Genome-Wide Epigenetic Regulation of miRNAs in Cancer

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Abstract

Aberrant microRNA (miRNA) expression contributes to tumorigenesis and cancer progression. Although the number of reported deregulated miRNAs in various cancer types is growing fast, the underlying mechanisms of aberrant miRNA regulation are still poorly studied. Epigenetic alterations including aberrant DNA methylation deregulate miRNA expression, which was first shown by reexpression of miRNAs upon pharmacologic DNA demethylation. However, studying the influence of DNA methylation on miRNA transcription on a genome-wide level was hampered by poor miRNA promoter annotation. Putative miRNA promoters were identified on a genome-wide level by using common promoter surrogate markers (e.g., histone modifications) and were later validated as such in different tumor entities. Integrating promoter datasets and global DNA methylation analysis revealed an extensive influence of DNA hyper- as well as hypomethylation on miRNA regulation. In this review, we summarize the current knowledge of the field and discuss recent efforts to map miRNA promoter sequences and to determine the contribution of epigenetic mechanisms to the regulation of miRNA expression. We discuss examples of tumor suppressive and oncogenic miRNAs such as the miR-34 and miR-21 family, respectively, which highlight the complexity and consequences of epigenetic miRNA deregulation. Cancer Res; 73(2); 473–7. ©2012 AACR.

Introduction

MicroRNAs (miRNA) are small noncoding RNAs that regulate gene expression posttranscriptionally by binding to complementary sequences in the 3’ untranslated region (UTR) of messenger RNAs (mRNA). The interaction results in mRNA cleavage or inhibited protein synthesis, leading to reduced protein expression of the targeted gene. Thus, up- or down-regulation of miRNAs influences the expression of oncogenes and tumor suppressor genes. The miRNA expression profiling experiments were carried out in a number of different cancer entities and identified a large set of aberrantly regulated miRNAs, of which many were deregulated in several tumor entities. The miR-21, for example, targets the tumor suppressor gene PTEN in lung cancer and other malignancies, and miR-15a/16-1 represses the antiapoptotic BCL2 in prostate carcinoma and chronic lymphocytic leukemia (CLL; ref. 1). The molecular mechanisms regulating normal miRNA expression or causing their deregulation in malignant diseases are mainly unknown.

Epigenetic modifications, such as aberrant CpG methylation, contribute to deregulated gene expression in cancer cells. Methyl groups are transferred to the carbon atom at position 5 of cytosines by DNA methyltransferases (DNMT1 or DNMT3A and DNMT3B). Methylation of promoter-associated CpG dinucleotides (especially in CpG islands) usually correlates with reduced transcription levels of the respective gene; promoter hypermethylation of tumor suppressor genes causes their silencing in cancer cells (2). In tumors, miRNAs were suggested to be deregulated by similar mechanisms, yet poor annotation of miRNA promoters hampered global analyses of their regulation (3). The miRNAs are either located outside of other transcripts and thus are transcribed independently (intergenic miRNAs) or reside within the intron of a host gene (intragenic) and can be cotranscribed. However, miRNA-specific promoters have also been described for intragenic miRNA (4).

Maturation of miRNAs involves several processing steps starting from a primary transcript, which may vary in length from several 100 base pairs (bp) to far more than 10 kb. Primary transcripts may contain several polycistronically arranged miRNAs (5) and introns and exons of protein-coding genes. For example, the 33.8 kb transcript including miR-34a contains one 30 kb intron, and only a small part of the second exon encodes the mature miR-34a (6). Most primary miRNAs are cleaved in the nucleus by the RNase complex Drosha–DGCR8 to an approximately 70 nt precursor miRNA (pre-miR). Pre-mirs are exported to the cytosol, where they are processed by the endonuclease Dicer giving rise to the mature miRNA (21 nt), which ultimately regulates protein expression (1). Defects in miRNA processing can contribute to aberrant expression of mature miRNAs in cancer. For example, DICER1 mutations were observed in pleuropulmonary blastoma (7) or tumors of the reproductive system including nonepithelial ovarian cancer and embryonal rhabdomyosarcomas (8). TARP2 is part of the Dicer-containing complex and carries mutations in hereditary carcinomas with microsatellite

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doi: 10.1158/0008-5472.CAN-12-3731
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instability (9). In the same tumor entities, miRNA maturation can be impaired by mutations in EXPORTIN-5, which mediates the nuclear export of precursor miRNAs (10).

