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Hans汉斯

新型微小RNA-9对乳腺癌增殖的影响及靶基因预测的生信分析

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摘　要

目的：通过对乳腺癌组织测序发现novel-miR-9在癌组织中呈现低表达。深入研究novel-miR-9对乳腺癌的调控机制及其生物学功能理论机制。方法：运用cck-8以及EdU实验检测novel-miR-9对人乳腺癌细胞增殖的影响；应用生物信息学分析预测novel-miR-9靶基因以及与乳腺癌相关基因；用Venny2.1.0绘制韦恩图得到靶基因集合；对靶基因集合进行GO功能注释分析，找出与细胞增殖相关基因；分析其基因在乳腺癌表达量；并对其进行蛋白交互作用，GO功能注释分析和KEGG Pathway分析。结果：novel-miR-9在人乳腺癌细胞呈低表达($P < 0.001$)；抑制乳腺癌细胞增殖能力($P < 0.001$)；通过Venny图以及GO功能注释分析和蛋白交互作用分析找出与细胞增殖相关的26个基因并发现其相互作用关系较复杂；GO分析发现靶基因可能参与细胞增殖、信号接收等生物过程；KEGG Pathway分析发现其靶基因主要富集在*PI3K-Akt*、*Ras*、癌症、癌症中的microRNA等信号通路。结论：novel-miR-9调控参与多种重要的生物学过程，为后续研究提供了线索。

关键词

novel-miR-9，细胞增殖，靶基因预测，信号通路，生物信息学分析

Effects of Novel MicroRNA-9 on Breast Cancer Proliferation and Bioinformatics Analysis of Target Gene Prediction

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Abstract

Objective: to study the low expression of novel-miR-9 in breast cancer by sequencing. For the further study of novel-miR-9 regulation mechanism of breast cancer and its biological function theory mechanism. Methods: cck-8 and EdU experiments were used to detect the effect of novel-miR-9 on human breast cancer cell proliferation; application of bioinformatics analysis to predict novel-miR-9 target genes and breast cancer related genes; a set of target genes was obtained by drawing Wayne map with Venny2.1.0; GO functional annotation analysis of target gene sets, Identification of genes related to cell proliferation; to analyze the expression of its gene in breast cancer; and protein interaction, GO function annotation analysis and KEGG Pathway analysis. Results: novel-miR-9 low expression in human breast cancer cells ($P < 0.001$); inhibition of breast cancer cell proliferation ($P < 0.001$); through Venny map and GO function annotation analysis and protein interaction analysis to identify 26 genes related to cell proliferation and found that the interaction relationship is more complex; GO analysis found that target genes may be involved in cell proliferation, signal reception and other biological processes; KEGG Pathway analysis found that its target genes are most enriched in *PI3K-Akt*, *Ras*, cancer, cancer microRNA and other signaling pathways. Conclusions: novel-miR-9 regulation is involved in many important biological processes, provided clues for the follow-up study.

Keywords

novel-miR-9, Cell Proliferation, microRNA Target Prediction, Signaling Pathways, Bioinformatics Analysis

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1. 前言

乳腺癌(breast cancer)是女性常见的恶性肿瘤之一，占女性癌死亡原因的首位[1]。增殖是控制乳腺癌发展的重要因素，但目前关于乳腺癌的增殖的机制尚未完全清楚。microRNA 是一类非编码小分子 RNA，长度为 18~26 个核酸的内源性小 RNA，其通过与靶基因 3 端非编码区域特异性结合的方式调节靶基因的表达[2]，参与多种生物调节途径，如细胞增殖和凋亡、脂肪代谢等[3]。最近有研究显示，microRNA 可以通过调控癌基因或抑癌基因表达，参与肿瘤细胞增殖等生物学过程[4] [5] [6]。

本研究在前期对乳腺癌组织测序中发现一批新型 microRNA。对这一批新型 microRNA 进行筛选，发现 novel-miR-9 (cgggggcuggcggcggc)对乳腺癌的增殖起到抑制作用。本研究运用生物信息学分析，预测 novel-miR-9 的靶基因，绘制韦恩图得靶基因集合，找出与增殖相关基因，并对其靶基因集合进行蛋白质互作网络分析，乳腺癌组织表达，GO (Gene Ontology)分析和 KEGG Pathway (Kyoto encyclopedia of genes and genomes)分析预测结果中的靶基因集合作用机制，并注释其靶基因的生物学功能，为展开 novel-miR-9 的靶基因鉴定及生物学功能研究提供理论基础。

2. 材料与方法

1.材料：正常细胞系 MCF-10A、人乳腺癌细胞系 MCF-7 和 MDA-MB-231 (中国科学院上海生命科学院细胞库)；DMEM 高糖培养基和澳洲胎牛血清(Gibco 公司)；转染试剂 Lipofectamine 2000 (Thermo Fisher 公司)；miDETECT A TrackTM miRNA RT-qPCR Starter Kit 和 microRNA 前后引物以及 mimic (广州锐博公司)；cck-8 (Biosharp 公司)；EdU (广州锐博公司)；Novel-miR-9 测序(华大基因)。

