[Frontiers in Bioscience S3, 970-979, June 1, 2011]

Role of miRNA in distinguishing primary brain tumors from secondary tumors metastatic to the brain

Matteo Fassan[1], Kris Sachsenmeir[2], Massimo Rugge[1], Raffaele Baffa[2]

[1] Surgical Pathology & Cytopathology Unit, University of Padova, Padova, Italy, [2] Research, MedImmune LLC, Gaithesburg, MD, USA

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1. ABSTRACT

Cancer is the result of complex processes that involve multiple molecular alterations. The understanding of such complexity has been improved by the advent of a new class of small, noncoding RNA gene products known as microRNAs (or miRNAs). miRNAs play an essential role in cancer development and progression by modulating gene expression binding to target mRNA, causing either mRNA degradation or translation inhibition. Several studies have shown that miRNAs can act either as tumor suppressors or as oncogenes, and that measurement of miRNA expression in malignancies may have diagnostic and prognostic implications. Beyond these valuable features, miRNAs could be potentially used in the future as innovative and targeted therapeutics. Recent *in vitro* and expression profiling studies have identified that specific miRNAs are directly involved in brain carcinogenesis and in the metastatic process. This review focuses on metastasis-related miRNAs and on the role of miRNAs in distinguishing between primary and metastatic brain tumors. In clinical practice, miRNAs could represent a promising new class of cancer biomarkers in the diagnosis and management of brain neoplastic lesions.

2. INTRODUCTION

Metastatic cancer is the main cause of mortality and the most significant process affecting the clinical management of cancer patients (1). Among the others, metastatic tumors of the central nervous system (CNS) are the most common intracranial neoplasms in adults.

It is estimated that 10% to 30% of patients with solid tumors are diagnosed with CNS metastases (2), and fraction rises to 25% in autopsy reports of patients who die of cancer (3). Moreover, in recent decades, an alarming increasing incidence of CNS secondary involvement has been observed due to the aging population and radiological detection of subclinical lesions.

In adults, the most common sources of brain metastases are (in descending order) lung cancer (especially small cell carcinoma and adenocarcinoma), breast cancer, melanoma, renal cancer, and colon cancer. By contrast, in the pediatric population, the most common sources are leukaemia, lymphoma, osteogenic sarcoma, rhabdomyosarcoma and Ewing sarcoma (4).

Invasion and dissemination through the haematogenous route is the preferential system used by metastatic cells to spread into CNS. Even rare, primary neoplasms in the head and neck region (e.g. squamous cell carcinoma, esthesioneuroblastoma) may extend intracranially by direct invasion (5).

In some types of cancers, brain metastases may occasionally be the first presenting feature. Moreover, in up to 10% of the patients, no primary tumor is found at presentation (4). From a prognostic point of view, the pathological characterization of brain malignancies (and the adequate differentiation between primary *versus* secondary CNS lesions above all) remains a diagnostic challenge. In fact, in spite of well established phenotypic and clinical profile, the molecular characterization of CNS lesions is inconsistent. In this view, expanding on such biological information might lead to new prognostic markers and targeted therapies.

3. miRNAs: NEW PRINCIPAL ACTORS ON THE NEOPLASTIC STAGE

In recent years, no biomarker has generated the excitement that accompanying the interest in microRNAs (or miRNAs). They were initially discovered in 1993 because of their role in controlling the timing of *Caernorhabditis elegans* larval development (6). Highly conserved across different species and throughout evolution, miRNAs are a family of endogenous small (19-22 nucleotides), noncoding RNA gene products, that modulate gene expression by binding to target mRNA (7-10).

In humans, miRNAs participate in fundamental biologic processes including cell cycle, differentiation, development, metabolism, and aging (7, 8, 11). Aberrant miRNAs expression is a hallmark of disease, including cancer (9). In fact, miRNAs are expressed in a tissue specific manner, and changes in miRNA expression within a tissue type can be correlated with disease status.

The human genome contains approximately 1,000 miRNA genes (http://microrna.sanger.ac.uk) that are estimated to regulate a third of all genes (7, 8, 11). Commonly, miRNA genes are located in cancer-associated genomic regions, including minimal regions of amplification, loss of heterozygosity, fragile sites, and common breakpoint regions at or near oncogenes or tumor suppressor genes (12).

