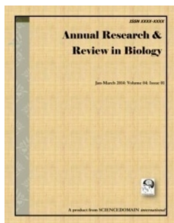

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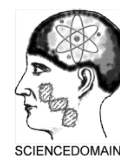

In Silico Mutagenesis Reveals Specific Binding Residues that Regulate KSRP – microRNA Precursor Interactions in Human

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Authors' contributions

Both the authors contributed equally towards the design of the study. Author SG performed the analyses and both authors approve of the manuscript.

Research Article

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ABSTRACT

Aims: The main aim of the study is to identify the key residues involved in interactions of KSRP and microRNAs of human.
Study Design: The KH type splicing regulatory protein acronymed as KSRP is a member of the FUSE binding protein family. The FUSE is an AT rich DNA element which is present 1.7kb upstream of the **c-Myc** oncogene promoter. KSRP also known as FUSE binding protein 2(FBP2). Various activities of KSRP has been reported by many workers over the years, however recent report suggest that KSRP is involved in a crucial step of the microRNA biogenesis pathway – the generation of precursor microRNAs from primary microRNAs. The premise of this work stems from the observation that much micro RNA has been implicated in numerous diseases and though antisense constructs have been projected as possible therapeutic agents against such microRNAs, protein inhibitors or Site directed mutagenesis (SDM) approaches should open up new intervention strategies.
Place and Duration: The work was done entirely at the DBT Centre for Bioinformatics, Presidency university, Kolkata for a period from June 2012 – May 2013.
Methodology: Comparative modeling followed by molecular dynamic simulation strategies based on flexible and rigid docking approaches were combined with *in silico* mutagenesis to analyze the interactions of KSRP and human "microRNAs"
Results: Results indicate that specific residues of the K type single stranded RNA binding domain play important roles in RNA binding and in their absence the binding affinities are affected.

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Conclusion: *In silico* mutagenesis in protein structures can serve as important initial steps to understand the interactions of proteins and their substrates. In context of KSRP the protein active site residues were identified and these could serve as important targets for future experiments.

Keywords: Micro-RNA; KSRP; in silico mutation; interacting residues.

1. INTRODUCTION

The generation of mature microRNAs is an evolutionarily conserved phenomenon in which primary miRNAs (pri – miRNAs) are initially processed to precursor microRNAs and finally to the mature micro RNAs. Ribonucleases such as Drosha and Dicer are involved in regulated endonucleolytic cleavages [1,2]. Over the years numerous experimental data have accumulated a large body of evidence towards the understanding of microRNA biogenesis [3,4]. As our understandings have increased it has become clear that numerous activators and coactivators are required to successfully accomplish the mature silent assassin production [5]. The identification of the microprocessor complex has helped us to understand the roles of dead box helicases such as p68 and p78, Drosha cofactors that have RNA binding activity – hnRNPA1 and KSRP [6–8]. Heo et.al. (2008) [9,10] have demonstrated that KSRP promotes processing of let7g precursors. Trabucchi et al. (2009) [11] have shown that KSRP is an essential component of the DICER complex in HELA cells. They have further indicated that this protein recognizes short G rich stretches (KH3) with high specific affinity. Knockout studies have also revealed that let7a mediated silencing pathway was affected when KSRP action was inhibited. Recombinant KSRP was also shown to increase the processivity of DICER.

The terminal loop (TL) acts as a pivotal structure as it is the very site where miRNA processing activators interact. They are also of the opinion that repressors for example lin 28 are regulated by the same mechanism [12]. It has been proposed that KSRP – RNA recognition depends both on the availability of single stranded RNA sequences and on the selectivity of sequence of the KH domains. The KH3 domain displays a strong affinity towards G containing sequences. It has also been implied that the recognition of the RE sequences by KSRP is dependent on the binding modes.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

The sequences of micro RNA precursors were derived from miRbase and initially the secondary structures were obtained using the MFold server [13]. This server utilizes a modified Zuker's algorithm for the prediction of RNA secondary structures and sorts the best structure according to the free energy. Once the secondary structures were obtained a symbolic programming approach was used at the MC SYM pipeline and the 3D structures of the precursors were obtained. These structures were then sorted according to their least free energies and a library of 3D structures belonging to the precursors was created. The creation of such a library was essential so as to sort the precursor structures according to their length and class. Once the library creation was completed the KSRP protein sequence was derived from the NCBI Genpept database and secondary structural analyses of the sequence were performed (Fig. 2). Following this a BLAST search was performed against the PDB database to identify suitable homologous structure.