In the cell, primary miRNAs have a very short half-life due to their rapid processing and, hence, are rather difficult to detect. However, to study epigenetic regulation of miRNAs, knowledge of the transcriptional start sites (TSS) of primary transcripts is required.

miRNAs Are Deregulated by Aberrant DNA Methylation

In cell lines, pharmacologic inhibition of DNMTs causes DNA demethylation, which leads to reactivated expression of some miRNAs, suggesting DNA methylation as the underlying cause of repression. As an example, treatment of the human bladder cancer cell line T24 with the DNMT inhibitor 5-aza-2'-deoxycytidine led to global demethylation. In combination with inhibition of histone deacetylases by 4-phenylbutyric acid, expression of 17 miRNAs could be restored. Among them, miR-127 displayed a prominent transcriptional upregulation, and was shown to target the proto-oncogene BCL6 (11). A similar approach identified epigenetically (de)regulated miRNAs by profiling miRNA expression in colorectal cancer (CRC) cells genetically deficient for DNA methyltransferase (DNMT)-1 and DNMT3B. In this study, miR-124 was identified as epigenetically silenced in CRC (12). Follow-up studies could show that miR-124 is epigenetically silenced in at least 10 different tumor entities including hematopoietic malignancies as well as gastric, liver, cervical, and breast cancer (13).

Because of the limited annotation of miRNA promoters, miRNA located in CpG islands or in close vicinity were among the first candidates to study epigenetic regulation. Mir-9-1 is associated with a CpG island 200 bp upstream, which is hypermethylated in breast cancer as well as in cell lines derived from lymph node metastasis of CRC, melanoma, and head and neck cancer (reviewed in ref. 13). CpG islands are also located adjacent to the miR-200 family members, which are master mediators of the epithelial phenotype. CpG island methylation correlated with downregulated miRNA expression in breast and prostate cancer cell lines (14). In addition, loss of miRNA expression was linked to acquisition of mesenchymal features as observed in epithelial–mesenchymal transition and tumor progression (15). Another early discovered example of silenced miRNAs is miR-1-1. The suspected primary transcript of miR-1-1 is C20orf166; the CpG island covering exon 1 and intron 1 of C20orf166 was found methylated in hepatocellular carcinoma. Ectopic overexpression of miR-1 reduced cell growth in vitro (16), which supported the function of miR-1-1 as a tumor suppressor. Although most of the studies focused on silenced tumors suppressor miRNAs so far, activation of oncogenic miRNAs by epigenetic mechanisms has recently been shown to be equally important. Let-7a-3, for example, was found methylated in normal lung tissue but not methylated in lung adenocarcinoma. The hypomethylation correlated with high expression levels of let-7a-3 in lung cancer cell lines (17). A systematic overview of candidate miRNAs that were identified as targets of epigenetic silencing is reviewed in refs. 13 and 18.

A common characteristic of most candidate-based studies is the focus on miRNAs that are closely associated with CpG islands. However, similar to protein-coding genes, DNA methylation–dependent regulation of miRNAs does not necessarily require the vicinity of CpG islands. An example is miRNA-199a. Upstream of miR-199a-2, located in the intron of DNMT3, a 390 bp stretch, not a CpG island, was differentially methylated in a testicular cancer cell line. The DNA methylation level in the identified region inversely correlated with expression of miR-199a-5p and with tumor malignancy (19).

To comprehensively characterize epigenetic regulation of miRNAs genome-wide and independent of CpG islands, a systematic approach for large-scale identification of primary miRNA transcripts and their promoters was required.

Identification of miRNA Promoters

The first primary miRNA transcripts were experimentally identified by northern blot or rapid amplification of cDNA ends (RACE) leading to the discovery of transcripts that are longer than the precursor miRNA and cover its sequence. For example, a 600 nt RNA molecule constituting the primary transcript of miR-30a was identified by northern blot using a set of probes surrounding miR-30a (5).

However, hybridization- or amplification-based approaches are restricted to individual, short, and highly abundant primary transcripts. To overcome these limitations, surrogate markers for promoter sequences were used in “miRome”-wide screens (4, 20, 21). Polymerase II (Pol II) transcribes not only protein coding genes but also a majority of known primary miRNA transcripts (4). Another surrogate marker for promoter presence (including that of miRNA promoters) is trimethylation of histone 3 at lysine 4 (H3K4me3; refs. 4, 20, 21). Both Pol II- and H3K4me3-associated genomic regions are detectable by chromatin immunoprecipitation (ChIP). Recent ChIP approaches allowed comprehensive detection of such regions on a genomic scale (4, 20). Integration of supporting data such as additional promoter features into bioinformatical models increases the confidence for miRNA promoter detection. These additional data include information on the overlap of putative promoters with start sites of expressed sequence tags, sequence conservation (20), or the annotation of cap analysis gene expression (CAGE) tags (21). Recently, the Encyclopedia of DNA Elements (ENCODE) consortium provided genome-wide profiles of DNA methylation and sensitivity sites (DHS), which are additional surrogate markers for regulatory elements such as promoters (23). As data are available from a large panel of cell lines, the promoters of cell type–specific miRNA can be interrogated. Examples are the promoters of the muscle–specific miR-206 and the endothelium–specific miRNA-126, which display DNA methylation profiles in cell lines derived from muscle cells or endothelial tissue, respectively (24).

