Advances in life science research

Prognostic and Clinicopathological significance of mir-3010b/130 cluster in breast cancer

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ABSTRACT

Breast cancer is the biggest cause of cancer-related mortality in women throughout the globe. Advanced breast cancer (BC) with distant organ metastases is deemed incurable, despite therapeutic attempts. It's crucial to get a deeper knowledge of BC molecular mechanisms in order to find novel treatment targets. Despite the fact that large-scale initiatives like The Cancer Genome Atlas (TCGA) have radically changed cancer medication research, diagnosis, and therapy, other important elements of tumor biology remain unknown. In this regard, post-transcriptional regulation of carcinogenesis is an area of cancer research that has received little attention. In this study we predicted and analysed various target and related pathways associated with miR-130b/301b cluster. The studies found the cluster is significantly associated with breast cancer and identified 8 prognostically important genes associated with this cluster. The gene expression analysis was performed through the *in silico* analysis and confirmed by the IHC analysis through human protein atlas. In this study we propose that the miR-130b/30lb cluster can used as a prognostic and diagnostic indicator in breast cancer.

KEY WORDS
Breast cancer, miR-130b/30lb cluster, TCGA, GEO

Introduction

Tumor development is thought to be a multistep process that involves the activation of oncogenes and the silencing of tumor suppressors (Pierotti et al., 2003). MicroRNAs (miRNAs) are noncoding RNAs with a 20-22 nucleotide sequence that are highly conserved (Ling et al., 2013). Endogenous miRNAs bind to the 3' untranslated region (UTR) of target rnRNAs to form the RNA-induced silence complex (RISC), which inhibit.-, the expression of target genes at the translational level (Gu et al., 2009). Recent research have demonstrated that miRNAs were involved in different physiological and pathological processes, and a growing number of evidences establish that the aberrant expression of miRNAs has a direct association with the onset and progression of malignancies (Condrat et al., 2020). The aberrant expression and malfunctioning of miRNAs may lead to cellular malfunction and eventually result in illnesses or even malignancies. Thus miRNAs may be utilized as new sorts of molecular targets for identifying and treating malignancies (Iorio & Croce, 2012).

The function of the miR-130b/301b gene cluster in carcinogenesis has been shown in a variety of cancer types, including breast, bladder, glioma, lung, ovarian, and pancreatic cancers (Gregorova et al., 2021). Indeed, this family belongs to a superfamily that has been identified as a pan-cancer oncogenic miRNA superfamily that targets key tumor suppressor genes (TSGs) including TGFBR2, SMAD4, and PTEN. However, as it has both oncogenic and tumor suppressive capabilities, the impact of its produced miRNAs in cancer seems to be tissue specific (Hamilton et al., 2013).

Breast cancer (BC) is one of the most common malignant neoplasms in women across the globe, and its death rate is continually rising as the world's population grows and ages (Momenimovahed & Salehiniya, 2019). The current year's global BC mortality trends are expected to be 656,000, with a female incidence of around 2.18 million (Sung et al., 2021). This shows that many kinds of BC may be more aggressive owing to their heterogeneity, resulting in a high female fatality rate. When a patient is diagnosed with BC, the first step is to learn about their prognosis so that they may get the best therapy possible, avoiding overtreatment of non-aggressive illness and under treatment of aggressive illness (Wahba & El-Hadaad, 2015). Clinical parameters such as tumor size, grade, number of lymph node (LN) metastases, patient age, and morbidity status are employed for this aim. Nonetheless, various research have concentrated on the development of novel prognostic biomarkers in tissues and biofluids (plasma, serum, urine) to establish clinical translatability

in the previous 20 years (Kamiyama et al., 2014).

The goal of this study was to get a better understanding of the clinical importance of the miR-130b/30Jb cluster in BRCA by combining the results of most published studies and accessible BRCA gene expression datasets (small RNA and rnRNA transcriptomic, methylomic and clinical data) (Fort et al., 2018). First, we collated all of the published studies that showed dysregulation of both miRNAs in separate BRCA cohorts, as well as the clinical significance provided in the original papers. Then, to gather further evidence of the involvement of the miR-130b/30lb cluster in this illness, we conducted further analyses of publically accessible BRCA datasets, with a focus on the biggest cohorts like The Cancer Genome Atlas (TCGA-BRCA). We also went through existing functional research on these miRNAs, with a focus on verified target genes described in the cancer literature. Overall, our data support the overexpression of both miRNAs in BRCA, suggesting that the cluster may have an oncogenic role in this illness.