However the BLAST search proved to be ineffective and no structure homologue having greater than 35% sequence – structure homology was found. Thus homology modelling was ruled out and using Modeller version 9.4 [15] loop modelling followed by threading was used to generate a 3D structure of the said protein. Along with this the sequence was submitted to the I-Tasser server for validation of the model obtained. I – Tasser [16] returned 5 best fit models and the model with the best sterically unhindered conformation was found to match with the structure that was generated using threading and loop modelling. Both these structures were then analyzed using Ramachandran plot at MOLPROBITY and no outliers were found indicating a successful model generation (Fig. 1).

2.1 *In Silico* Mutation Analyses

The selected protein model was now loaded to DEEP VIEW [14] and *in silico* mutations were introduced at reported interacting sites (nucleic acid binding site and GXXG motifs), The induced mutations were performed according to table 2

Flow Chart of Work:

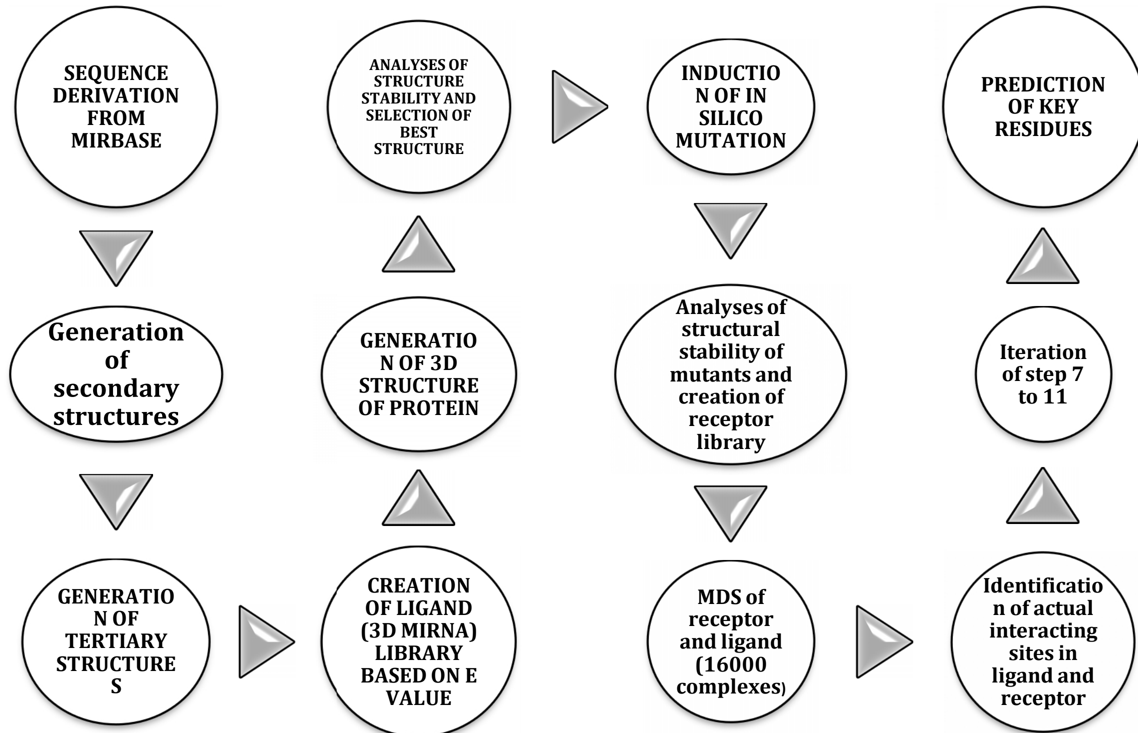

Fig. 1. Flow chart of work (MDS = Molecular Dynamic Simulation)

The dataset of microRNAs were let7 precursor sequences of Homo sapiens derived from mirBASE [17]. Multiple sequence alignment was performed using SEAVIEW program [18] using clustal W method with gaps and 100 bootstraps were performed leading to Cladogram generation.