These noncoding RNAs are initially synthesized for the most part by RNA polymerase II as long primary transcripts (pri-miRNAs), which are subsequently capped and polyadenylated. pri-miRNAs are subsequently processed in the nucleus into stem-loop precursors of ~70 nucleotides (pre-miRNAs) by Drosha RNase III endonuclease. pre-miRNAs are transported out of the nucleus by Ran-GTP/exportin 5 and are further processed by the RNAse III Dicer in the cytoplasm to yield a ~19-22 nucleotides duplex, named miR/miR*. The mature single-stranded miRNA product is then incorporated into the complex known as miRNA-containing ribonucleoprotein complex (miRNP), miRgonaute, or miRNA-containing RNA-induced silencing complex (miRISC) and is used to regulate the expression of target genes. Binding of miRNAs to the 3' UTR of mRNA with perfect or near-perfect complementary sequences induces mRNA degradation, whereas imperfect complementarity often induces translational repression. The seed sequence of miRNAs, representing 7-8 nucleotides in the 5' end, is critical for efficient targeting and miRNAs harboring similar seed sequences can theoretically regulate the expression of a similar subset of genes (7, 8, 12).

In vitro studies have demonstrated that aberrant expression of miRNAs contributes to carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes. The dysregulation of such "oncomirs" has been observed in a variety of hematologic and solid malignancies and miRNA expression has been correlated with biomolecular and prognostic characteristics, indicating that miRNA signatures could be used to ascertain the biological or clinical features of human cancers (7, 8, 12).

The altered miRNA expression observed in human cancer is the result of several different mechanisms: i) chromosomal abnormalities (13); ii) inherited mutations (14); iii) polymorphisms (SNPs) (15); iv) defects in the miRNA biogenesis machinery (e.g. alteration in Drosha or Dicer activity) (16-19); v) epigenetic changes (e.g. altered DNA methylation) (20-28). To complicate this scenario, miRNAs themselves can regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism (21, 29, 30).

Of interest, miRNAs are, in contrast to most mRNAs, long-lived *in vivo* and very stable *in vitro*, which might be critical in a clinical setting and allow analysis of formalin-fixed paraffin-embedded (FF-PE) samples. Several reports have already demonstrated the high reproducibility and accuracy of miRNA expression profiling in archived FF-PE human specimens (9, 31, 32). It has to be emphasized that if FF-PE specimens are considered as an invaluable tool for biomarker discovery and validation, on the other hand they are not always compatible with modern genomic techniques such as gene expression arrays due to mRNA degradation and modification during fixation and processing. The "unlocking" of our histopathology archives of well-annotated FF-PE tissue specimens for miRNA expression profiling will allow the beginning of a new era of systematic molecular evaluation supported by the advanced histopathological and clinical characterization of the specimens: a new intriguing revolution in translational research.

Beyond histopathological applications miRNA shows offer a number of practical advantages. Practical considerations include their relative stability within the circulation (33-36), including their presence in exosomes (37). miRNAs are generally more stable than messenger or total RNA, do not require specific antibody-linked detection reagents of protein biomarkers, and offer the

specificity of nucleic acid detection methods such as RT-PCR. Obtaining miRNA samples suitable for biomarker analysis is relatively non-invasive. In addition to plasma samples, miRNAs have been quantitatively detected from urine (38), stool (39) and sputum (40) samples. In the report of the use of lung cancer patient sputum, the diagnostic sensitivity and specificity of a miRNA was greater than that of sputum cytology (40).

From a therapeutic viewpoint, advances in the understanding of the molecular role of miRNAs in the neoplastic process will significantly contribute to the identification of alternative molecular pathways upon which new therapeutic approaches can be designed and will undoubtedly influence the selection of new therapeutic modalities. In addition, miRNAs have been proposed as new attractive therapeutics: the intrinsic characteristics that permit to miRNAs to be very stable and resistant in FF-PE tissues, allow a longer molecular/structural resistance and activity *in vivo*. This is further supported by the fact that miRNA-based shRNAs inhibit gene expression more potently than traditional stem-loop shRNA in mammalian cells (7-9, 12). Sequence-specific inhibition of miRNAs can be achieved by antisense oligonucleotides that have undergone chemical modifications designed to enhance their stability and specificity. These cholesterol-conjugated antisense RNAs "antagomirs" and/or LNA-modified "antimirs" have been found to function as efficient and specific silencers of endogenous miRNAs in cancer derived cell lines and in mice (7-9, 12). Although exciting, the use of miRNA-based gene therapy in human cancers must still demonstrate high efficiency of target inhibition, with significantly improved patient survival and minimal toxicity. Moreover, development of miRNA-based therapies requires effective pharmacological delivery.

