arrest and apoptosis; consequently, its down-regulation may play a key role in the development of cancer [15]. Down-regulation of *DIRAS1* protein was significantly correlated with Esophageal Squamous Cell Carcinoma (ESCC) lymph node metastasis, which was associated with its function in inhibiting cell migration and invasion by down-regulation of MMP-2 and MMP-9 via ERK- and p38-dependent signaling pathway. Findings suggest that *DIRAS1* plays an important suppressive role in the development and progression of ESCC [16]. Therefore, the importance of aberrant *RAS* protein function in human cancers may be greater than expected [17], and makes it an object of study.

Materials and Method:

Review of literature and Gene Cards database facilitated the selection of *RAS* subfamily proteins, which have their expressions in cell proliferation, tumor suppression, growth and survival. Five members of *RAS* subfamily: *HRAS*, *NRAS*, *KRAS*, *RASSF2*, and *DIRAS1* were selected for their characterization, and sequences were retrieved from National Centre for Biotechnology Information (NCBI) database. Sequence analysis was performed using BLAST tool to probe the human protein *HRAS*, *NRAS*, *KRAS*, *RASSF2* and *DIRAS1* homology in different vertebrates. Multiple sequence alignment revealed the conserved regions among selected protein sequences using Clustal-X and T-coffee. To analyze the evolutionary relationship among selected sequences, Clustal-W tool has been used. Primary structure prediction of selected *RAS* proteins was performed using ProtParam tool of the ExPASy proteomics server. Determination of molecular weight, isoelectric point, Grand Average of Hydropathicity (GRAVY value) and number of positive and negative residues revealed physicochemical nature of proteins under study. Secondary structure prediction was done using SOPMA tool, in which characteristics such as α -helix, β -turn, extended strand, and random coil predicted the secondary structure of *RAS* proteins. Tertiary structure prediction of *RAS* proteins was performed using three bioinformatic tools: CPH, HH-pred, and Phyre. RASMOL visualization provided 3D structure of selected PDB ids. To analyze the functional domains, protein-protein interaction, and functional motifs, several *in-silico* tools such as SMART, STRING, and Motif Scan have been used respectively. Disordered regions in the sequences were examined using tools: DisEMBL, Glob Plot, RONN, and DISOPRED. Q-site finder, Pocket finder, and CASTp provided ten ligand binding sites for each protein and amino acids with maximum surface area have been noted. Pubchem and ChEBI facilitated the search for prophylactic chemical compound for cancer. Ligands have been selected and screened using Lipinski rule. Docking study was performed using HEX. Ligand binding affinity with the receptor protein was

analyzed, and comparative analysis yielded the best drug candidate, based on the best binding affinity, which can be used as best oncogenic *RAS* inhibitor, thus for cancer treatment, after considerable clinical trials.

Results and Discussion:

RAS Protein Sequences: The sequence of Human *RAS* proteins: *HRAS*, *NRAS*, *KRAS*, *RASSF2* and *DIRAS1*, consisting of 189, 189, 189, 157, and 198 amino acids respectively, was retrieved from NCBI database and converted into FASTA format. BLAST result provided the degree of homology among higher vertebrates. Organisms that are found to be the best homolog to the selected human *RAS* proteins are: *Rattus norvegicus* in *HRAS* with 100% identity; *Heterocephalus glaber* in *NRAS* and *KRAS* with 100% identity; *Macaca fascicularis* in *RASSF2* and *DIRAS1* with 97% and 99% identity respectively.

