In silico Meta-Analysis of Circulatory microRNAs in Prostate Cancer

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Abstract: Circulatory microRNAs (miRNAs) have emerged as a new class of non coding RNA molecules which regulate many crucial molecular and biological processes. We have aimed to shed light on the roles of circulatory miRNAs in Prostate Cancer (PCa) using an integrative *in silico* bioinformatics approach. We have described a new protocol for target prediction and functional analysis which was applied to 40 highly differentially dysregulated circulatory miRNAs in PCa. This framework comprises: (i) evidence of involvement of these circulatory miRNAs from previous literature and microarray analysis (ii) overlap of prediction results by target prediction tools, including miRTarBase, miRDB, DIANA-microT 4.0 and TargetScan (combining computational learning, alignment, interaction energy and statistical tests for minimization of false positives), (iii) gene ontology (GO) along with pathway enrichment analysis of the miRNA targets and their pathways and (iv) linking these pathways to oncogenesis and cancer hallmarks. More than 200 target genes and 40 regulatory pathways were retrieved and analysed which was followed by associating their roles with cancer hallmark processes. Wnt signalling, Cell cycle, MAPK signalling, Cadherin signalling, Integrin signalling and Ras pathways were some of the identified regulatory pathways during bioinformatics analysis. These signalling and developmental pathways crosstalk and regulate stem cell renewal thus indicating a definite role of circulatory miRNAs in PCa development. Our study identified miR-181, miR-9, Let-7 family, miR-26b circulatory miRNAs, to be contributing majorly in the oncogenic pathways, thus proposing their role as potential biomarkers in PCa initiation and progression.

Keywords: Prostate Cancer, microRNA, Target prediction, Cancer hallmarks.

INTRODUCTION

MicroRNAs are ~22 nucleotides long with 5' phosphate and 3' hydroxyl group, non protein coding endogenous small single stranded RNAs which were originally discovered in *Caenorhabditis elegans* [1]. Evidences have shown miRNAs to play crucial cellular roles in apoptosis, cell cycle differentiation, proliferation and stem cell maintenance [2]. Various studies have shed light on correlation of miRNA mutations or its dysregulation with different cancers highlighting the role of miRNAs as tumor suppressors and oncoMIRs [3, 4].

Circulatory microRNAs have emerged as promising non-invasive biomarkers for PCa as they can be easily and readily detected from bodily fluids including serum, plasma, urine, semen and tears (Figure **1**) [5]. MiRNAs have been shown to act robustly against external factors like enzymatic degradation, pH conditions and freeze thaw cycles, further vouching for their potential as ideal biomarkers for cancer [6]. Researchers worldwide are focussing on exploring the potential role of miRNAs as diagnostic, prognostic and treatment targets for PCa [7].

MiRNA target prediction and their biological validation has been a major challenge to the research community worldwide. Till date, more than thousand microRNAs have been discovered, but the complete exploration and validation of their targets and their contribution to metabolic pathways is still awaited. A number of computational algorithms have been developed based on thermodynamic stability of miRNA-mRNA duplex [8], base pairing pattern, site accessibility, UTR context and multiple target site evaluation [9,10]. Other developed programmes are based on techniques like Bayesian classifier, Artificial Neural Networks and Support Vector machines [11, 12]. However, the sensitivity and specificity of these software and databases are still under evaluation. Thus we have tried to explore an improved approach for target prediction based on consensus of tools and multiple statistical steps to provide consensual data for the biological and functional annotation of the circulatory microRNAs in PCa (Figure **2**).

Prostate cancer (PCa) has emerged to be a major health burden in elderly men worldwide. Clinically, PCa is diagnosed at local or in advanced stage, and prescribed treatments include active surveillance, radiotherapy, androgen deprivation and radical prostatectomy [13]. Till date, oncologists employ techniques like Transurethral ultrasound, Digital rectal ultrasound and PSA (Prostate Specific Antigen) screening for pathological diagnosis of PCa patients. PSA screening remains to be the current gold standard biomarker for diagnosis which however lacks in specificity and sensitivity leading to a high rate of

