Screening the microRNA-25 target database revealed FBXW7 as one of its top onco-target hits towards potential anti-cancer therapeutic design.

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Finding anti-cancer therapeutics is a continuous process focusing on the improvement of the therapeutic efficacy and quality for the benefit of patients. Among various approaches to treat cancer, RNA therapeutics play a key role due to their regulatory functions in controlling the expression of various oncogenes. In the current study, the micro-RNA (MiR) database was used to search for possible targets of MiR-25. Out of the total hits (>900), the top five ranked targets with 100% score were considered to check their relevance to Oncology. The "F-box and WD repeat domain containing 7" (FBXW7) gene was identified to be highly relevant to various cancers. Hence FBXW7 was chosen as the final target and was further explored to be a regulator of several critical proteins such as cyclin-E thus playing a role in regulating at least seven different types of cancers. Based on the above findings, the FBXW7 was identified to be a potential drug target to design MiR-25-based RNA therapeutics that can possibly be used in pan-cancer by targeting the FBXW7.

Keywords: HIV/AIDS, HIV protease, protease inhibitors, qsar, analogs, binding affinities.

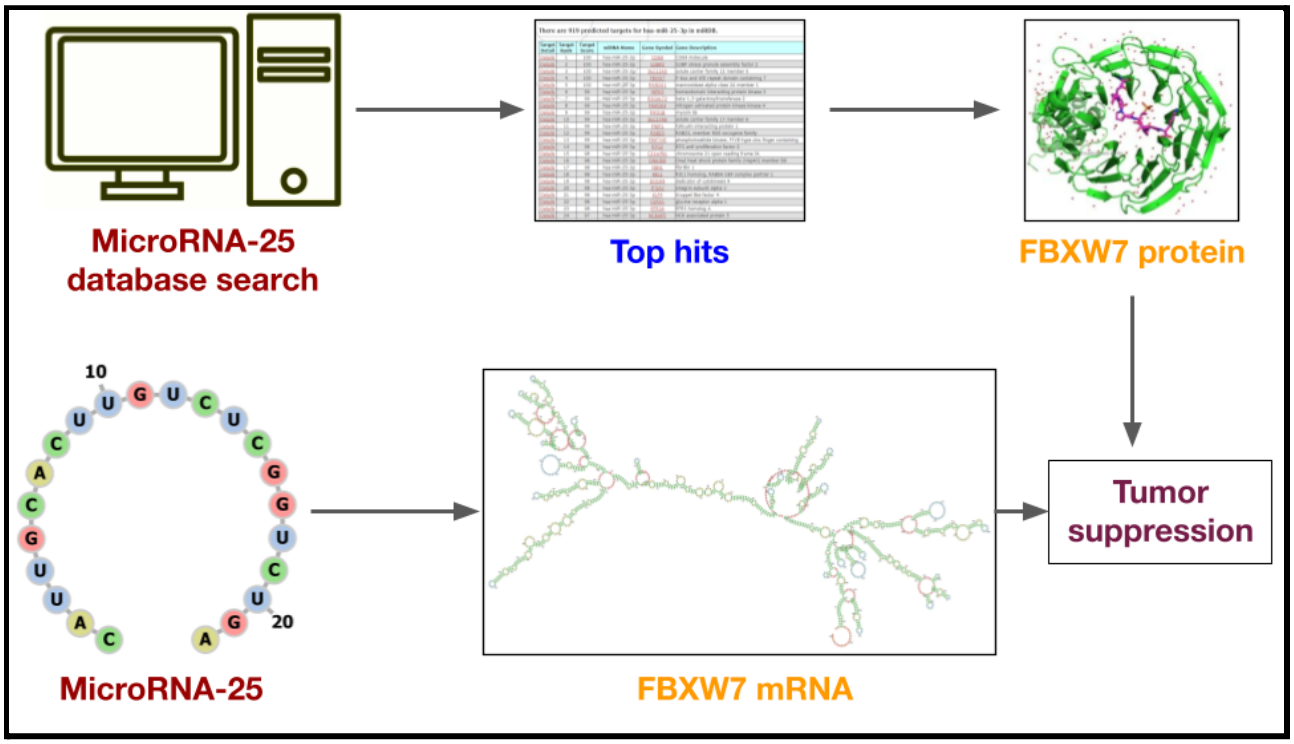

Figure 1. Overview of the microRNA-25 screening and identification of onco-target, FBXW7 that is a tumor suppressor.