3. RESULTS AND DISCUSSION

Over the years there has been much debate as to how the amino acid sequence of the protein actually influences the biological property. An accepted standard measure of the importance of protein residues has been sequence conservation [19-22]. Halabi et al. [23] working on S1A serine proteases have indicated that “decomposition of the protein into three quasi-independent groups of correlated amino acids” which they have termed as protein sectors. However these sectors are actually basically structural evolutionary units and are instrumental in maintenance of protein structures. The question however still remains that conserved protein residues actually have influence on function or is the function of the protein dependent on the spatial arrangement of a group of amino acids.

Fig. 2. Secondary Structure details of KSRP

Through this work with KSRP and its interactions with precursor miRNA structures we have been able to locate key interaction sites in both the protein and RNA structures (Fig. 3). As is indicated in the methodology the identification involved an exhaustive pipeline of *in silico* mutation analyses. All mutants except six (406 MET/CYS; 424 GLY/PRO; 461 SER/THR; 473 LYS/ARG; 477 ILE/LEU; 619 THR /TYR;) exhibited differences in the binding energies which were on the higher side (Table 1&2). The six mentioned complexes exhibited lower binding energy when compared with the wild type.

The second set of docking runs which involved the mutant analyses of nucleotides showed a similar trend to the protein mutants.

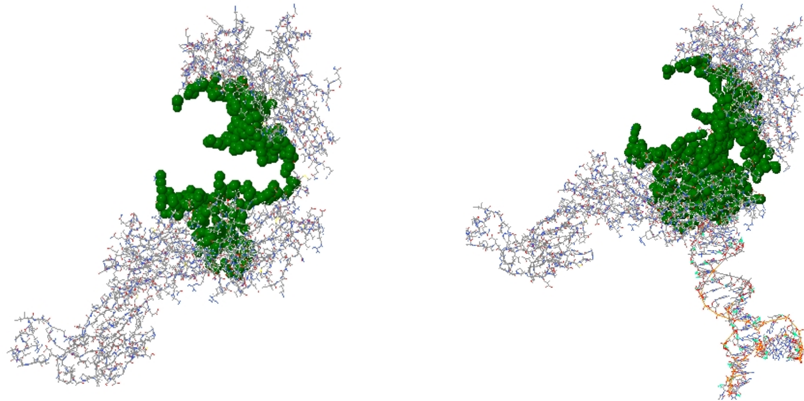

Fig. 3. Interactions of KSRP and human microRNA precursor (left – binding pocket marked in green in the protein; right – microRNA precursor bound to the specific pocket)

However all the mutants of nucleic acids were docked with the wild type protein to keep one aspect of the complex constant.

Table 1. Results of wild type (both RNA and protein wild type structures)

Serial number	Docking mode	E- value	Average e-value	Remarks
1	FLEXIBLE	-1679.86		Both molecules flexible
2	RIGID	-1680.65	-1680.255	ksrp protein receptor kept rigid and ligand (precursor microRNA) kept flexible.

The Cladogram generated using SEAVIEW (Fig. 4) showed that a large part of the interacting region was conserved across the homologues indicative of a sequence and structure based functional assignment of this particular protein group.

