Musashi1 expression is negatively correlated with numb expression in brain metastases

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Abstract
The expression of tumor stem cell markers musashi1 (msi1) and numb in brain metastases were detected to explore their roles in the development of brain metastases.

A total of 51 cases of brain metastasis, 29 cases of primary tumor and 15 cases of normal brain tissue were selected. Immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) were used to detect msi1 and numb expression at the protein and mRNA levels. Correlation between msi1 and numb in brain metastases was evaluated.

Immunohistochemistry and RT-PCR showed that no significant difference in the expression of msi1 and numb between brain metastases and primary tumors was observed (P > .05); the expression of msi1 and numb in brain metastases was significantly higher than that in normal brain tissues (P < .05); and the expression of msi1 and numb in primary tumors was significantly higher than that in normal brain tissues (P < .05). In general, the expression of msi1 gene was negatively correlated with the expression of numb at mRNA level by Pearson correlation analysis (r = −0.345, P < .05). Additionally, the expression of msi1 and numb in brain metastases was not related to gender, age, and tissue origin (P > .05).

Msi1 is highly expressed in brain metastases and primary tumors, while numb is lowly expressed in brain metastases and primary tumors; msi1 and numb are negatively correlated in brain metastases, suggesting that msi1 and numb may have regulatory mechanisms in the development of brain metastases.

Abbreviations: 3′-UTR = 3′-untranslated regions, BM = brain metastases, DAB = 3, 3′-Diaminobenzidine, HE = hematoxylin-eosin, HER2 = human epidermal growth factor receptor-2, Msi1 = Musashi1, SIF = staining intensity fraction, TSCs = tumor stem cells.

Keywords: brain metastases, musashi1, numb, tumor stem cells

1. Introduction
Brain metastases (BM) are one of the most common intracranial malignant tumors in adults, which could cause great disasters to human health, and are also major threats to peoples life and economy. The incidence of BM is increasing over the years, but the treatment, prognosis and basic research of brain metastases are still insufficient. The normally accepted theory of brain metastases is stem cell theory of cancer. It is believed that brain metastases are caused by the migration and homing of tumor stem cells (TSCs). Up to now, TSCs in brain metastases have not been completely isolated and identified and the specific mechanism between TSCs and the development of brain metastasis is still not clear. Therefore, to distinguish specific surface marker of TSCs in brain metastases is still of particular significance.

Musashi1 (msi1) is an evolutionarily conserved RNA binding protein which has been considered as a broad-spectrum marker of TSCs. Msi1 is mainly involved in the regulation of cell proliferation, differentiation, cell cycling and apoptosis. At the early stage of tumor proliferation, the expression of msi1 was obviously increased. The overexpression of msi1 in many malignant tumors suggests that msi1, as a broad-spectrum marker of TSCs, may be valuable for the isolation and identification of TSCs. Moreover, the high expression of msi1 in all kinds of malignant tumors predicts a poor prognosis.

mRNA of numb is the target of msi1, and is known as “cell fate determinant.” It is found that numb might change the biological characteristics of daughter cells by regulating Notch signaling pathway. The RNA binding protein msi1 can inhibit the translation of numb, activate Notch signaling pathway, and finally induce tumor formation by specifically binding with 3′-untranslated regions (3′-UTR) of numb. So far, the correlation between msi1 and numb has not been verified in brain metastases. In this study, immunohistochemistry and RT-PCR were used to detect the expression of msi1 and numb at the protein and mRNA levels in brain metastases, and to explore their internal connection, so as to prove the molecular biological mechanism of the development of brain metastases.
2. Materials and methods

2.1. Tissue samples

The tissue samples of brain metastases were collected from 51 patients in Department of Neurosurgery, the Fourth Hospital of Hebei Medical University from 2014 to 2016. The patients included 24 males and 27 females, aging from 24 to 76 years, with a median age of 52 years. The primary tumor tissue samples were collected from the biological specimen bank of the Institute of Oncology, the Fourth Hospital of Hebei Medical University. The normal brain tissue samples were taken from the brain tissues of 15 patients (10 males and 5 females) with brain injury who underwent internal decompression and resection. All experimental protocol was under the approval of Ethics Committee of the fourth hospital of Hebei Medical University. Written consent was obtained from the patients.

Part of the tissue samples were fixed in 10% formalin solution, preserved in wax blocks, stained with HE and specific antibodies; the other part was stored in a refrigerator at −80°C for RNA extraction. All tumor tissues were stained with hematoxylin-eosin (HE) staining and diagnosed as brain metastasis and primary tumor by Department of Pathology of the Fourth Hospital of Hebei Medical University. The classification of brain metastasis tissue sources were listed in Table 1.

