Mol Diagn Ther (2016) 20:97–110
DOI 10.1007/s40291-016-0186-3

REVIEW ARTICLE

miR-21 Might be Involved in Breast Cancer Promotion and Invasion Rather than in Initial Events of Breast Cancer Development

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Published online: 18 February 2016

Abstract Breast cancer (BC) is a heterogeneous disease that develops into a large number of varied phenotypes. One of the features used in its classification and therapy selection is invasiveness. MicroRNA-21 (miR-21) is considered to be an important element of BC invasiveness, and miR-21 levels are frequently increased in different tumor types compared with normal tissue, including the breast. Experimental and literature research has highlighted that miR-21 was always significantly elevated in every study that included invasive breast carcinomas compared with healthy breast tissue. The main goal of this research was to specify the predominant role of miR-21 in the different phases of BC pathogenesis, i.e. whether it was involved in the early (initiation), later (promotion), or late (propagation, progression) phases. Our second goal was to explain the roles of miR-21 targets in BC by an in silico approach and literature review, and to associate the importance of miR-21 with particular phases of BC pathogenesis through the action of its target genes. Analysis has shown that changes in miR-21 levels might be important for the later and/or late phases of breast cancerogenesis rather than for the initial early phases. Targets of miR-21 (*TIMP3*, *PDCD4*, *PTEN*, *TPM1* and *RECK*) are also primarily involved in BC promotion and progression, especially invasion, angiogenesis and metastasis. miR-21 expression levels could perhaps be used in conjunction with the standard diagnostic parameters as an indicator of BC presence, and to indicate a phenotype likely to show early invasion/metastasis detection and poor prognosis.

Key Points

MicroRNA-21 (miR-21) is more likely to be connected to the processes of breast cancer (BC) propagation and progression than to the initial phases of malignant transformation.

miR-21 might be used as a future biomarker for early screening of invasion/metastasis.

Target genes of miR-21 regulate processes associated with the later and/or late phases of BC pathogenesis rather than the early phases.

1 Introduction

More than 95 % of malignant breast tumors originate from the transformed breast epithelia. One of the parameters and features used for classification of breast carcinomas refers to its invasiveness. Non-invasive, in situ carcinomas characterize the presence of the myoepithelial cell layer [1], while invasive breast carcinomas have the ability to invade the surrounding tissue and are not coated with the myoepithelial layer [2]. Breast carcinomas rise mostly from ductal and/or lobular cells and, based on their origin and invasiveness, are classified as ductal carcinomas in situ (DCIS), invasive ductal carcinomas (IDC), lobular carcinomas in situ (LCIS), and invasive lobular carcinomas (ILC) [1].

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Breast cancerogenesis represents a series of events resulting in the formation of cells with malignant phenotype. From the aspect of the stepwise model, cancer pathogenesis might be observed through three main phases: initiation, promotion, and progression [3]. Accumulation of genetic and epigenetic changes initiate formation of a malignant tumor, promote clonal expansion and survival of transformed cells [4]. During the phase of tumor progression, the tumor enlarges due to the intensive proliferation and absence of apoptosis. Breast cancer (BC) disease propagates through the acquisition of invasive phenotype and initiation of angiogenesis, and metastasizes in order to form a secondary tumor. Neoplastic transformation of the breast cells represents the result of numerous molecular changes, such as activation of oncogenes, inactivation of tumor suppressor genes [5], changes in complex signaling network among cells and the microenvironment of a tumor [6, 7], and epigenetic changes via ten main events described by Hanahan and Weinberg as hallmarks of cancer [8, 9]. Epigenetic changes might be important events in BC pathogenesis [10–12]; the most important epigenetic changes are histone modifications [13] and DNA methylation—hypomethylation and hypermethylation [10]. Another important epigenetic phenomenon is microRNA interference, a process of gene silencing on the post-transcriptional level [11, 14]. Transcriptional or translational repression of tumor suppressors involved in cell-cycle regulation, apoptosis, or cell migration caused by an epigenetic event frequently cause tumorigenesis and tumor propagation and/or progression. BC invasion, as well as invasion of carcinoma cells in general, is a multi-step process. After malignant transformation, accumulation of genetic and epigenetic changes, and changes in the microenvironment of a tumor, cells might acquire the ability to break through the basal membrane and to infiltrate the surrounding tissue. In malignancies originating from epithelial cells (such as ductal and lobular cells of the breast glandular tissue), cells develop invasive ability through a series of events known as epithelial–mesenchymal transition (EMT), which is a precursor step to the process of invasion in breast cancerous tissue [1, 15].

2 MicroRNAs

MicroRNAs have introduced new sights of observation and, likewise, new levels of studying the complexity of gene expression regulation, especially in cancer research. MicroRNAs are minuscule, non-coding RNAs that silence target genes by translational repression, most likely in three ways: (1) by enhanced degradation of messenger RNA (mRNA) [16] (increased deadenylation of mRNAs [17]); (2) by cleaving mRNA; or (3) by means of the site-specific cleavage (probably not so frequent in mammals) [16]. MicroRNAs bind to the 3′-UTR (3′-untranslated region) of the target mRNAs, mostly in an incomplete complementary way that allows 'finding' and binding more than 100 potential targets each [18]. Approximately 30 % of human genes might be regulated by miRNAs due to the in silico estimation of Rajewsky [19]. Moreover, Selbach et al. [20] suggested that the synthesis of 30 % of proteins might be directly or indirectly altered by miRNAs. miRNA regulation seems to be one of the most frequent mechanisms of post-transcriptional gene regulation [21]. All of the facts mentioned above emphasize the importance of small epigenetic mRNA/protein regulators, miRNAs. Although it is necessary to perform screening for miRNAs involved in physiological or pathological mechanisms, it is also important to get into the core of particular miRNAs related to a disease or process.