The distance of miRNA promoter sequences to the miRNA coding sequence of the precursor miRNA can be up to 50 kb
The difficulty to characterize miRNA promoters and their epigenetic regulation is illustrated by members of the miR-34 family. These members are downstream targets of the p53 pathway (25) and were among the first miRNAs for which aberrant promoter DNA methylation was characterized (6, 25, 26). Three family members are present in the mammalian genome: miR-34a is located on chromosome 1, miR-34b/c reside on chromosome 11 as dicistronic cluster within transcription unit BC021736. Initially, BC021736 was considered the hosting gene of the miR-34b/c cluster. The BC021736 promoter contains 2 p53-binding motives, which were suspected to account for the p53 dependent regulation of miR-34b/c (25). However, only miR-34c resides in one of 2 BC021736 exons, whereas miR-34b covers the exon–intron junction and, hence, is neither part of the fully processed BC021736 transcript nor the spliced intron (Fig. 1A). Moreover, only the expression of miR-34b/c but not of BC021736 was reactivated upon pharmacologic demethylation in the CRC cell line HCT116. As an alternative to the BC021736 promoter, a CpG island, 4.5 kb upstream of the miRNA-coding sequence and overlapping with the TSS of BTG4, was suggested as the promoter for miR-34b/c. Although BTG4 is transcribed in the opposite direction of miR-34b/c, the upstream CpG island locus has promoter function in both directions (26). Hypermethylation of the CpG island was found to be a common feature of leukemias, lymphomas, and solid tumors (e.g., gastric cancer; ref. 13).

The third member of the miR-34 family, miR-34a, resides in the second exon of a 33.8 kb transcript (EF570049), which contains p53-binding sites and a CpG island in the promoter region (Fig. 1B; refs. 6). Hypermethylation of this CpG island in a variety of solid tumors (breast, lung, colon, kidney, bladder, and pancreatic carcinoma) correlated with silenced expression (27). In CLL, miR-34a was upregulated, despite same DNA methylation levels of the EF570049 promoter CpG island in healthy B cells and CLL samples (28). Instead, loss of DNA methylation in patients with CLL and enrichment of H3K4me3, a marker for promoter presence, in cell lines derived from patients with CLL was observed in a region located 12 kb upstream of the miR-34a coding sequence (28). The suspected promoter does not contain a known p53-binding motif and, thus, leaves room for speculation of a p53-independent regulation of miR-34a.

MiR-21 is oncogenic and is upregulated in a large panel of proliferative malignancies (1). MiR-21 is processed from a 3 kb primary transcript overlapping with the terminal genomic sequence of the protein-coding gene VMP1 (Fig. 1C). Yet, VMP1 is not the host gene, as the coding sequence of miR-21 is located downstream of VMP1. The promoter of miR-21 has been well studied in different cell systems, but different putative promoters were defined depending on the analyzed tissue. In the cervical cancer cell line, HeLa, a region spanning from part of intron 10 into part of intron 11 of VMP1 displayed promoter activity (29). A more upstream region only in the tenth intron revealed promoter activity in HEK293T cells, derived from human embryonic kidney, or the melanoma cell line UACC62
(4, 30). The apparently contradictory results indicate different tissue-specific miR-21 promoters. Tissue specificity of promoters should be taken into consideration when analyzing aberrant promoter methylation in disease.

In our CLL study, the promoter sequence closer to miR-21, overlapping with the promoter identified in HeLa, was completely unmethylated in all CLL samples but showed significant DNA methylation in B-cell controls. This was in line with upregulation of miR-21 in CLL cases compared with their healthy counterparts. The fact that miR-21 is overexpressed in different tumor entities suggests DNA hypomethylation as a major mechanism for miR-21 regulation.