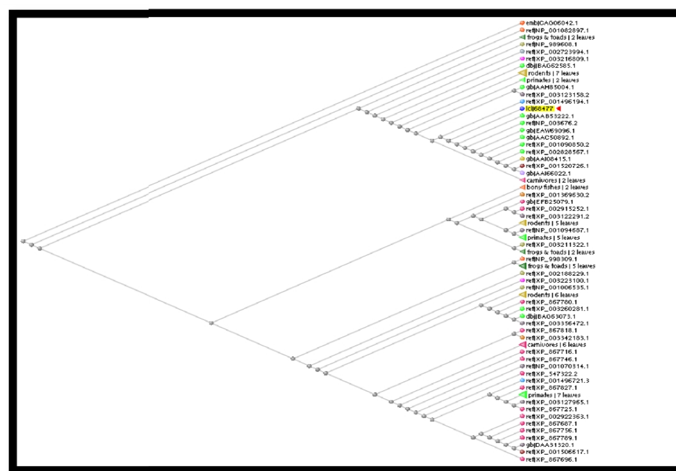

Fig. 4. Cladogram generated using Seaview of all the KSRP homologues

Ganguli et al. [5] has earlier indicated that major microRNA binding proteins such as dicer and argonautes possess specific functional residues which serve as important binding motifs for RNA. The current work further strengthens the concept that micro RNA – protein interactions are modulated specifically by the capacity of targeted residues in the protein and in the RNA molecule as well.

Table 2. Induced mutations and free energy values of docked complexes

Serial number	Residue number	Original residue identity	Altered residue identity	E value	Remarks
1.	1	U	A	-1577.61	HIGH
2.	2	G	C	-1551.71	HIGH
3.	3	G	C	-1546.74	HIGH
4.	4	G	C	-1547.78	HIGH
5.	5	A	U	-1567.67	HIGH
6.	6	U	A	- 1568.56	HIGH
7.	7	G	C	-1569.78	HIGH
8.	8	A	U	-1544.87	HIGH
9.	9	G	C	-1543.67	HIGH
10.	10	G	C	-1548.67	HIGH
11.	11	U	A	-1546.67	HIGH
12.	12	A	U	-1547.78	HIGH
13.	13	G	C	-1566.78	HIGH
14.	65	U	A	-1556.68	HIGH
15.	66	C	G	-1566.78	HIGH
16.	67	U	A	-1566.78	HIGH
17.	68	A	U	-1545.78	HIGH
18.	69	C	G	-1535.67	HIGH
19.	70	U	A	-1545.67	HIGH
20.	70	U	A	-1565.46	HIGH
21.	71	G	C	-1546.76	HIGH
22.	72	U	A	-1566.87	HIGH
23.	73	C	G	-1546.76	HIGH
24.	74	U	A	-1546.76	HIGH
25.	75	U	A	-1566.87	HIGH
26.	76	U	A	-1576.76	HIGH
27.	77	C	G	-1556.67	HIGH
28.	78	C	G	-1556.67	HIGH
29.	79	U	A	-1566.76	HIGH
30.	80	A	U	-1545.76	HIGH
31.	145	MET	Tryptophan	-1656.78	HIGH
32.	147	GLU	Asparagine	-1656.78	HIGH
33.	157	GLY	Phenylalanine	-1656.78	HIGH
34.	158	LEU	Valine	-1666.72	HIGH
35.	159	ILE	Leucine	-1666.77	HIGH
36.	161	GLY	Phenylalanine	-1673.34	HIGH
37.	162	ARG	Aspartic acid	-1675.45	HIGH
38.	165	GLU	Aspartic acid	-1676.38	HIGH
39.	166	GLN	Histidine	-1654.28	HIGH
40.	168	ASN	Cysteine	-1656.78	HIGH
41.	169	LYS	Arginine	-1686.36	HIGH
42.	170	ILE	Leucine	-1677.56	HIGH
43.	196	GLY	Valine	-1645.87	HIGH
44.	198	PRO	Glycine	-1654.42	HIGH