2.1 MicroRNAs and Breast Cancer (BC)

The majority of miRNAs involved in cancer pathogenesis are tumor suppressors [21]. In their review, Chuang and Jones [22] listed and described the most frequently downregulated miRNAs in cancer, such as let-7, miR-127, miR-16-1, miR-145 and miR-15a, as well as several important upregulated oncomiRs such as miR-21, miR-155, miR-372, miR-373, miR-17-92 cluster, and miR-146, most of which are downregulated via alterations in their biogenesis pathway, genetic loss [21], or epigenetic silencing. CpG islands in their promoter regions can also be hypermethylated [23], and miRNA genes can also be epigenetically silenced. It is very unusual that promoters of almost one-third of the examined miRNAs showed hypermethylated status, a proportion that was much higher than methylation frequency in protein-coding genes [24]. Oncogenic microRNAs can be upregulated via the DNA hypomethylation process [21, 25, 26]. Promoter regions of their genes, or genes that they share promoter regions with, are frequently associated with CpG islands. Epigenetic changes that are characteristic of cancer pathogenesis might influence the levels of miRNA expression in both ways, i.e. promoter hypomethylation that inhibits expression of suppressor miRNAs, and vice versa; DNA hypomethylation could loosen up the transcription of oncomiRNAs. Hypomethylation can also be the cause of miR-21 overexpression [25].

microRNAs extracted from serum, plasma, and saliva (circulating microRNAs) could become very powerful tools for early screening and personalized approaches among patients with cancer, and likewise for miRNAs extracted from solid tissue. Hundreds of circulating microRNAs can be measured from human blood samples

[27]. It has already been shown that circulating miRNAs (miR-21, miR-222, miR-155, members of the let-7 family, miR-29a, miR-195, miR-202, etc.) may be promising biomarkers for the early detection, diagnosis, prognosis and prediction of therapy response of BC, and also for the presence of metastatic disease [28, 29]. Mar-Aguilar et al. [30] reported that expression levels of seven investigated miRNAs (miR-10b, miR-21, miR-125b, miR-145, miR-155, miR-191, and miR-382) extracted from the blood of BC patients were significantly elevated compared with healthy individuals. It should be noted that miR-21 may potentially be a very important tool (future stabile biomarker) for the screening and diagnosis of BC, and determining disease progression, response to therapy and the likelihood of metastasis [30], which is one of the goals of this research.

3 MicroRNA-21 (miR-21) in BC Formation, Promotion, Invasion, and Metastasis

miR-21 is a well-known oncomiR involved in BC pathogenesis. It regulates various processes such as development, cell differentiation, regulation of the cell cycle, migration, motility, proliferation, apoptosis and senescence [31]. Apart from its role in the various physiological processes, miR-21 is present in disorders such as vascular disease, hypertrophy, and ischemic heart disease [32]. The miR-21 expression level also changes the influence on pathological conditions such as autoimmune, metabolic and inflammatory diseases (asthma, ulcerative colitis, eosinophilic esophagitis) [33]. miR-21 is the most frequently investigated miRNA in human neoplasia; its expression levels were elevated in almost every human cancer examined [34]. In ovarian cancer, it is suggested that miR-21 might be involved in cell growth and invasion [35, 36], and in colorectal [37] and hepatocellular carcinomas [38] miR-21 has been shown to be a very important promoter of cellular outgrowth, migration, invasion, and metastasis. In breast carcinoma cell lines, miR-21 was connected to cell proliferation and migration [39, 40], while in regard to patients with breast carcinomas, miR-21 was associated with advanced clinical stage, metastasis, and poorer outcome [41–45]. According to Kalluri and Weinberg, miR-21 is also connected to the process of EMT [46]; it is one of several key signaling molecules participating in the regulation of EMT [46]. After changes in EMT characteristics, epithelial cells become motile migratory myoepithelial cells with the ability to move through degraded extracellular matrix (ECM) into the surrounding tissue.

Between 2006 and 2008, miR-21 gained its deserved epithet ‘onco’. Volinia et al. [47] linked miR-21 with the syntagma ‘universal overexpression in cancers’. Real-time polymerase chain reaction (RT-PCR) quantification of miRNA expression was first established by Chen et al. [48]. miR-21 was among the first described and experimentally confirmed oncomiRNAs, and was overexpressed in all examined types of tumors compared with healthy tissue [31, 47]. This fact was later supported by the revelation of miR-21 targets—*TIMP3* (tissue inhibitor of matrix metalloproteinases 3) [49], *PDCD4* (programmed cell death receptor 4) [50, 51], *PTEN* (phosphatase and tensin homolog) [50, 52–54], *TPM1* (tropomyosin 1) [55, 56], and *RECK* [57], whose protein products were known to be antitumorigenic, antiproliferative, anti-invasive, and antiangiogenic in and outside of the breast epithelial or stromal cells. In the study by Hatley et al. [58] in a K-ras^{LA2} murine model, miR-21 overexpression significantly increased the number of tumors; however, these investigators implied that miR-21 overexpression itself might have been sufficient for the initial process of cancerogenesis. Moreover, it appeared that miR-21 overexpression in human tumors did not have a direct influence on cancer pathology but enhanced processes related to non-small cell lung cancer (NSCLC) pathogenesis [58].

