MicroRNAs as biomarkers for the diagnosis and prognosis of human cancer

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Abstract

miRNAs are small-noncoding RNA molecules that regulate gene expression on a post-transcriptional level. A number of oncogenes and tumor suppressors were found to be targets of miRNAs and global miRNA expression signatures were able to distinguish between cancerous and non-cancerous tissues. Therefore it was not surprising that some miRNAs could be linked to the pathogenesis of cancer. In this review we provide an overview of the use of microRNAs as diagnostic and prognostic tools in cancer and focus on the use of miRNA expression as biomarker for disease activity.

Introduction

Although miRNAs were already discovered in the early 90s[1] they did not become the focus of cancer research until the pioneering work of Calin and colleagues was published in 2002.[2] The great novelty of this work was the finding that miR-15 and miR-16 are encoded on chromosome 13q14 in B-cells derived from patients with chronic lymphocytic leukemia (CLL). The 13q14 locus is frequently deleted in human CLL (>50%) and associated with a good prognosis which led the authors to the hypothesis that miRNAs might be involved in the pathogenesis of human cancers. Starting with this report, the number of publications on miRNAs listed in Pubmed has nearly exponentially increased with every year. Nowadays, there is no doubt that miRNAs not only represent novel tools for the characterisation of human cancers that might help to better understand cancer development and maintenance, but also offer new therapeutic options. As a result, one novel treatment concept is the miRNA replacement therapy that introduces miRNA mimics to restore miRNA function in cancers that are dysfunctional for this miRNA.[3] This strategy might offer new perspectives in cancer treatment in the future. Furthermore, there is evidence to suggest that microRNAs are not only involved in tumor biology but can also serve as predictors for cancer progression or therapy response, and are biomarkers for the activity of pathways important in tumorigenesis as has been reported by our group. The importance of miRNAs in this respect and the future perspectives in clinical practice are reviewed here.

miRNAs and human cancer

MicroRNAs are small non-protein coding RNA molecules that regulate gene expression on a post-transcriptional level via binding to the 3' UTR of mRNA targets thus resulting in mRNA degradation or repression.[4-8] More than 5300 human genes are thought to be regulated by miRNAs, amounting to about 30% of all genes and over 60% of all protein-coding genes being known miRNA targets. Many of these miRNAs are expressed in a highly tissue-specific manner and contribute to the establishment and/or maintenance of the characteristic tissue-related gene expression signature.[9-12] Moreover, miRNAs were shown to regulate the fate of cell lineages as nicely demonstrated for the hematopoietic system. During hematopoiesis every step from the multipotent progenitor to mature myeloid and lymphoid cells is regulated by miRNAs.[13]

It is not suprising that miRNAs also play a major role in cancer development and progression. The commonly accepted view is that cancer results from a disturbed balance between factors that either promote or suppress tumor formation.[14] Important oncogenes that are frequently involved in carcinogenesis such as Ras or c-Myc deliver growth signals and increase cellular proliferation or disable tumor suppressor signals (like MDM2), while tumor suppressors like p53 or PTEN counteract these stimuli. For this review we have used a restricted set of cancer-related genes in order to create a graphical representation of the potential roles of miRNA in the establishment of a cancer phenotype. The choice of displayed genetic targets was influenced by the availability of data on miRNA interaction. Needless to say, such a representation greatly oversimplifies the picture, however this illustration is designed to demonstrate proof of concepts rather than create an adequate picture of real transformation processes (Figure 1A) shows a schematic representation of the chosen oncogenic and tumor suppressor genes and their proposed interaction on the levels of mitogenic response or DNA damage. After achieving mutations in these genes and/or after enhanced production of growth signals or mutations in the corresponding signal transduction molecules the imbalance between cellular growth and growth suppression can be disturbed and tumor cells might be generated (Figure 1B). In already established tumors, alterations in these genes might also contribute to a bad prognosis as shown for p53 and ATM loss of function aberrations or increased MDM2 activity in (*see below*). A novel concept now integrates miRNAs in this tumor formation concept and shows that an aberrant expression of key miRNAs that regulate those oncogenes and tumorsuppressors might also contribute to tumor formation (Figure 1C).

