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MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer

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ABSTRACT

Despite advances in detection and therapies, breast cancer is still the leading cause of cancer death in women worldwide. The etiology of this neoplasm is complex, and both genetic and environmental factors contribute to the complicated scenario.

Gene profiling studies have been extensively used over the past decades as a powerful tool in defining the signature of different cancers and in predicting outcome and response to therapies.

More recently, a new class of small non-coding RNAs, microRNAs (miRNAs), able to regulate gene expression binding seed sequences on the 3′UTR of mRNA targets, has been linked to several human diseases, including cancer. An increasing amount of experimental evidence shows that miRNAs are aberrantly expressed in different tumour types, and that they can have a causal role in tumourigenesis.

Here, we describe and discuss the evidence supporting the association between miRNAs and breast cancer, underlining their role in the development of this neoplasia, and the impact on putative innovative therapeutical approaches.

1. Introduction

Profiling of tumours through microarray technologies has greatly improved over the past decades, and it has been used as a powerful tool in expanding knowledge on cancer etiology. Gene expression profiles provide specific molecular signatures containing information able to explain the mechanisms of tumour development and progression, and they open up new possibilities in predicting clinical outcome or response to treatment. However, although these technologies have provided most of the new biomarkers with potential use for diagnosis, drug development, and tailored therapy, they have not exhaustively elucidated all the detailed mechanisms at tumour origin, thus suggesting that tumourigenesis may occur through novel or poorly characterised pathways.

In recent years new players have been revealed in the biology of cancer: microRNAs (miRNAs), a small class of non-coding RNAs able to regulate gene expression at post-transcriptional level, binding through partial sequence homology the 3′ untranslated region (3′ UTR) of target mRNAs, and causing a block of translation and/or mRNA degradation.[1] Several studies have indeed demonstrated that miRNAs are highly specific for tissue and developmental stages, that they play important roles in essential processes, such as differentiation, cell growth, stress response and cell death[2,3], and are involved in several human diseases, including cancer. Microarray technologies have been applied to this new class of molecules as a tool to recognise miRNAs differentially expressed between normal and tumour samples, as reviewed by Calin and Croce.[4] Moreover, recent exciting and

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encouraging data demonstrated how miRNAs can be associated with well-defined clinico-pathological features and outcome in different tumour types.

We will describe the experimental evidence underlying the involvement of miRNAs in breast cancer, and we will discuss their possible use as markers of diagnosis and prognosis, and eventually as new targets or tools of a specific therapy.

2. MiRNAs are aberrantly expressed in human breast cancer

Genome-wide miRNA expression studies have been providing over recent years an increasingly detailed portrait of the involvement of these small regulatory molecules in human cancer.

The first reports describing the existence of a miRNA signature characterising human breast cancer were published in 2005[5–7], suggesting the involvement of miRNAs in the pathogenesis of this human neoplasm. Lu and colleagues[7] used a beads-based flow cytometric technique to evaluate the miRNA profiling in different tumour types, including breast cancer. The authors reported that miRNA expression, globally down-regulated in tumours in comparison to normal tissues, classifies human cancers according to developmental lineage and differentiation status more accurately than mRNA expression profiling. Our group[5] described the first breast cancer-specific miRNA signature performing a genome-wide miRNA expression analysis on a large set of normal and tumour breast tissues, resulting in the identification of a list of 29 microRNAs differentially expressed, and able to classify tumours and normal tissues with an accuracy of 100%. Among the miRNAs differentially expressed, miR-10b, miR-125b and miR-145 were down-modulated, while miR-21 and miR-155 were up-modulated, suggesting that these miRNAs could exert a role as tumour suppressor genes or oncogenes. From the same group, Volinia et al.[6] further examined the miRNA expression signature in a panel of different solid tumours, including breast, and found a list of miRNAs deregulated in common among the different signatures, as miR-21 and miR-191. More recently, Blenkiron et al.[8] did not observe a perfect separation between breast carcinomas and normal tissue according to miRNA expression, but a subset of miRNAs were differentially expressed in the subgroups of tumours originally described by Sorlie[9]: luminal, normal-like, HER2-positive and basal, with clinical implications.

Despite some discrepancies in the results described by different studies, which could be due to the application of different microarray platforms, techniques or analytical tools, these evidences underline the role of miRNAs in the biology of breast cancer, and provide the starting point to investigate new molecular mechanisms of breast cancer initiation, progression and metastasis.