2.细胞培养与分组：DMEM 高糖培养基 + 质量分数为 10%澳洲胎牛血清 + 质量分数为 1%的双抗，置于 37℃、5% CO_2、饱和湿度的细胞培养箱中培养。无菌操作，3 *d* 传代培养 1 次。取传 2 代，对数增殖，状态良好的细胞用于后续实验。将对数增殖的 MCF-7 和 MDA-MB-231 细胞收集，均匀铺于 6 孔板上，5 × 10^5 万/孔，放入 37℃、5% CO_2、饱和湿度的细胞培养箱中培养。待细胞铺满 6 孔板后，应用 Lipofectamine 2000 将 mimic 及 mimic-NC 瞬时转染 MCF-7 与 MDA-MB-231 细胞。放入 37℃、5% CO_2、饱和湿度的细胞培养箱中培养 6~8 h，将 6 孔板液体换为完全培养基后继续放入 37℃、5% CO_2、饱和湿度的细胞培养箱中培养。

3.RNA 提取与 RT-qPCR 检测：用 Trizol 法提取收集 MCF-10A、MCF-7、MDA-MB-231 细胞 RNA，置于−80℃冰箱保存待检。用 RNeasy Maxi 试剂盒纯化 RNA，在 NanoDrop 2000c 上测定 RNA 浓度。RNA 样品的 A 值 260/280 比 1.8~2.1 之间用于进一步研究。按反转录试剂盒操作说明书进行操作，将提取的每种细胞的总 RNA (1 μg)反转录成 cDNA。使用罗氏 LightCycler 480 Real-Time PCR 系统检测 novel-miR-9 的表达水平。使用 U6 作为内参，U6 上游引物：5’-CTCGCTTCG GCAGCACA-3’；下游引物：5’-ACGCTTCACGAATT TGCGT-3’。RT-qPCR 热循环条件为：第一步：95℃ 10 min；第二步：95℃ 2 s，60℃ 20 s，70℃ 10 s，共 40 个循环；第三步：融解曲线生成。采用相对定量法，以 U6 为内参，各种细胞中 10 个 microRNA 的水平为它与同样本中的 U6 的比值为相对表达水平，由公式 Flods = 2 − ΔΔCt 计算获得，ΔΔCt 为 10 个 microRNA 的相对表达水平。

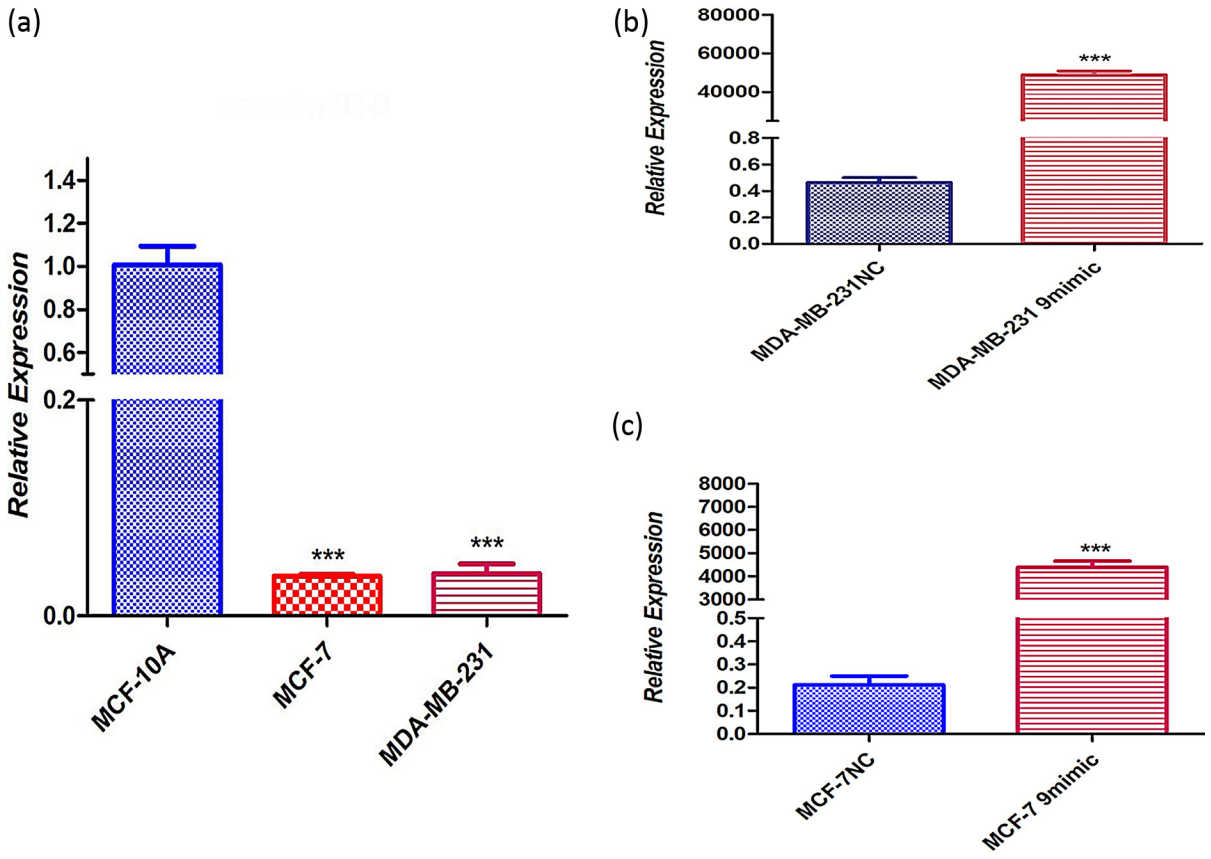
4.cck-8 和 EdU 检测增殖：cck-8：对照组和实验组乳腺癌细胞以质量分数为 10%的胎牛血清高糖培养基配制成 2 × 10^3 个细胞/孔的细胞悬液，96 孔板培养(37℃、5% CO_2、饱和湿度的细胞培养箱)，分别于 24、48 和 72 h 后，加入 cck-8 溶液，每孔 10 μL，用全波长酶标仪在 450 nm 处测量各孔的吸光度值。EdU：对照组和实验组乳腺癌细胞以质量分数为 10%的胎牛血清高糖培养基配制成 2 × 10^4 个细胞/孔的细胞悬液，24 孔板培养(37℃、5% CO_2、饱和湿度的细胞培养箱)，培养 24 h，按照 protocol 加入 EdU 试剂，并在正置荧光显微镜拍照。