2.2. Experimental groups

Normal brain tissue was set as control group. Brain metastasis and primary tumors were the experimental group. According to different tissue sources, it can be divided into lung cancer source group and other cancer groups.

2.3. HE staining

The tissue samples were fixed in 10% formalin solution, preserved in wax blocks, stained with HE following the standard protocol as described previously.[13] Briefly, the tissues were washed with running water for several hours, dehydrated with ethanol, and xylene, and then were embedded in paraffin and sectioned. The paraffin section was baked, dewaxed and hydrated and placed into the aqueous solution of hematoxylin for dyeing for 3 minutes, and eosin for 3 minutes. The slides were sealed and examined under light microscope.

2.4. Immunohistochemistry

Immunohistochemistry was conducted as previously described.[14] Briefly, the tissues were fixed in 10% formalin solution, preserved in wax blocks, and sectioned. After heat recovery, the sections were incubated with primary antibodies against msi1 (1:200, ab52865, abcam) and numb (1:500, TAS01614, ORIGENE). After that, the tissues were incubated with the secondary antibody. The staining was assisted by 3, 3’-Diaminobenzidine (DAB).

Msi1 was mainly located in the cytoplasm and nucleus. The staining was uniform and brownish yellow. The staining of numb was mainly located in the cytoplasm. The images were taken by a light microscope with a magnification of 400×. According to the standard of staining intensity fraction (SIF), the scores were evaluated: the positive staining <10% was counted as 1 point, 10% ~ 50% counted as 2 points, 50% ~ 75% counted as 3 points, and >75% counted as 4 points; no staining counted as 0 point, light yellow count as 1 point, brown yellow count as 2 points, and brown counted as 3 points. The above 2 scores were added and supposed as SIF: 0 score was “−−”, 1–2 score was “++”, 3–4 score was “+++”, 5–6 score was “++++”. “++” and “+++” are defined as positive expression, while “−−” and “++” are defined as negative expression.

2.5. RT-PCR

Total RNA was extracted from the tissues using the Trizol kit and the purity was confirmed by optical density (OD) 260/OD280. cDNA was synthesized using the Reverse Transcrip Script Kit (GeneCopoeia). The PCR system included 10μl GoldStar Tap MasterMix, 2μl Primer, 1μg cDNA, and 7μl RNase-free dH2O. The reaction condition included: initial denaturation 10 minutes at 95°C, denaturation 45 second at 95°C, annealing 45 second at 95°C, annealing 45 second, extension 5 second at 72°C of 35 cycles. After reaction, DNA was underwent agarose gel electrophoresis. The primer sequences were listed in Table 2.

The mRNA expression level of msi1 and numb in the electrophoretic image was semi quantitatively analyzed by Genesys software, and the average value was obtained after 3 times of experiment repetition. The gray value of the target gene was measured by the software gel Pro analyzer 3.1, and the relative expression was obtained by referring to the OD value of

| Types          | Primer sequence          | Annealing temperature (°C) | Product size (bp) |
|----------------|--------------------------|----------------------------|-------------------|
| Msi1           | F: 5’- TTCGCGGTGTTGTTGACGTATGTTGAG-3’ | 59                         | 250               |
|                | R: 5’- GCCCTGTTAATACTCGGTGT-3’       |                            |                   |
| Numb           | F: 5’TGGTTGATTGATGGCGATTTGCA-3’      | 57                         | 155               |
|                | R: 5’- CAATCCTAAGAGGGGTTGATGGCCG-3’  |                            |                   |
| GAPDH          | F: 5’- AGGTGAAGGCTGTCACACAG-3’       | 57                         | 104               |
|                | R: 5’- AGGCGCTTGGATGGCAACA-3’        |                            |                   |
GAPDH to standardize the OD value of the gene. The formula is as follows: Relative mRNA expression = OD value of target gene/OD value of GAPDH as previously described.\(^{15}\)

2.6. Statistical analyses

The data analysis was performed by SPSS21.0 statistical software. Unpaired \(t\) test was used in the analysis of mRNA expression. Chi test was used to analyze msi1 and numb expression at protein level. Spearman correlation analysis was used for grade data. Pearson correlation analysis was used for continuous variable data. \(P < .05\) showed the significant difference.

3. Results

3.1. Expression of msi1 at protein and mRNA levels in brain metastasis, primary tumor and normal brain

All tumor tissues were stained with HE staining and diagnosed as brain metastasis and primary tumor tissue (Fig. 1). The information of the cases was listed in Table 3.

The results of immunohistochemistry demonstrated that the positive rates of msi1 protein in brain metastasis, primary tumor and normal brain tissue were 60.8% (31/51), 65.5% (19/29), and 13.3% (2/15), respectively (Fig. 2, Table 4). There was no significant difference in the expression of msi1 protein between brain metastases and primary tumors (\(P > .05\)); the expression of msi1 protein in brain metastases was significantly higher than that in normal brain tissues (\(P < .05\)); the expression of msi1 protein in primary tumors was significantly higher than that in normal brain tissues (\(P < .05\)).