3. miRNA expression regulation in breast cancer: Genomic changes and epigenetic mechanisms

Several studies have investigated the molecular alterations leading to an aberrant miRNA expression in cancer, identifying chromosomal aberrations[10,11], epigenetic mechanisms or abnormalities in miRNA-processing genes.

In our miRNA expression study[5], we suggested the involvement of genomic alterations as the mechanism of miRNA expression regulation, analysing their chromosomal localization: miR-125b, for example, down-modulated in breast cancer, is located at chromosome 11q23-24, one of the regions most frequently deleted in breast, ovarian and lung tumours, while miR-21, overexpressed in tumours, is located at chromosome 17q23, amplified in breast cancer. These suggestions were then confirmed by a genomic study published in 2006, where Zhang and colleagues[12] performed a high-resolution CGH array of three epithelial tumours (breast carcinoma, ovarian carcinoma and melanoma), showing that 73% of miRNA genes in breast cancer are located in regions with DNA copy number abnormalities. Notably, their genomic data were in agreement with our expression profiling, being that 82% of the miRNAs overexpressed and amplified, and 60% down-modulated and deleted. A strong correlation between expression and genomic alteration for miR-33 and miR-320 was also reported by Blenkiron et al.[8] This group also investigated possible alterations in the miRNAs processing machinery in human breast cancer, observing significant changes in the expression of DICER1 and AGO, enzymes involved in miRNA maturation and function, in different tumour subtypes.

Recently, evidence has shown that miRNAs may also be regulated by epigenetic mechanisms, as changes in genomic DNA methylation pattern, in different tumour types.[13–15] In a recent study performed on epithelial ovarian cancer[16], we also suggest that the overexpression of some miRNAs, such as miR-21 and miR-203, could be due to DNA hypomethylation. In breast cancer, a recent paper by Lehmann et al.[17] shows that miR-9-1 is hypermethylated and consequently down-modulated in breast cancer. Evidence also proves that chromatin remodelling exerts a role in miRNA dysregulation in breast cancer: Scott and colleagues[18] showed that in SKBR3 breast carcinoma cells histone deacetylase inhibition is followed by extensive and rapid alteration of miRNA levels.

In summary, both genomic instability and epigenetic changes cooperate in leading to an aberrant miRNA expression in breast cancer.

4. Biological effects and targets: New players in the biology of breast cancer

Having selected candidate miRNAs likely to be involved in the biology and development of breast cancer, the important issue then is to investigate their functional role.

Being that miRNAs are able to explicate their function through regulation of specific mRNAs, a great interest has always been addressed to the identification of target molecules: let-7 suppresses RAS[19] and HMGA2[20,21]; miR-15 and miR-16 target Bcl-2[22]; miR-372 and miR-373 are novel oncogenes in testicular germ cell tumours that numb the p53 pathway.[23]

One of the first miRNAs identified as a potential oncogene was miR-21, which was shown to promote cell survival and proliferation initially in glioblastoma cells.[24] In breast cancer, where miR-21 is overexpressed[5], the inhibition of its function using antisense molecules suppressed both *in vitro* and *in vivo*

growth, and led to increased apoptosis, the effect mediated by the direct down-modulation of the anti-apoptotic factor Bcl-2.[25] Zhou and colleagues[26] identified another molecule targeted by miR-21 in breast cancer, Tropomyosin 1 (TPM1), which was able to exert an anti-oncogenic function binding microfilaments and regulating cytoskeleton. More recently, the oncosuppressor PDCD4, previously supposed as a putative target in pancreatic tumours by Roldo et al.[27] according to a comparison between gene profiling and miRNA signature, and confirmed in colorectal cancer[28], has been validated in breast carcinoma MCF7 cell line by Frankel et al..[29] TGFβ has also been predicted as a potential target by multiple methods[5]: the suppression of the inhibitory signalling TGFβ-mediated could be responsible for cell growth enhancement and breast cancer development.