miRNAs as biomarkers in cancer

Nearly every human cancer has been extensively microarrayed in recent years. Although, the biological readout of such miRNA signatures, that do not often correspond to each other, might be debatable, it was clear already very early that miRNA expression clearly differs between healthy and malignant tissue[15] and that several oncogenes and tumor suppressors are targets of miRNAs. Therefore, it was an obvious issue to analyse the diagnostic, prognostic and predictive value of microRNAs in human cancer.[16,17] In the following section we will focus on i) the emerging use of circulating miRNAs in cancer diagnosis, ii) two selected miRNAs with an impact on cancer progression, as paradigm for oncogenic miRNAs (miR-21) and tumorsuppressive miRNAs (let-7), and iii) review the role of miR-34a as biomarker for p53 activity, as a prototypic model for a new way to exploit information from miRNA expression studies.

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Circulating miRNAs as biomarkers for cancer diagnosis and metastasis

It was demonstrated, recently, that the signatures of circulating miRNAs can be informative in cancer diagnosis. The first evidence that miRNAs expressed in various tissues might enter the blood stream and remain detectable came from Lawrie *et al.*[18] The authors found that the tumor-associated miRNAs miR-155, miR-210 and miR-21 were increased in serum from diffuse large B-cell lymphoma patients as compared to healthy controls.[18] Using a human prostate cancer xenograft mouse model Mitchell *et al.* could validate the technique of cancer classification by miRNA serum level detection.[19] Subsequent studies found differential miRNA expression in serum and plasma of lung, ovarian and colorectal cancer patients as compared to healthy individuals.[20-22] Also for breast cancer a panel of circulating differentially expressed miRNAs was discovered.[23-25] Among those miR-21 was identified,[26] a miRNA that is frequently deregulated in various types of cancer and often related to cancer progression and prognosis as described in the section below. Not only in solid tumors, but also in leukemias, circulating miRNAs might be useful as biomarkers for cancer diagnosis. In the case of AML the miR-92a/miR-638 ratio in blood serum could best discriminate between leukemic and healthy blood.[27] Such a technology may be especially helpful for the diagnosis of leukemia in patients displaying a so-called aleukemic presentation of AML, where the tumor cells are not present in the peripheral blood, but only in the bone marrow.

Moreover, circulating miRNAs may be informative regarding metastasis. In prostate cancer several circulating miRNAs were correlated to the occurence of metastatic versus localized tumors, with mir-375 and miR-141 as the most pronounced markers for high-risk tumors in general.[28] Also for breast cancer a very recent publication demonstrated the correlation of certain miRNA (miR-34a and miR-155) serum levels to the presence of metastasis.[29] If the diagnostic impact of certain circulating miRNAs can be validated in the future and in large patient cohorts, this would offer the great opportunity to detect cancer and/or metastasis very early during blood routine examiniations, without the need for biopsies, and with a greater chance for successful treatment for each individual patient.

miRNAs as Biomarkers for cancer progression

The first report that linked cancer progression and miRNA expression came from Calin *et al.*[30] The authors found a miRNA signature that discriminated between high and low risk CLL patients. Subsequent analysis revealed a number of further miRNAs that were correlated to disease progression in a great variety of different human cancers, which was excellently reviewed by Croce *et al.*[31,32] In this section we will therefore just focus on let-7 as paradigm for a tumorsuppressive miRNA and miR-21 as paradigm for an oncogenic miRNA.