RT-PCR results revealed that the relative expression of msi1 mRNA in brain metastasis, primary tumor and normal brain tissue was 0.78 ± 0.50, 0.60 ± 0.36, and 0.38 ± 0.29, respectively (Fig. 3, Table 4). The expression of msi1 mRNA in brain metastases was not significantly different from that in primary tumors (\(P > .05\)); the expression of msi1 mRNA in brain metastases was significantly higher than that in normal brain tissues (\(P < .05\)); the expression of msi1 mRNA in primary tumors was significantly higher than that in normal brain tissues (\(P < .05\)).

3.2. Expression of numb at protein and mRNA levels in brain metastasis, primary tumor and normal brain tissues

The results of immunohistochemistry displayed that the positive expression rate of numb protein was 33.3% (17/51), 31.0% (9/29), and 73.3% (11/15), respectively (Fig. 4, Table 5). There was no significant difference in the expression of numb protein between brain metastases and primary tumors (\(P > .05\)); the expression of numb protein in brain metastases was significantly lower than that in normal brain tissues (\(P < .05\)); the expression of numb protein in primary tumors was significantly lower than that in normal brain tissues (\(P < .05\)). RT-PCR showed that the relative expression of numb mRNA was 0.34 ± 0.21, 0.47 ± 0.31, and 2.42 ± 1.82 in brain metastases, primary tumors and normal brain tissues, respectively (Fig. 5, Table 5). There was no significant difference in the expression of numb mRNA between brain metastases and primary tumors (\(P > .05\)); the expression of numb mRNA in brain metastases was notably lower than that in normal brain tissues (\(P < .05\)); the expression of numb mRNA in primary tumors was lower than that in normal brain tissues with significance (\(P < .05\)).

3.3. Correlation analysis of mRNA expression of msi1 and numb in brain metastasis

Msi1 and numb mRNA relative expression were 0.78 ± 0.50 and 0.34 ± 0.21, respectively. Through Pearson correlation analysis, msi1 and numb mRNA expression were negatively correlated (\(r = -0.258, P < .05\)) (Fig. 6).

3.4. Analysis of the expression of msi1 and numb in brain metastasis and clinicopathological data

No significant difference was found between the relative expression of msi1 mRNA in the group of less than or

![Table 3](attachment://Table_3.pdf)

| Group          | N (%) |
|----------------|-------|
| Age            |       |
| <52 years      | 26 (50.0%) |
| ≥52 years      | 25 (40.0%) |
| Gender         |       |
| Male           | 22 (43.1%) |
| Female         | 29 (56.8%) |
| Histogenesis   |       |
| Lung cancer    | 29 (56.8%) |
| Other Cancers  | 22 (43.1%) |

Figure 1. Morphology of brain metastases, primary tumor, and normal brain tissue. HE staining with the magnification of 200×.
equal to 52 years old and that in the group of more than 52 years old ($P > .05$); Meanwhile, there was no significant difference between the relative expression of numb mRNA in the group of less than or equal to 52 years old and that in the group of more than 52 years old ($P > .05$) (Table 6).

In addition, there was no significant difference in the relative expression of msi1 mRNA between male group and female group ($P > .05$); and there was no significant difference in the relative expression of numb mRNA between male group and female group ($P > .05$) (Table 6).

Besides, there was no significant difference in the relative expression of msi1 mRNA between the lung cancer source group and other source groups ($P > .05$); similarly, there was no significant difference in the relative expression of numb mRNA between the lung cancer source group and other source groups ($P > .05$) (Table 6).

4. Discussion

In this study, immunohistochemical staining and RT-PCR were used to detect the expression of tumor stem cell marker msi1 and

### Table 4

The expression of Msi1 in different tissues at both mRNA and protein levels.

| Tissue               | Protein expression | $X^2$  | $P$      | mRNA expression | $t$       | $P$     |
|----------------------|--------------------|--------|----------|-----------------|----------|---------|
|                      |                    |        |          |                 |          |         |
| Group                |                     |        |          |                 |          |         |
| Brain metastases     | 31                  | 20     | 0.177    | 0.78 ± 0.50     | 1.876    | .065    |
| Primary tumor        | 19                  | 10     | .60 ± 0.36|                 |          |         |
| Group                |                     |        |          |                 |          |         |
| Brain metastases     | 31                  | 20     | 10.439   | 0.78 ± 0.50     | 3.929    | <.001   |
| Normal brain tissue  | 2                   | 13     | 0.38 ± 0.29|                 |          |         |
| Group                |                     |        |          |                 |          |         |
| Primary tumor        | 19                  | 10     | 10.791   | 0.60 ± 0.36     | 2.096    | .042    |
| Normal brain tissue  | 2                   | 13     | 0.38 ± 0.29|                 |          |         |
Figure 3. The expression of Msi1 gene in brain metastases, primary tumor, and normal brain tissue. A) Brain metastases tissues. B) Primary tumor tissues. C) Normal brain tissues. MA: 100 bp DNA Maker.