The expression of miR-10b, found down-modulated in breast carcinoma in comparison to normal tissue[5], has subsequently been analysed in correlation with clinical progression and presence of metastasis by Weinberg's group.[30] They observed that miR-10b was indeed down-modulated in all the breast carcinomas from metastasis-free patients, but, surprisingly, 50% of metastasis-positive patients had elevated miR-10b levels in their primary tumours. Induced by transcription factor Twist, miR-10b inhibits the translation of mRNA encoding homeobox D10, releasing the expression of the pro-metastatic gene RHOC and thus leading to tumour cell invasion and metastasis.

miR-17-5p is part of the polycistron miR-17-92[31] and, through E2F1 modulation, it participates in the control of the balance between cell death and proliferation driven by c-Myc.[32] In breast cancer, it seems to exert the role of a tumour suppressor: binding to the 3′UTR of AIB1 (Amplified in Breast Cancer-1 protein), and inhibiting its trans-activation function, miR-17-5p suppresses the oestrogen-dependent and independent breast cancer cell proliferation.[33]

miR-27a is another miRNA that seems to play an important role in breast cancer: suppressing the expression of the transcription factor ZBTB10/RINZF, as first demonstrated by Scott and co-workers[18], miR-27a leads to the overexpression of Sp factors, and to the consequent increase of several survival and angiogenic molecules, such as Survivin, VEGF and VEGFR1.[34]

miR-125a and -b, down-regulated in breast cancer[5], are involved in the regulation of ERBB2 and ERBB3, two important tyrosine kinase receptors often deregulated in breast cancer[35,36], impairing the downstream signalling pathway and the ability of SKBR3 cell line to grow and invade.[37]

Another crucial pathway in breast cancer is oestrogen mediated signalling: miR-206, differentially expressed between ER+ and ER– breast carcinomas[5], has subsequently been validated by another group, who also demonstrated that miR-206 is able to directly target ERα.[38]

Even though no investigations have yet been performed in a model of breast carcinoma, it is mandatory to underline the important evidence that recently connected miRNAs to the well known 'genome guardian' p53: members of the miR-34 family are direct proapoptotic transcriptional targets of p53[39,40], and they mediate its regulatory function by turning off survival molecules.[41] Considering the existence of breast cancers carrying a WT p53, the loss of miR-34a could be one of the mechanisms used by tumour cells to escape the control of a functioning p53 and to survive oncogenic stimuli. Restoring the ability of tumour cells to undergo apoptosis, the enforced re-expression of miR-34 in breast cancer could also contribute to improving the responsiveness to chemotherapy or other treatments.

The cartoon reported in Fig. 1 illustrates the involvement of miRNAs in the complicated network of the molecules previously described.

Nevertheless, to make the story even more complicated, another group recently reported the quite shocking evidence that miRNAs can also lead to the overexpression of target genes, subverting the idea of an exclusive, inhibitory role, and thereby introducing new challenging perspectives in the miRNA scenario.[42]

5. miRNAs as diagnostic and prognostic tools

An increasing and encouraging number of evidences demonstrate how miRNAs can correlate with well-defined clinicopathological features and disease outcome: in CLL, a unique signature of 13 miRNAs could discriminate tumours according to prognosis and disease progression[43]; in lung cancer, poor prognosis correlates to the expression of miR-155 (high) and let-7a-2 (low)[44]; in human pancreatic cancer, Bloomston and colleagues[45] identified a miRNA, miR-196a-2, that may significantly impact survival.

Considering that miRNA profiling seems to correlate with cell differentiation and development more accurately than gene profiling, it is very interesting and important to evaluate if and how miRNAs could be used as cancer biomarkers. In our profiling[5], we analysed miRNA expression in different groups of tumours classified according to specific biopathologial features, such as expression of hormone receptors, grade and stage of the disease, vascular invasion and proliferation index. While we could not identify miRNAs differentially expressed according to HER2 receptor expression, another group[46] showed that the miRNA signature was actually able to discriminate between HER2+ and HER2– tumours. Blenkiron et al.[8], on the contrary, could not find any miRNA related to either HER2 status or tumour stage and vascular invasion, but, according to their study, miRNA signatures are able to classify the five molecular breast tumour subtypes (Luminal A, Luminal B, Basal-Like, HER2+ and Normal-Like), which are characterised by different prognoses.