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The human body is made of trillions of cells which grow old or get damaged in the course of time and they will be replaced by new cells. The cells multiply through a process called cell division whenever new cell production is required by the body [1]. When this process gets disturbed, it causes abnormal cell growth which may further lead to tumor formation. Cancer is a disease where there is abnormal growth of cells in the body and also poses a potential risk of being spread or invading other parts of the body[1]. This abnormal cell growth or a disturbance in the regular cell division cycle is usually triggered by the genetic changes. Genetic changes are considered to be one of the biggest reasons for the development of cancer [1][2]. These genetic changes can be caused due to several external factors like physical carcinogens (UV rays), chemical carcinogens (tobacco, asbestos, arsenic etc), biological carcinogens (viruses, bacteria etc) and inherited gene mutations [2][7][8]. Inherited gene mutations also account for a small percentage of cancer cases recorded every year [8]. These factors induce changes in the genetic sequences termed as mutations. Since the expression of genes and its products regulate the cell cycles, there are high chances for mutations to bring imbalance in these processes and develop into cancer. The tumors formed can either be cancerous which have the potential to spread to other parts of the body or be benign which do not spread to other body parts, but the tumor can grow irrespective of being benign or cancerous [3]. Symptoms like a lump or abnormal bleeding, loss of appetite and unexplained weight loss, prolonged illness like cough, change in bowel movements etc are few possible signs of cancer [4]. Based on the symptoms and screening tests like colonoscopy, mammogram, PAP test, diagnosis of cancer can be done [3][4]. Based on the screening test reports, further investigations are made by medical imaging tests and biopsies are used as confirmation tests for cancer detection [4]. Based on the location of origin of cancer they are broadly classified by the doctors into four types namely- carcinomas, sarcomas, lymphomas and leukemias [3]. The development of cancer tumors and their spread to other body parts is termed as metastasis. This spread of cancer to other parts is usually by the translocation of cancerous cells through the bloodstream from one part of the body to other body parts. Genetic factors and lifestyle choices, such as smoking, can contribute to the development of the disease. Several elements affect the ways that DNA communicates with cells and directs their division and death. Annually, cancer causes around 15.7% of total deaths in the world (approximately 8.8 million deaths per year) [4][5]. In the year 2020, cancer caused 10 million deaths worldwide, becoming the leading cause of death in the world [6][7]. The most common types of cancer cases seen in the year 2020 include- breast cancer (2.26 million cases), lung cancer (2.21 million cases), colon and rectal cancer (1.93 million cases), prostate cancer (1.41 million cases), non melanoma skin cancer (1.20 million cases) and stomach cancer (1.90 million cases) [7]. If we observe the types of cancers which caused most of the deaths in the year 2020, they include- lung cancer (1.80 million deaths), colon and rectum cancer (935000 deaths), liver cancer (830000 deaths), stomach cancer (769000 deaths) and breast cancer (685000 deaths) [7]. The risk factors of cancer which are known to increase the chances of a person getting cancer include - age, habits and lifestyle, family history, health conditions and environment [8]. Such risk factors do not necessarily cause cancer in a person but they make a person more likely to get cancer in their lifetime. With proper lifestyle and care, cancer can be prevented. Following a healthy diet and exercise, maintaining a healthy body weight, avoiding exposure to carcinogens, avoiding smoking and drinking of alcohol, taking regular health checkups and checking out for any required

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immunizations and getting them done in time are known to help prevent cancer to a large extent [8].

Even though cancer is one of the dreadful diseases, it does have treatment methods which can help to cure if they are in early stages or try to extend the lifetime of a person if the disease is in advanced stages. The type of treatment to be given to the patient is decided based on the type of cancer they have, the stage of cancer and the symptoms the patient is experiencing. So the treatment can be one mode of treatment or a combination of treatments and it also depends on the diagnostic reports of the screening tests, medical imaging tests and biopsies. The treatments which are primarily used in cancer treatment include- surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy, hormonal therapy, stem cell transplant and palliative care [4][9]. In a surgery in cancer patients, the cancerous tumor or part is removed by the surgeon by the surgical procedures [9]. Surgeries are suggested for patients in whom the tumor is solid and contained in one area [10]. Surgery cannot be alone suggested as the entire treatment for cancer but can work better with a combination of other therapies [9][10]. In case of chemotherapy, anti-cancer drugs are used for treatment. These drugs help treat cancer by killing cancer cells but they are also known to cause many side effects during their use [10][11]. Immunotherapy helps the immune system to fight cancer by helping them to recognise cancer cells [11]. There are several types of immunotherapies and immuno therapy works only for a few patients not for all. When high doses of radiation are used to kill cancerous cells or shrink tumors, it is radiation therapy [10]. In targeted therapy, the factors which help the cancer cell growth and spread are identified and thus identified targets are treated [10]. For those cancers which use the hormones to grow, hormone therapy is used where this treatment stops or slows down the associated hormones [10]. High doses of chemotherapy and radiation therapy may destroy the blood forming stem cells in cancer patients and in such cases, stem cell therapy may be used to restore the destroyed blood forming stem cells [10]. As many of these cancer treatments cause severe side effects to the patients undergoing the treatment, to help relieve the side effects of these treatments, palliative treatment is done [12]. These cancer treatments and therapies along with cancerous cells, also show their effect on healthy cells and other parts of the body and thus many side effects can be seen in cancer patients undergoing treatments like chemotherapy, radiotherapy etc. The cancer treatment side-effects which are usually observed are - anaemia, loss of appetite, bleeding and bruising, constipation, delirium, diarrhea, edema, fatigue, fertility issues, flu symptoms, hair loss, infection and neutropenia, lymphedema, memory or concentration problems, mouth and throat problems, nausea and vomitings, nerve related issues, organ related inflammation, sexual health issues, skin and nail changes, insomnia, etc [13]. These side effects can be observed as either long term effects or short term effects and they change from patient to patient depending on the type of cancer, location of the cancer in the body, combination of treatments administered and also the past health history of the patient.