Sequence Analysis: Multiple sequence alignment of all homolog sequences revealed significant conserved regions. For *HRAS*, *NRAS*, and *KRAS*, sequences The patterns

MTEYKLVVVGAGGVGKSAITITQLIQNHVDEY
DPTIDSYRKQVVIDGETCLLDLDTAGQEEYSAM
RDQYMRTGEGFLCVFAINN, YREQIKRVKDS,
DVPMLVLVGNKCDL, IETSAKTRQ, VEDAFY, and
LVREIR are present in all 6 sequences; for *RASSF2*,
sequences RTRSD, HRFSINGHFYNHKTSVFTPA,
and GSVTNVR are present in all selected sequences;
for *DIRAS1*, MPEQSNDYRVVVFVGGAGGVGAGGVG
KSSLVLRFRD, YIPT, EDTYRQVISCCKS,
CTLQITDTTGSHQFPMQRLSISKGFHAFILV, SVTS,
NKCDE, WKCAFMEETSAK, and NVKELFQELL are
found in all 6 sequences, which indicate that these
sequences are conserved. BLAST result, multiple
sequence alignment, and phylogenetic analysis
discovered highest homology of human *HRAS* sequence
with *M. musculus* and *R. norvegicus*; of human *NRAS*
and *KRAS* with *H. glaber*, and of *DIRAS1* with *M.*
fascicularis, which has its implications in clinical trials
in future. Significant conserved regions suggest that
sequences are maintained by evolution despite
speciation. *RASSF2* was most divergent among
selected proteins; therefore it was not taken into
further consideration.

Structural Analysis: Primary structure analysis provided the physicochemical properties of *RAS* proteins. Molecular weight of *HRAS*, *NRAS*, *KRAS*, and *DIRAS1* is 21298.1, 21229.1, 21655.8, and 22328.7 respectively. Most abundant amino acid is Valine in *HRAS*, *NRAS*, *DIRAS1* (8.5%, 9.0%, and 9.1% respectively), and Valine and Lysine in *KRAS* (8.5%). A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable [18]. ProtParam server predicted that *HRAS* and *DIRAS1* are unstable whereas *NRAS* and *KRAS* are stable proteins. The isoelectric point of a protein is an important property, because it is at this

point that the protein is least soluble [19]. Computed isoelectric point of *HRAS*, *NRAS*, and *KRAS* is below 7 that are likely to precipitate in acidic buffers, and for *DIRAS1* it is 8.94, soluble in basic buffers. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains: alanine, valine, isoleucine, and leucine, which is 81.96, 82.96, 85.03, and 81.62 for *HRAS*, *NRAS*, *KRAS*, and *DIRAS1* respectively. It may be regarded as a positive factor for the increase of thermo-stability of globular proteins [20]. The Grand Average Hydropathy (GRAVY values) showed that all proteins are hydrophilic, ranging from -0.432 to -0.317, supports the soluble nature of *RAS* proteins. The secondary structure of alpha helix predicted values 40.74%, 45.50%, 38.62%, 41.41% for *HRAS*, *NRAS*, *KRAS*, and *DIRAS1* respectively. Secondary structure of β -turn predicted values: 6.35%, 5.29%, 6.35%, and 5.05% for *HRAS*, *NRAS*, *KRAS*, and *DIRAS1* respectively. Values for random coil ranged from 30.69% to 36.36% in four proteins. From tertiary structure analysis PDB ID: 3CON was selected as ideal representative for the structure of *HRAS*, *NRAS*, and *KRAS*, and 2FN4 for *DIRAS1*. 3D structures of all PDB IDs were generated through RASMOL.

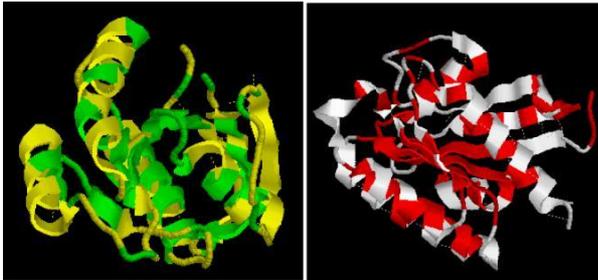


Figure: 1, PDB ID: 3CON (left), 2FN4 (right)

Functional Analysis: *RAS* domain is conserved in all four proteins, whose cellular function is GTP-hydrolysis which when mutated gets stuck in the "on" mode, prevent GTP-hydrolysis, and causing wild, uncontrolled growth leading to tumors [21]. Thus, targeting this region will be substantial in cancer treatment. Furthermore, overlapping domains such as, Arf, Miro, and GTP_EFTU, which are involved in GTP-hydrolysis, mitochondrial homeostasis and apoptosis, and elongation factor respectively, are also found to be conserved. However, additional research is required to understand their interactive roles in causing cancer.