Materials and Methods

Data collection and pre processing

The expression data of individual members in the miR-30lb/130b cluster were retrieved from the data available in the gene expression omnibus database (https://www.ncbi.nlm.nih.gov/geo/ accessed on 12, April, 2021), UALCAN (Chandrashekar et al., 2017) and cBioportal for Cancer Genomics (Ceramil et al., 2012). The tumour and normal sample data from the above databases were used in this study. The UALCAN dataset contain 76 normal and 749 tumour sample data in TCGA-BRCA dataset. The EVmiRNA (Liu et al., 2019) tool is used for the detection of miR-301b/l 30b in extracellular vesicle. The position of the miRNAs in the miR-301b/l30b cluster were identified and confirmed by miRbase (Kozomara et al., 2019) and UCSC genome browser (http: //genome.ucsc.edu/).

Differential expression ofmiR-17/19 in BRCA

The differential expression of the miR-301b/130b cluster members were analysed through an open tool called UALCAN. It employed to analyse and compare the miRNA and miRNA gene expression patterns in the dataset. The dataset (GSE4566) was retrieved from GEO portal. The individual members of the cluster were called using the script available in the portal and the miRNA showing p <0.05 were found as statistically significant. The MEXPRESS (Koch et al., 2015) tool was used to plot clinical correlation attributes ofmiR-301b/130bexpressions.

miR-301b/130b target prediction

The target genes for the miR-30lb/130b cluster were predicted using miRTarBase (Huang et al., 2020). lt serves as comprehensively annotated, experimentally validated miRNA-target interactions databases in the field of miRNA related research. The identified target genes were further screamed through GEPIA2 (Tang et al., 2019) tool to get specific targets for Breast cancer.

miR-301b/130b network construction and target

We used miRNet (Chang et al., 2020) online platform for the prediction and design of the miR-30lb/130b cluster network. mirNet facilitates the development of miRNA-protein coding gene, miRNA-lncRNA, miRNA-circRNA and miRNA-sncRNA networks, all of which are guided by statistical and functional data analysis. The correlation of expected targets with the potential acquisition of different cancer hallmarks was predicted using CHG (Zhang et al., 2020). In addition, starBase (Li et al., 2014) used to classify the competitive endogenous RNAs. BRCA specific miRNA interactions were performed by CoMeTa (Gennarino et al., 2012) and miRSig (Nalluri et al., 2017).

Functional enrichment

Biological functions, ontological processes and related pathways controlled by miR-301b/130b were predicted using WebGestalt (Liao et al., 2019). The GO analysis was performed for the prediction of cellular component (CC), molecular function (MF) and biology process (BP). Pathway prediction was done by using Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. P<0.05 was considered to be statistically relevant and is included in this study. The PPIN of rniR-l5a/16-l-MC target genes was predicted and constructed using the STRING v.11 (Szklarczyk et al., 2019) and NetworkAnalyst 3.0 (Zhou et al., 2019) tools. We only included the differentially expressed target of miR-301b/130b observed in the TCGA-BRCA dataset for PPIN production, with an interaction score >0.9 and a minimum level of interactions= 3. In addition, the cytohubba tool in Cytoscape software (Shannon et al., 2003) was used to identify the top hub genes. Using the UALCAN database, tbe Kaplan-Meier curve (KM) and the Log-rank system were used to determine the prognostic significance of the miR- 301b/l30b hub genes. HCMDB (Human Cancer Metastasis Database) (Zheng et al., 2018) was used to analyse the interaction between members of the rniR-301b/130b cluster and BRCA metastases

Protein expression analysis

Protein Atlas version 18.l was used to derive immunohistological levels of identified hub genes in normal and BC tissues. Normal tissue expression levels were obtained from glandular cells, whereas a consensus level for BC tissues was manually determined based on tissue level frequency. The cellular expression of the identified genes are also validated using Human Protein Atlas.

