Reduced expression of microRNA-206 regulates cell proliferation via cyclinD2 in gliomas

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Abstract. MicroRNAs are short single-stranded non-coding RNA molecules that function as regulators of tumor progression, including regulation of glioblastoma multiforme, which is a World Health Organization grade IV glioma. Based on the results of a microRNA microarray, which included 198 patients with glioma from the Chinese Glioma Genome Atlas data set, it was observed that microRNA-206 (miR-206) was downregulated in high-grade (grades III and IV) gliomas compared with grade II gliomas. In addition, high expression of miR-206 was associated with longer overall survival time in glioma patients. The present study aimed to investigate the biological functions of miR-206 in glioma progression in vitro using the LN229 glioma cell line. Cell proliferation was observed to be inhibited subsequent to transfection with miR-206. It was suggested that miR-206 induced cell cycle G1/S phase arrest by suppressing the expression of cyclinD2. The results of the present study concluded that miR-206 inhibits glioma progression via the regulation of cyclinD2 and that miR-206 may be a novel biomarker with potential for use as a therapeutic target in gliomas.

Introduction

Glioblastoma multiforme (GBM) is the most prevalent lethal intracranial tumor in adults (1). It is characterized by extensive intracranial invasion and the patients have been reported to tolerate conventional and advanced treatments during therapy (2). Despite the improved strategies for diagnosis and the aggressive tumor treatments used, the median survival of patients with GBM remains to be approximately one year (3,4). Therefore, more effective targeted therapies are crucial for improving the prognosis of patients with GBM.

MicroRNAs are small non-coding RNA molecules that are comprised of 16~22 nucleotides and downregulate translation by targeting mRNAs (5). These microRNAs bind with the 3' untranslated regions (3'UTRs) to block complementary sites on their mRNA targets and therefore serve important inhibitory functions in the post-transcription of gene expression, in a similar capacity to that of RNAi (6). Previous studies have indicated that this novel class of gene regulators has an involvement in human cancer progression and tumorigenesis (7).

Previous studies reported that microRNA-206 (miR-206) expression was markedly reduced in osteosarcoma and lung cancer, and that it was necessary for cell growth, migration, apoptosis and invasion (8,9). In human breast cancer and rhabdomyosarcoma, downregulated miR-206 was associated with cell proliferation, migration and metastasis (10-13). However, the function of miR-206 in gliomas remains to be fully elucidated. In the present study, the aim was to investigate the functional role of miR-206 in gliomas and to further elucidate the mechanism of miR-206 in tumorigenesis and progression. The present study hypothesized that miR-206 acts as a tumor suppressor and suppresses glioma cell proliferation via cyclinD2.

Materials and methods

Patients and tissue collection. All patient information was obtained from the Chinese Glioma Genome Atlas (CGGA; www.cgga.org.cn/). The microRNA microarray analysis was conducted on 198 patients with glioma and based on the gene expression microarray and cyclinD2 expression was analyzed in 225 patients with grade II glioma or high-grade gliomas (HGGs), of which 158 patients also underwent microRNA microarray analysis. In the present study, the patients underwent a resection operation between January 2006 and December 2010 and subsequently received adjuvant treatment of temozolomide combined with radiotherapy. The present study was approved by the Beijing Tiantan Hospital (Beijing,
miR-206 is downregulated in GBM and associated with poor prognosis in patients with glioma. To investigate the tumorigenesis-associated molecular alterations in glioma, the microRNA expression levels were analyzed in 63 patients with grade II glioma and 135 patients with HGGs by microarray analyses. Among these microRNAs, miR-206 expression was observed to be downregulated as the degree of malignancy in gliomas increased (P<0.0001; Fig. 1A). The overall survival time was assessed using Kaplan-Meier survival curve analysis and all survival information used was from the CGGA. The results demonstrated that patients with glioma grade II or HGGs with high expression of miR-206 had a markedly increased rate of progression-free survival as compared with those with low miR-206 expression (P<0.05, P<0.05; Fig. 1B and C). The overall survival curves together with the the miR-206 expression levels in the 198 patients demonstrated that reduced expression levels of miR-206 were associated with poor prognosis in patients with glioma.
Cyclin D2 is a direct target of miR-206. Based on the above analysis, the possible targets of miR-206 were searched with TargetScan (http://www.targetscan.org/), leading to the identification of cyclin D2. Cyclin D2 was observed to share seven imperfect complementary sites with miR-206 and was identified to be important in the cell cycle; therefore, a luciferase reporter assay was designed to verify this. Subsequent to co-transfection with the miR-206 mimics and cyclin D2-3’UTR-plasmids, relative luciferase activity was observed to be significantly reduced (P<0.01; Fig. 2A). The luciferase reporter experiment suggested that cyclin D2 may be a potential target of miR-206. Western blot analysis was also conducted in order to confirm the role of cyclin D2. The results confirmed that miR-206 mimics-transfected cells exhibited reduced cyclin D2 expression corresponding to that in the negative control cells (Fig. 2B). Finally, correlation analysis was conducted to investigate the association between the expression of miR-206 and cyclin D2 in glioma. The results demonstrated an inverse correlation between the expression of cyclin D2 and miR-206 (Spearman's rank: r=-0.201, P=0.012, n=158). miR-206, microRNA-206; UTR, untranslated region; NC, negative control; hsa, Homo sapiens.
Based on these results, cyclinD2 was suggested to be a direct target of miR-206 in gliomas.