The usage of drugs for a longer time develops a phenomenon called drug resistance in cancer patients. Drug resistance is the tolerance developed by the disease to the drugs or pharmaceutical treatments [14]. This phenomenon of drug resistance is not only found in cancer but also in many other diseases when the disease develops resistance to the drugs being used. In cancers, the mechanisms like DNA mutations and metabolic changes will promote the processes like drug inhibition and drug degradation which will lead to the drug resistance in the patient [14]. The several factors that lead to drug resistance include the mechanisms like - drug efflux, DNA damage repair, drug inactivation, cell death inhibition,

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epithelial-mesenchymal transition, epigenetics, drug target alteration etc [14]. The drugs need metabolic activation for the drugs to function and any down regulations or mutations in these activation pathways can either decrease the activation or may lead to drug inactivation [14]. For example, if we consider the treatment of acute myelogenous leukemia with cytarabine (AraC), a nucleoside drug activation can be done only after multiple phosphorylation events that convert it to AraC-triphosphate [14][15][16]. A drug's molecular target defines the drug's efficacy [14]. Any mutations in the genes or any kind of modifications in their expression levels cause alterations in the targets, thus causing drug resistance in cancer patients [14]. For example, if we see topoisomerase II inhibiting drugs, certain cell lines have become resistant to these drugs due to mutations in the topoisomerase II gene that alters the drug target [14][16][18]. The transport of various substances across the plasma membranes is regulated by the transmembrane proteins. The ABC (ATP Binding Cassette) transporters are the most important regulators and enable the efflux of substances through the plasma membrane [14]. The mechanism of efflux is important to prevent accumulation of toxins in the cell and this mechanism causes drug resistance by reducing the accumulation of drugs by enhancing their efflux [14][19]. DNA damage repair mechanisms have been known to cause drug resistance in cancer patients by reversing the damage induced by the drugs used in chemotherapy [14]. This drug resistance due to DNA damage response can be seen in this example where cisplatin (platinum containing anticancer drug), which causes harmful crosslinks in DNA and lead to cell apoptosis, but because of primary DNA repair mechanisms involved in reversing the damage caused due to platinum, drug resistance to cisplatin can be observed [14][20][21]. Hence, it can be said that the efficacy of chemotherapy drugs whose purpose is to cause DNA-damage to cancerous cells and to apoptize them, depends on the failure of the cancer cell's DNA Damage Response mechanisms [14][22]. The drug resistance in cancer therapy has been the biggest challenge of cancer treatment and the approaches like deep analysis and assessment of the tumor, early detection and precise monitoring, mapping cancer dependencies, monitoring response and adaptive interventions and the combined assessment of these approaches, drug resistance can be controlled to some extent [23]. Since these approaches are highly costly and are also very much exhaustive processes, it is necessary to bring up new methods to address these problems.

MicroRNAs are small single stranded non coding RNA molecules which are usually of 22 nucleotides in length. They are found in plants, animals and viruses. The main function of these microRNAs is post-transcriptional regulation of gene expression and RNA silencing [24]. Messenger RNAs (mRNAs) are silenced by miRNAs by either cleavage of the mRNA strand to two pieces or destabilization of the mRNA through shortening of its polyA tail or by less efficient translation of the mRNA into proteins by ribosomes and this occurs when miRNAs base pair with complementary sequences in the mRNA and thus cause gene silencing [24]. MicroRNAs also function as the controller of many biological processes like cell development, cell differentiation, cell proliferation and cell apoptosis [25]. The first microRNA was discovered in the early 1990's by the Ambros and Ruvkun groups in Caenorhabditis elegans [25][26]. Most of the miRNAs lie in the introns or exon regions of other genes and are usually regulated together with their host genes [24][27]. The sequence encoded by the RNA polymerase II (Pol II) when bound to a promoter found near the DNA sequence forms the hairpin loop of the pre-miRNA [24][28]. The pre-miRNA is capped with a specifically modified nucleotide at the 5' end, polyadenylated with multiple adenosines and spliced [28]. miRNA's with upstream Alu sequences, tRNA's and mammalian wide

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interspersed repeat (MWIR) promoter units are transcribed by RNA polymerase III (Pol III) [24][29]. One pri-miRNA can have from one to six miRNA precursors whose hairpin loop structures are composed of about 70 nucleotides each, where each hairpin is flanked by sequences necessary for efficient processing [24]. DGCR8 or Pasha (in invertebrates) or DiGeorge Syndrome Critical Region 8, a nuclear protein recognises the dsRNA of hairpins in pri-miRNA and when associated with Drosha enzyme, it forms a microprocessor complex [30][31]. This complex results in the formation of a precursor-miRNA or pre-miRNA. If these pre-miRNA's are directly spliced out of introns,by not undergoing the microprocessor complex, they are termed as Mirtrons [32]. Thus formed pre-miRNAs are exported from the nucleus by nucleocytoplasmic shuttler Exportin-5 involving process, which aids in the transport of the pre-miRNA from the nucleus to cytoplasm and binds the pre-miRNA with RNase III enzyme dicer which yields an imperfect miRNA-miRNA duplex which is about 22 nucleotides in length [33][34]. Either of the strands can act as functional miRNA. Further research on miRNA expression profiles and miRNA signaling pathways shows an evidence that miRNAs play key role in the pathogenesis of cancer by functioning as oncogenes or tumor suppressors, proving them to be a potential targets for the development of anti-cancer therapeutics [35]. In a wide range of cancer types, some important molecular pathways which are involved in tumor progression, invasion, angiogenesis and metastasis are known to be influenced by regulation of miRNAs [36]. MicroRNA usage as a therapeutic has two major hurdles which are the stability of the microRNA and its delivery system. The miRNA delivery systems for using miRNA as an anti-cancer therapeutic include: virus based delivery, non-viral delivery (artificial lipid based vesicles, polymer based or chemical structures), and extracellular vesicle (EV) based delivery system [36]. Studies show that miRNA-based gene therapy provides an attractive anti-tumor approach for integrated cancer therapy [37].