Results

Analysis of miRNA datasets

The validation of the miR-301b/130b cluster was done through the UCSC genome browser (http://genome.ucsc.edu/). The miR-130 gene family is exclusive to vertebrates. Four miRNA precursor genes are found in the human genome: mir-30la (chr17), mir-130a (chr11), mir-130b, and mir-30Jb (at chr22). The seed regions of the miRNAs produced from the miR-130b/30lb gene cluster precursors are identical. The 3' arm of the hairpin is preferentially processed to create mature miRNAs bsa-miR-l30b-3p and hsa-miR-30lb-3pfrom the precursorRNAshsa-mir-13<liandhsa-miR-30lb-3p

from the precursor RNAs hsa-mir-130b and hsa-mir-30lb. Their present transcript annotation indicates that they are transcribed as a di-cistronic RNA transcript with 7 noncoding exons, classed as a "known processed transcript."

Expression data analysis of miR-30lb/130b cluster

The expression analysis was done by using publicly available databases for breast cancer named TCGA-BRCA. The dataset contains many microarrays and clinical data of BRCA patients. Each member of the miR-30lb/130b cluster was quired into UALCAN and the miRNA expression was analyzed in TCGA-BRCA. The expression analysis of the TCGA-BRCA dataset reveals that the 301b/130b cluster is significantly higher in the tumour samples (n=749) when compared to normal samples (n=76) (Figure I A). The exosomal expression analysis of miR-30lb reveals that it significantly expressed in bone, adrenal glands, lung and uterus exosomes were as miR-130b found to be associated with bone, brain, lung, nerve and ovary exosomes (Figure I B). The clinical correlation analysis indicates the miR-301b/130b cluster expression is correlated with BRCA estrogen receptor status, BRCA progesterone receptor status, histological type, lab proc her2 neu IHC receptor status, lab procedure her2 neu in situ, menopause status, new tumor event, gender, tumor stage and sample type (Figure l (C)).

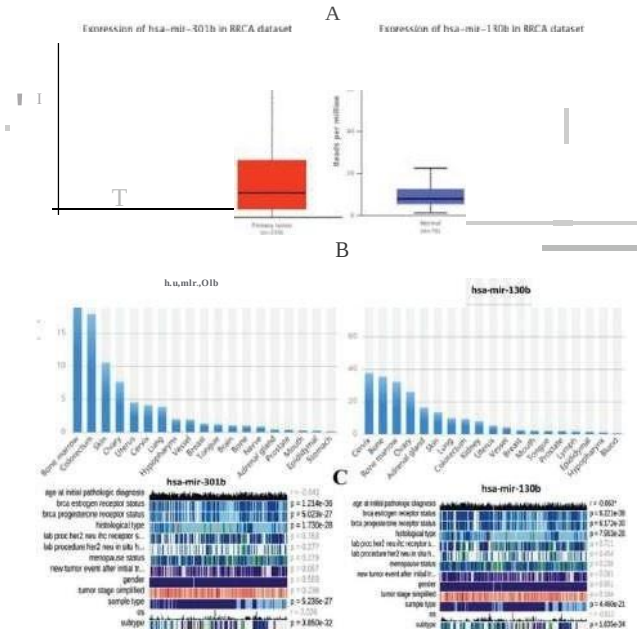

Figure 1. A) miR-30lb/130b cluster members expression in TCGA-BRCA. B) miR-301b/130b is expressed in extracellular vesicles such as bone, brain, Jung, nerve, ovary and many more. C) Clinical correlation analysis of miR301b and miR130b.

Target prediction and cluster network analysis

The expressjon network of the miR-301b/l 30b was predicted using the Co-expression Meta-analysis of miRNA Targets (CoMeTa) tool and found co-expressed with miR454. The miRNA-miRNA interactions were predicted through the miRsig tool specific to lung cancer (Figure 2 A). The analysis found that many miRNAs from the interaction cluster were in the OncomiR LUAD database. The breast-specific rniRNA-lncRNA, miRNA-circRNA and miRNA-sncRNA networks were predicted using miRNet and the predicted network contains 2198 edges and nodes of 54 sncRNA, 1179 circRNA, 44 lncRNA and 1284 genes (Figure 2 B). The targets for the miR-214/l 99a-2 cluster were retrieved from the miRTAR Base and identified 1630 genes. The overlapping analysis revealed that 582 genes are differentially expressed in the TCGA-BRCA database. Further analysis found that among 582 differentially expressed genes (DEGs) 108 genes are found up-regulated and 474 genes are found down-regulated. ThemiRnet tool is used for the construction of the miR-301b/l30b cluster gene targets. The interaction network consists of the top 90-100 predicted gene targets for each member in the miR-301b/l30b cluster (Figure 2 C). The other miRNAs for DEGs were analysed and summarized in (Figure 2 D).