**CyclinD2 is increased in HGGs and is correlated with poor prognosis.** According to the gene expression microarray, it was observed that with a higher degree of malignancy, cyclinD2 expression was significantly increased (P<0.0001; Fig. 3A). In addition, the correlation between overall survival time and the expression of cyclinD2 was analyzed in 225 patients. The results demonstrated that, independent of the glioma grade, glioma patients with low levels of cyclinD2 expression exhibited a significantly greater survival time, while the survival time of patients with high levels of cyclinD2 was lower (P<0.05, P<0.05; Fig. 3B and C).

**miR-206 inhibits cell proliferation and arrests G1/S transition in the cell cycle via targeting cyclinD2 in glioma cell lines.** To investigate the biological function of miR-206 in the progression of glioma, a series of overexpression assays were conducted in the GBM cell line LN229. A colony formation assay indicated a significant reduction in cell formation (P<0.05; Fig. 4A). In addition, the MTT assay demonstrated a significant reduction in cell growth.
with cells transfected with miR-206 mimics compared with those of negative control cells at 120 h (P<0.05; Fig. 4B). These assays indicated that miR-206 was associated with glioma cell proliferation. Cell cycle assays demonstrated that the miR-206-transfected cells had a significantly increased percentage of cells in the G1/G0 phase, whereas a significant reduction in cells in the G2 and S phases was observed compared with that in negative control cells (P<0.001, P<0.01 and P<0.01, respectively; Fig. 4C). In conclusion, the results suggested that miR-206 may arrest G1/S transition in glioma cell lines via targeting cyclinD2.

Discussion

Previous studies have indicated that microRNAs regulate gene expression and may also function as tumor suppressors or oncogenes (14). By binding to 3’UTRs, microRNAs suppress the expression of their respective target gene prior to translation, similar to the the mechanism of RNAi (6,15). Furthermore, previous studies have demonstrated that these microRNAs are critical in tumorigenesis and are significant targets for the development of clinical treatments (16,17). Previous studies have identified that miR-206 is downregulated in lung cancer, breast cancer and osteosarcoma (8-10). In breast cancer, miR-206 levels were shown to be correlated with cell growth, clinical stage and lymph node metastasis, and affected the overall survival of patients with breast cancer (10). In lung cancer, as a tumor suppressor, miR-206 was associated with tumor cell migration and invasion (9). Similar effects were also observed in osteosarcoma and in miR-206-transfected cells, where a reduction in cell viability, promotion of cell apoptosis and inhibition of cell invasion and migration were identified (11). However, the function of miR-206 in glioma remains to be fully elucidated.

In the present study, miR-206 was observed to be downregulated in glioma based on microRNA microarray analysis. In addition, the overall survival of patients varied significantly depending on the expression levels of miR-206, suggesting that patients with a high expression of miR-206 had an improved prognosis, based on separate statistical analyses in grade II gliomas and HGGs. Therefore, it was hypothesized that miR-206 may be important in tumorigenesis and the progression of glioma.