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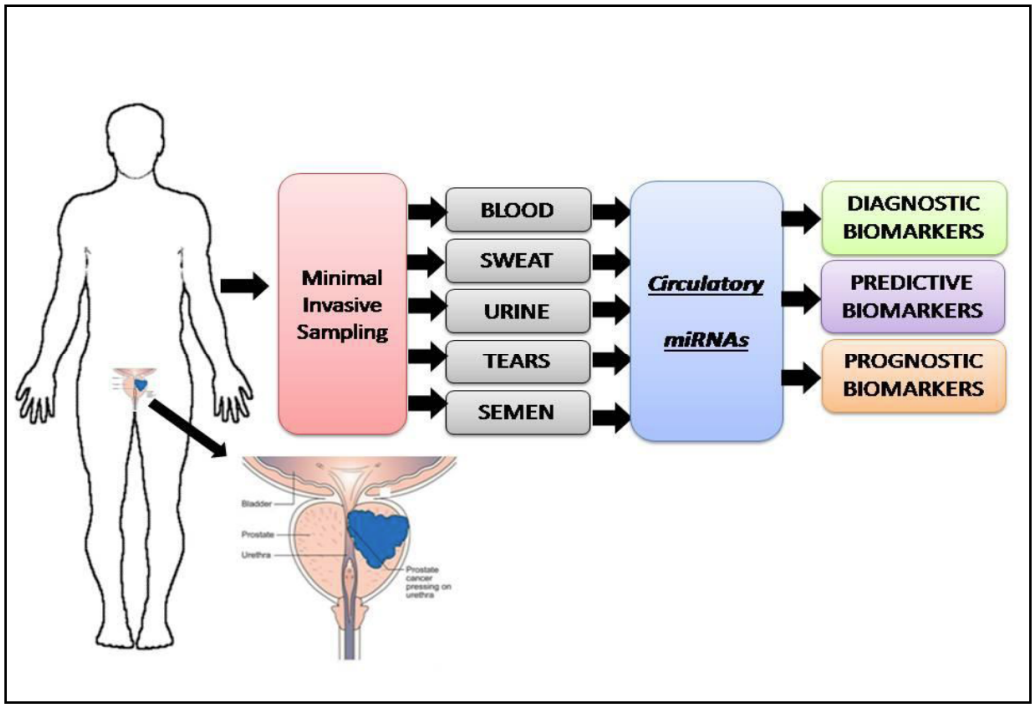

Figure 1: Potential role of circulatory miRNAs in Pca.