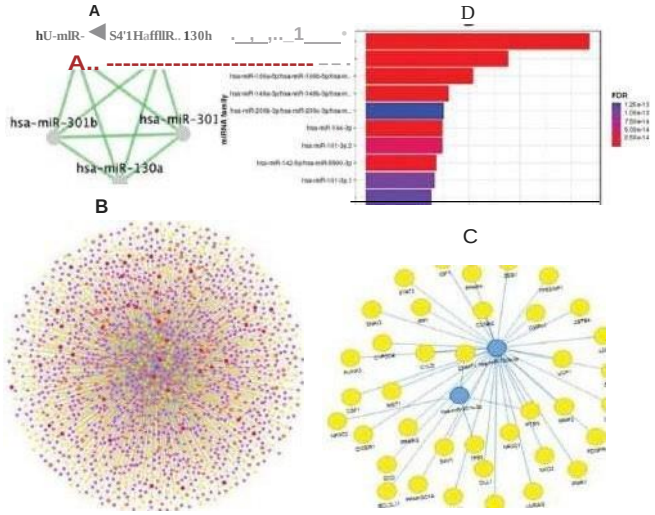

Figure 2. A) Co-expression network. **B)** Breast specific miRNA-lncRNA, circRNA, sncRNA uetwork. C) Top 90-100 predicted gene targets for miR-301b/130b D) Other predicted miRNAs. cluster.

GO analysis of DEGs

The patl1way and gene ontology analysis were done through a web-based gene set analysis toolkit. The interesting list contains 582 user IDs in which 582 user IDs are unambiguously mapped to 582 unique Entrez gene IDs. Among 582 unique Entrez gene IDs, 498 IDs are annotated to the selected functional categories and also in the reference list, which are used for the enrichment analysis. The reference list can be mapped to 61506 Entrez gene IDs and 7469 IDs are annotated to the selected functional categories that are used as the reference for the enrichment analysis. The top enriched pathways include **MAPK** signaling pathway, Apelin signaling pathway, Pathways in cancer, Transcriptional misregulation in cancer, Calcium signaling pathway, Melanogenesis, Glucagon signaling pathway, Axon guidance, Hippo signaling pathway

and Renin secretion pathways (Figure 3 A). The top GO terms enriched for the biological process include Regulation of developmental process, Cellular developmental process, Anatomical structure morphogenesis, Tube morphogenesis, Cell differentiation, Tube development, Regulation of multicellular organismal development, Regulation of multicellular organismal process, Animal organ development and Nervous system development (Figure 3 B). The cellular component enrichment includes Nuclear lumen, Neuronal cell body, Membrane raft, Membrane microdomain, Membrane region, Cell body, Cell junction, Somatodendritic compartment, Cell projection and Nucleoplasm (Figure 3 C). The molecular function includes molecular function regulator, kinase activity, anion binding, phosphotransferase activity, alcohol group as acceptor, DNA-binding transcription activator activity, DNA-binding transcription activator activity, RNA polymerase II-specific, protein kinase activity, Transferase activity, transferring phosphorus-containing groups, regulatory region nucleic acid binding and Cis-regulatory region sequence-specific DNA binding (Figure 3 D).

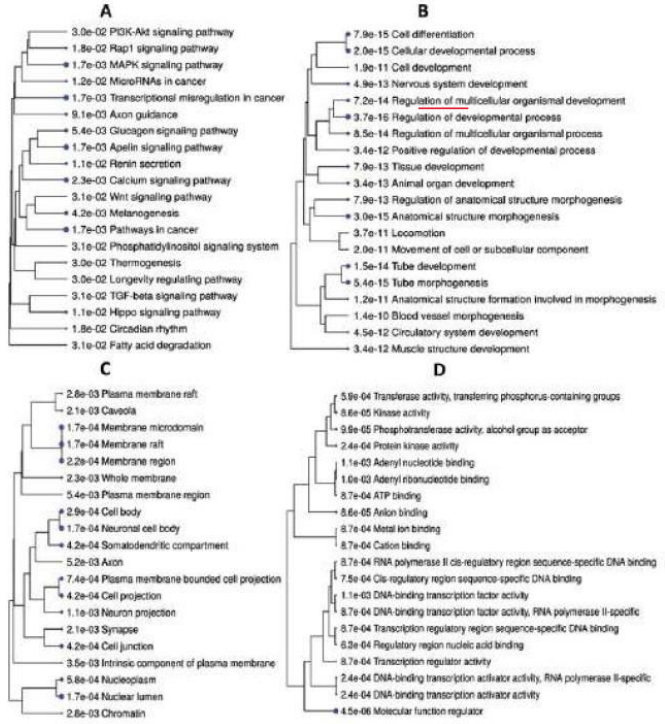

Figure 3. A) GO Pathway enrichment. 8) GO biological process. C) GO Cellular component. D) molecular function analysis