5.生物信息学网站和软件：利用 TargetScan [7] (http://www.targetscan.org/)，miRDB [7] (http://mirdb.org/)，miRWalk [7]和 miRbase [7] 4 个数据库预测 novel-miR-9 的靶基因；GeneCards (http://www.genecards.org/) 可查询到与乳腺癌相关的致病基因；两者取交集，获得同乳腺癌相关的预测靶基因；运用 UCSC [8] (https://xena.ucsc.edu/)在线工具分析靶基因在乳腺癌患者中的差异表达；DAVID6.8 数据库[9] (https://david.ncifcrf.gov/)对预测的靶基因集合进行 GO 分析和 KEGG 信号传导通路的富集分析；STRING [10] (http://string-db.org/)在线工具分析靶基因之间的交互作用。

6.统计学分析差异：计量资料用均值±标准差($\chi \pm s$)表示，统计方法采用配对 *t* 检验，$P < 0.05$ 表明差异具有统计学意义。

3. 结果

1) novel-miR-9 在细胞中的表达水平 利用 RT-qPCR 检测 novel-miR-9 在正常细胞 MCF-10A 和人乳腺癌细胞 MCF-7、MDA-MB-231 中的表达量，发现 novel-miR-9 在乳腺癌细胞的表达量低于正常细胞(图 1)。