4. miRNAs AND THE ACQUISITION OF THE METASTATIC PHENOTYPE

The establishment of the metastatic phenotype is a multifactorial process and the result of largely unexplained molecular interactions. Over recent decades, molecular genetic studies have been conducted to investigate genes and gene products that drive the metastatic process. However, due to the heterogeneity of the metastatic process and the focal nature of oncogene/tumour suppressor gene alterations, the role of these genes in the onset of metastases and the diagnostic and/or prognostic value of such gene alterations is still limited (41).

Recently, several lines of evidence show how miRNAs are involved in the regulation of biologic processes leading to the acquisition of metastatic potential such as adhesion, migration and invasion, and angiogenesis [Tables 1 and 2, for review (9, 20, 31, 42-52)].

The first published article on this topic by Ma and colleagues (42), described the supporting role of miR-10b in breast cancer invasion and metastasis. Whereas miR-10b was down-regulated in most breast cancers in comparison with normal breast tissue, it was over-expressed in about a half of metastatic breast cancers. *In vitro*, the ectopic expression of miR-10b had no effect on cell proliferation, but increased transwell migration and Matrigel invasion. What is more, *in vivo* studies demonstrated that miR-10b over-expressing tumors exhibit invasive behaviours and were highly vascularised. The up-regulated miR-10b inhibits the translation process of the messenger RNA encoding homeobox D10 (*HOXD10*), resulting in an increased expression of a well-characterised pro-metastatic gene, *RHOC*. These data were supported by the fact that HOXD10 expression disappears progressively in breast tumours showing increasing degrees of malignancy. However, the association of miR-10b with breast cancer metastasis has not been confirmed in a large series of early-stage breast cancers (43).

Following this initial finding, the deregulation of miRNA expression in metastatic human cancer has been demonstrated in other instances too. For example, miR-21, a ubiquitously over-expressed miRNA in human cancer, has been implicated in the acquisition of invasive and metastatic properties by colon and breast cancer cell lines, by targeting multiple tumour suppressor genes, such as *PTEN*, *PDCD4*, *RECK*, *TPM1* and *MASPIN* (31, 44, 45). Moreover, miR-21 over-expression has been associated with an advanced clinical stage and lymph node metastasis in human breast cancer and hepatocellular carcinoma (46, 47).

Similarly to miR-10b, miR-373 and miR-520c did not affect cell proliferation, but promoted migration and invasion of cancer-derived cell lines *in vitro* (50). The two miRNAs possess similar seed sequences, suggesting that they could regulate an overlapping set of gene targets. In fact, among nine shared potential gene targets, CD44 (which encodes a cell surface receptor for hyaluronan) was found to be a direct target of both miRNAs. In cancer samples, miR-373 was upregulated in tumors exhibiting lymph node metastases, and its expression showed an inverse correlation with CD44 expression.

In contrast to the other studies, Tavazoie and colleagues (48) identified, for the first time, miRNAs with metastasis-suppressive characteristics. Comparing miRNA expression of the metastatic nodule versus the unselected breast cancer parental cells, they found miR-335, miR-126, and miR-206 as consistently down-regulated in metastatic samples. In support of these data, miR-335 or miR-126 low expression correlated with poor metastasis-free survival in clinical samples. *In vitro* analysis demonstrated that miR-335 acts as metastasis-suppressor gene by targeting the transcription factor *SOX4* and *TNC* (encoding tenascin C, an extracellular matrix component).

Subsequently, also miR-146a and miR-146b have been demonstrated to inhibit invasion and migration of breast cancer cells by down-regulating NFkB by targeting *IRAK1* and *TRAF6* (53). Of interest, these two miRNAs are encoded on different chromosomes (Table 2), but their mature sequence differs by only two nucleotide at the 3' region, predicting similar and overlapping mRNA targets.