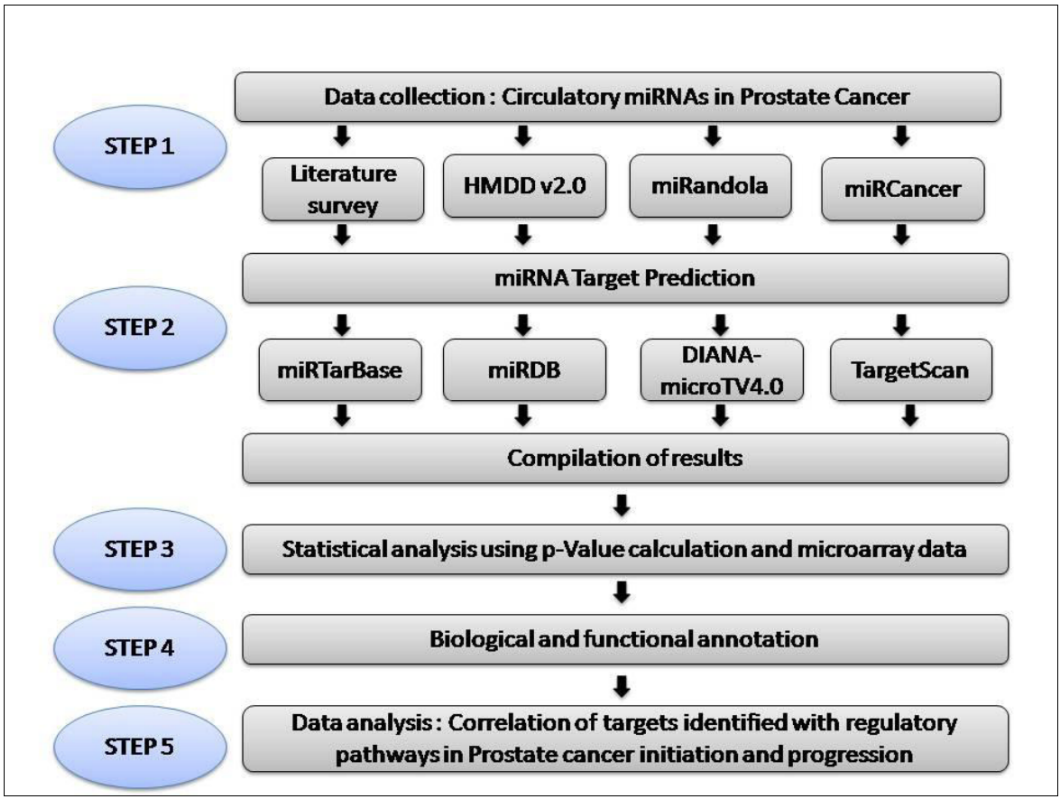

Figure 2: The protocol followed for miRNA target prediction and functional analysis.

overdiagnosis and overtreatment in patients, thus demanding for immediate intervention for discovery of novel biomarkers for early diagnosis and prognosis [14].

Several studies indicate that some miRNAs are differentially expressed in PCa and can be potential biomarkers; however there has been a controversy in the literature about the exact role and expression levels

of miRNAs in different cancers [15]. The expression profiling and high throughput data generated in studies in PCa requires investigation into the exact functional roles of miRNAs in PCa [16]. Here, we aim to investigate the functional roles of dysregulated circulatory miRNAs in PCa by performing an integrated *in silico* meta-analysis.

The analysis was initiated by identification of key circulatory miRNAs dysregulated in PCa, followed by identification of their target genes using an integrated *in silico* approach. Identification of the key enriched pathways and gene ontology annotations which affected cancer hallmarks were then conducted.

Our analysis revealed that the circulatory miRNAs are crucial regulators of signalling pathways such as Wnt signalling, Cadherin signalling, Integrin signalling, Platelet-Derived Growth Factor Receptor (PDGFR) signalling, Epidermal Growth Factor Receptor (EGFR) signalling, Mitogen-Activated Protein Kinase (MAPK) signalling and RAS pathway. These pathways are reported to be integral part of major hallmarks of cancer viz. evading apoptosis, sustained angiogenesis, self sufficiency to growth signals, insensitivity to anti growth signals, limitless replication potential, tissue invasion and metastasis. Our analysis confirmed that circulatory miRNAs are differentially regulated and can act as either oncoMIRs or tumor suppressors during carcinogenesis and can be used as diagnostic, prognostic or predictive biomarkers for PCa.

MATERIALS AND METHODS

The miRNA data was consolidated by thorough literature survey and online databases miRandola [17], miRCancer [18] and HMDD [19] to compile a comprehensive list of circulatory microRNAs associated with PCa initiation and progression.

Search Strategy and Inclusion Criteria

To compile a comprehensive list of circulatory microRNAs we carefully searched and reviewed online literature databases – Pubmed, Medline and EMBASE till 30th October, 2016. The search terms employed in the literature survey included (1) “Prostate cancer”, “ PCa”, “Prostate Adenocarcinoma”, “Prostate neoplasms” (2) “ miRNAs”, “microRNAs”, “circulatory microRNAs” (3) “diagnosis”, “prognosis” (4) “cancer initiation”, “cancer progression”. Studies included analysis performed on serum, plasma, urine, saliva and semen. Exclusion criteria included miRNA studies based on tissues, non English articles, reviews and articles without complete data.

miRNA Target Prediction

This was the most crucial part of the analysis for which we employed four databases – miRTarBase [20], miRDB [21], TargetScan [22] and DIANA-microTV 4.0 [23] to ensure high specificity for miRNA target prediction. Each of the databases employs different approach for target prediction. miRTarBase provides more than 360000 miRNA-target interactions and is among the most updated databases which are compiled by manually surveying literature related to functional miRNA studies. miRDB predicts miRNA targets using miRTarget by analysing miRNA target interactions from high throughput sequencing data. TargetScan uses modelling of RNA-RNA duplex interactions with sequence analysis based on thermodynamic modelling. Diana-micro T 3.0 was the first database to provide experimentally validated miRNA gene interactions and uses UTRs search based approach for stringent seed pairing to the miRNAs. Targets from each of the databases were retrieved to make a comprehensive list and then common targets were filtered using Venn diagram approach.