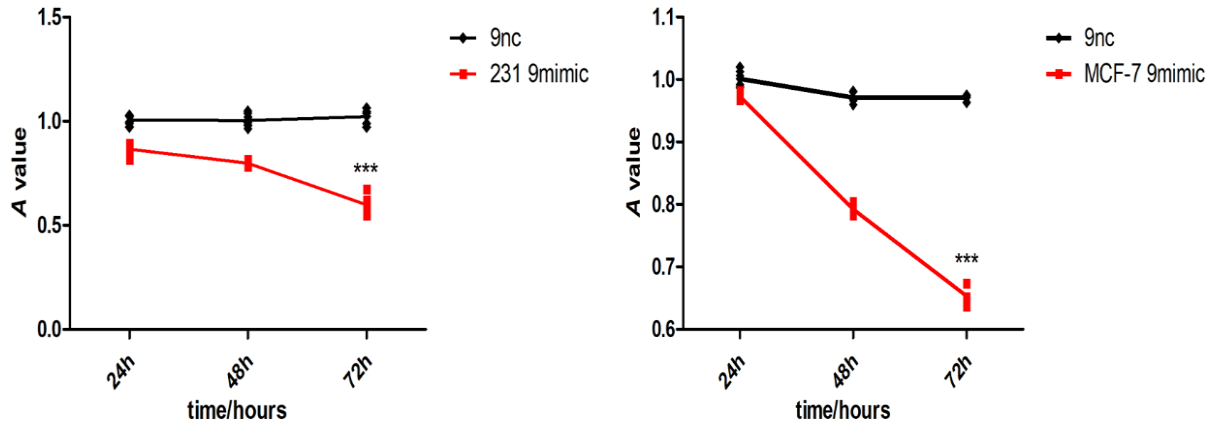


***$P < 0.001$ compared with MCF-10A, MDA-MB-231 NC and MCF-7 NC.

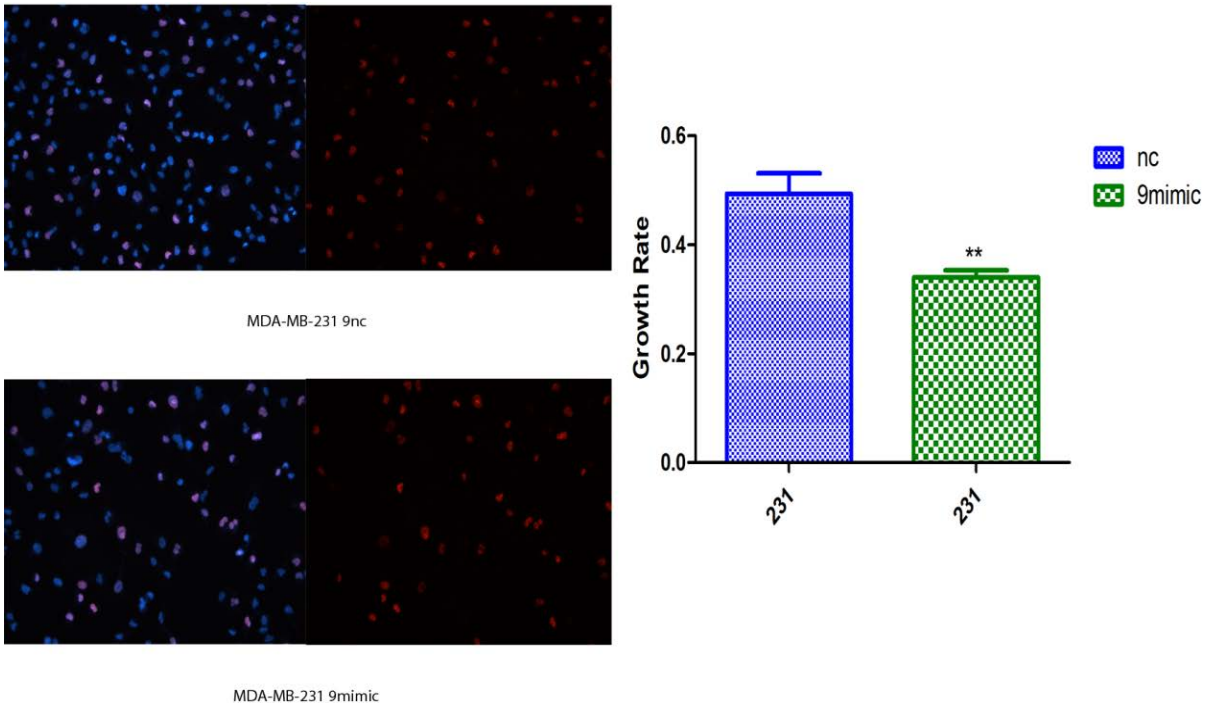
Figure 1. Expression level of novel-miR-9 in cells. (a) Novel-miR-9 in normal cells and in human breast cancer cells; (b) (c) to verify whether novel -miR-9 was introduced into human breast cancer MDA-MB-231 and MCF-7 cells
图 1. novel-miR-9 在细胞中的表达水平。(a) novel-miR-9 中正常细胞 MCF-10A 和人乳腺癌细胞 MCF-7、MDA-MB-231；(b) (c) 验证 novel-miR-9 是否导入人乳腺癌 MDA-MB-231 和 MCF-7 细胞

2) novel-miR-9 对乳腺癌细胞增殖影响 将 novel-miR-9 类似物导入 MCF-7 和 MDA-MB-231 细胞，运用 cck-8 以及 EDU 检测乳腺癌细胞增殖能力(图 2)。

3) 预测 novel-miR-9 靶基因 通过 TargetScan，miRDB，miRWalk 和 miRanda 预测 novel-miR-9 的靶基因与 GeneCard 查询到与乳腺癌相关的致病基因取交集得到同乳腺癌相关的 1188 个预测靶基因(图 3)。



(a)



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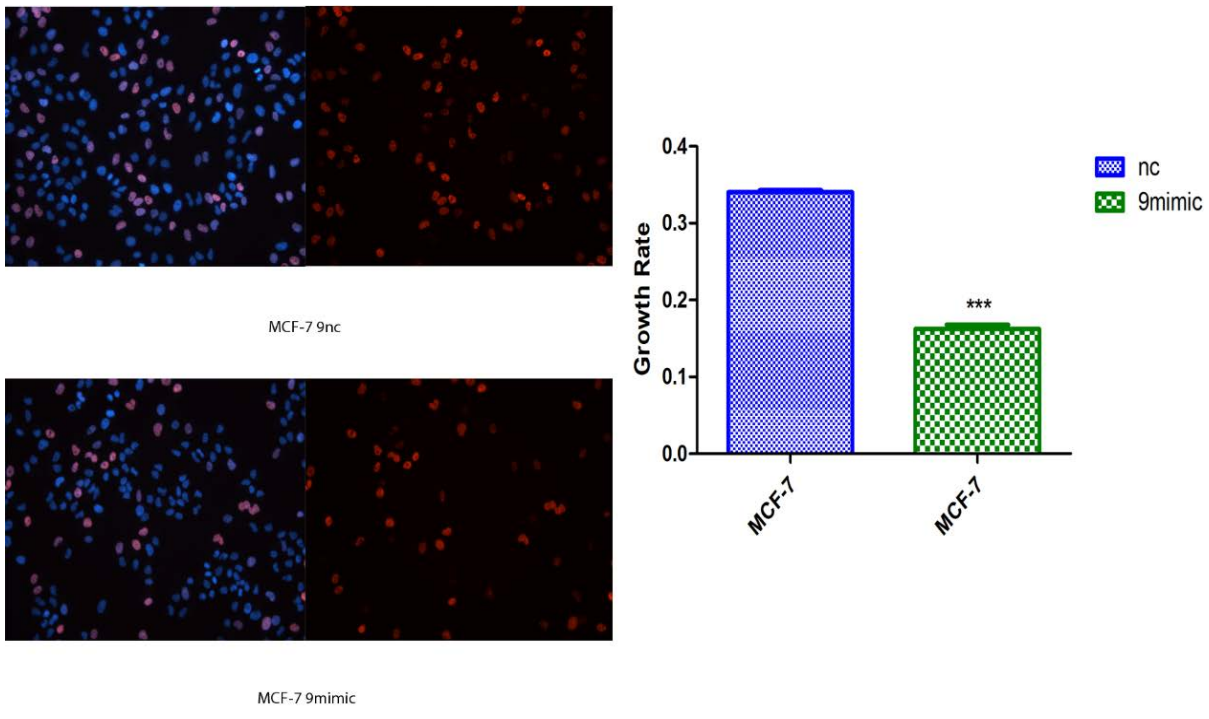