Table 1. Representative miRNAs aberrantly expressed in metastatic cancer and their most significant putative role in the process

Process	Anti-metastatic	Pro-metastatic
EMT	miR-141 miR200a/b/c miR-205 miR-429	miR-10b
Migration & Invasion	miR-146a/b miR-206 miR-335 miR-31	miR-10b miR-21 miR-143 miR-182 miR-373 miR-520c miR-183
Apoptosis	miR-31 miR-206	miR-29a/b/c miR-182
Angiogenesis	miR-15b miR-16 miR-20a/b miR-126	miR-17-92 miR-27a/b miR-19a/b miR-130a miR-221 miR-222 miR-378 let-7f

Table 2. miRNAs involved in tumor metastasis and their most significant putative target genes

miRNA	Locus	Expression in metastasis	Target genes	References
let-7 family	Ch 9, 11, 21, 22	Down-regulated	*HMGA2, MYC, RAS*	81, 82
miR-7	9q21.32	Down-regulated	*PAK1*	83-85
miR-10b	2q31.1	Up-regulated	*HOXD10*	31, 42, 86
miR-17-92	13q31.3	Up-regulated	*CTGF, TSP-1*	87, 88
miR-21	17q23.1	Up-regulated	*PDCD4, PTEN, RECK, TPM1, MASPIN, NFIB, ARCKS, TIMP3, SPRY2*	31, 44-47
miR-31	9p21.3	Down-regulated	*RHOA*	54
miR-34a	1p36	Down-regulated	*MET*	89
miR-34b	11q23.1	Down-regulated	*C-MYC, CDK6*	20
miR-34c	11q23.1	Down-regulated	*E2F3*	20
miR-122	18q21	Down-regulated	*ADAM17, RHOA, RAC1*	90, 91
miR-126	9q34.3	Down-regulated	*IRS1, VCAM1, CRK*	48, 92, 93
miR-130a	11q12	Up-regulated	*GAX, HOXA5*	94
miR-141	12p13.31	Down-regulated	*ZEB1, SIP1*	49
miR-143	5q33.1	Up-regulated	*FNDC3B*	95
miR-146a/b	5q33.3; Ch 10	Down-regulated	*IRAK1, TRAF6*	53
miR-148a	7p15.2	Down-regulated	*TGIF2*	20
miR-182	7q32.2	Up-regulated	*MITF, FOXO3*	96
miR-183	7q32.2	Down-regulated	*EZRIN*	97
miR-200 family	1p36.33; 12p13.31	Down-regulated	*ZEB1, SIP1*	49
miR-205	1q32.2	Down-regulated	*ZEB1, SIP1*	49
miR-206	6p12.2	Down-regulated	*NOTCH3*	98
miR-221/222	Xp11.3	Down-regulated	*CDKN1B*	85, 99
miR-335	7q32.2	Down-regulated	*SOX4, TNC, PTPRN2, MERTK*	48, 86
miR-373	19q13.41	Up-regulated	*CD44, LATS2*	50, 86
miR-378	5q33.1	Up-regulated	*SUFU, FUS-1*	100
miR-429	1p36.33	Down-regulated	*ZEB1, SIP1*	49
miR-520c	19q13.41	Up-regulated	*CD44*	50

miR-31 has been proposed as important metastasis-specific miRNA. In fact, this miRNA has been demonstrated to inhibit multiple steps in the metastatic process, including invasion, anoikis, and tissue colonization, leading to a 95% reduction in lung metastasis in an orthotopic model of breast cancer (54, 55). Moreover, miR-31 levels have been observed significantly lower in breast cancer patients with metastatic disease (54).

Gregory and co-workers (49) found that all five members of the miRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 target E-cadherin repressors *ZEB1* and *SIP1* (known also as *ZEB2*). Moreover, these miRNAs have been shown to be markedly down-regulated in cells that had undergone epithelial-to-mesenchymal transition (EMT) (49, 56-58), underlying the early involvement of miRNAs de-regulation in the acquisition of the metastatic phenotype. Actually, EMT is a process whereby epithelial tumor cells are stimulated by extracellular cytokines, such as TGF-beta, or intracellular cues, such as oncogenic Ras, to lose their epithelial polarity and gain mesenchymal phenotypes with increased migratory and invasive capabilities. Therefore, EMT is considered to be an initiating and essential early step in the tumour metastatic process and an interesting topic for investigating the prevention and targeted therapies of metastastic spread. These findings were confirmed by four other independent studies (56, 58).