Mir-25 belongs to the miR-106b/25 cluster. The mir-106b/25 is highly conserved in vertebrates and the cluster includes miR-106b, miR-93 and miR-25, all located in a 515 bp region on chromosome 7q22 in intron 13 of the host gene minichromosome maintenance protein 7 (MCM7) [38][39]. The MCM7 belongs to the MCM complex of DNA helicases, which are essential in the initiation of the DNA replication process [38][40]. Mir-25 has a different seed sequence from the other two miR's belonging to the same cluster - miR-106b/25. The sequence of miR-25 can be obtained from the miR database website - http://www.mirbase.org/ or http://www.mirdb.org/. The mature miR-25 sequence has 22 nucleotides and 1163 predicted target mRNA transcripts with conserved sites [38]. Mir-25 is known to function as an oncogenic miRNA and also plays an important role in development of cardiac hypertrophy and heart failure under pathophysiological conditions [38]. Since the targets of miR-25 are involved in processes like differentiation of cells, cell proliferation, inflammation, migration, apoptosis, oxidative stress, calcium handling etc., many studies have shown that miR-25 functions as a key regulator in many cancerous and non-cancerous diseases. MiR-25 ia also known to have a significant role in regulating the pathophysiological conditions of acute myocardial infarction, left ventricular hypertrophy, heart failure, diabetes mellitus, diabetic nephropathy, tubulointerstitial nephropathy, asthma bronchiale, cerebral ischemia/reperfusion injury, neurodegenerative diseases, schizophrenia, multiple sclerosis [38]. In cancer types like brain tumors, lung, breast, ovarian, prostate, thyroid, oesophageal, gastric, colorectal, hepatocellular cancers miR-25 is known to play a crucial role as an oncogene [38].

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Materials & Methods:

The miRNA database search for MiR-25 targets: MiR-25 sequence was searched in the miRNA database (mirdb.org) [41][42] in order to obtain the MiR-25 related targets. The search option includes hsa-miR-25-3P and hsa-miR-25-5P for which the search results are displayed separately under two different web links. The current study is focused only on the "hsa-miR-25-3P" variant because of its high target score in the search results. Targets were then shortlisted based on cancer relevance, ranking and score. The targets that were ranked among the top five with 100% score were considered first among which only cancer related targets were given priority. Other targets that were among the top 5 with 100% score that were not cancer relevant were considered for other disease relevance towards potential therapeutic development. Further, such analysis may result in the discovery of novel drug targets in general other than Oncology. None of the search results for the "5p" variants were seen in the top 5 target ranks with 100% target score. In fact there were only 51 targets in total as hits for the "5p" variant with the highest target score of 87%. Once the top five targets have been explored, the next round of selection will focus on the target score of 99% which gives more targets and so forth which is beyond the scope of this report. However, as the target score decreases, more literature-based data/evidence is needed in order to confirm the specificity of MiR-25 in binding the target. Statistical analysis may also be needed only for such low score targets if analyzed in the context of disease. Due to high target score in this study, no statistical analysis was performed for FBXW7 gene to validate it as a genuine MiR-25 target. Alternately the FBXW7-mRNA secondary structure analysis was performed to confirm MiR-25 binding.

NCBI Search: Further details of the targets were obtained using NCBI genbank and nucleotide databases. The nucleotide sequence of the target and MiR-25 binding site on the sequence were identified using both mirdb.org and NCBI. The one Oncotarget hit that was identified among the top 5 was the FBXW7 gene. This gene has been already implicated in several types of cancers such as the ovarian and breast cancers to begin with. The gene ID for FBXW7 is 55294. It was RefSeq reviewed which avoids any further confusion in the sequence. The FBXW7 gene was found to be a part of the chromosome no. 4 and more than 5 alternate splice variants were observed that give rise to different isoforms. These isoforms may not pose any threat to this study as MiR-25 binds downstream to the splicing sites at the 3'-UTR of FBXW7 mRNA.

Computing the RNA secondary structure: In order to understand the complexity of the FBXW7-3'-UTR, the mRNA sequence was submitted to the RNA folding server (http://rna.tbi.univie.ac.at/) [43-46]. Computatio -ns were performed using RNA fold 2.4.18 that yielded a thermodynamic ensemble with a total energy value of -423.32 kcal/mol. while the most stable secondary structure was found to have a minimum free energy of -392.20 kcal/mol. These predictions must be considered with caution because the algorithms may or may not completely be able to predict the most stable structure that exists in nature, especially given the highly flexible nature of single stranded mRNA molecules. The positional entropy was seen to fluctuate between 0.0 and 3.5 units with an average of 1.75 units.

Secondary structure analysis of FBXW7 protein: The 3D structures of FBXW7 (PDB IDs: 2OVP, 2OVQ and 2OVR) were downloaded from the protein data bank (www.rcsb.org). These structures were analyzed using a molecular graphics program, PyMOL [47] to understand the secondary structural elements and protein-protein interactions. PyMOL has 2 win -dows, one allows us to type commands and choose options to control display while the other window is the actual graphics window in which 3D structural details of molecule are displayed.