Si et al. [59] showed that anti-miR-21 has the ability to inhibit cell growth in vitro and reduce tumor growth in vivo. They found that oligonucleotide anti-miR-21 reduced breast tumor cell growth in a dose-dependent way by knocking down miR-21 molecules. In 2009, Yang et al. [50] silenced the expression of endogenous miR-21 in MCF-7 BC cells and noted an increase of 58 proteins, potential targets of miR-21. They performed luciferase assays and extracted six potential direct targets of miR-21. *PDCD4* was one of the candidates quantified by their proteomic approach. In 2010, Medina et al. [60] definitively established the title ‘oncogene’ for miR-21. They induced overexpression of miR-21 in transgenic line, which resulted in lymphoma development. In their 2012 review, Jansson and Lund [21] framed research on miR-21 and noted that tumor propagation might be dependent on miR-21 overexpression (because in the case of lowered rates of miR-21, the tumor shrank, probably due to apoptosis). Jansson and Lund [21] proposed miR-21 inhibition as a potential novel model for BC therapy.

To date, it seems that most of the research has suggested that miR-21 is associated mainly with advanced clinical stages, grades, invasiveness, lymph node metastasis, and poor prognosis [41–45]. Our goal was to research available data on miR-21 and its association with the different phases in BC disease. For the needs of this research, the whole process of BC pathogenesis was divided into three phases: early, later, and late. Another goal of this research was to review the potential relevance of miR-21 molecules in the formation, neoplastic transformation (early), promotion,

proliferation, growth (later) or propagation/progression (through grades), invasion, angiogenesis, and metastasis (late) phases of breast pathogenesis.

First, Iorio et al. [61] showed that miR-21 levels were significantly changed in breast carcinomas compared with normal breast tissue. miR-21, whose expression could have discriminated cancer from normal tissues was also differentially expressed in cancers with a different proliferation index and tumor stage. It is still unclear whether or not miR-21 was involved in the initial phases of BC pathogenesis. In the same research, it was shown that miR-21 had a certain impact on the late or later phases (Table 1).

Si et al. [59] transfected MCF-7 cells with the anti-miR-21 molecule and injected them into the pads of nude mice. Anti-miR-21 was associated with lower cell proliferation rates. It was also shown that the inhibitory effect of anti-miR-21 was stronger on tumor growth than on cell growth. This might indicate that microenvironment and ECM events that induce overexpression of miR-21 significantly influence BC promotion and propagation (Table 1). These investigators showed that miR-21 might also be involved in the initial phases of BC development. miR-21 overexpression decreases Bcl-2 protein levels, which reflected on decreased rates of apoptosis. Decreased rates of apoptosis increase the chance of cancer formation.

According to Buscaglia and Li [62] it appears that miR-21 displays its oncogenic activity mainly through inhibition of cellular apoptosis. Hannafon et al. [63] showed that miR-21 was significantly overexpressed in DCIS samples compared with histologically normal samples, and likewise in the samples taken after a reduction mammoplasty. Nevertheless, in their previous work, Emery et al. [64] pointed out that DCIS samples were obtained from tumor samples that also contained IDC component. In the same research, the investigators also mentioned that gene expression studies showed that DCIS which originated from IDC–DCIS tumors shared more similar features with IDC components than with solo DCIS component [64], which might be the reason for significantly higher miR-21 levels in DCIS compared with non-tumorous tissue.

In the data of Chen et al. [65], miR-21 levels were significantly increased in atypical ductal hyperplasia (ADH) samples compared with normal samples. No difference was found between normal and DCIS tumors or between ADH and DCIS. On the other hand, the difference was also high between DCIS and IDC, ADH and IDC, and normal and IDC tumors. ADH, DCIS, IDC and normal tissue were obtained mostly from the same sample via the laser capture microdissection method. Increased levels of miR-21 could be one of the factors/mechanisms that might be responsible for ADH formation. It is very interesting that the difference in miR-21 levels between ADH and DCIS has not been detected. According to these findings, miR-21 molecules might not be significant for malignant transformation, while in IDC samples, miR-21 levels were the highest. According to their observations, the difference in miR-21 levels was higher between DCIS and IDC than between ADH and IDC. According to the results of Chen et al. [65], miR-21 overexpression might be more significant to earlier, premalignant events and for late events such as invasion rather than malignant transformation itself (Table 1). In this research, DCIS lesions were examined that were not part of IDC–DCIS component, IDC–DCIS tumors, pure IDC tumors and their five matched normal tissue samples. It is noted that the difference between DCIS and pure invasive tumors (IDC, ILC, and IDC–ILC) was high. The results have not shown the presence of significant differences between non-transformed (even though they were obtained from patients with invasive breast carcinoma) and DCIS tissue. According to these facts, miR-21 might have a preferential role in BC invasion than in tumor formation [66] (Table 1). Volinia et al. [67] measured significantly higher levels of miR-21 in DCIS compared with healthy tissue. They also mentioned that levels of miR-21 in IDC tumors remained the same, or were even lower than in DCIS. If the process of invasion is divided into early and later/late sections, it might be supposed that in the period of time before the invasion occurs (which is still an in situ carcinoma), miR-21 levels could increase. Furthermore, during and after the process of invasion, levels of miR-21 molecules might decrease [68]. According to the results of Volinia et al. [67], miR-21 could be involved in the early phases of BC formation (Table 1).

Yan et al. [44] showed that miR-21 overexpression in human BC was associated with advanced clinical stage, lymph node metastasis, and poor patient prognosis. They noted that the majority of patients with overexpressed miR-21 were in advanced clinical stages. These findings might indicate that miR-21 levels were related to the later stages of BC progression (Table 1). Our research indicates that miR-21 levels are the highest in grade II tumors (compared with grade I and grade III groups) [68]. The data also show that miR-21 is not significantly related to lymph node metastasis [68] (Table 1). Additionally, it is important to mention the research of Walter et al. [41], in which miR-21 was not moderately or highly expressed in grade I tumors but only within grade II and III tumors. This result also suggests its involvement in the late phases of BC pathogenesis (Table 1).