As for protein-coding genes and as observed in primary tumors, miRNA expression has been demonstrated to be influenced by epigenetic regulation in the metastatic

Table 3. Representative miRNAs aberrantly expressed in CNS tumors. In bold miRNAs shared with the metastatic signature

Tumor	miRNAs
GBM	**let-7 family**, **miR-7**, **miR-10b**, **miR-15b**, **miR-21**, miR-26a, **miR-34a**, miR-124, miR-126 miR-128, miR-137, miR-137, miR-181a, miR-181b, **miR-221**, **miR-222**, miR-425, miR-451, miR-486
Medulloblastoma	**let-7g**, miR-9, miR-19a, miR-20, miR-92, miR-106b, miR-124, miR-125a, miR-125b, miR-191, miR-199b-5p, miR-324-5p, miR-326
Oligodendroglioma	miR-9, miR-124, miR-137
Pituitary adenoma	**let-7 family**, miR-15a, **miR-16**, miR-21, miR-144, miR-152, miR-153, miR-181b, miR-191
Embrional Tumor with Abundant Neuropil and True Rosettes (ETANTR)	miR-372, **miR-373**, miR-517, miR-518b, miR-519d, miR-520-3p

process as well. Lujambio and colleagues (20) investigated the role of DNA methylation-associated silencing of tumour suppressor miRNAs in human cancer metastasis, finding a specific metastatic miRNA hypermethylation profile.

Other important miRNAs have been implicated in the metastatic process [Table 2; for review (51, 52, 56, 58, 59)]. Overall, these reports strongly confirm that specific miRNA genes are directly involved in cancer metastasis, and highlight the translational impact of this new class of non-coding RNAs as new diagnostic and prognostic biomarkers in metastatic cancers. miRNAs that strongly correlate with malignant behaviours (e.g. miR-10b) might be useful as diagnostic molecules, and miRNAs that predict future metastatic relapse (e.g. miR-335 and miR-126) might serve as highly useful prognostic markers. Both groups of miRNAs could represent potential therapeutic targets for cancer treatment. However, extensive *in vivo* studies involving miRNA transgenics and knockouts will be required to determine whether manipulating the levels of a specific miRNA (by using miRNA mimics or inhibitors) can indeed reverse or prevent metastasis. Nevertheless, some other main issues have yet to be investigated, such as the influence of the secondary metastatic microenvironment on tumor cells' miRNAs expression.

5. miRNAs AND BRAIN TUMORS

Poorly differentiated metastatic carcinomas may be difficult to distinguish histologically from high-grade astrocytic malignant neoplasms, particularly on small biopsy specimens. Moreover, despite considerable advances, including multi-modal treatments with surgery, radiotherapy, and chemotherapy, the overall prognosis remains dismal for patients diagnosed with primary and secondary CNS tumors. Thus, the identification of new promising diagnostic and prognostic biomarkers is an impellent requirement in CNS cancer research.

As previously observed in different organ settings, different miRNA expression profiles have been characterized for CNS tumors [Table 3; for review (60-67)].

Among the others, glioblastoma multiforme (GBM) is the most common primary intracranial malignancy and the most aggressive type of CNS tumor (68). Ciafre and colleagues (69) examined the global expression of 245 miRNA genes in GBM by microarray technology. This approach allowed the identification of dysregulated miRNAs in GBM compared with peripheral normal tissue, with miR-221 upregulated and miR-128, miR-181a, miR-181b, and miR-181c downregulated in GBM tissues.

Chan and colleagues (70) identified miR-21 as markedly elevated in human primary GBM tissues and cell lines. Moreover, the knockdown of miR-21 in cultured GBM cells triggers activation of caspases and an increased apoptotic cell death. Also the cluster miR-221/222 has been found to be up-regulated and to have a pro-tumorigenic effect in GBM by targeting $p27^{kip1}$ (69, 71). On the other hand, several miRNAs have been observed as weakly expressed in GBM compared with normal brain, including miR-124, miR-7, and miR-128 (64, 69).

Medulloblastoma is considered another important aggressive CNS neoplasm. A recent report described alterations of 248 miRNAs in 14 medulloblastomas compared with 7 adult and fetal normal cerebellar controls (72). This study revealed some similarities with gliomas, with miR-21 upregulated, and downregulation of miR-124, miR-128, and miR-7 among many others. Of interest, miR-127, which has been described as downregulated in GBM was upregulated in medulloblastomas. Garzia and colleagues (73) identified miR-199b-5p as downregulated in tumor samples. Moreover, they found that the dysregulation of this miRNA in cancer derived cell lines was associated with metastatic spread and significantly affected stem cell signalling through inhibition of the Notch target Hes1.