(c)

$^{**}P < 0.01$, $^{***}P < 0.001$ compared with MDA-MB-231 NC and MCF-7 NC

Figure 2. Effects of Novel-miR-9 on the proliferation of breast cancer cells. (a) CCK-8 assayed the effect of Novel -miR-9 on the proliferation of MCF-7 and MDA-MB-231 breast cancer cells; (b) EDU detection of the effect of Novel-miR-9 on the proliferation of MDA-MB-231 breast cancer cells; (c) EDU was used to detect the effect of Novel-miR-9 on the proliferation of breast cancer cells MCF-7

图 2. novel-miR-9 对乳腺癌细胞增殖影响。(a) cck-8 检测 novel-miR-9 对乳腺癌细胞 MCF-7 和 MDA-MB-231 增殖影响；(b) EdU 检测 novel-miR-9 对乳腺癌细胞 MDA-MB-231 增殖影响；(c) EdU 检测 novel-miR-9 对乳腺癌细胞 MCF-7 增殖的影响

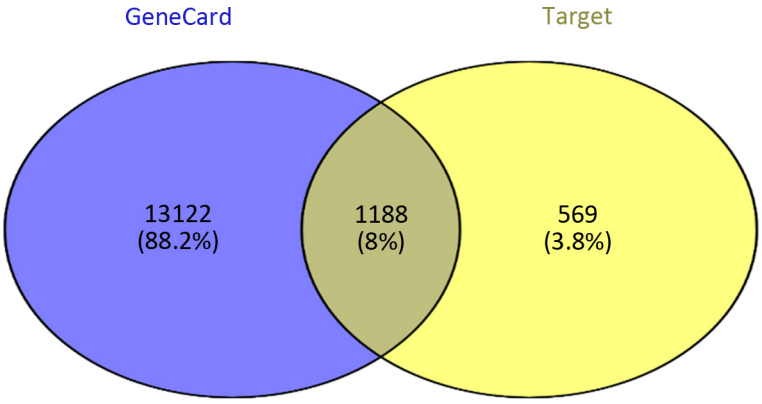


Figure 3. Predicts target genes associated with Novel -miR-9 and breast cancer
图 3. 预测 novel-miR-9 同乳腺癌相关的靶基因

4) 找出与增殖相关靶基因 运用 DAVID 线上网站找出 1188 个靶基因中与增殖相关的靶基因。发现 *BRCA1*、*CDH1*、*ERBB2*、*EGFR* 等 26 个基因直接调控细胞增殖(表 1)并列出 26 个基因信息(表 2)。

Table 1. GO analysis of DEGs in Breast Cancer
表 1. GO 分析乳腺癌的差异基因

Term	Description	Count in gene set	P-value	Benjamini
GO:0019899	enzyme binding	209	1.5696323496283148E−15	2.4091839634365897E−12
GO:0001067	regulatory region nucleic acid binding	122	6.3502055620786575E−15	2.4522606167920458E−12
GO:0044877	macromolecular complex binding	162	3.453964263902479E−14	1.0703660180411134E−11
GO:0000989	transcription factor activity, transcription factor binding	92	5.709065010437362E−13	8.84858852856496E−11
GO:0008092	cytoskeletal protein binding	114	2.3582441895158947E−12	3.0460400868292936E−10
GO:0016301	kinase activity	109	1.809990556789057E−8	9.04994877370946E−7
GO:0019838	growth factor binding	26	1.552211434415833E−6	6.014643089469196E−5
GO:0008013	beta-catenin binding	19	8.190108187078926E−6	2.4888509503584455E−4
GO:0035326	enhancer binding	20	2.2386715257633048E−5	5.980934338446486E−4
GO:0005102	receptor binding	134	3.0316297765182315E−4	0.006989990769231791