45.	**199**	**GLU**	**Aspartic acid**	**-1656.78**	HIGH
46.	**201**	**VAL**	**Glycine**	**-1676.88**	HIGH
47.	**202**	**GLN**	**Histidine**	**-1646.82**	HIGH
48.	**203**	**LYS**	**Aspartic acid**	**-1667.98**	HIGH
49.	**204**	**ALA**	**Valine**	**-1656.78**	HIGH
50.	**205**	**LYS**	**Aspartic acid**	**-1666.88**	HIGH
51.	**206**	**MET**	**Tryptophan**	**-1656.88**	HIGH
52.	**207**	**MET**	**Tryptophan**	**-1636.84**	HIGH
53.	**209**	**ASP**	**Glutamic acid**	**-1656.48**	HIGH
54.	**210**	**ASP**	**Glutamic acid**	**-1666.84**	HIGH
55.	**212**	**VAL**	**Proline**	**-1656.78**	HIGH
56.	**213**	**SER**	**Tyrosine**	**-1656.88**	HIGH
57.	**214**	**ARG**	**Lysine**	**-1656.88**	HIGH
58.	**215**	**GLY**	**Proline**	**-1656.78**	HIGH
59.	**291**	**LYS**	**Arginine**	**-1646.88**	HIGH
60.	**294**	**GLN**	**Asparagine**	**-1676.88**	HIGH
61.	**294**	**GLN**	**Asparagine**	**-1634.65**	HIGH
62.	**295**	**ALA**	**Tyrosine**	**-1676.88**	HIGH
63.	**298**	**MET**	**Tryptophan**	**-1676.88**	HIGH
64.	**299**	**VAL**	**Glycine**	**-1656.78**	HIGH
65.	**300**	**MET**	**Tryptophan**	**-1634.67**	HIGH
66.	**301**	**ASP**	**Glutamic acid**	**-1646.34**	HIGH
67.	**303**	**LEU**	**Isoleucine**	**-1666.54**	HIGH
68.	**304**	**ARG**	**Lysine**	**-1676.88**	HIGH
69.	**305**	**GLU**	**Aspartic acid**	**-1636.56**	HIGH
70.	**306**	**ARG**	**Lysine**	**-1676.52**	HIGH
71.	**307**	**ASP**	**Glutamic acid**	**-1678.51**	HIGH
72.	**308**	**GLN**	**Asparagine**	**-1678.32**	HIGH
73.	**312**	**GLY**	**Proline**	**-1656.78**	HIGH
74.	**314**	**ARG**	**Aspartic acid**	**-1665.32**	HIGH
75.	**329**	**VAL**	**Phenylalanine**	**-1665.62**	HIGH
76.	**330**	**PRO**	**Glycine**	**-1638.32**	HIGH
77.	**331**	**ARG**	**Lysine**	**-1676.88**	HIGH
78.	**332**	**HIS**	**Serine**	**-1678.52**	HIGH
79.	**333**	**SER**	**Threonine**	**-1648.62**	HIGH
80.	**334**	**VAL**	**Proline**	**-1654.71**	HIGH
81.	**336**	**VAL**	**Proline**	**-1656.78**	HIGH
82.	**337**	**VAL**	**Proline**	**-1656.74**	HIGH
83.	**338**	**ILE**	**Leucine**	**-1638.32**	HIGH
84.	**341**	**SER**	**Threonine**	**-1643.32**	HIGH
85.	**355**	**ARG**	**Lysine**	**-1626.22**	HIGH
86.	**356**	**ILE**	**Leucine**	**-1646.36**	HIGH
87.	**358**	**PHE**	**Alanine**	**-1657.32**	HIGH
88.	**368**	**LYS**	**Arginine**	**-1667.22**	HIGH
89.	**370**	**ALA**	**Leucine**	**-1646.44**	HIGH
90.	**373**	**MET**	**Tryptophan**	**-1676.56**	HIGH
91.	**384**	**ARG**	**Aspartic acid**	**-1654.16**	HIGH
92.	**386**	**ILE**	**Leucine**	**-1644.18**	HIGH
93.	**387**	**ASN**	**Histidine**	**-1626.82**	HIGH
94.	**388**	**ASP**	**Histidine**	**-1665.42**	HIGH
95.	**389**	**LEU**	**Isoleucine**	**-1658.72**	HIGH
96.	**390**	**LEU**	**Isoleucine**	**-1655.92**	HIGH
97.	**391**	**GLN**	**Serine**	**-1648.34**	HIGH