Let-7

Let-7 has become a prototype for a miRNA that functions as a tumor suppressor as it regulates the expression of the Ras oncogene frequently mutated in cancer.[33] Downregulation of let-7 may lead to overexpression of Ras and thus constitutive active prosurvival and pro-proliferation signalling cascades that lead to cancer (Figure 1C). Indeed, it was reported that low let-7e[34] and let-7-2a[35] expression were associated with decreased overall survival in squamous cell carcinoma[34] and adenocarcinoma[35] patients, respectively, as well as with shortened postoperative survival.[36] In human glioblastoma and prostate cancer cells let-7a replacement therapy has led to decreased proliferation and migration *in vitro* and reduced tumor growth *in vivo*.[37,38] Furthermore, it was established that let-7 was one of 5 miRNAs (miR-25, miR-191, let-7e, miR-34c-5p and miR-34a) that could discriminate between the occurrence of squamous cell carcinoma and adenocarcinoma, two subtypes of non-small cell lung cancer.[34] These data suggest that let-7 can serve both as a biomarker for lung cancer subtypes as well as a prognostic marker that predicts disease progression (and response to treatment).

miR-21

The prototype of an oncogenic miRNA that was frequently found to be overexpressed in human cancer[39-46] (prostate, stomach, breast, pancreatic, colon, glioblastoma and squamous cell carcinoma) is miR-21. This miRNA was shown to regulate a variety of tumor suppressors such as Pdcd4,[47,48] Cdc25A,[49] tropomyosin,[50] BTG2[51] and PTEN.[52] miR-21 overexpression *in vitro* has led to increased proliferation, invasion, migration, metastasis and chemoresistance in pancreatic, ovarian, colon and hepatocellular cancer cells.[41,47,52-54] Knockdown of miR-21 on the other hand could reconstitute normal proliferation and invasion rates[47,51,54] and induced apoptosis.[45,46] *In vivo* elevated levels of miR-21 were associated with advanced tumor stages and the occurrence of metastasis in colon[44] and breast cancer.[55,56] In head and neck,[46] as well as lung cancer,[40] high miR-21 expression could predict shorter overall survival times. The important role of miR-21 in cancer development and progression was reported by Medina *et al.*[57] who generated a mouse model conditionally expressing miR-21. These mice developed a pre-B malignant lymphoid-like phenotype including lymphadenopa-