GO，基因数据库；DEGs，差异表达基因。

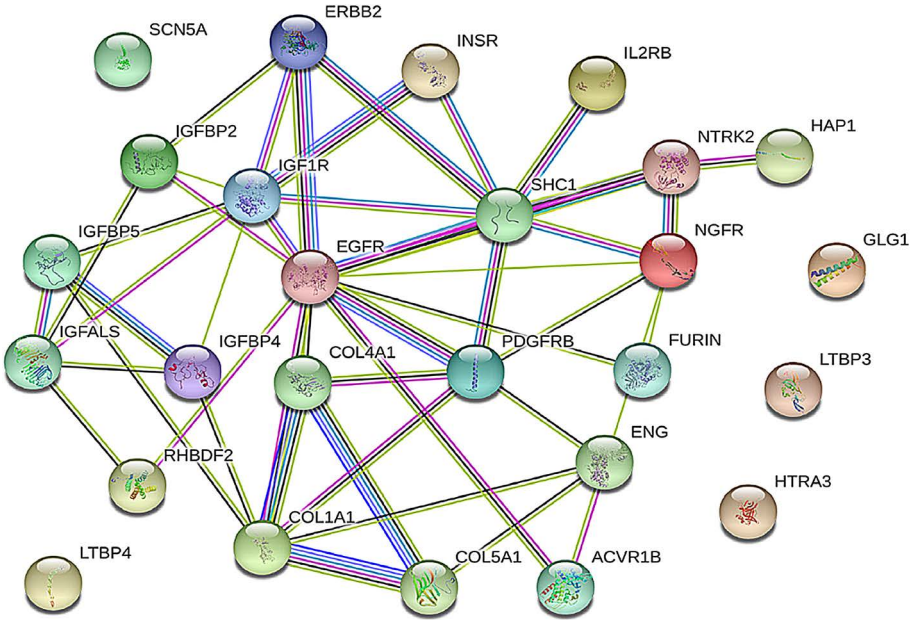
Table 2. Functional roles of 26 genes
表 2. 26 个基因的功能

No.	Gene symbol	Full name	Function
1	*GLG1*	Golgi Glycoprotein 1	Binds fibroblast growth factor and E-selectin. Membrane sialo glycoprotein of the Golgi apparatus, binding fibroblast growth factor and E selectin, ubiquitous.
2	*LTBP3*	Latent Transforming Growth Factor Beta Binding Protein 3	Key regulator of transforming growth factor beta that controls TGF-beta activation by maintaining it in a latent state during storage in extracellular space.
3	*ERBB2*	Erb-B2 Receptor Tyrosine Kinase 2	Protein tyrosine kinase that is part of several cell surface receptor complexes, but that apparently needs a coreceptor for ligand binding. Essential component of a neuregulin-receptor complex, although neuregulins do not interact with it alone.
4	*EGFR*	Epidermal Growth Factor Receptor	Receptor tyrosine kinase binding ligands of the EGF family and activating several signaling cascades to convert extracellular cues into appropriate cellular responses.