Conserved Domains	<i>HRAS</i>	<i>NRAS</i>	<i>KRAS</i>	<i>DIRAS1</i>
Ras	1-166	1-166	1-166	9-169
Miro	5-119	5-119	5-119	9-124
Arf	1-163	1-163	1-163	1-167
GTP_EFTU	2-166	20-166	30-167	-

Table: 2, Conserved domains and their positions.

RAS proteins regulate a wide range of biological processes by interacting with a broad assortment of effector proteins [15]. Hence, the prediction of *RAS* interactions facilitates the understanding of complex *RAS* signaling network, which may be critical in cancer treatment. *Raf1*, *SOS1*, *BRAF*, *PIK3CA* proteins are found to be functionally associated with *H-RAS*, *N-RAS* and *K-RAS*. *RASSF1* effector, mediate apoptosis and cell cycle arrest, displays interaction with *H-RAS* and *KRAS*, whereas *PTPN11*, *EFGR* and *MAP2K1* with *N-RAS* and *K-RAS*.

Conserved regions/ Residues	Position			
	<i>Hras</i>	<i>Nras</i>	<i>Kras</i>	<i>Diras1</i>
M	1	1	1	1
Y	4	4	4	8
VV	7-8	7-8	7-8	11-12
GAGGVGKS	10-17	10-17	10-17	14-21
L	19	19	19	23
F	28	28	28	32
D	30	30	30	34
Y	32	32	32	36
PTIED	34-38	34-38	34-38	38-42
YR	40-41	40-41	40-41	44-45
D	47	47	47	51
C	51	51	51	55
L	53	53	53	57
I	55	55	55	59
DT	57-58	57-58	57-58	61-62
G	60	60	60	64
AM	66-67	66-67	66-67	70-71
G	75	75	75	79
F	78	78	78	82
VF	81-82	81-82	81-82	85-86
S	89	89	89	93
I	100	100	100	104
K	104	104	104	108
S	106	106	106	110
D	108	108	108	113
P	110	110	110	115
LVGNKCD	113-119	113-119	113-119	118-124
R	123	123	123	128
V	125	125	125	130
A	130	130	130	135
A	134	134	134	139
ETSAK	143-147	143-147	143-147	148-152
V	152	152	152	157
F	156	156	156	161
L	159	159	159	164
E	162	162	162	168
R	164	164	164	170
C	186	186	186	195

Table: 1, Conserved regions in 4 *RAS* proteins.

The present analysis yielded certain functional sites which are critical in normal *RAS* protein functions. Phosphorylation has functional impact on *RAS* proteins, as many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation [22]; protein myristoylation is critical in signal transduction, apoptosis, or alternative extracellular protein export [23]; altered glycosylation

sites are universal features of cancer cells, and certain types of glycan structures are well-known markers for tumor progression [24], also, the *RAS* transforming activity is critically dependent on prenylation for its

signal transduction abilities [25] Hence, computational analysis availed the identification of all functionally significant motifs which can be targeted to block the *RAS* mutation.