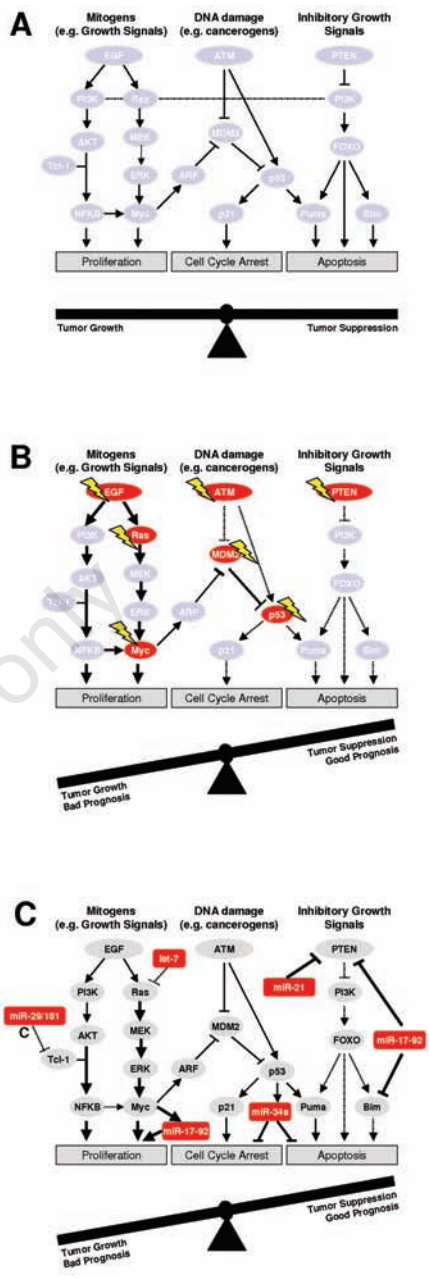

Figure 1. Schematic representation of cancer development. (A) In a normal environment cellular proliferation and growth arrest are tightly controlled and kept in balance to prevent tumor development caused by external stimuli like cancerogenes or internal stimuli like growth signals. (B) Mutations or deletions in genes involved in cell cycle control and/or apoptosis may disturb the balance between tumor growth and tumor suppression and might cause cancer development in response to tumor promoting signals. (C) In the current view, not only classical oncogenes and tumorsuppressors are invovled in cancer development and progression but also miRNAs that are regulated by them, or itself regulate tumor associated genes.

thy and splenomegaly. After miR-21 inhibition tumor cells rapidly died upon apoptosis and tumors regressed in between a few days.[57] A similar study was provided by Hatley *et al*.[58] The authors generated miR-21 gain- and loss-of function mice and found enhanced tumorigenesis in miR-21 gain-of function mice, while miR-21 loss-of function mice were partially protected against tumor formation.[58]

In conclusion, miRNAs can act as tumor suppressors as well as in an oncogene-like manner. In these ways they seemingly can influence disease outcome and may be useful markers to predict survival times. Development of targeting strategies for those miRNAs with distinct contributions to pathogenesis will be very interesting.

miRNAs as Biomarkers for gene activity

Not all miRNAs displaying prognostic power in human cancer necessarily have a unequivocal role in disease pathogenesis. It may also be possible that miRNAs may be useable as readouts of the complex genetic pathways involved in cancer and that assessment of their quantity may allow to define interesting surrogate markers for tumor behaviour. We present the example of miRNA-34a in CLL. A clearly defined individual role of this miRNA in the pathophysiology of CLL has not yet been elucidated, but the information yielded by its analysis still yields extremely valuable information. We use the example as a paradigm for additional future exploration in the field of miRNAs.

miR-34a is a reporter of p53 pathway activity

miR-34a belongs to the miR-34 family that consist of two further members: miR-34b and miR-34c. While miR-34a is encoded on chromosome 1 (1p36), mir-34b and c share one transcript on chromosome 11 (11q23), and are expressed in a more tissue specific manner compared to miR-34a. In 2007 five groups in parallel could show that miR-34a is regulated by p53 on a transcriptional level.[59-63] miR-34a induction upon DNA damage and oncogenic stress was highly dependent on the presence of functional p53, which binds to evolutionary conserved binding sites upstream of the miR-34a coding sequence.[59-63] Recently, a direct connection between miR-34a expression levels and aberrations in p53, namely del17p13 and p53 mutations, could be drawn in CLL.[64-67]

It is well established that p53 is one of the most important cancer associated genes and this is reflected in the fact that 50% of all human cancers show deletions or mutations in p53. The tumor suppressor role of p53 is mediated via its central position in signalling pathways that induce apoptosis, DNA damage response, and stop cellular proliferation (Figure 1B). In CLL approximately 5-12 % of patients carry either mutations or deletions in p53.[68,69] Both defects are strong independent prognostic markers and associated with a worse overall and treatment free survival.[68,70] Moreover, CLL patients with p53 mutations or deletions are often refractory to standard chemotherapy e.g. fludarabine, which is reflected in a relative increase of p53 del/mut patients to 40% in the fludarabine refractory patient group.[71] The significantly inferior efficacy of even the currently best chemoimmunotherapy regimes (CLL8), and the fact that some treatment modalities (such as Alemtuzumab) may have a relatively better rate of response even in this high risk group, makes the identification of patients that harbor p53 defects of particular importance in clinical practice.[72,73]