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There are 919 predicted targets for hsa-miR-25-3p in miRDB.

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	100	hsa-miR-25-3p	CD69	CD69 molecule
Details	2	100	hsa-miR-25-3p	G3BP2	G3BP stress granule assembly factor 2
Details	3	100	hsa-miR-25-3p	SLC12A5	solute carrier family 12 member 5
Details	4	100	hsa-miR-25-3p	FBXW7	F-box and WD repeat domain containing 7
Details	5	100	hsa-miR-25-3p	MAN2A1	mannosidase alpha class 2A member 1
Details	6	99	hsa-miR-25-3p	HIPK3	homeodomain interacting protein kinase 3
Details	7	99	hsa-miR-25-3p	B3GALT2	beta-1,3-galactosyltransferase 2
Details	8	99	hsa-miR-25-3p	MAP2K4	mitogen-activated protein kinase kinase 4
Details	9	99	hsa-miR-25-3p	MYO1B	myosin IB
Details	10	99	hsa-miR-25-3p	SLC17A6	solute carrier family 17 member 6
Details	11	99	hsa-miR-25-3p	FNIP1	folliculin interacting protein 1
Details	12	99	hsa-miR-25-3p	RAB23	RAB23, member RAS oncogene family
Details	13	99	hsa-miR-25-3p	PIKFYVE	phosphoinositide kinase, FYVE-type zinc finger containing
Details	14	98	hsa-miR-25-3p	BTG2	BTG anti-proliferation factor 2
Details	15	98	hsa-miR-25-3p	C21orf91	chromosome 21 open reading frame 91
Details	16	98	hsa-miR-25-3p	DNAJB9	DnaJ heat shock protein family (Hsp40) member B9
Details	17	98	hsa-miR-25-3p	FBN1	fibrillin 1
Details	18	98	hsa-miR-25-3p	RIC1	RIC1 homolog, RAB6A GEF complex partner 1
Details	19	98	hsa-miR-25-3p	DOCK9	dedicator of cytokinesis 9
Details	20	98	hsa-miR-25-3p	ITGAV	integrin subunit alpha V
Details	21	98	hsa-miR-25-3p	KLF4	Kruppel like factor 4
Details	22	98	hsa-miR-25-3p	GLRA1	glycine receptor alpha 1
Details	23	98	hsa-miR-25-3p	EFR3A	EFR3 homolog A
Details	24	97	hsa-miR-25-3p	NCKAP5	NCK associated protein 5

Figure 2. List of microRNA-25 targets, top 24.

FBXW7 F-box and WD repeat domain containing 7 [*Homo sapiens* (human)] Download Datasets

Gene ID: 55294, updated on 18-May-2021

Summary

Official Symbol FBXW7 provided by HGNC
Official Full Name F-box and WD repeat domain containing 7 provided by HGNC
Primary source HGNC:HGNC:16712
See related Ensembl:ENSG00000109670 MIM:606278
Gene type protein coding
RefSeq status REVIEWED
Organism Homo sapiens
Lineage Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo
Also known as AGO; CDC4; FBW6; FBW7; hAgo; FBX30; FBXW6; SEL10; hCdc4; FBXO30; SEL-10
Summary This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination. The F-box proteins are divided into 3 classes: Fbws containing WD-40 domains, Fbls containing leucine-rich repeats, and Fbxs containing either different protein-protein interaction modules or no recognizable motifs. The protein encoded by this gene was previously referred to as FBX30, and belongs to the Fbws class; in addition to an F-box, this protein contains 7 tandem WD40 repeats. This protein binds directly to cyclin E and probably targets cyclin E for ubiquitin-mediated degradation. Mutations in this gene are detected in ovarian and breast cancer cell lines, implicating the gene's potential role in the pathogenesis of human cancers. Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Mar 2012]
Expression Broad expression in skin (RPKM 23.3), brain (RPKM 19.1) and 24 other tissues See more
Orthologs mouse all

Figure 3. Screenshot of NCBI-FBXW7 page.

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The display in the graphics window can be changed according to the user requirements. For example, by default all the structures that are loaded into the graphics window are shown as lines, each line indicating a bond between two atoms in the structure. In the line display it is difficult to identify the secondary structural elements of the protein hence, the user can choose to display cartoon mode in which the secondary structural elements are clearly displayed as alpha-helices and beta-strands. A combined view of both cartoons and lines allows the user to display both the secondary structural elements at the protein backbone level as cartoon and the side chains of all amino acids are displayed as lines which gives a better perspective of the overall structure for further analysis. Additionally, PyMOL also has other functionalities such as measurement of distance between two atoms that allows the user to calculate both intra- and inter-molecular hydrogen bonds along with their bond lengths and bond angles for accuracy. Small molecules such as drugs, peptides, etc. can be displayed differently from the protein for easy identification. Mostly the small molecules are displayed as sticks instead of lines so that each of the bonds are displayed as sticks while the protein is displayed in cartoons and lines for clear distinction. The graphics window also allows the user to display the protein amino acid sequence so that these amino acids can be precisely located on the 3D structure for accurate interpretation.