Correlating Microarray Data

Various studies showing differentially expressed genes in PCa using microarray analysis [20-23] were also comprehensively reviewed to correlate and validate the genes identified from online databases which further verified the reliability of predicted miRNA targets in our study.

Biological and Functional Annotations

The functional annotation of the differentially regulated predicted miRNA targets was carried out using Panther [24], GeneCodis [25] and KEGG [26] for analysing their regulated pathways, biological processes, cellular localisation and molecular functions. REACTOME database [27] was further used for interactive pathway enrichment analysis.

RESULTS

Thorough literature survey and online databases like miRandola, miRCancer and HMDD v2.0 were used for identification of differentially regulated circulatory microRNAs in PCa as shown in Table **1**. 33 upregulated and 7 downregulated microRNAs were shortlisted for further enrichment analysis.

Table 1: Summary of Dysregulated Circulatory microRNAs in PCa

Down regulated miRNAs	Upregulated miRNAs	Methods	Patients numbers	Reference
Let-7e, let-7c	1285, 622, 346, 940	miR- microarray	25 metastatic PCa vs. 17 BPH, 80 PCa, 44 BPH, 54 HI	[28]
181-a2	141,375	qRT-PCR	78 PCa vs. 28 controls	[29]
	21,221	qRT-PCR	18 localised PCa, 8 local advanced PCa, 25 metastatic PCa	[30]
	20a,21	qRT-PCR	82 PCa	[31]
	375, 298, 141, 346	qRT-PCR	25 metastatic PCa vs. 25 controls	[32]
24, 223, 30c, 26b	1207, 874 , 106a, 93	qRT-PCR	36 PCa vs. 12 controls	[33]
	16, Let 7i, 195, 26a	qRT-PCR	45 PCa vs. 38 controls	[34]
	200b, 516a, 375, 141, 9	qRT-PCR	7 metastatic PCa vs. 14 localised PCa	[35]
	296,143,125b, 141, 100	qRT-PCR	25 metastatic PCa vs. 25 controls	[36]
	107, 766, 640, 574, 328, 485, 16, 92a, 103, 636, 766, 885, 197, 34b, 486	miR- microarray	5 PCa vs. 8 controls	[37]

miRNA Target Prediction

To ensure specificity and sensitivity of the miRNA target approach, we decided to employ four extensively used miRNA databases: miRTarBase, miRDB, DIANA-microT 4.0 and TargetScan for target gene prediction for minimum false positive and false negative results. Once the targets were shortlisted, we identified the overlapping genes using Venn diagram approach. To further validate our findings, the targets were compared and correlated with PCa microarray data available online.

Our analysis identified some of the important metabolic pathways regulated by the circulatory miRNAs and their target genes (Table **S1**) such as *CCND1,CAV1, MAPK1, CDKN1B, AR, AKT2, PTEN, BCL2, DAPK2, NOTCH1, SMAD4, WNT5* and *BIRC5* which are known to play crucial role in sustaining hallmarks of cancer

Biological and Functional Annotations

PANTHER and GENECODIS server were used to carry out functional enrichment analysis, where the significant threshold value was set to 0.05 for the p-value. The analysis revealed pathways which are known to play crucial role in cancer development. Maximum genes were seen to be involved in cellular and metabolic processes such as cell communication and cell cycle. Other important molecular functions regulated by the predicted miRNA targets include Protein binding (GO: 0005515; 2854), Metal ion binding (GO 0046872; 1768) and nucleotide binding (GO: 000166; 1328) (Figure **3a**; Table **S2**). In parallel, regulated biological functions include Transcriptional regulation (GO: 0006355; 1003), Signal transduction (GO: 0007165; 747) and Organismal development (GO: 0007275; 585) (Figure **3b**; Table **S3**).

Functional enrichment analysis revealed that pathways associated with tumor initiation and progression were seen to be most significantly regulated which included Angiogenesis, Wnt signalling, Cadherin signalling, Endothelian signalling, FGF signalling and cell cycle. Similarly Panther pathways analysis also revealed that Wnt signalling (P00057: 175), Inflammation mediated by chemokine and cytokine signalling (P00031: 120), and Angiogenesis (P0005: 118) to be the most commonly regulated pathways (Figure **3c**; Table **S4**). Pathways in cancer (Kegg05200: 236), MAPK signalling (Kegg04010: 197) and Focal adhesion (Kegg04510: 150) were seen to be most commonly regulated by KEGG analysis (Figure **3d**; Table **S5**).