Other evidence that contributes to the idea that miR-21 is preferentially involved in invasion is the fact that miR-21 has often been mentioned in the context of biomarkers for EMT (expressed in myoepithelial cells but not in epithelial cells) [46, 69, 70]. The findings and conclusions from this review do not exclude the potential role of miR-21 in BC formation. This process might be individually based and is

Table 1 Potential role of miR-21 in the phases of breast cancer pathogenesis examined in tumor tissue and tumor cells

Type of sample groups	Effect of miR-21	Method	Study, year	Event in BC pathogenesis
MDAMB231, HeLa cells	miR-21 promotes anchorage-independent tumor growth	qRT-PCR, Northern blot, transfections, infections and luciferase assays	Orso et al. 2013 [73]	Later[c]
MCF-7 cells	Forced re-expression of miR-21 resulted in higher motility, migration and invasion via induced epithelial–mesenchymal transition, and in self-renewal and increased forming of cell colonies	Wound healing assay, migration assay, invasion assay, qRT-PCR, mammosphere-forming assay, Western blot	Han et al., 2012 [72]	Later, late[d]
MDA-MB-231 cells	Anti-miR-21 reversed EMT, decreased migration and invasion	Cell proliferation assay, migration assay, invasion assay, qRT-PCR, Western blot mammosphere formation assay, anti-miR-21 transfection and re-expression	Han et al., 2012 [70]	Later, late
MCF-7 cells	Anti-miR-21 inhibits cell growth in vitro and in vivo	qRT-PCR, anti-miR-21 transfection	Si et al., 2007 [59]	Later, late
Breast cancer tissue compared with paired normal tissue	Higher miR-21 levels associated with advanced clinical stages and worse prognosis	miRNA microarray analysis, qRT-PCR	Yan et al., 2008 [44]	Later, late
Fibroadenoma compared with paired normal tissue	Increased in fibroadenoma	qRT-PCR, colony formation assay, wound healing assay, luciferase reporter assay, Western blot, mRNA array, locked nucleic acid in situ hybridization for miRNA	Yan et al., 2011 [40]	Earlier (before malignant transformation)[a]
Breast cancer tissue compared with paired normal tissue	Increased in BC			Not clear (not specified what type of tumor specimens, according to their invasiveness, were analyzed)
MCF-7, MDA-MB-231 cells, miR-21 knockout mice	Anti-miR-21 inhibited migration, invasion and in vivo tumor growth			Later, late
Normal compared with ADH	Precancerous tissue showed increased levels	qRT-PCR	Chen et al., 2013 [65]	Early[b]
ADH compared with DCIS	Not changed			Early
DCIS compared with IDC-DCIS	Statistical trend towards higher levels in IDC–DCIS	qRT-PCR	Petrović et al., 2014 [66]	Late
Invasive (ILC, IDC and ILC–IDC) compared with DCIS	Increased			
Normal compared with DCIS	Not changed			
Normal compared with DCIS	Increased	Deep sequencing and microarray analysis	Volinia et al., 2012 [67]	Early
DCIS compared with IDC	Not changed or lowered			Early
IDC compared with normal matched tissue	Increased in BC, associated with aggressive tumor characteristics and poor prognosis	qRT-PCR	Lee et al., 2011 [42]	Late

Table 1 continued

Type of sample groups	Effect of miR-21	Method	Study, year	Event in BC pathogenesis
IDC	Higher in grade II compared with grade I	qRT-PCR	Petrović et al., 2014 [68]	Late
IDC compared with normal matched tissue	Increased in BC, higher miR-21 levels associated with higher proliferation index, lymph node-positive status, later clinical stages (II and III) and lower *PTEN* protein expression rates	qRT-PCR	Huang et al., 2009 [43]	Late
Pregnancy-associated breast carcinomas	Overexpression found in high-grade and lymph node-positive tumors, ER, PR, associated with progression through grades and metastasis and poor clinical outcome	qRT-PCR, IHC for miR-21 targets	Walter et al., 2011 [41]	Late
Breast cancer tissue compared with normal non-matched tissue	Increased in BC, associated with advanced clinical stages and proliferation index	miRNA microarray analysis, qRT-PCR	Iorio, 2005 [61]	Not clear (not specified what type of tumor specimens, according to their invasiveness, were analyzed)
Breast cancer samples	Higher expression levels associated with vascular invasion, higher grade, but not with lymph node status, clinical stage and progression of the disease	qRT-PCR	Qian et al., 2008 [45]	Late
Normal tissue, FEA of the breast, DCIS and IDC; the most cases were in IDC	Increase in the percentage of positive cases of miR-21 from normal to FEA, DCIS and IDC	miR-21 ISH	Qi et al., 2009 [56]	Early, later, late (mostly)
IDC		miR-21 ISH	Rask et al., 2011 [39]	Later

BC breast cancer, *ER* estrogen receptor, *FEA* flat epithelial atypia, *IHC* immunohistochemistry, *ISH* in situ hybridization, *DCIS* ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *PR* progesterone receptor, *qRT-PCR* quantitative real-time polymerase chain reaction, *EMT* epithelial-mesenchymal transition, *mRNA* messenger RNA, *miRNA* micro RNA

[a] Earlier events before malignant transformation

[b] Early initiation of tumorogenesis, malignant transformation

[c] Later events–tumor promotion (growth and cell proliferation)

[d] Late events–propagation through grades and stages (EMT, invasion, and metastasis)

probably rare compared with its role in invasion as BC is a very heterogeneous disease.