enrichment comparing with the KEGG pathway. The enricher analysis identified that the hub genes are involved in the pathways such as Proteoglycaos in cancer, EGFR tyrosine kinase inhibitor resistance, Pathways in cancer, MicroRNAs in cancer, Transcriptional misreguJation in cancer, MAPK signaling pathway, PI3K-Akt signaling pathway, Adherens junction, Melanoma and Endocrine resistance.

Prognostic significance of miR-301b/130b cluster in BRCA

The survival analysis conducted for the miR-301b/130b cluster reveals that the cluster expression in the BRCA patient sample is negatively correlated with the overall survival of the patients. The target prediction analysis for the rniR-30Ib/130b cluster identified 1630 genes; among them, 582 genes are differentially expressed in the TCGA-BRCA database. The PPI network analysis revealed 8 hub genes (CSFI, SMAD4, PTEN, ESRI, MET, ERBB3, ZEB!, IGFI) and were analysed for its prognostic significance through Kaplan Meier survival analysis. The further analysis of the hub genes CSFI, SMAD4, PTEN, ESRI, ERBB3 and ZEBI are significantly associated with the overall survival of the BRCA patients (Figure 4). The 6 prognostically important genes and that they are integrated into the hallmark of cancer genes and the analysis revealed that the genes are involved in cancer hallmarks such as Genome Instability and Mutation, Tumor-Promoting Inflammation, Reprogramming Energy Metabolism, cell death resistance, Sustaining Proliferative Signalling and Evading Immune Destruction. The GEPIA2 analysis of the 8 hub genes identified that 2 genes ESRI and ERBB3 was found to be upregulated and 6 genes CSFT, SMAD4, PTEN, MET, ZEBl and IGFI are down-regulated.

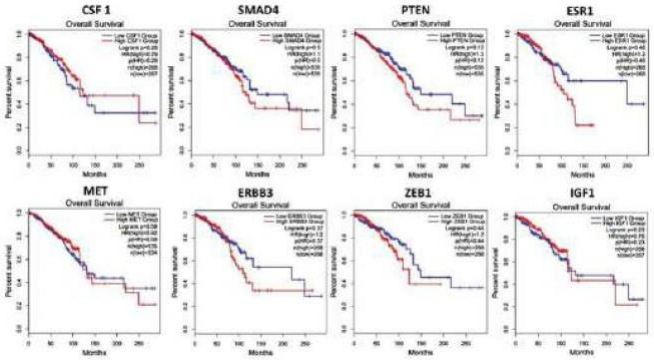

Figure 4. Kaplan Meier survival analysis of bub genes

Protein-protein interaction (PPI) network analysis

The differentially expressed targets of the 301b/130b cluster were used to construct the PPI construction through the STRING tool for protein-protein interaction and Cytoscape for the visualisation. The constructed network consists of 329 nodes 580 edges with an enrichment P-value of <1.0 e-16. The Cytoscape tool was used to visualise the hub genes and the predicted bub genes are CSFI, SMAD4, PTEN, ESRl, MET, ERBB3, ZEBland lGPl. The identified hub genes were analysed by the Enricher tool for GO

Identification of hub genes in breast tumor tissues

Breast cancer molecular research has shown the disease's inherent variability, opening new avenues for individualized treatment. Despite the development of a number of gene expression-based methods, current clinical practice is still mainly dependent on traditional clinical and pathologic criteria. The creation of integrated multi-lHC indices to describe the tumors' biology and clinical behavior may fill this gap. The Human Protein Atlas **(HPA)** is a significant effort to study protein expression in human tissues, both healthy and tumoral. As a result, we discovered hub genes