Reactome analysis further supported the findings of GeneCodis, PANTHER and KEGG analysis and reported interactions among the identified pathways such as Angiogenesis, Wnt signalling, Cadherin signalling, Endothelian signalling, FGF signalling and cell cycle. Reactome analysis further revealed that the important target genes regulated crucial pathways are those of signal transduction, circadian clock, protein metabolism, cell-cell communication, diseases including cancer, extracellular matrix organisation, cell cycle and programmed cell death (Figure **4**).

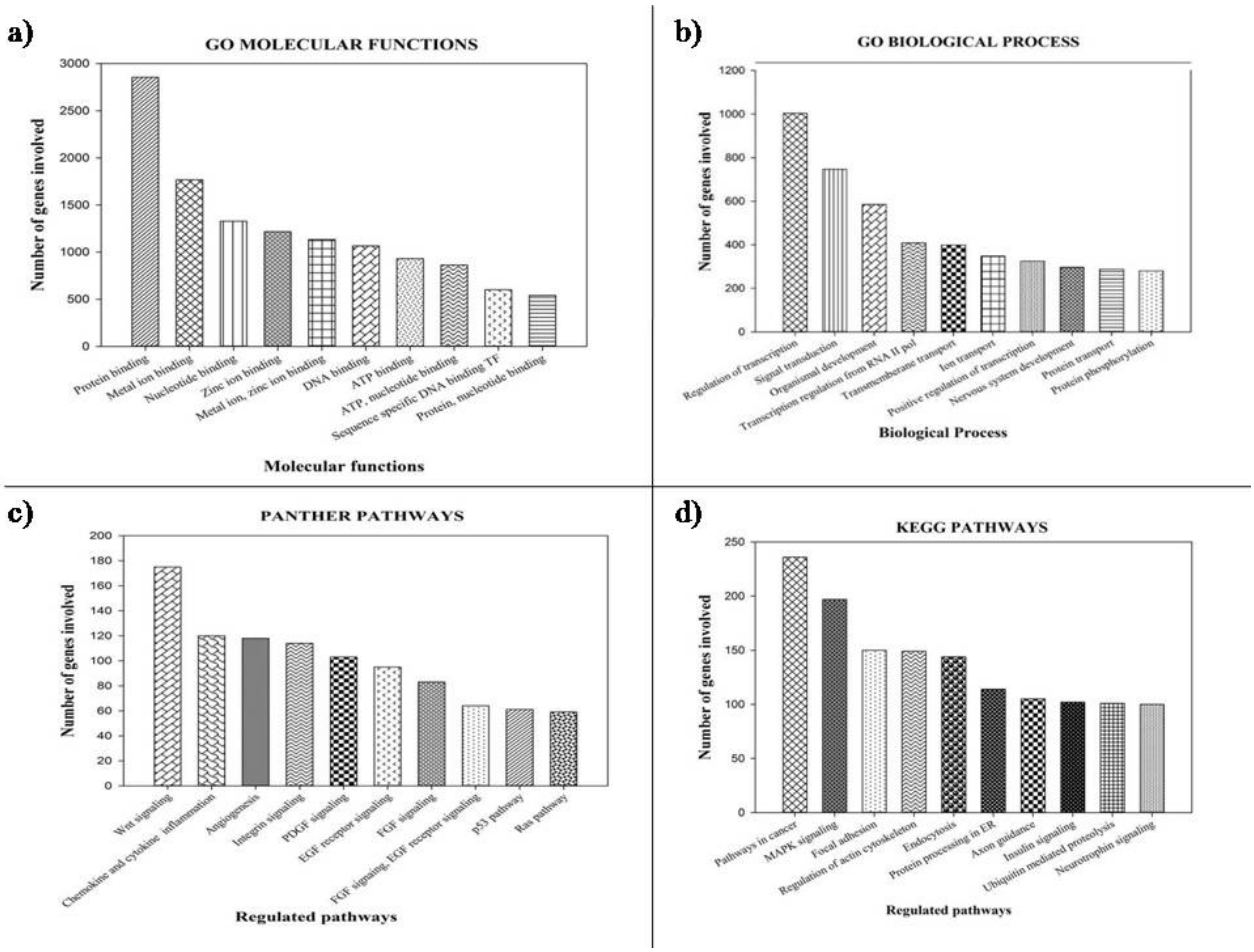

Figure 3: Biological and functional annotation of predicted miRNA target genes (**a**): Graphical representation of GeneCodis results showing molecular functions regulated by predicted miRNA target genes; (**b**): Graphical representation of GeneCodis results showing biological functions regulated by predicted miRNA target genes; (**c**): Graphical representation of PANTHER results showing pathways regulated by predicted miRNA target genes; (**d**): Graphical representation of KEGG results showing pathways regulated by predicted miRNA target genes.