In vivo experiments reported that miR-21 was not included in the initial phases of cancerogenesis but was included in propagation [21, 58] and later events related to tumor progression [71]. Han et al. [72] showed the potential relationship between changes in miR-21 expression level and the features of cancer stem cells (CSCs). Higher levels of miR-21 expression reflected on BC progression, cell migration, invasion, and metastasis via EMT. CSCs are a subpopulation of tumor cells that have the ability to enhance tumor growth. They have been described as the boost elements of cancer progression and metastasis, and may cause recurrence of the tumor. Han et al. [72] described the role of miR-21 overexpression in tumor promotion propagation (Table 1). First, they showed that re-expression of miR-21 promoted cell motility, migration and invasion in MCF-7 cells. According to these investigators, miR-21 overexpression in BC cells leads to acquisition of migratory and invasive abilities. In addition, cells with forced miR-21 expression gained an EMT phenotype with downregulated E-cadherin and upregulated N-cadherin and vimentin. Additionally, MCF-7 cells showed reduced cell–cell contact. Their results emphasized the role of miR-21 and its downstream signaling pathways on the CSC phenotype in BC cells [69, 72].

The results of Orso et al. [73] also described the role of miR-21 in tumor promotion, i.e. in tumor growth (Table 1). Yan et al. [40] showed that anti-miR-21 inhibited growth and migration of two cell lines (MDA-MB-231 and MCF-7) in vitro, and also reported that anti-miR-21 inhibited tumor growth in nude mice. Enhanced miR-21 levels in BC compared with matched healthy tissue, and in fibroadenoma compared with controls, shows the potential role of miR-21 in early tumorigenesis (Table 1). Rask et al. [39] noted that miR-21 expression was induced by the neoplastic tissue. Their results suggested that miR-21 was highly expressed in stromal fibroblast-like cells, and that miR-21 was predominantly expressed in the stroma of IDC, and increased cell proliferation rates. As cancer-associated fibroblasts have a strong impact on the growth, promotion, and progression of tumors [74], this might be further evidence of its predominant involvement in the later or late phases of BC progression (Table 1).

A number of researchers have shown overexpressed miR-21 in BCs compared with the levels of expression in normal, non-tumorous tissue [44, 49, 59, 61]. None of these researchers explained the difference between in situ, invasive with non-invasive component, and pure invasive tumors. Li et al. [75] proposed high invasion and metastasis, and low invasion and metastasis groups, and showed that the high invasion metastasis group showed the highest rates of miR-21 expression, while the normal group showed the lowest rates. It is important to emphasize that both groups were IDCs (Table 1). Nassar et al. [76] also compared invasive ductal carcinomas, with healthy adjacent tumor tissue, with significant differences between the healthy and the IDC groups. In the majority of the research, different types of breast carcinomas were examined together. Most of the examined tumors were predominantly the invasive BC forms, this is only to be expected as these are the most frequent type, this would explain why research does not show a clear picture of the role of miR-21 in the process of breast pathogenesis, and why the results of work trying to identify an association of miR-21 with various parameters showed no coherent results in research that compared normal and all cancerous tumors with normal and subgrouped tumors. Given these facts, miR-21 might be used as a factor that separates health tissue from tumorous tissue (mostly invasive) [44, 49, 59, 61] and, on the other hand, a factor that separates different BC forms (such as pure invasive from invasive associated with non-invasive tumors) [77]. Neither research nor this review can exclude the role of miR-21 in some early phases of BC formation. Moreover, it could be an individual incidence. In some cases, increased miR-21 levels in precancerous tissue might trigger some mechanisms for malignant transformation (via silencing *PTEN* or *PDCD4*); in other cases (probably more frequently), miR-21 (via its targets such as *TIMP3*, *TPM1* or *RECK*) influences invasion and metastasis. Although miR-21 levels are usually increased in invasive breast carcinomas and in carcinomas with poor prognosis, it would not be unusual to sometimes detect very high miR-21 expression levels (compared with normal tissue) in in situ carcinomas. Overexpression of miR-21 could silence its targets, such as *TIMP3* [75], or *SMAD-7* (miR-21 promotes the transforming growth factor (TGF)-β pathway by silencing *SMAD-7* [65]), which could initiate epithelial–mesenchymal transformation and invasion. Si et al. [78] detected significantly higher levels of both circulating and non-circulating miR-21 in serum and tissue samples, respectively, compared with samples taken from healthy individuals. In stage 3 BC samples the highest miR-21 levels were detected. The interesting and exciting results of Yang et al. [79] and Asaga et al. [80] suggested strong evidence for a correlation between miR-21 and metastasis (Table 2). Yang et al. [79] observed abnormally high concentrations of miR-21 molecules in 98 % of patients with metastatic BC (Table 2). When they combined the levels of miR-21 molecules with circulating tumor cells (CTCs), the specificity of metastasis detection reached 100 %. No differences were observed between the primary and metastatic BC sample groups. It is important to note that all samples were taken from females with the invasive BC form (from stage I to III). Asaga et al. [80] showed that circulating miR-21 levels can separate patients

Table 2 Potential role of miR-21 in the phases of breast cancer pathogenesis according to blood samples and review studies