The expression of several members of the let-7 family was found to be down-modulated in breast cancer samples with either lymph node metastasis or higher proliferation index, suggesting that low levels of this miRNA could be associated with poor prognosis[5]; remarkably, a recent report shows that let-7 expression is markedly reduced in BT-IC (breast tumour-initiating cells) and increased with differentiation, and that this miRNA is also able to regulate stem cell-like properties of breast cancer cells.[47] In our expression study, we also highlighted two miRNAs, miR-145 and miR-21, whose expression discriminated between cancer and normal tissues, and that were also differentially expressed in cancers with different proliferation indexes or tumour stage. In particular, miR-145 was progressively down-regulated

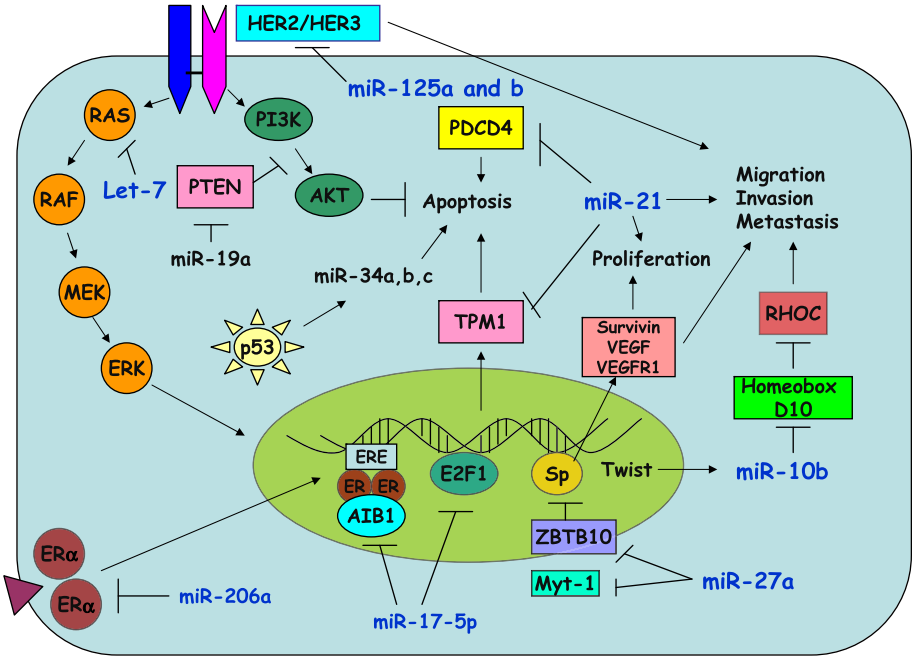

Fig. 1 – miRNAs take their place in breast cancer biology. Summary of the interconnections between miRNAs and tumour suppressor genes and oncogenes in breast cancer.

from normal breast to cancer with a high proliferation index. Similarly, but in the opposite direction, miR-21 was progressively up-regulated from normal breast to cancer with high tumour stage. Another miRNA potentially involved in cancer progression is miR-9-3, down-regulated in breast cancers with either high vascular invasion or presence of lymph node metastasis, suggesting that its down-regulation could be acquired in the course of tumour progression and acquisition of cancer metastatic potential. miR-9-1, the same mature sequence but located on a different chromosome, is also down-modulated in breast cancer, as recently shown by Lehmann et al.[17] miR-10b, as described above, has been correlated with metastatic potential in breast cancer.[30]

Table 1 summarises the information available to date regarding some of the most important miRNAs involved in human breast cancer.

6. miRNAs as possible targets or tools for therapy

The potential usefulness of a miRNA-based therapy in cancer is now being exploited: in breast cancer, the reduced migration and invasion capacities induced by miR-125 or the use of anti-miR-21 to elicit a pro-apoptotic response are important examples.

Moreover, miRNAs involved in specific networks, such as the apoptotic pathway, the HER family-driven or ER-mediated signalling, could likely influence the response to chemotherapy or to targeted therapies, such as Trastuzumab, the monoclonal antibody directed against HER2, or anti-oestrogens, such as Tamoxifen.

Considering the evidences that associate the mechanism of action of Trastuzumab to the perturbation of signalling pathways in a cancerous cell, several miRNAs could mediate

Table 1 – miRNAs in human breast cancer.