DISCUSSION

Pathways Regulated By Predicted miRNA Gene Targets

Our *in silico* analysis exhibited the crucial role played by miRNA dysregulation during cancer initiation and progression. Our study revealed 40 dysregulated circulatory miRNAs (Table **1**) and more than 200 targets (Table **S1**) which have been reported in carcinogenesis. Some of the identified targets have been experimentally validated in PCa [28-37]. The crosstalk between the pathways dysregulated by the core miRNA targets further shed light on the contribution of circulatory miRNAs in PCa. The functional and biological annotation showed that these miRNA targets affected several cellular and molecular processes including Angiogenesis, Wnt signalling, Cadherin signalling, Endothelian signalling, FGF signalling and cell cycle.

Wnt Signalling and Cadherin Signalling

Wnt signalling was found to be the most significantly regulated pathway (Genes: 175; p: 5.78827e-27) in our functional enrichment analysis. Studies have reported Wnt signalling to play crucial role in neural patterning, planar cell polarity, cell fate decisions, stem cell self renewal, proliferation, differentiation, migration, apoptosis and many hereditary neurodegenerative disorders, coronary diseases and malignancies including PCa [38,39]. Evidences suggest that Wnt signalling regulates androgen activity, tumor aggression, maintenance of EMT and stem cell self renewal in PCa for example *BCL2* and *BCL9*, target genes of miR-30c and miR-26b validated by miRTarBase are modulators of Wnt transcription and is reported to be associated with PCa progression [40, 41]. *BCL2* as suggested by Gene Ontology annotation is a crucial contributor in programmed cell death or apoptosis in variety of cell systems including