Figure 4. The expression of Numb protein in brain metastases, primary tumor, and normal brain tissue (400×). A) Brain metastases tissues. B) Primary tumor tissues. C) Normal brain tissues.
its downstream gene numb in brain metastasis, primary tumor and normal brain tissue, and to further explore the relationship between them.

Msi1 protein and mRNA were highly expressed in brain metastases and primary tumors, but low in normal brain tissues, indicating that msi1-labeled tumor stem cells are possibly existing in both brain metastases and primary tumors. A large number of experiments have proved that tumor stem cell marker msi1 is indispensable in the process of proliferation, invasion and metastasis of lung cancer, renal cancer, breast cancer, and other malignant tumors, indicating that tumor stem cell marker msi1 is likely to play a positive role in the process of brain metastasis of malignant tumors. It was found that msi1 was highly expressed in primary breast cancer, especially in metastatic breast cancer, and msi1 was also highly expressed in metastatic lymph nodes. Moreover, the expression of msi1 was closely related to human epidermal growth factor receptor-2 (HER2) gene. HER2 was a high risk factor for brain metastasis in breast cancer. At the same time, this experiment found that msi1 was highly expressed in CD133 positive breast cancer cells, indicating that msi1 was enriched in breast cancer stem cells. Like CD133, CD44, ALDH1, and nestin, msi1 could be another tumor stem cell marker. Ravindran et al found the co-expression of CD133 and msi1 in oral squamous cell carcinoma, and the expression of CD133 and msi1 increased gradually from normal tissue to atypical hyperplasia tissue to cancer tissue, manifesting that msi1 is related to the invasion and differentiation of oral cancer cells.

In this study, the expression of numb was significantly lower in brain metastasis and primary tumor tissues, but higher in normal brain tissues. A large number of experiments have proved that the lack of numb may induce the activation of Notch signaling, which may lead to the occurrence, invasion and metastasis of tumor. Hence, low expression of numb may be related to brain metastasis of primary tumor. Numb is a tumor suppressor gene and the low expression of numb may lose its inhibitory effect on tumor. Lack of expression of numb in triple-negative breast cancer activated the Notch signaling, thus enhancing the process
and metastasis of epithelial mesenchymal transition (EMT).\cite{26}

Triple-negative breast cancer is a common type of breast cancer with brain metastasis. EMT is an important biological process of invasion and metastasis of epithelial cell-derived malignant tumors. Therefore, low expression of numb may be related to brain metastasis of primary tumors. Moreover, EMT interacts with tumor stem cells.\cite{27,28} Koch et al has reported that numb is located in the upstream of notch, antagonizing Notch signaling pathway, and its inactivation leads to abnormal differentiation and proliferation of cells.\cite{28}

Our present study also indicated that the expression of msi1, numb at protein and mRNA levels was negatively correlated in brain metastases, which indicated that msi1 and numb might promote the occurrence of brain metastases through signaling pathway regulation mechanism, or there was direct interaction between msi1 and numb in the brain metastases. A large number of experiments have proved that msi1 elicits invasion and metastasis of tumor cells by inhibiting the expression of numb in malignant tumors,\cite{29,30} and this relationship is also likely to exist in brain metastases. It has been proved that msi1 activates Notch signaling pathway by inhibiting numb at the translation level.\cite{15}

Its biological significance is to maintain the self-renewal and vitality of neural stem cells, as well as diseases (such as tumors) that msi1 may involve. The specific molecular mechanism of msi1 controlling numb is not clear, so it cannot be ruled out that msi1 can induce other RNA binding proteins to regulate numbers of differentiation by regulating the Musashi/Numb/Notch pathway. Cell 2001;21:3888–900.

5. Conclusion

In summary, msi1 is highly expressed in brain metastases and primary tumors, while numb is low in brain metastases and primary tumors; msi1 and numb are negatively correlated in brain metastases and primary tumors, suggesting that msi1 and numb may have regulatory mechanisms in the development of brain metastases.

Author contributions

DYL and CK conceived and designed the study. DYL, LJJ, and WSB performed the study. DYL and LJJ analyzed the data. DYL wrote the paper. CK revised the paper. All authors had reviewed and agreed on the contents of this paper.

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