Conserved Motifs	HRAS	NRAS	KRAS	DIRAS1
<i>N</i> -glycosylation site	88-85	88-85, 172-175	85-88	172-175
ATP/GTP-binding site motif A (P-loop)	10-17	10-17	10-17	10-17
CK2 phosphorylation site	35-38, 89-92,	35-38, 89-92, 173-176	35-38, 89-92, 172-175	31-34, 39-42, 93-96, 110-113, 126-129
N-myristoylation site	13-18, 151-156, 180-185	13-18, 151-156, 177-182	13-18	17-22
PKC phosphorylation site	39-42, 145-147, 183-185	39-41, 145-147	39-41, 145-147	31-33, 43-45, 89-91, 126-128, 150-152, 169-171, 182-184, 188-190
Prenyl group binding site	186-189	186-189	186-189	195-198
Tyrosine Kinase Phosphorylation site	88-96, 149-157	88-96, 149-157	88-96, 149-157	Not found

Table: 3, Conserved Motifs found in 4 RAS subfamily proteins at respective positions.

Disorder and Ligand binding site prediction: From disorder prediction, most mutation prone regions within domain have been predicted for each protein, through verification using different *in silico* tools, and by analyzing the mutational scores. In *HRAS* these amino acids are Glycine, Serine, and Glutamic acid; in *NRAS*: Glycine, Glutamine, Cysteine, and Methionine; in *KRAS*: Glycine, Proline, and Cysteine; and in *Di-RAS1* it is Aspartic acid. Glycine was predicted to have greatest surface area in *HRAS*, *NRAS* and *KRAS*, whereas for *DIRAS1* it is Aspartic

acid, which can be demonstrated as best ligand binding sites.

Ligand Screening and Docking Analysis: Ligand screening was performed following “Lipinski Rule”, Based on this rule, 25 ligands were selected from Pubchem and ChEBI. Among all 25 Ligands, 5 chemicals were screened on the basis of Benzene rings. Docking analysis was performed using Hex and energy was noted. Trimetrexate showed the least docking energy for both receptors proteins (*3CON* and *2FN4*).

SN	Ligand Name	Chemical ID	Molecular Formula	Docking Energy (K cal/mol)		Molecular weight [g/mol]	LogP value	H. bond donor	H. bond Acceptor
				3CON	2FN4				
1	Trimetrexate	5583	C ₁₉ H ₂₃ N ₅ O ₃	-216.46	-210.14	369.41762	2.3	3	1
2	Ifosfamide	5864	C ₇ H ₁₅ Cl ₂ N ₂ O ₂ P	-135.56	-138.82	261.085962	0.9	1	4
3	Methyl5-aminolevulinate hydrochloride	60641	C ₆ H ₁₂ ClNO ₃	-136.74	-126.69	181.61700	-0.24	2	4
4	6-Mercaptopurine	667490	C ₅ H ₄ N ₄ S	-114.55	-109.04	152.17706	0	2	4
5	Methyl5-aminolevulinate	724125	C ₆ H ₁₁ NO ₃	-137.30	-133.09	145.15640	-1.3	1	3

Table: 4, List of screened ligands and their properties.

Conclusion:

The present analysis entails 5 members of *RAS* subfamily: *HRAS*, *NRAS*, *KRAS*, *RASSF2*, and *DIRAS1*, selected from NCBI database. *HRAS*, *NRAS*, *KRAS* show high conservation which suggests their functional similarity, while *DIRAS1* is relatively distinct subgroup as concluded from the overall analysis. Sequence alignment revealed that *RASSF2* is most divergent among selected members. From the present analysis it can be concluded that selected

human *RAS* proteins have high degree of homology with higher vertebrates. Physicochemical properties of *RAS* proteins presented significant data which will avail in protein purification to further unveil the dimensions of *RAS* functionality. Secondary structure revealed that all 4 protein are largely alpha helical. *RAS*-domain is found to be the functional domain, conserved in all 4 proteins, and responsible for GTP-hydrolysis. Phosphorylation, glycosylation, myristoylation, prenylation, and ATP/GTP binding

sites are critical to the normal RAS functioning which are found to be conserved in all four proteins. From the present *in silico* drug designing, it can be concluded that Trimetrexate can be an effective inhibitor for RAS oncogenic activity, which will in turn arrest the RAS over expression and metastasis of cancerous cells. It can be further incorporated into drug development proceedings and clinical trials.

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