98.	**392**	**SER**	**Threonine**	**-1626.38**	HIGH
99.	**393**	**LEU**	**Isoleucine**	**-1658.49**	HIGH
100.	**394**	**ARG**	**Lysine**	**-1648.55**	HIGH
101.	**395**	**SER**	**Threonine**	**-1628.66**	HIGH
102.	**396**	**GLY**	**Proline**	**-1652.68**	HIGH
103.	**397**	**PRO**	**Glycine**	**-1634.32**	HIGH
104.	**398**	**PRO**	**Glycine**	**-1668.32**	HIGH
105.	**399**	**GLY**	**Proline**	**-1647.46**	HIGH
106.	**406**	**MET**	**Cysteine**	**-1688.32**	**LOW**
107.	**407**	**PRO**	**Glycine**	**-1618.62**	HIGH
108.	**409**	**GLY**	**Proline**	**-1647.46**	HIGH
109.	**411**	**ARG**	**Lysine**	**-1658.72**	HIGH
110.	**413**	**ARG**	**Lysine**	**-1628.33**	HIGH
111.	**415**	**ARG**	**Lysine**	**-1628.64**	HIGH
112.	**416**	**GLY**	**Proline**	**-1647.46**	HIGH
113.	**417**	**GLN**	**Asparagine**	**-1656.32**	HIGH
114.	**418**	**GLY**	**Leucine**	**-1645.63**	HIGH
115.	**419**	**ASN**	**Glutamine**	**-1636.23**	HIGH
116.	**420**	**TRP**	**Serine**	**-1664.34**	HIGH
117.	**421**	**GLY**	**Proline**	**-1627.26**	HIGH
118.	**424**	**GLY**	**Proline**	**-1682.46**	**LOW**
119.	**425**	**GLY**	**Proline**	**-1626.46**	HIGH
120.	**426**	**GLU**	**Aspartic acid**	**-1624.46**	HIGH
121.	**427**	**MET**	**Tryptophan**	**-1628.46**	HIGH
122.	**428**	**THR**	**Cysteine**	**-1644.46**	HIGH
123.	**429**	**PHE**	**Leucine**	**-1646.63**	HIGH
124.	**430**	**SER**	**Threonine**	**-1626.26**	HIGH
125.	**431**	**ILE**	**Leucine**	**-1645.63**	HIGH
126.	**433**	**THR**	**Tyrosine**	**-1636.26**	HIGH
127.	**435**	**LYS**	**Asparagine**	**-1668.26**	HIGH
128.	**437**	**GLY**	**Proline**	**-1657.42**	HIGH
129.	**438**	**LEU**	**Isoleucine**	**-1646.28**	HIGH
130.	**439**	**VAL**	**Glycine**	**-1636.29**	HIGH
131.	**461**	**SER**	**Threonine**	**-1686.25**	**LOW**
132.	**463**	**GLN**	**Asparagine**	**-1628.26**	HIGH
133.	**464**	**LEU**	**Isoleucine**	**-1624.26**	HIGH
134.	**472**	**PHE**	**Leucine**	**-1645.63**	HIGH
135.	**473**	**LYS**	**Arginine**	**-1686.26**	**LOW**
136.	**474**	**LEU**	**Isoleucine**	**-1626.26**	HIGH
137.	**475**	**PHE**	**Leucine**	**-1646.56**	HIGH
138.	**476**	**ILE**	**Leucine**	**-1664.58**	HIGH
139.	**477**	**ILE**	**Leucine**	**-1682.58**	**LOW**
140.	**478**	**ARG**	**Aspartic acid**	**-1622.76**	HIGH
141.	**479**	**GLY**	**Valine**	**-1612.48**	HIGH
142.	**479**	**GLY**	**Valine**	**-1632.56**	HIGH
143.	**480**	**SER**	**Threonine**	**-1612.48**	HIGH
144.	**481**	**PRO**	**Glycine**	**-1645.76**	HIGH
145.	**482**	**GLN**	**Histidine**	**-1646.78**	HIGH
146.	**483**	**GLN**	**Histidine**	**-1656.77**	HIGH
147.	**484**	**ILE**	**Leucine**	**-1667.76**	HIGH
148.	**485**	**ASP**	**Glutamic acid**	**-1646.86**	HIGH
149.	**487**	**ALA**	**Valine**	**-1626.21**	HIGH
150.	**488**	**LYS**	**Arginine**	**-1636.56**	HIGH
151.	**490**	**LEU**	**Isoleucine**	**-1646.44**	HIGH