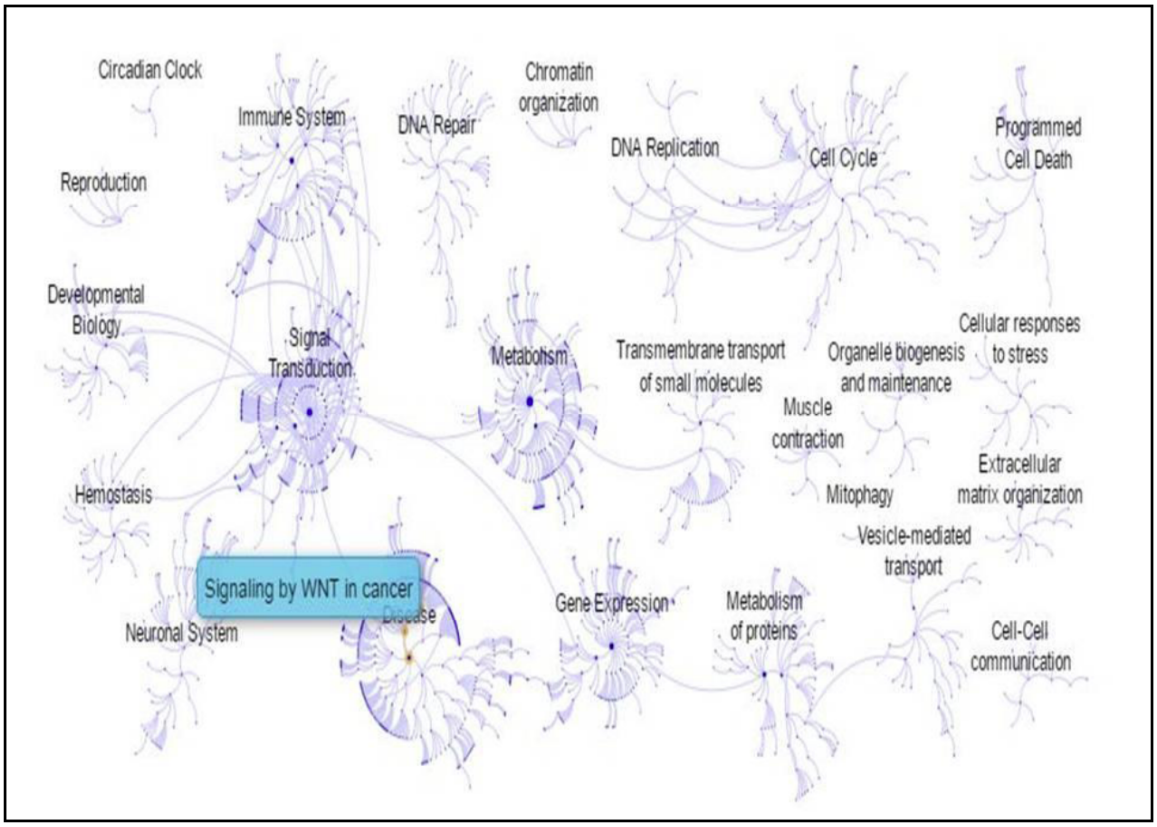

Figure 4: Reactome analysis showing crosstalk among the pathways regulated by predicted miRNA gene targets.

lymphohematopoietic and neural cells correlating the differentially regulated circulatory miRNAs and their target genes regulating multiple cancer hallmarks [41-44].

miR-141, another commonly reported circulatory miRNA in PCa known to be aberrantly expressed in various tumors was also identified by our functional gene enrichment analysis. miR-141 is known to play an integral role in various cancer hallmarks including epithelial to mesenchymal transition, proliferation, migration, invasion and drug resistance. *Zeb1* and *Zeb2*, target genes of miR-141 as reported by miRTarBase are integral components of Wnt signalling and are often dysregulated during tumorigenesis. According to COMETA analysis, the major targets of miR-141 include *PSEN1*, *CXCL2*, *NCAM1*, *CCND2*, *GRB2* and *UBE2Z* which play significant role in regulating cell cycle (0.0012), Ubiquitin mediated proteolysis (p=0.0016), p53 signalling (p=0.0036), axon guidance (p=0.0181) and cell-adhesion (p=0.0099).

Another important miRNA identified in our analysis viz. miRNA-26b is known to inhibit *GSK3-beta* expression which in turn alters the β-catenin pathway in many cancers [45]. miR-20 has been reported to directly target β -catenin to inhibit cell proliferation in meningiomas [46]. Similarly miR-200, a tumor suppressor miRNA as identified in our analysis is also reported to play active role in promotion of cell adhesion and differentiation by inhibiting *Zeb1* and *SIP1* (validated by miRTarBase)which eventually leads to increased cell adhesion due to increase in expression of *E-cadherin* [47]. Similarly miR-30a-5p identified has also been reported to suppress *PRDM1*, a transcriptional regulator which can inhibit Wnt / β-catenin signalling which eventually suppresses the proliferation of glioma cells [48].

Other signalling pathways identified such as Cadherin signalling are known to cross talk with Wnt signalling pathway [49, 50]. Our analysis identified miR-200b, miR-200c, miR-200, miR-192, miR-9, miR-655 which are known to regulate “Cadherin switching i.e. *E-cadherin* to *N-cadherin* and vice-versa” during EMT by targeting multiple transcriptional regulators including *Zeb1*, *Zeb2*, *EP300*, *NF-κB1* and *PTTG1* (validated by COMETA and miRTarBase) [51-53] thus indicating their role in regulating EMT.

Angiogenesis

Another significantly regulated pathway identified by our functional enrichment analysis was Angiogenesis

(Genes: 118; p-value: 3.30659e-30), a major hallmark of cancer and an integral part of tumor initiation, growth and metastatic spread of tumors (Table **S4**). Angiogenesis in tumors occur by regulation of proangiogenic and antiangiogenic factors and is a dynamic process of degradation of basement membrane surrounding capillaries, endothelial migration into Extracellular matrix towards stimuli, endothelial cells proliferation, capillary diffusion and fusion into tubular network of blood cells [54-56]. The highly expressed miRNAs known to regulate angiogenesis "Angiomirs" include miR-15b, -16, -20, -21, -23a and -b, -24, -29a and -b, -31, -99a, -100, -103, -106, 125a and -b, -126, -130a, -181a, -191, -221, -222, -320, let-7, let-7b, let-7c, and let-7d [57, 58].