Results and Discussion:

FBXW7 was identified in the top five targets: The search results from mirdb gave more than 900 targets that are regulated by MiR-25. As mentioned earlier, there are MiR-25-3p and MiR-25-5p. In this study only the search results from the "3p" variant were considered because of the high score of targets identified in the hit list compared to the target score obtained in the hit list with the "5p" variant. In fact the targets that ranked among the top five in the hit list with 100% score were seen for the "3p" variant but not for the "5p" variant. Among these targets, FBXW7 was identified as one of the cancer relevant targets in the top ranked targets with 100% score. MiR-25 was found to bind the 3'-UTR region of FBXW7 which contains 1641 bases. It was identified that the MiR-25 binds to the 3'-UTR of FBXW7 at positions 286 (5'-GUGCAAU-3'), 407 (5'-UGCAAUA-3') and 1596 (5'-GUGCAAUA-3') that would further block the translation of FBXW7 mRNA into FBXW7 protein. Formation of the RISC (RNA-induced silencing complex) at the 3'-UTR will inhibit the protein synthesis thus regulating the protein expression at the mRNA level. It is noteworthy that the entire sequence of MiR-25 may not bind to the FBXW7 mRNA as a single stretch because MiR-25 itself may have stem loop secondary structures but even partial binding can regulate the translation of FBXW7 mRNA. This observation gives us an opportunity to design an MiR-based RNA therapeutic to regulate FBXW7. Although there are three binding sites that were mapped onto the sequence of 3'-UTR region of FBXW7 for MiR-25, all these were mapped onto the linear mRNA sequence. However, it is very common for the mRNA molecules to have various secondary structures with multiple stem loops, etc. Hence, the secondary structure of the FBXW7-3'-UTR sequence was predicted using the RNA folding server (http://rna.tbi.univie.ac.at/). Mapping the MiR-25 sites on the secondary structure of FBXW7-3'-UTR sequence would yield a much better understanding of how efficiently MiR-25 can control the expression of FBXW7 in the real cellular context. Currently available robust computer algorithms have been shown to miss target prediction sometimes, most probably due to this reason.

The secondary structure of FBXW7-3'-UTR: The secondary structure of 1641 bases containing FBXW7-3'-UTR looks very complicated with multiple (almost close to 100) stem-loop structures.

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MicroRNA and Target Gene Description:

miRNA Name	hsa-miR-25-3p	**miRNA Sequence**	CAUUGCACUUGUCUCGGUCUGA
Previous Name	hsa-miR-25		
Target Score	100	**Seed Location**	286, 407, 1596
NCBI Gene ID	55294	**GenBank Accession**	NM_033632
Gene Symbol	FBXW7	**3' UTR Length**	1641
Gene Description	F-box and WD repeat domain containing 7		

3' UTR Sequence

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   1 agagcagaaa agatgaattt gtccaattgt gtagacgata tactccctgc ccttccccct
  61 gcaaaaagaa aaaaagaaaa gaaaaagaaa aaaatccctt gttctcagtg gtgcaggatg
 121 ttggcttggg gcaacagatt gaaaagacct acagactaag aaggaaaaga agaagagatg
 181 acaaaccata actgacaaga gaggcgtctg ctgtctcatc acataaaagg cttcactttt
 241 gactgagggc agctttgcaa aatgagactt tctaaatcaa accaggtgca attatttctt
 301 tattttcttc tccagtggtc attgggcagt gttaatgctg aaacatcatt acagattctg
 361 ctagcctgtt cttttaccac tgacagctag acacctagaa aggaactgca ataatatcaa
 421 aacaagtact ggttgacttt ctaattagag agcatctgca acaaaaagtc atttttctgg
 481 agtggaaaag cttaaaaaaa ttactgtgaa ttgtttttgt acagttatca tgaaaagctt
 541 tttttttttt ttttttgcca accattgcca atgtcaatca atcacagtat tagcctctgt
 601 taatctattt actgttgctt ccatatacat tcttcaatgc atatgttgct caaaggtggc
 661 aagttgtcct gggttctgtg agtcctgaga tggatttaat tcttgatgct ggtgctagaa
 721 gtaggtcttc aaatatggga ttgttgtccc aaccctgtac tgtactccca gtggccaaac
 781 ttatttatgc tgctaaatga aagaaagaaa aaagcaaatt atttttttt atttttttc
 841 tgctgtgacg ttttagtccc agactgaatt ccaaatttgc tctagtttgg ttatggaaaa
 901 aagacttttt gccactgaaa cttgagccat ctgtgcctct aagaggctga gaatggaaga
 961 gtttcagata ataaagagtg aagtttgcct gcaagtaaag aattgagagt gtgtgcaaag
1021 cttattttct tttatctggg caaaaattaa aacacattcc ttggaacaga gctattactt
1081 gcctgttctg tggagaaact tttctttttg agggctgtgg tgaatggatg aacgtacatc
1141 gtaaaactga caaaatattt taaaaatata taaaacacaa aattaaaata aagttgctgg
1201 tcagtcttag tgttttacag tatttgggaa aacaactgtt acagttttat tgctctgagt
1261 aactgacaaa gcagaaacta ttcagttttt gtagtaaagg cgtcacatgc aaacaaacaa
1321 aatgaatgaa acagtcaaat ggtttgcctc attctccaag agccacaact caagctgaac
1381 tgtgaaagtg gtttaacact gtatcctagg cgatcttttt tcctccttct gtttattttt
1441 ttgtttgttt tatttatagt ctgatttaaa acaatcagat tcaagttggt taattttagt
1501 tatgtaacaa cctgacatga tggaggaaaa caacctttaa agggattgtg tctatggttt
1561 gattcactta gaaattttat tttcttataa cttaagtgca ataaaatgtg ttttttcatg
1621 ttaaaaaaaa aaaaaaaaaa a
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Figure 4. Screenshot of FBXW7 3' UTR with MiR locations.