	Effect of miR-21	Method	Study, year	Event in BC pathogenesis
Serum/tissue	In patients with tumors, significantly higher levels of miR-21 in serum and tissue compared with healthy individuals. Significantly increased in stage 1 compared with normal, increases through stage, the highest in stage 3 BC	qRT-PCR	Si et al., 2013 [78]	Later,[b] late,[c] not clear for early (stage 0 tumors were not examined)
Serum	98 % of cases with abnormally increased miR-21 levels in patients with metastasis compared with healthy individuals	qRT-PCR	Yang et al., 2015 [79]	Late
Plasma	miR-21 levels distinguish patients with local or regional metastasis from patients with distant metastasis	qRT-PCR	Asaga et al., 2011 [80]	Late
Review	miR-21 listed as characteristic for myoepithelial cells after the epithelial–mesenchymal transformation		Kalluri and Weinberg, 2009 [46]	Late
Review	miR-21 in BC involved in maintaining proliferative signaling and activation of invasion and metastasis		Goh et al., 2015 [18]	Later, late
Review	miR-21 involved in the promotion of cell proliferation, migration, invasion, and metastasis via tumor suppressor targets and apoptosis		Buscaglia and Li [62]	Early,[a] later, late (rather than early)

BC breast cancer, *qRT-PCR* quantitative real-time polymerase chain reaction

[a] Early initiation of tumorogenesis, malignant transformation

[b] Later events–tumor promotion (growth and cell proliferation)

[c] Late events–propagation through grades and stages (EMT, invasion, and metastasis)

with BC from healthy individuals, and can also separate subjects with distant metastases from patients with a local and regional state of breast carcinoma.

The results of this study are provided in Tables 1 and 2, and are presented in the Venn diagram illustrated in Fig. 1. Two of 23 studies included in these results showed that miR-21 might be important in the early phases of BC, and two studies associated miR-21 with later events only. Nine studies associated miR-21 overexpression with late events alone, while two studies showed that relatively high miR-21 levels were associated with early, later and late phases simultaneously. Eight studies associated miR-21 overexpression with later and late events simultaneously. To summarize, 19 reports connected miR-21 overexpression with the late phase, 12 reports connected miR-21 overexpression with the later phase, and only six reports (from only four studies) associated miR-21 overexpression with the early phase of BC development.

4 miR-21 Targets in BC and their Role in Tumor Pathogenesis

By performing an in silico analysis of miR-21 target prediction, the role of the most important miR-21 targets is described in the following section. Targets were selected and characterized by the phases in BC pathogenesis that they were involved in, according to the available literature and to (1) in silico analysis using five online prediction tools, and (2) experimentally confirmed data. First, the hallmarks of cancer used are described by Hanahan and Weinberg [9], and miR-21 targets are then connected with the hallmarks of cancer from the research by Buscaglia and Li [62]. Furthermore, targets that were experimentally proven to be the targets of miR-21 in breast cancer are also used, according to the research of Buscaglia and Li [62] and Walsh et al. [81]. Identification of potential miR-21 target genes was performed using five online software tools: TargetScan Human 7.0 (http://www.targetscan.org/) [82], microRNA.org [83], PicTar (http://pictar.mdc-berlin.de/) [83], miRDB (http://mirdb.org/miRDB/) [84, 85], and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/search.php) [86]. TargetScan Human version 7.0 predicts target genes based on 14 different features of the microRNA site that recognizes target and mRNA sequence near the target site [82]. According to the newest update, miRDB identifies functional miRNAs (by name or target mRNA) according to four criteria (scores): literature mining, sequence conservation, miRNA expression profile, and functional annotation by miRBase [84, 85]. microRNA.org is a tool for microRNA target prediction that uses the miRSVR target scoring system [83]. PicTar identifies mRNA targets of miRNAs based on alignment on 3′UTR on the predicted target sites [87]. Finally, miRTarBase uses miRNA target interactions experimentally validated by reporter assay, Western blot, microarray and next-generation sequencing methods, and compares results with other databases [86].