To further investigate the function of miR-206 in glioma progression, the target-predicting database Targetscan was searched and cyclinD2 was identified as a potential target of miR-206, which is associated with the cell cycle. miR-206 was previously reported to regulate cyclinD2 in rhabdomyosarcoma (11,12), breast cancer (10,13) and gastric cancer (18). To support this association, a luciferase reporter assay was conducted in the present study, which identified cyclinD2 as a target of miR-206 in gliomas. The results of the western blot assay were also in agreement with this, as cyclinD2 expression was found to be negatively correlated with miR-206 expression. CyclinD2 is a member of D-type cyclins and is crucial in the progression of the cell cycle (19). G1 cyclins, including cyclinDs and cyclinEs, combined with cyclin-dependent kinases CDK4 and CDK6, have been reported to be activated in the late G1 phase and regulate G1/S transition (20). In the process of tumor formation, disruption of cell cycle progression from G1 to S phase is commonly observed (21). Based on the results of previous studies, the overall survival was analyzed separately in patients with grade II gliomas and HGGs in regard to the expression of cyclinD2. The results demonstrated that in gliomas, low levels of cyclinD2 may be associated with lower glioma grades and longer survival time, and further confirmed that cyclinD2 may act as a positive regulator in tumorigenesis and function as a tumor oncogene in gliomas. However, miR-206 exhibited the opposite effect, indicating that cyclinD2 is inversely correlated with miR-206 and is negatively associated with the prognosis of gliomas. The correlation between the expression levels of these miR-206 and cyclin D2 is therefore likely to be important in the development of gliomas. Thus, in order to investigate the function of miR-206 in cell proliferation, MTT and colony formation assays were conducted and the results demonstrated that increased miR-206 expression inhibited cell proliferation in GBM. Cell cycle analysis was also conducted in order to detect the percentage of cells in different stages of the cell cycle. This analysis demonstrated that miR-206-transfected cells exhibited a significantly increased G1/G0 population and a reduction in the S phase population as compared with negative control cells. These results further demonstrated that miR-206 acted as a cell cycle inhibitor, as an increase in the levels of miR-206 expression significantly inhibited transition of LN229 cells from G2/M to S phase. In conclusion, cyclinD2 was a direct target of miR-206 and miR-206 regulated the cell cycle by promoting G1/S arrest and suppressing cell proliferation via targeting cyclinD2 in gliomas.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate that miR-206 suppresses glioma formation and possibly targets the downstream complementary sites of cyclinD2 to inhibit cancer cell proliferation. In addition, the low expression of miR-206 in patients with glioma was demonstrated to be correlated with poor prognosis. Therefore, it was concluded that miR-206 acts as a tumor suppressor in glioma and regulates cell proliferation and cell cycle arrest by targeting cyclinD2. On the basis of observation and data analysis, miR-206 was suggested to be a novel candidate for use as a prognostic marker in patients with glioma and to have potential for use as a therapeutic target in gliomas.

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References

1. Parsons DW, Jones S, Zhang X, et al: An integrated genomic analysis of human glioblastoma multiforme. Science 321: 1807-1812, 2008.
2. Furnari FB, Fenton T, Bachoo RM, et al: Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 21: 2683-2710, 2007.
3. Wang Y, Li S, Chen L, et al: Glioblastoma with an oligodendrogliaoma component: distinct clinical behavior, genetic alterations, and outcome. Neuro Oncol 14: 518-525, 2012.
4. Zhang W, Zhang J, Yan W, et al: Whole-genome microRNA expression profiling identifies a 5-microRNA signature as a prognostic biomarker in Chinese patients with primary glioblastoma multiforme. Cancer 119: 814-824, 2013.
5. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
6. Hannon GJ: RNA interference. Nature 418: 244-251, 2002.
7. Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
8. Bao YP, Yi Y, Peng LL, et al: Roles of microRNA-206 in osteosarcoma pathogenesis and progression. Asian Pac J Cancer Prev 14: 3751-3755, 2013.
9. Wang X, Ling C, Bai Y and Zhao J: MicroRNA-206 is associated with invasion and metastasis of lung cancer. Anat Rec (Hoboken) 294: 88-92, 2011.
10. Kondo N, Toyama T, Sugiu H, Fujii Y and Yamashita H: miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. Cancer Res 68: 5004-5008, 2008.
11. Miyachi M, Tsuchiya K, Yoshida H, et al: Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma. Biochem Biophys Res Commun 408: 89-93, 2010.
12. Yan D, Dong Xda E, Chen X, et al: MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J Biol Chem 284: 29596-29604, 2009.
13. Li Y, Hong F and Yu Z: Decreased expression of microRNA-206 in breast cancer and its association with disease characteristics and patient survival. J Int Med Res 41: 596-602, 2013.
14. Carmell MA, Xuan Z, Zhang MQ and Hannon GJ: The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev 16: 2733-2742, 2002.
15. Wightman B, Ha I and Ruvkun G: Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-862, 1993.
16. Gao J, Wang WY, Mao YW, et al: A novel pathway regulates memory and plasticity via SIRT1 and miR-134. Nature 466: 1105-1109, 2010.
17. Shi ZM, Wang XF, Qian X, et al: MiRNA-181b suppresses IGF-1R and functions as a tumor suppressor gene in gliomas. RNA 19: 552-560, 2013.
18. Zhang L, Liu X, Jin H, et al: miR-206 inhibits gastric cancer proliferation in part by repressing cyclinD2. Cancer Lett 332: 94-101, 2013.
19. Dehay C and Kennedy H: Cell-cycle control and cortical development. Nat Rev Neurosci 8: 438-450, 2007.
20. Sherr CJ: Mammalian G1 cyclins. Cell 73: 1059-1065, 1993.
21. Sherr CJ and Roberts JM: Living with or without cyclins and cyclin-dependent kinases. Genes Dev 18: 2699-2711, 2004.