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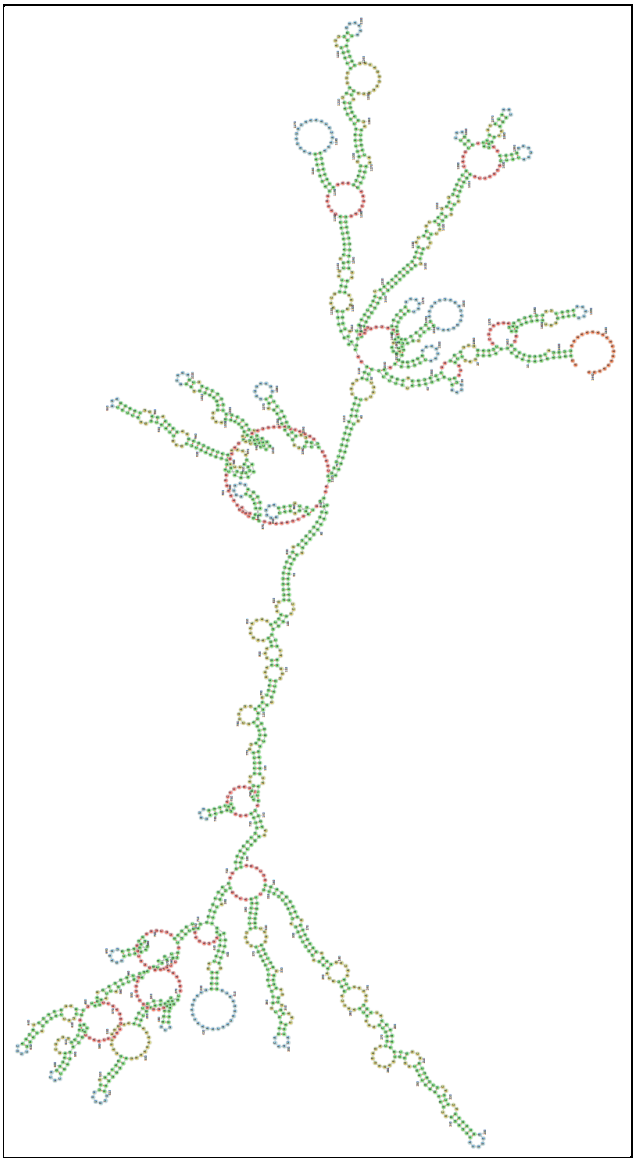

Figure 5. Predicted secondary structure of FBXW7 mRNA.

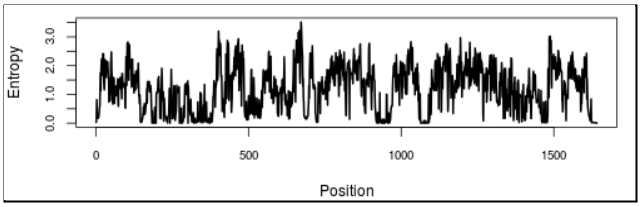

Figure 6. Entropic variation throughout the predicted secondary structure of FBXW7 mRNA.

All three MiR-25 binding sites, at positions 286 (5'-GUGCAAU-3'), 407 (5'-UGCAA UA-3') and 1596 (5'-GUGCAAUA-3') were successfully mapped onto partial loops. In this context, it is noteworthy that not the entire stretch of MiR-25 needs to bind the target mRNA; only a part of MiR-25 binding would suffice. Additionally, it is very difficult to predict the accurate secondary structure of mRNA of the targets because it depends on multiple variables such as the overall thermodynamic stability, sequence complementarity, length of the sequence, etc. Although robust force fields based on NMR data are used in the prediction algorithms, the final results should always be considered with caution. Moreover, mRNA is single stranded and is not as stable as double stranded DNA helix which implies that the secondary structure of mRNA may not be stable over a period of time again due to thermodynamic factors such as entropy fluctuations. As calculated by the RNA folding server, the average positional entropy fluctuations was 1.75 units. Together the knowledge of the FBXW7-3'-UTR secondary structure in combination with the positional entropy provides us deeper insights into the overall binding affinity of MiR-25 in the real cellular milieu rather than just a computational prediction. This information aids greatly in designing the RNA-based therapeutics and their predicted binding *in vivo* with confidence.

FBXW7 is a part of cellular protein degradation process: The FBXW7 protein is a part of the ubiquitin-proteasome system (UPS) that degrades the unwanted proteins in cells in a "TIMELY" fashion in order to maintain cellular homeostasis. Ubiquitin is a small protein that acts as a signal for various proteins' destinies when covalently attached. Mostly ubiquitin is attached as a single unit (monoubiquitin) to the proteins via post-translational modifications but polyubiquitin tail is always attached via ubiquitin ligase enzymes such as FBXW7.