6. PERSPECTIVES: miRNAs, FROM BENCH TO THE BEDSIDE

At this time, aberrant miRNA expression signatures have been found to be involved in human metastatic and CNS tumors (31, 47, 74). Moreover, miRNA profiling holds potential for differentiating between different pathological lesions and identifying gene expression signatures that are associated with particular cancer phenotypes (12). In this way, genome-wide profiling (improved by the advent of global microarray technology) integrated by functional studies that involve over-expression and down-regulation of miRNAs represent the current approach that is most likely to yield advances in the new field of non-coding RNA research (7-9, 12). Beyond these valuable features, miRNAs could be potentially used in the future as innovative and targeted therapeutics.

Differentiation between primary and metastatic tumors in the CNS is often encountered in pathological practice, but still remains a diagnostic challenge for brain pathologists. Regardless of adequate morphological and immunohistochemical tools, and despite the progress

recently made for molecular evaluation of brain tumors, some neoplastic brain specimens still cannot be definitively classified. The accurate diagnosis of CNS malignancies is essential for selection of proper treatment and patient's management. Recently, Nass and colleagues (75) profiled miRNA expression levels in 15 gliomas and 237 epithelial tumors including 50 brain metastases. In this seminal work, the authors observed that overexpression of miR-92b and miR-9/9* could consistently distinguish primary brain tumors from brain metastases with a sensitivity of 88% and specificity of 100%. Thus, the combined expression of these two specific miRNAs could be considered as a useful diagnostic tool to aid the pathologist in the diagnostic assessment of primary and/or secondary CNS neoplasms. It should be emphasized that both miRNAs have been reported to be over-expressed in neuronal-specific stem cells and to exhibit dynamic expression patterns in the developing brain (76-79). These data suggest that the over-expression of the two brain-specific miRNAs in primary brain tumors could be due to a higher number of transformed brain stem cells, leading to speculation about stem cells' role in brain carcinogenesis.

The characterization of metastases of unknown origin still represents an intriguing pathological and diagnostic dilemma. Recent studies have suggested that, unlike with mRNA expression, a modest number of ~200 miRNAs might be sufficient to classify human cancers and the original tissue in which cancer developed (80). In their seminal work, Rosenfeld and colleagues (74) demonstrated that primary tumors and metastasis from the same tissue consistently show a similar pattern of miRNA expression, confirming the promising role for miRNAs analysis in the investigation of metastatic lesions of unknown origin.

Similar results were obtained by our group (31). In order to search for a specific miRNA expression signature characterising the metastatic phenotype of solid tumours, we performed miRNA micro-array analysis on a series of 43 paired primary tumours (ten colon, ten bladder, 13 breast, and ten lung cancers) and their relative metastases. We identified a global metastatic miRNA signature comprising 15 over- and 17 under-expressed miRNAs and four different organ-specific miRNA signatures, confirming that miRNA profiling could discriminate between different histotypes. Among the miRNAs identified in our analysis, some were previously described to be significantly associated with cancer progression (e.g. miR-10b, miR-21, miR-141, miR-200b, miR-200c, and miR-205). Moreover, we performed an immunohistochemical study for three well-defined miRNA gene targets (PDCD4, DHFR, and HOXD10 genes) on a small series of paired colon, breast, and bladder cancers, and one of their metastatic lymph nodes. As expected, we found that the immunohistochemical expression of these targets correlated significantly with corresponding miRNA deregulation.

In conclusion, we are facing to a new fascinating era in cancer research. Further functional and molecular studies will be essential for elucidating the significance of abnormal miRNA expression in CNS neoplastic lesions. Nonetheless, the miRNA-revolution has begun and will definitively influence in the next few years the diagnostic routinely management of CNS lesions.

7. ACKNOWLEDGMENTS

The authors apologize to those colleagues whose outstanding work could not be cited due to space constraints or to unintended omissions. There is no conflict of interest to declare.

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Key Words: Metastatic Cancer, Mirna, Biomarkers, SNC, Review

Send correspondence to: Raffaele Baffa, Research MedImmune LLC, Gaithersburg, MD, Tel: 301-398-4415, Fax: 301-398-9415, E-mail: BaffaR@MedImmune.com

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