with distinct protein expression profiles in breast carcinoma tissues. To do so, we evaluated the immunohistochernistry levels of 8 prognostically important genes in normal and malignant breast tissues. From the analysis it is found that the genes SMAD4, PTEN, ESRl,MET, ERBB3 and ZEBI are found to be expressed in BRCA pathology samples were as CSFI and ZEBI are found to be little expression or negligible (Figure 5). The protein atlas were also used for the gene localization. From the analysis the gene CSFI found to be mainly localized to the plasma membrane, SMAD4 is localized to the nucleoplasm, cytosol and in centrosome, PTEN is found to be localized to the nucleoplasm and in cytOsol, ESRJ is mainly localized to vesicles and nucleoplasm. The MET gene detected in Plasma membrane and Cytosol; and is predicted to be secreted. CSF1 found to be localized to the plasma membrane and actin filaments. ZEB! gene is detected in Nucleoplasm and Nucleoli (Figure 6).

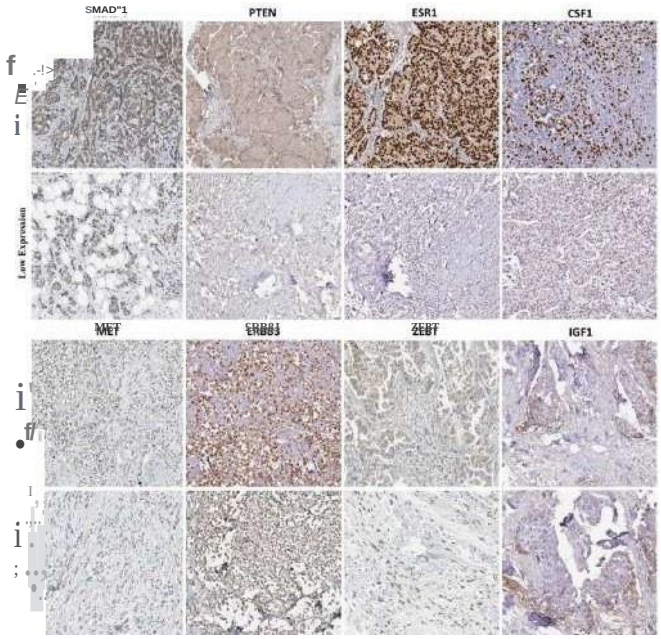

Figure 5. Representative immunohistochemical stains of eight predicted huh genes

Discussion

MicroRNAs (miRNAs) are tiny RNA molecules that have emerged as key post-transcriptional regulators of gene expression (Catalanotto et al., 2016). They've been shown to be involved in the control of all of the cell's fundamental activities, including differentiation, proliferation, and apoptosis (Macfarlane & Murphy, 2010). Each miRNA has hundreds of target genes, and a single gene may be targeted by several miRNAs, resulting in complicated interaction networks that arc only partly understood (Bartel, 2009). Multiple studies shows the significance of miRNAs in all the cancer hallmarks identified by Hanahan and Weinberg and suggested that they could act as oncogenes or tumor suppressors (Hanahan & Weinberg, 20 L1). Further experimental evidences indicated that certain miRNAs may potentially have a function beyond the cancer start and directly engage in cancer invasiveness and metastasis. Jndeed, miRNA profiles may differentiate not only between

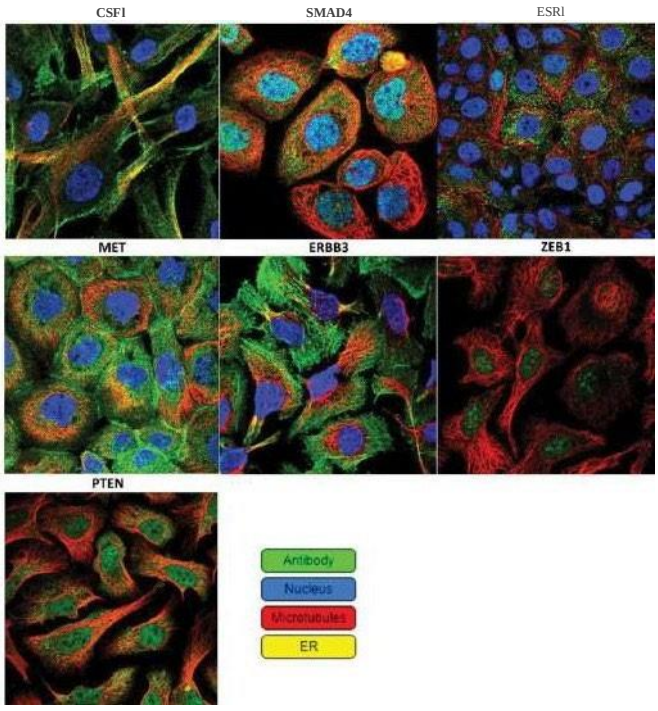

Figure 6. **Annotated subcellular location(s) for the encoded protein(s) based on the human cells assay**