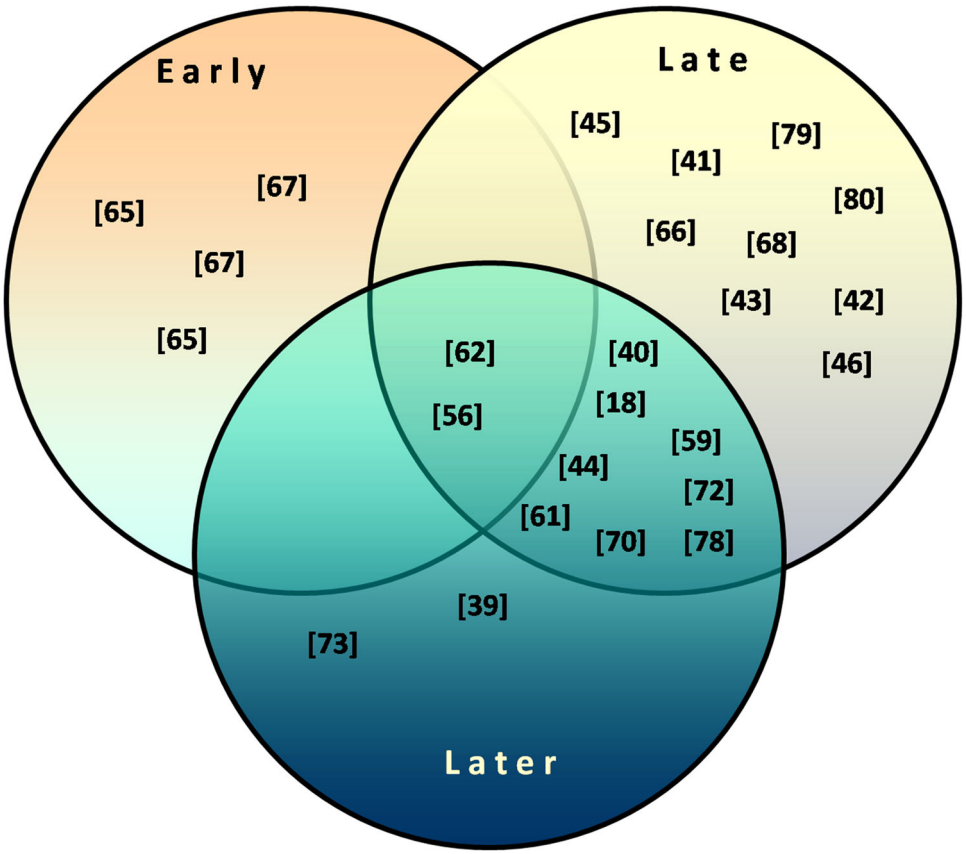

Fig. 1 Venn diagram representing particular research with evidence regarding miR-21 involvement in BC pathogenesis. According to two studies, miR-21 might be important in the early phases of BC. Two studies associated miR-21 with later events only, nine studies associated miR-21 with late events, two studies associated miR-21 with early, later and late phases simultaneously, while eight studies associated miR-21 overexpression with later and late events simultaneously. Nineteen reports associated miR-21 overexpression with the late phase, 12 associated miR-21 overexpression with the later phase, and only six associated miR-21 overexpression with the early phase of BC pathogenesis. *Numbers* in *square brackets* represent the numbers of the references, as per the reference list. *BC* breast cancer

The described online tools selected a large number of candidate genes. Based on experimental and literature research, the selected genes were informative for BC pathogenesis (*TIMP3*, *PDCD4*, and *RECK*), and some genes were added that were experimentally proven to be targets of miR-21, such as *TPM1* and *PTEN*.

4.1 Software that Recognized a Particular Target of miR-21

Based on in silico analysis *TIMP3*, *PDCD4*, *RECK* and *TPM1* mRNAs were extracted as targets of miR-21 in BC. Cited programs use parameters such as 3′ base pairing, the type of target site, adenine uracil dinucleotide (AU) density, and location of the target site as parameters for scoring [88]. All five software tools used in our analysis recognized *TIMP3*, *PDCD4* and *RECK* as potential targets of miR-21 (Table 3). What is very interesting is that only one software program (MiRTarBase) recognized *TPM1* and *PTEN* (Table 3), although they have been the most researched genes in association with miR-21 (in comparison with *TIMP3*, *RECK* and *PDCD4*). They were also experimentally proven as targets of miR-21. To date, no research has experimentally proven that *RECK* was the target of miR-21 in BC cells, although it was described as an important tumor suppressor involved in BC propagation [89]. All the online tools used recognized *RECK* as a potential target of miR-21.

4.2 Targets of miR-21 and Phases of BC Pathogenesis That They are Involved In

TIMP3, a member of the metalloproteinase inhibitor family, represents one ECM-binding protein that locally inhibits matrix metalloproteinases (MMPs). TIMP3 activity suppresses angiogenesis [90], invasion, and metastasis formation [91, 92], and several studies described its tumor suppressor role in promoter methylation-based studies [93]. *TIMP3* promoter methylation was detected in 21–27 % of patients with invasive ductal carcinomas [94].

PDCD4 is a tumor suppressor involved in invasion, tumor promoting inflammation, and avoiding cell proliferation and cell death, according to Buscaglia and Li [62]. In general, PDCD4 is connected to resisting cell death, tumor-promoting inflammation, and evading growth suppressor in cancer. It was explained that human epidermal growth factor receptor 2 (HER-2) stimulated expression of miR-21 which silenced *PDCD4* in BC cells [95, 96].

Table 3 Software and online tools used for miR-21 target prediction

miR-21 target	Software tool for target prediction				
	TargetScan Human 7.0	microRNA.org	PicTar	miRDB	MiRTarBase
TIMP3	+	+	+	+	+
PDCD4	+	+	+	+	+
PTEN	-	-	-	-	+
TPM1	-	-	-	-	+
RECK	+	+	+	+	+

Symbol + is present if the software program recognized the target of miR-21, while symbol – is present if the software program did not recognize the target of miR-21

Table 4 The role of miR-21 targets molecule in cancer pathogenesis

	The role of miR-21 target in cancer pathogenesis					
Hallmarks of cancer *Protein product of miR-21 target gene*	Maintenance of proliferative signaling	Tumor-promoting inflammation	Induction of invasion and metastasis	Angiogenesis	Avoiding cell death	Evading growth suppressors
TIMP3			+	+	+	
PDCD4		+	+		+	+
PTEN	+					
TPM1			+			
RECK			+	+		

Representation of the involvement of miR-21 target genes described by Buscaglia and Li [62] in the hallmarks of cancer described by Hanahan and Weinberg [9] in breast cancer. Most of the target genes protein products [TIMP3, PDCD4, TPM1 and RECK (four of five described in this article)] were involved in the late phases of breast cancer pathogenesis invasion and metastasis (the darkest column). Two target genes were important for early to later events (avoiding cell death), i.e. *TIMP3* and *PDCD4* (moderately shaded column), and two were linked to angiogenesis, i.e. *TIMP3* and *RECK* (also moderately shaded column). *PDCD4* was connected with evading tumor suppressor events and tumor-promoting inflammation, while *PTEN* was involved in the maintenance of proliferative signaling (the lightest columns)

PTEN, as a tumor suppressor, inhibits the Akt pathway by phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) dephosphorylation. In BC cells, PTEN has the ability to control the cell cycle, induce apoptosis, and regulate cell-cycle arrest. *PTEN* overexpression results in a cell death and cell-cycle arrest [97, 98]. Inhibition of *PTEN* by miR-21 interference may cause neoplastic transformation, and may also facilitate migration and enable invasion and metastasis [36]. Protein products of *PDCD4* and the *PTEN* gene are regulators of cell proliferation, apoptosis, and stimulation of tumor-promoting inflammation. These events are responsible for tumor progression and propagation through stages and grades, and for invasion and metastasis [62, 63].