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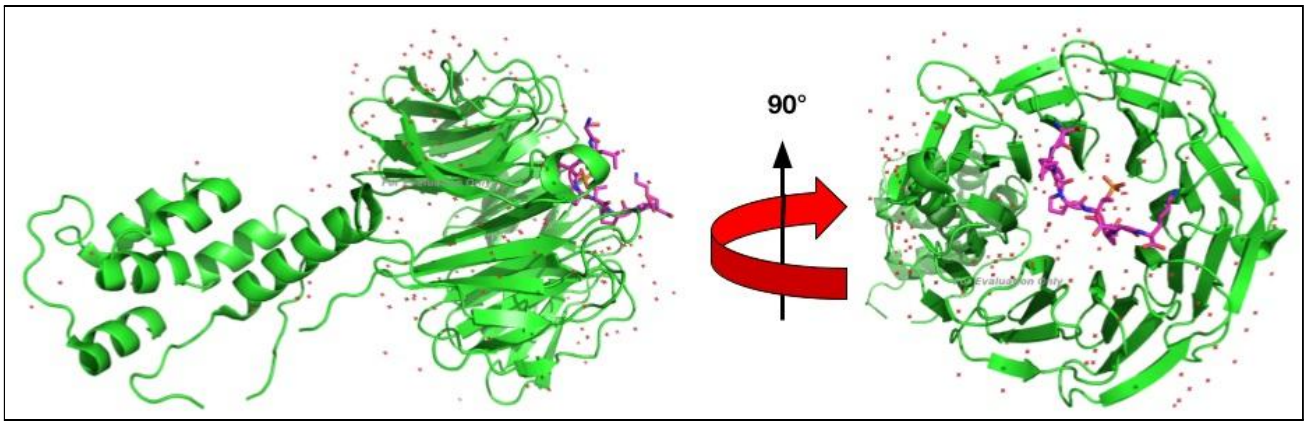

Figure 7. Three-dimensional protein structure of FBXW7.

There are several ubiquitin ligase enzymes in humans that create diversity to bind diverse substrates in order to degrade them in a timely fashion to maintain cellular homeostasis. FBXW7 protein binds to the substrate (protein that is targeted for degradation by the UPS, for example, c-Myc, cyclin-E, etc.) and facilitates the other proteins in UPS to add the polyubiquitin tail onto the substrate [48]. It has also been proposed that FBXW7 can dimerize and target the substrates to the UPS thus increasing the efficiency of cellular protein degradation [48]. Once the substrate is attached with a polyubiquitin tail, it is then recognized by the proteasome for degradation. FBXW7 in complex with SKP1, RBX1, CUL1 and UBC is efficiently involved in attaching the polyubiquitin tail to the unwanted substrate that is targeted to the 26S proteasome [49].

The beta-propeller domain of FBXW7 protein has versatility: Secondary structure analysis of FBXW7 protein revealed that its beta-propeller domain has versatility in binding various substrates and is promiscuous in substrate binding sites. For example, the consensus sequence on the substrate that binds the beta-propeller domain of FBXW7 is "-X---TPPXS". However, FBXW7 can bind to Cyclin-E (CSLIPTPDKE), c-Myc (FELLPTPPLS), c-Jun (EMPGETPPLS) and Notch1 (APLGGTPTLS) substrates to successfully target them to the 26S-proteasomal degradation for maintaining the cellular homeostasis. Additionally, FBXW7 binds to multiple other substrate proteins.

Structural analysis of FBXW7: The secondary structure analysis of FBXW7 bound to the substrate peptide from Cyclin-E was analyzed. The overall structure contains 5-6 alpha helices that bind to the Skp1 protein as a part of the multi-protein complex for substrate degradation and also contains a typical beta-propeller type domain with multiple anti-parallel beta-sheets. It is clearly seen that the variable flexible loops extending out of this beta-propeller like domain causes the versatility in substrate binding. The substrate binds across the beta-propeller domain due to which multiple hydrogen bonding networks are established between FBXW7 and the substrate molecules. This type of binding affinity is needed in order for the substrate to get the polyubiquitin tail attached. In addition to the hydrogen bonds, FBXW7 is also involved in hydrophobic contacts and van der Waals interactions with various substrates during the process of ubiquitination. The combination of different types of interactions offers the beta-propeller domain of FBXW7 more versatility not only in substrate specificity but also in reversibly binding multiple substrates. This is critical because the substrates once ubiquitinated must be released from this multi-protein complex in order for it to be degraded by the 26S proteasome.

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Conclusion: The outcome of this study is very intriguing because we unexpectedly stumbled upon a very critical onco-target that can be probed with the MiR-25 as an RNA-therapeutic for various cancers. It is concluded that MiR-25 binds with the 3'-UTR of FBXW7 mRNA both on the primary sequence and the secondary structure assuring that MiR-25 has high affinity and selectivity for FBXW7. Additionally, from the protein structural analysis it is evident that the beta-propeller domain of FBXW7 protein has high versatility in binding various substrates that are involved in multiple types of cancers. So, by regulating the FBXW7 at its mRNA level using MiR-25 as a therapeutic, one can gain control on multiple cancers in the context of RNA-therapeutic development. In future, the MiR-25 will be synthesized, HPLC-purified and tested against its capability in regulating the expression of FBXW7 mRNA both *in vitro* and *in vivo*. Once confirmed, then the MiR-25 will be tested against various cancers and will be further developed for human trials.

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