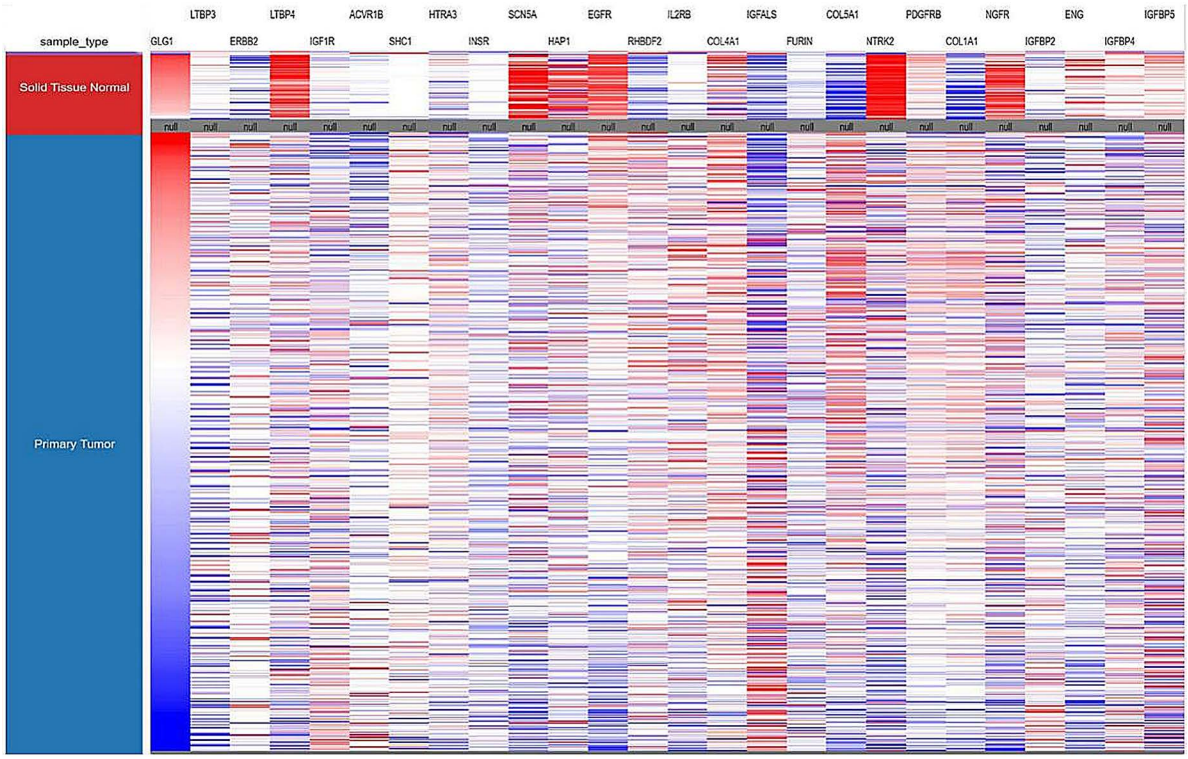
Continued

5	*LTBP4*	Latent Transforming Growth Factor Beta Binding Protein 4	Key regulator of transforming growth factor beta that controls TGF-beta activation by maintaining it in a latent state during storage in extracellular space.
6	*IGF1R*	Insulin Like Growth Factor 1 Receptor	Function: receptor tyrosine kinase which mediates actions of insulin-like growth factor 1. Binds IGF1 with high affinity and IGF2 and insulin (INS) with a lower affinity.
7	*ACVR1B*	Activin A Receptor Type 1B	Transmembrane serine/threonine kinase activin type-1 receptor forming an activin receptor complex with activin receptor type-2. Regulating a many physiological and pathological processes.
8	*SHC1*	SHC Adaptor Protein 1	Signaling adapter that couples activated growth factor receptors to signaling pathways. Participates in a signaling cascade initiated by activated KIT and KITLG/SCF.
9	*HTRA3*	HtrA Serine Peptidase 3	Serine protease that cleaves beta-casein/CSN2 as well as several extracellular matrix (ECM) proteoglycans such as decorin/DCN, biglycan/BGN and fibronectin/FN1.
10	*INSR*	Insulin Receptor	This protein mediates the voltage-dependent sodium ion permeability of excitable membranes.
11	*SCN5A*	Sodium Voltage-Gated Channel Alpha Subunit 5	To be deleted, mutated, or overexpressed in several kinds of cancers.
12	*HAP1*	Huntingtin Associated Protein 1	Originally identified as neuronal protein that specifically associates with HTT/huntingtin and the binding is enhanced by an expanded polyglutamine repeat within HTT possibly affecting HAP1 interaction properties.
13	*RHBDF2*	Rhomboid 5 Homolog 2	Regulates ADAM17 protease, a sheddase of the epidermal growth factor (EGF) receptor ligands and TNF, thereby plays a role in sleep, cell survival, proliferation, migration and inflammation.
14	*IL2RB*	Interleukin 2 Receptor Subunit Beta	Receptor for interleukin-2.
15	*COL4A1*	Collagen Type IV Alpha 1 Chain	Type IV collagen is the major structural component of glomerular basement membranes (GBM), forming a ‘chicken-wire’ meshwork together with laminins, proteoglycans and entactin/nidogen.
16	*IGFALS*	Insulin Like Growth Factor Binding Protein Acid Labile Subunit	Involved in protein-protein interactions that result in protein complexes, receptor-ligand binding or cell adhesion.
17	*FURIN*	Furin, Paired Basic Amino Acid Cleaving Enzyme	Ubiquitous endoprotease within constitutive secretory pathways capable of cleavage at the RX(K/R)R consensus motif.
18	*COL5A1*	Collagen Type V Alpha 1 Chain	Type V collagen is a member of group I collagen.
19	*NTRK2*	Neurotrophic Receptor Tyrosine Kinase 2	Receptor tyrosine kinase involved in the development and the maturation of the central and the peripheral nervous systems through regulation of neuron survival, proliferation, migration, differentiation, and synapse formation and plasticity.
20	*PDGFRB*	Platelet Derived Growth Factor Receptor Beta	Tyrosine-protein kinase that acts as cell-surface receptor for homodimeric PDGFB and PDGFD and for heterodimers formed by PDGFA and PDGFB, and plays an essential role in the regulation of embryonic development, cell proliferation, survival, differentiation, chemotaxis and migration.
21	*COL1A1*	Collagen Type I Alpha 1 Chain	Type I collagen is a member of group I collagen.
22	*NGFR*	Nerve Growth Factor Receptor	Low affinity receptor which can bind to NGF, BDNF, NTF3, and NTF4. Forms a heterodimeric receptor with SORCS2 that binds the precursor forms of NGF, BDNF and NTF3 with high affinity, and has much lower affinity for mature NGF and BDNF.
23	*IGFBP2*	Insulin Like Growth Factor Binding Protein 2	Inhibits IGF-mediated growth and developmental rates.
24	*ENG*	Endoglin	Vascular endothelium glycoprotein that plays an important role in the regulation of angiogenesis.
25	*IGFBP4*	Insulin Like Growth Factor Binding Protein 4	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture.
26	*IGFBP5*	Insulin Like Growth Factor Binding Protein 5	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture.