Name	Localisation	Expression and role	Targets
MiR-21	17q23.2	Overexpressed[5–7] amplified[12] oncogenic role[25,26,29]	BCL2[25] TPM1[26] PDCD4[29]
MiR-155	21p21.3	Overexpressed[5,6]	
MiR-206	6p12.2	Overexpressed[5,6]	ERα[38]
MiR-125a	19q13.41	Down-modulated[5,6] oncosuppressor[37] down-modulated[5,6]	ERBB2, ERBB3[37]
MiR-125b	11q24.1	Deleted[12] oncosuppressor[37]	ERBB2, ERBB3[37]
MiR-145	5q32	Down-modulated[5,6]	
miR-10b	2q31.1	Down-modulated[5,6],but associated with metastatic potential[30]	Homeobox D10[30]
MiR-9-1	1q22	Down-modulated, hypermethylated[17]	
MiR-27a	19p13.12	Oncogenic role in MDA-MB-231 cells[34]	ZBTB10[34]
MiR-17-5p	13q31.3	Oncosuppressor in breast cell lines[33]	AIB1[33]
Let-7	[a]	Down-modulated[5,6] reduced in BT-ICs[47]	RAS[19] HMGA2[20,21]

a Members of the let-7 family have different chromosomal localisation.

the sensitivity to this drug: one could be miR-21, which directly targets the oncosupressor PTEN in hepatocellular carcinoma[48], and which has also been demonstrated by the same group to influence the response to chemotherapy, in particular to gemcitabine, in human cholangiocarcinoma cell lines.[49] Considering that PTEN has been described as a modulator of responsiveness to Trastuzumab[50], it would be of great interest to evaluate if it is also regulated by miR-21 in breast cancer. Even though evidence from Frankel et al.[29], as well as some preliminary data from our lab, does not support this hypothesis, it is worth investigating the possible correlation between miRNAs and PTEN. Another molecule that seems to play a role in the Trastuzumab-mediated effects is the cell cycle regulator $p27^{kip1}$[51], a target of miR-221 and -222, as recently demonstrated by independent groups in different tumour types.[52–54] The likely hypothesis that these miRNAs are also able to exert their regulatory role on p27 in breast cancer would open the possibility of a therapeutic intervention to improve responsiveness to Trastuzumab.

Besides targeted therapies and chemotherapy, miRNAs could also alter sensitivity to radiotherapy, as recently reported by Slack's group[55]: in lung cancer cells, the let-7 family of miRNAs can suppress the resistance to anticancer radiation therapy, probably through RAS regulation.

The results obtained to date offer the experimental bases for the use of miRNAs as both targets and tools in anti-cancer therapy, but there are at least two primary issues to address to translate these fundamental research advances into medical practice: to develop 'engineered' animal models for cancer-associated miRNAs, and to improve the efficiency of miRNA/anti-miRNA delivery *in vivo*. To overcome this obstacle, modifier miRNA molecules with longer half-lives and increased efficiency have been recently developed, such as the anti-miRNA oligonucleotides (AMOs)[56], the locked nucleic acid (LNA)-modified oligonucleotides[57], and the cholesterol-conjugated antagomirs.[58] To improve the *in vivo* delivery, the methods that have been tested in pre-clinical studies over the past decades for short-interfering RNAs (siRNA) or short heteroduplex RNA (shRNA)[59] could also be applied to miRNAs. Moreover, the advantage of miRNAs over siRNA/shRNA is their ability to affect multiple targets with a single hit, thus regulating a whole network of interacting molecules.

7. Concluding remarks

Over the past decades, great efforts have been spent to elucidate the molecular mechanisms involved in breast cancer and to identify molecules useful as bio-markers of diagnosis or prognosis.

Gene profiling studies have represented a powerful tool to address this issue: different prognostic tests are available according to gene expression, such as the MammaPrint, developed by Van't Veer and colleagues[60], which is able to predict relapse according to the expression of genes regulating cell cycle, invasion, metastasis and angiogenesis. However, miRNAs, which are able to target multiple proteins and are involved in different cellular functions, could provide the information missing in all the signatures described to date according to gene expression.

Moving forward from miRNA profiles to biological studies, results obtained to verify the importance of miRNAs differentially expressed between breast and normal tissue, or among different groups of tumours, are encouraging: even if our knowledge regarding the molecular mechanisms which can explain the role of miRNAs in breast cancer is still incomplete, the evidence available to date clearly shows the involvement of miRNAs in the etiology of this neoplasm.

Moreover, the available data strongly suggest a possible use of miRNAs as markers of diagnosis and prognosis, and eventually as new targets or tools of a specific therapy.

Conflict of interest statement

According to the rules of this journal, I declare that:

(1) All the authors declare that they participated in the planning, execution, or analysis of the study.
(2) All the authors declare that they have seen and approved the final version.
(3) All the authors declare that they have no conflict of interest in connection with this paper.

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