Epigenetic Regulation of miRNAs on a Genome-Wide Level

The number of known miRNAs has increased from around 400 in the first promoter characterization in 2008 (20) to more than 1,400 in the recent miRBase 19 release (3). Thus, many of the recent genome-wide studies of miRNA regulation included extended screening for miRNA promoters.

In mammary fibroblasts and epithelial cells, promoters for 232 miRNAs were identified through H3K4me3 profiling. Thirty-eight percent of these promoters showed tissue-specific silencing by DNA methylation. For example, miR-200c/141 was only expressed in epithelial cells and promoter methylation was evident in fibroblasts. Other epigenetic-silencing mechanisms were found to be more frequent at the promoters of tissue specifically repressed miRNAs. 58% were silenced by histone 3 lysine 27 trimethylation, and redundancy of both repressive marks was limited to 21% of tissue specifically silenced miRNAs (31).

H3K4me3 profiling of the CRC cells line HCT116 and its derivative with a DNMT1 and DNMT3B double knockout identified promoter data for 233 miRNAs. For 22 intragenic miRNAs, an independent, miRNA-specific promoter was suggested and for the majority of intergenic miRNAs, the identified promoter was within a 2 kb window. Comparison of the DNMT1 and DNMT3B double knockout and the wild-type cell line revealed epigenetic silencing of 37 miRNA promoters of which 22 contained a CpG island (32). DNA methylation was present at the promoter of miR-124 and miR-34b/c, which was previously identified to be silenced in HCT116 (12, 26) but also for novel candidates, such as miR-338 or miR-944 (32). The function of both miRNAs and other recently identified silenced miRNAs (13) is poorly understood, but their epigenetically silencing could point towards a function as tumor suppressor.

In our study, investigating epigenetic regulation of miRNAs in CLL, promoters for 781 miRNAs were identified by upstream H3K4me3 enrichment using a panel of leukemic cell lines, primary CLL cells, and healthy B cells (28). One hundred and twenty eight miRNAs were targets of recurrent aberrant methylation in CLL cells compared with their healthy counterparts. Hypomethylation accounted for more than 60% of differential methylation and was almost exclusively found adjacent to CpG islands or in CpG-free promoters. The high impact of hypomethylation is in concordance with the recent observations of extensive genome-wide DNA hypomethylation in CLL (33). As previous studies used reactivation through pharmacologic DNA demethylation to assess epigenetic regulation of miRNA, the impact of aberrant hypomethylation in reactivation of miRNAs might have been underestimated.

Outlook

Large collaborations such as the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA) currently create extensive data sets of genetic, epigenetic, and transcriptome profiles of different tumor entities and cell lines. Furthermore, the ENCODE consortium profiled a variety of cell lines for 12 histone modifications and variants including H3K9me3 and acetylation of histone 3 at lysine 9 (H3K9ac) to disclose regulatory regions in the human genome. The resulting data will add to extend our knowledge on tissue-specific and ubiquitous miRNA promoters. As part of the ENCODE project, chromatin accessibility, a hallmark of promoters, and other regulatory elements, was defined by DHS in 125 diverse cell and tissue types. Analysis of 329 miRNA promoters revealed that 300 overlapped with or were close to a DHS (24). The broad variety of tissues analyzed for DHS will not only allow characterizing promoters of tissue-specific miRNAs but might also permit to estimate the number of tissue-specific miRNAs promoters as suggested for miR-21. The importance of tissue specificity is underlined by results from parallel expression profiling, which revealed that only 28% of the known miRNAs were detectable in at least one of 15 cell lines, and 59 miRNAs were expressed in one cell line only (34). The ENCODE consortium is about to publish the genomewide DNA methylation data completing analysis of epigenetic regulation of all gene classes including miRNAs in cell lines. In addition, cancer methylomes are analyzed and will be made publicly available by the ICGC, which, for example, provided the methylomes of patients with CLL (33). In conclusion, integrating data sets from different sources will enable scientists to estimate the global influence of DNA methylation on the regulation of miRNA and their aberrant behavior in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C. Baer, R. Claus, C. Plass
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Claus
Writing, review, and/or revision of the manuscript: C. Baer, R. Claus, C. Plass
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Claus

Acknowledgments

The authors thank Dieter Weichenhan for critically reading the manuscript and Yoon Jung Park for continued support and inspiring discussions. The authors apologize to those whose work was not included because of space limitations.

Grant Support

The authors are supported by the German Carreras Foundation (DJUCS R 10/27). C. Baer holds a stipend of the Helmholtz International Graduate School for Cancer Research.

Received September 23, 2012; revised November 19, 2012; accepted November 27, 2012; published OnlineFirst January 10, 2013.
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