5) 增殖相关基因相互作用及乳腺癌组织表达 运用 STRING 线上网站分析相关基因的交互作用；运用 UCSC 线上网站分析 26 个基因在乳腺癌组织中的表达量(图 4)。



(a)



(b)

Figure 4. Interaction of 26 genes and their expression levels in breast cancer tissues. (a) Analysis of the interactions of 26 genes by String; (b) the expression levels of 26 genes in breast cancer tissues were analyzed by UCSC

图 4. 26 个基因相互作用及在乳腺癌组织的表达量。(a)用 STRING 分析 26 个基因的相互作用；(b)用 UCSC 而分析 26 个基因在乳腺癌组织的表达量

6) 相关基因的 GO 功能注释及 KEGG 信号传导通路的富集分析 运用 DAVID 线上网站分析 26 个基因的 GO 功能注释以及 KEGG 信号传导通路的富集分析(表 3)。

Table 3. GO and KEGG pathway enrichment analysis of 26 Genes
表 3. GO 和 KEGG 富集化分析 26 个基因

Term	description	Count in gene set	P-value	Benjamini
GO:0019838	growth factor binding	26	7.177095130023525E−54	1.4713045016548225E−51
GO:0019199	transmembrane receptor protein kinase activity	9	4.3968967310014277E−13	4.5063952569535104E−11
GO:0005520	insulin-like growth factor binding	7	3.4171081240130494E−12	2.335056592528417E−10
GO:0004714	transmembrane receptor protein tyrosine kinase activity	6	5.5603020389256557E−8	2.2797213016234608E−6
GO:0005102	receptor binding	12	6.568087664376786E−6	1.3463717490713734E−4
GO:0016301	kinase activity	9	6.796957977711867E−5	8.193251938890533E−4
GO:0004872	receptor activity	11	1.554075440077943E−4	0.001516041185204764
GO:0032403	protein complex binding	7	0.001135535736491165	0.008589457035824144
hsa04510	Focal adhesion	8	1.775015314267736E−7	1.1892532947421763E−5
hsa04151	PI3K-Akt signaling pathway	9	3.3386255161809444E−7	1.1184334800629081E−5
hsa04014	Ras signaling pathway	6	1.1890305517812948E−4	0.002652136307670472
hsa04015	Rap1 signaling pathway	5	0.001150670572651469	0.01095940585115629
hsa05200	Pathways in cancer	5	0.011027515410980928	0.011157926385015005
hsa05206	MicroRNAs in cancer	4	0.02664017560230009	0.010776432474605246

GO，基因库；KEGG，基因富集化。

4. 讨论

乳腺癌为临床常见的恶性肿瘤之一，其发病率在不断的升高且有向年轻化发展的趋势，这对乳腺癌的临床防治造成极大的困难。近年来，miRNA 在癌症中的作用机制已经成为研究热点，基因芯片有助于探究 miRNA 在多种癌症中的表达模式[11]。多项研究表明，miRNA 参与细胞生长、增殖、分化和凋亡等多个生理过程[12] [13]，在癌症发生和发展中有重要作用，甚至能够预测肿瘤分类[14]。

miRNA 主要是通过结合 mRNA 的 3'UTR 区，转录后调控靶基因[15]，其体现在一方面可与沉默核糖蛋白复合物结合，通过序列完全互补的方式降解目的蛋白，另一方面可通过非完全互补结合发挥转录后调控。因此明确 microRNA 作用的靶基因尤为重要。

运用生物信息学软件预测 miRNA 靶基因，已经成为生物学研究的一种重要手段。现已开发出多种 miRNA 靶基因预测软件，但不同软件的算法不同，优缺点也有所侧重。本研究使用 TargetScan，miRDB，miRWalk 和 miRbase 在线分析软件进行靶基因的预测，由于结合了多个靶基因预测软件的结果，大大排除了假阳性的影响，准确性增高。近年来将 Genecards 数据库查询到的疾病相关因子的调控网络与生物信息学软件所获基因进行联合，为后续实验验证提供大量非常有价值的线索[16]。本研究将 Genecards 数据库中与乳腺癌相关的基因同预测出的潜在靶基因交集，共获得 1188 个基因；通过 DAVID 数据库 GO 功能分析所获得的 1188 个基因，得到与增殖相关基因 26 个，包括 *EGFR*、*IGF1R*、*IGFBP2* 等。其中可以看出在乳腺癌明显高表达的有 *LTBP4*、*SCN5A*、*HAP1*、*EGFR*、*NTRK2*、*NGFR* 和 *ENG*；明显低表达的有 *RHBDF2*、*IGFALS*、*COL5A1*、*COL1A1*、*ERBB2*。通过 STING 分析 26 个蛋白相互作用，发现 *ERBB2*、

EGFR、*COL1A1*、*COL5A1* 在 26 个基因中起着关键作用。

综上所述，生物信息学在线分析软件所获得的信息能够在 miRNA 与疾病发生相关的研究中发挥很好的前瞻性作用。本研究对 Novel-miR-9 进行增殖实验、靶基因预测、GO 富集分析和 KEGG 通路富集分析，结果表明在乳腺癌发病过程中 Novel-miR-9 可能通过调控多个与乳腺癌发病相关的靶基因，并可能参与多条信号通路的调节，从而在乳腺癌中发挥抑癌基因的作用。通过对 Novel-miR-9 的靶基因预测，我们有机会在这些与增殖相关的 26 个靶基因中，找到能被 Novel-miR-9 靶向调节的基因。从而可以进一步对该 microRNA 对人乳腺癌细胞增殖影响的机制研究，为将来逆转乳腺腺癌的化疗耐受提供了新的靶点。

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