TGF- β Signalling

TGF- β signalling emerged as another important signalling identified in our analysis and has been reported to be employed as another potential chemotherapeutic target for many malignancies including PCa [59-61]. TGF-β signalling and its receptor inhibitors are being extensively investigated *in vivo*, *in vitro* and in PCa patients worldwide. It works in a dual fashion during Prostate tumorigenesis and also works as a promoter during tumor metastasis to advanced stages of PCa [62, 63]. Regulation of miR-200, miR-181a and miR-21 are being explored to study their effect on TGF-β signalling during proliferation and metastasis [64-66]. According to our analysis, miR-200 (p=0.0018) and miR-21 (p= 0.0081) target genes such as *SMAD2, SMAD4, CREBBP, SMAD5, TGFBR1* and *THBS1* which are known to be crucial regulators of TGF-β signalling during tumor initiation and progression [64-66].

Other Signalling Pathways

Other important signalling identified in our analysis was Integrin signalling. Integrins are heterodimeric cell surface receptors which mediate adhesion to the extracellular matrix and cell-cell interactions [67, 68]. Regulatory functions of integrins include migration, differentiation, proliferation and apoptosis. Our analysis showed gene targets of miR-31, miR-375, miR-17, miR-148, miR-29b, miR-124, miR-183, miR-29a and miR-184 to be crucial regulators of Integrin signalling (Genes: 114; p-value: 9.50443e-26) [69, 70].

EGFR signalling was another significant pathway identified in our analysis (Genes: 95; p-value: 1.83569e-29). Reports have shown the major contribution of EGFR in carcinogenesis and progression of various malignancies including PCa [71, 72]. Increase in expression of EGFR has been correlated with increase in tumor aggressiveness and poor prognosis of the tumor [73]. Scientists have shown more than 40%-80% of PCa with increased levels of EGFR [74]. Studies have shown miR-145, miR-34, Let-7, miR-321, Let-21, miR-221/222, miR-30b and miR-30c to be regulators of EGFR signalling in many cancers (Table **2**) [75-77].

Our functional enrichment analysis showed PDGF pathway to be among the most statistically significant regulatory pathways (Genes: 103; p-value: 2.59615e-29) which is known to regulate angiogenesis. The expression of PDGF has been correlated with increased vascularity and vascular wall maturation [79]. Reports have suggested PDGF to be attractive targets for chemotherapy in various malignancies. Various experiments have shown reduction in interstitial fluid pressure in tumors during treatment which can be caused by inhibition of PDGF expressions [80, 81]. Besides, factors such as PDGF-D (Platelet-derived growth factor D) have been known to play crucial roles in cell proliferation, transformation, migration, metastasis and apoptosis. Many reports have correlated the expressions of PDGF-D with miR-34a, miR-200a, miR-200b, miR-200c, miR-141 and miR-429 which corroborate our functional enrichment analysis results [82, 83].

Table 2: MicroRNAs known to regulate EGFR Signalling [78]

miRNA	MiR regulator	Targets
miR-21	EGFR, ErbB2,c-MET,AP-1	PTEN. SPRTY, PDCD4
Let-7	EGFR, C-Myc, LIN28,	Ras, C-Myc
miR-7	EGFR	EGFR, IRS1, IRS2, RAF-1
miR-34	P53	c-MET
miR-221/222	EGFR, c-MET	PTEN, Apaf-1
miR-30b/c	EGFR, c-MET	BIM

CONCLUSION

In conclusion, our in silico analysis have identified circulatory miRNAs and their target genes dysregulated during PCa initiation and progression. This was followed by identification of the signalling pathways regulated by these circulatory miRNAs and their target genes. The most significantly dysregulated pathways found in our analysis were Wnt signalling, Cadherin signalling, TGF-β signalling, Cell cycle regulation, angiogenesis, apoptosis and MAPK signalling which are known to be essential in maintaining the hallmarks of cancer. Further, based on our analysis, targets of miR-181, miR-9, Let-7 family, miR-26b were seen to contribute majorly in these oncogenic pathways, probing their potential roles in PCa initiation and progression.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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SUPPLEMENTARY TABLES

The supplementary tables can be downloaded from the journal website along with the article.

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