normal and malignant tissue but tbey can also effectively identify various subtypes of a given disease, especially of breast cancer. In the era of enormous genomic data, the study of public patient gene expression data is a vital resource to determine the definitive significance of a gene for a disease. Although miR-130b/30lb has been previously quantified in many BRCA cohorts, the biggest and most complete BRCA cohort publically accessible, the TCGA-BRCA, has not been probed yet (Kurozumi et al., 2017). We nex.t examined this dataset for correlations between the genomic status and expression of miR-130b/301b cluster and variousfeatures of the disease.

In this study, we focused at the, diagnostic and prognostic importance of the miR-130b/30lb cluster in breast cancer. We identified that the miR-130b/301b cluster members and its paralogues is significantly associated with BRCA. Furthermore, the expression of miR-130b/301bcluster members has the ability to be used as an independent prognostic marker in BRCA. In addition, we have also screened the targets of the miR-l30b/30 Ib BRCA with a bioinformatic method for predicting pathways and biological functions. The current analysis showed that miR-130b/301b cluster is significantly associated with BRCA tissues compared to the normal tissues. The gene cluster analysis of the miR-!30b/301b cluster identified 8 hub genes (CSFI, SMAD4, PTEN, ESRI, MET, ERBB3, ZEBI, IGFI) is significantly associated with the overall survival of the BRCA patients. The genes further analysed for the correlation between the cancer hallmarks for the suggestion of potential prognosis and development of disease by altering different differend cancer hallmarks traits. The analysis identified the hub genes were involved in the cell death resistance, activating invasion and metastasis, evading growth suppressors, sustaining proliferative signaling and enabling repl.icative immortality. We think that this analysis will help to find the potential drug targets. To get the deep knowledge of the biological pathways and molecular function a bio informatics method was employed.

This analysis revealed that the miR-17/19 cluster and its paralouges can regulate many cell signalling and cell biochemical pathways including MAPK signaling pathway, Apelin signaling pathway, Pathways in cancer, Transcriptional misregulation in cancer, Calcium signaling pathway, Melanogenesis, Glucagon signaling pathway, Axon guidance, Hippo signaling pathway and Renin secretion pathways. The further molecular biological studies are needed to confirm *this* identified molecular functions in BRCA. One of the most serious issues of cancer care and patient survival is therapy resistance. The survival analysis of the hub genes also reveals the cluster is prognostically significant. The immunohisto-chernistry analysis of 8 prognostically important genes in normal and malignant breast tissues found that the genes SMAD4, PTEN, ESRl,MET, ERBB3and ZEB1 are found to be expressed in BRCA pathology samples. The gene localisation analysis reveals the hub genes are most commonly located in Plasma membrane, Cytosol, Nucleoplasm, Nucleoli, Microtubules and centrosome. Despite the fact that our research is extensive, it also has certain limits. In light of the significance of the genes and pathways found in our research and other previous studies. More research into the function of these genes and pathways in the pathophysiology of BRCA could be undertaken in the future.

Conclusion

The best end points for evaluating advanced treatments are debatable. Patient-reported outcome measures should be used in studies to assess the best standardized end points. There is an immediate need for dedicated quality-of-life resources to assess metastatic disease. miR-130b/301b is one of the best-known miRNA clusters. Members of the cluster play essential roles in normal growth, and their expression is dysregulated in a number of diseases, including hematopoietic and solid cancers, as well as immune, neurodegenerative, and cardiovascular diseases. miRNAs in the miR-l30b/301b cluster is significantly associated with breast cancer eel] lines. This study predicted the potential targets for the differentially expressed miRNAs in breast cancer in comparison with normal and tumor samples and found CSFI, SMAD4, PTEN, ESRl, MET, ERBB3, ZEBl and IGFl genes are significantly associated with the overalJ survival of the BRCA patients. In thjs study we propose that the miR-130b/30lb cluster can used as a proganostic and diaganostic indicator in breast cancer. Furthermore, detecting miR-130b/301b in BRCA extracellular vesicles will aid in the production of liquid biopsy-based minimal invasive biomarkers.

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