TPM1 (tropomyosin 1) is a protein necessary for cellular migration due to its role in muscle contraction by regulating mechanisms of actin [62]. Elements of cytoskeleton, in addition to its basic role in cell shape and structure maintenance, or the role in cell motility, might also have a crucial place in neoplastic transformation, apoptosis, cell growth, proliferation, and contact inhibition, which all are processes related to tumor formation and progression [99]. *TPM1* was experimentally proven to be a target of miR-21 in the MCF-7 cell line [55]. As further evidence that miR-21 might be more significant for invasion rather than formation, we note that Buscaglia and Li [62] reported that miR-21, by silencing *TPM1*, emphasizes its role in tumor

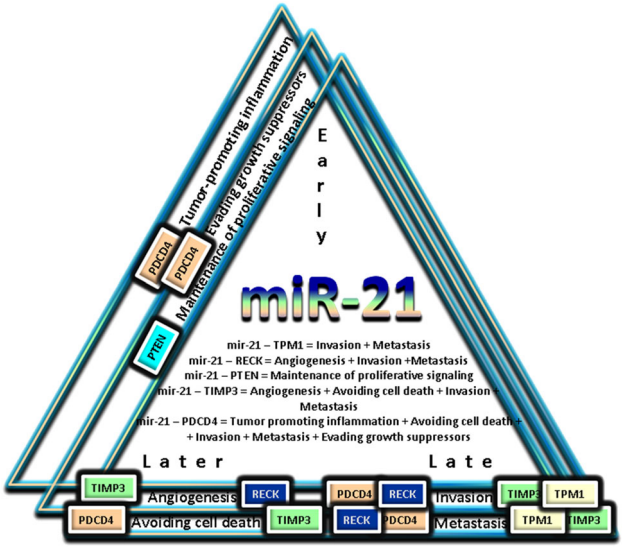

Fig. 2 Schematic view of miR-21 targets and their involvement in early, later, and/or late phases of BC pathogenesis. *Left sides* of the equations represent translational repression of particular targets mediated by miR-21, while the *right side* shows potential consequences

progression by influencing cellular migration rather than apoptosis; therefore, the actions of TPM1 might be more important for the process of invasion rather than tumor formation.

RECK, similar to TIMP3, is an unusual inhibitor of metalloproteinases because it is bound to ECMs. Downregulation of *RECK* lacks inhibition of MMPs and promotes invasion and metastasis, which was shown in different BC cell lines [89]. miR-21 might silence *RECK* mRNA and promote later events during BC pathogenesis, rather than initial phases, but *RECK* is still not confirmed in vitro to be a direct target of miR-21 in BC cells, but was proven to be an important negative regulator of BC progression [89]. There is evidence that *RECK* is a direct target of miR-21 in both NSCLC [100] and glioblastoma cells [101].

During this research, hallmarks of cancer were conjoined with early, late, or later phases of BC pathogenesis. Three hallmarks of cancer (avoiding cell death, maintenance of proliferative signaling, and tumor promoting inflammation) were classified into the early-to-later group as it was impossible to assign such a complex event with a single-time determinant. Angiogenesis was linked with early and late events. Evading growth suppressors hallmark was preferably connected to early events, while invasion and metastasis were related to late events of BC pathogenesis. Targets of miR-21 (significant for BC pathogenesis) are more likely to be involved in invasion than in the initial phases of cancerogenesis, according to the analysis described in Table 4 and Fig. 2. A potential role of miR-21 target genes was analyzed (described in Buscaglia and Li [62]) in the hallmarks of cancer described by Hanahan and Weinberg [9]. Four of five miR-21 target genes (*TIMP3*, *PDCD4*, *TPM1* and *RECK*) were involved in the late phases of BC pathogenesis, invasion and metastasis (Table 4; Fig. 2). Two target genes, *TIMP3* and *PDCD4*, were associated with early-to-later events, such as avoiding cell death, while two (*TIMP3* and *RECK*) were associated with early and late event angiogenesis (Table 4; Fig. 2). PDCD4 is also important with regard to evading tumor suppressor events and tumor-promoting inflammation, while PTEN regulates maintenance of proliferative signaling (Table 4; Fig. 2). It could also be noted that *TIMP3* (as one of the most often-described targets of miR-21 in BC) was associated with the following events: resisting cell death (early to later event), induction of invasion and metastasis (late), and angiogenesis (early and late) (Table 4; Fig. 2).

5 Conclusions

According to this review, literature research, and the role of its target genes, miR-21 overexpression might be described as a signature of later (promotion) and late (progression, especially invasion and metastasis) phases of BC rather than early events, such as neoplastic transformation. miR-21 might also be proposed as a factor whose levels significantly change during BC propagation and progression (through the EMT, migration, invasion, and metastasis). miR-21 expression levels could be used as a factor for the presence of BC, as well as the presence of metastatic state, in combination with other standard and non-standard diagnostic/prognostic parameters and tools. On the other hand, it might also be used as a factor for early invasion detection (a biomarker of EMT) and a factor for prognosis in patients with invasive BCs. Targeting miR-21 in the future might reverse, slow down, or stop progressive tumor activities, especially tumor growth, invasion, and metastasis.

Acknowledgments The author would like to thank Ivana Radulović for editing and proofreading this work.

Compliance with Ethical Standards

Conflicts of interest Nina Petrović declares no conflicts of interest.

Funding This work was supported by the Ministry of Education and Science, Republic of Serbia (grant number ON173049).

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