However, not only direct defects in p53 strongly affect disease progression and therapy response, but also indirect ones, as described for the p53 upstream target ATM, which is induced in response to DNA damage, or the negative p53 inhibitor MDM2, which controls p53 degradation via the proteasome in unstressed cells. The impact of both genes have been described in CLL. Deletions and mutations of ATM (del11q) have a similarly poor prognosis and response to therapy as defects in p53 itself.[69,74,75] Similarly, increased MDM2 transcriptional activity due to the SNP-309 polymorphism in the MDM2 promoter[76] leading to high p53 degradation in stressed and unstressed cells, indeed negatively influences overall- and treatment-free survival in CLL as has been described by our laboratory.[77] This, however, means that 5 independent analyses (p53 deletion, p53 mutation, ATM deletion, ATM mutation, SNP-309) would be needed to predict whether an individual CLL patient belongs to a high risk group with respect to defects along the p53 axis and thus should be treated accordingly (Figure 2A).

In our report we established miR-34a to be an effective and reliable surrogate marker for p53 activity.[67,78] Interestingly, nearly all CLL patients that were classified as having low miR-34a expression showed defects along the p53 axis, i.e. they were also positive for p53 mutation, p53 deletion, ATM deletion, or the SNP-309 in the MDM2 promoter. The predictive value of miR-34a expression with respect to p53 dysfunction was therefore calculated to approximately 90%. Furthermore, the single analysis of miR-34a expression as a prognostic marker could effectively predict treatment-free survival intervals accurately and with a power that was comparable to the combination of the multiple FISH, PCR, and sequencing assays generally used to establish the status of the p53 axis. In summary our work suggested, that miRNA 34a could be used as a reliable biomarker for p53 pathway integrity in p. Given that clinically information about the integrity of the p53 response in CLL is thought to be the most important factor that determines treatment outcome and survival, a prospective analyses validating of the power of miRNA-34a determination in predicting prognosis and treatment outcome is eagerly awaited.

Conclusions

miRNA profiling is proving invaluable in the study of human cancers. The analysis of miRNA expression patterns may aid in the unraveling of individual lesions central to tumorigenesis, as seems to be the case for mir-15/16, Let-7 or miR-21 to name but a few.

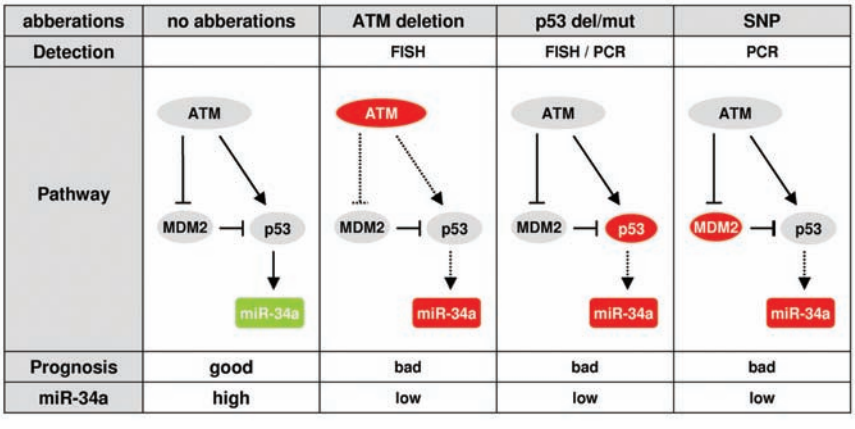

Figure 2. miR-34a is a biomarker for p53 activity. In CLL miR-34a is expressed at high levels if the p53 pathway is intact. Aberrations along the p53 axis (e.g. deletion/mutation of ATM or p53, SNP-309 in MDM2) that are associated with a bad prognosis cause decreased miR-34a expression. Thus the miR-34a expression level serves as biomarker for p53 activity and is related to bad prognosis in CLL.

However, miRNA expression patterns may also reflect the outcome of complex interactions along a more conventional tumor suppressor/oncogene pathway, thus allowing the definition of parameters that impact that pathway as well. Our work with miR-34a in CLL provides proof of principle for the latter,[67,78] thus opening the field for further discovery, clinical application, and ultimately, improved management of disease.

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