152.	**526**	**GLY**	**Valine**	**-1641.76**	HIGH
153.	**528**	**PRO**	**Glycine**	**-1641.76**	HIGH
154.	**529**	**GLY**	**Valine**	**-1642.77**	HIGH
155.	**530**	**ALA**	**Leucine**	**-1642.46**	HIGH
156.	**532**	**PRO**	**Glycine**	**-1641.56**	HIGH
157.	**538**	**PRO**	**Glycine**	**-1642.66**	HIGH
158.	**539**	**PRO**	**Glycine**	**-1643.66**	HIGH
159.	**540**	**HIS**	**Serine**	**-1644.88**	HIGH
160.	**541**	**GLN**	**Asparagine**	**-1628.26**	HIGH
161.	**542**	**TYR**	**Threonine**	**-1656.35**	HIGH
162.	**543**	**PRO**	**Glycine**	**-1626.36**	HIGH
163.	**544**	**PRO**	**Glycine**	**-1644.36**	HIGH
164.	**545**	**GLN**	**Asparagine**	**-1656.36**	HIGH
165.	**547**	**TRP**	**Tyrosine**	**-1624.36**	HIGH
166.	**548**	**GLY**	**Valine**	**-1668.36**	HIGH
167.	**549**	**ASN**	**Glutamine**	**-1624.36**	HIGH
168.	**557**	**PRO**	**Valine**	**-1635.18**	HIGH
169.	**558**	**ALA**	**Leucine**	**-1630.46**	HIGH
170.	**559**	**PRO**	**Valine**	**-1631.26**	HIGH
171.	**560**	**HIS**	**Serine**	**-1666.16**	HIGH
172.	**561**	**ASP**	**Glutamine**	**-1622.56**	HIGH
173.	**563**	**SER**	**Threonine**	**-1622.26**	HIGH
174.	**564**	**LYS**	**Arginine**	**-1628.16**	HIGH
175.	**573**	**ASN**	**Histidine**	**-1677.26**	HIGH
176.	**574**	**ALA**	**Leucine**	**-1655.96**	HIGH
177.	**575**	**ALA**	**Leucine**	**-1622.16**	HIGH
178.	**576**	**TRP**	**Tyrosine**	**-1634.26**	HIGH
179.	**577**	**ALA**	**Leucine**	**-1628.26**	HIGH
180.	**580**	**TYR**	**Threonine**	**-1624.46**	HIGH
181.	**618**	**TYR**	**Threonine**	**-1644.16**	HIGH
182.	**619**	**THR**	**Tyrosine**	**-1686.16**	**LOW**
183.	**620**	**LYS**	**Arginine**	**-1654.28**	HIGH
184.	**621**	**ALA**	**Valine**	**-1614.16**	HIGH
185.	**651**	**TYR**	**Threonine**	**-1648.16**	HIGH
186.	**682**	**TYR**	**Threonine**	**-1638.26**	HIGH
187.	**683**	**ARG**	**Aspartic acid**	**-1628.16**	HIGH
188.	**684**	**GLN**	**Serine**	**-1664.66**	HIGH
189.	**685**	**GLN**	**Serine**	**-1646.88**	HIGH

4. CONCLUSION

We have been able to demonstrate through our analyses that KSRP interacts with multiple human miRNA precursors through specific nucleic acid binding motifs. Specific binding residues serve as prime interactors of these interactions. When mutated they cause a reduction in the binding efficacy and complex formation. The precursor miRNA molecules also possess some interacting hotspots. These data prove that interaction hotspots exist in protein RNA interactions as well and can be utilized for the generation of aptamers or antisense constructs as well as deletion experiments.

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CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

The authors declare that there are no competing interests.

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