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*Original Article*

# Neurotrophin-3 upregulates HER-2 to promote the growth of brain metastasis from colorectal cancer

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**Abstract:** Brain metastasis (BM) from colorectal cancer (CRC) is not common but prognosis is poor. Overexpression of neurotrophin-3 (NT-3) and autocrine NT-3 signaling can promote tumor growth and metastasis. Similarly, the human epidermal growth factor receptor 2 (HER-2) is overexpressed in many cancers and this upregulation is associated with poor prognosis. Moreover, NT-3 and HER-2 signaling pathways may interact in HER-2+ metastasis cases. The aim of this study was to assess the relationship between CRC metastasis risk and expression levels of NT-3 and HER-2 as revealed by immunohistochemical staining. We investigated metastasis tissue specimens from 47 CRC cases (19 with BM, 11 with liver metastasis (LM), 17 with other metastases (OM)), and primary CRC lesion specimens from 21 cases with no organic metastasis, all treated surgically from 2000 to 2013. Positive HER-2 staining was found in a significantly greater proportion of CRC with BM specimens (7 of 19, 36.8%) than primary CRC (3 of 21, 14.3%), CRC with LM (3 of 11, 27.3%), or CRC with OM (0/17) specimens ($P<0.05$). Moreover, a higher proportion of CCR with BM specimens were NT-3-positive (73.7%) than CRC with LM or CRC with OM ($P<0.05$). Expression levels of NT-3 and HER-2 in CRC with BM specimens were linearly correlated ($R = 0.614$, $P<0.05$), suggesting that NT-3 overexpression may upregulate HER-2. Kaplan-Meier survival curves revealed significantly longer survival in both SER-2(-) vs. SER-2(+) and NT-3(-) vs. NT-3(+) patients ($P<0.05$). Dual expression of NT-3 and HER-2 may confer increased risk of BM from CRC and concomitant poorer prognosis.

**Keywords:** Neurotrophin-3, HER-2, colorectal cancer, brain metastasis

## Introduction

Brain metastasis (BM) from colorectal cancer (CRC) was rare in past decades, with reported incidences ranging from 0.3% to 9% [1]. Such patients may complain of headache, cognitive deterioration, aphagia, motor disturbances, and visual disturbance as well as symptoms unrelated to function of the brain area involved [2, 3]. However, the incidence of BM from CRC has risen to 10% during the course of treatment due to prolonged survival resulting from advances in drugs, radiotherapy, and surgical treatments [4-6]. Timely resection of BM along with whole-brain radiation can promote survival [7]. Thus, patients may benefit from the identification of factors predictive of BM to facilitate early treatment.

Human epidermal growth factor receptor 2 (HER-2) is a proto-oncogene (17q21) encoding a 185-kDa transmembrane tyrosine kinase receptor. HER-2 gene amplification and protein overexpression have been linked to tumorigenesis in many tissues and to poorer prognosis [8, 9]. For instance, HER-2 amplification or strong overexpression increases risk for BM from many tumors [10, 11], although whether this is also true for CRC remains unclear. Aprile et al. reported a HER-2(+) rate of 8.1% for the primary CRC tumor and 12% for the corresponding BM, with concordance of 89% [12], suggesting that HER-2 expression by the primary CRC has potential negative prognostic value for BM. Neurotrophin-3 (NT-3) plays an important role in the development of the neural-crest-derived peripheral and central nervous systems [13]. NT-3 acts directly on precursor cells and in combination with other neurotrophic factors to promote the survival and differentiation of enteric neurons and glia [14]. Tauszig-Delamasure and colleagues suggested that the abnormal expression of NT-3 and autocrine signaling may increase the aggression of neuroblastoma [15]. Moreover, NT-3 and HER-2 expression levels may be interdependent; ectopic expression of NT-3 increased the expression of HER-2 and E-cadherin at the cell-cell junction in BM from

**Table 1.** Clinical characteristics of the enrolled patients (n = 68)

| | N | % |
|---|---|---|
| Sex | | |
| Male | 32 | 47.1 |
| Female | 36 | 52.9 |
| Position of lesions | | |
| Primary lesions | 21 | 30.9 |
| Brain | 19 | 27.9 |
| Liver | 11 | 16.2 |
| Other | 17 | 25.0 |
| Position of BM in colon cancer | | |
| Right Supratentorial | 3 | 27.27* |
| Left Supratentorial | 5 | 45.46* |
| Right Subtentorial | 2 | 18.18* |
| Left Subtentorial | 1 | 9.09* |
| Position of BM in rectal cancer | | |
| Right Supratentorial | 3 | 37.50* |
| Left Supratentorial | 1 | 12.50* |
| Right Subtentorial | 3 | 37.50* |
| Left Subtentorial | 1 | 12.50* |

* = the rate is calculated on the BM population of colon/rectal cancer.

breast cancer [16]. It is unclear, however, whether NT-3, HER-3, or dual overexpression contributes to the risk of BM from CRC.

In this retrospective study, we estimated the expression levels of NT-3 and HER-2 in CRC and associated metastases by immunohistochemistry of preserved specimens from 68 CRC patients treated surgically over the past 14 years (2000-2013).

## Materials and methods

### *Patients and specimens*

We collected paraffin-embedded tissue blocks from 68 patients with CRC, including 19 with BM, 11 with liver metastasis, 17 with other single organ metastases, and 21 with no metastasis, all treated surgically from 2000 to 2013 at Huashan Hospital, Fudan University (Shanghai, China). The average age of the patents is 61 (41-87). The "other" organic metastases group included 7 cases of peritoneal metastasis, 4 of ovarian metastasis, 3 of greater omentum metastasis, and one each of gum, osseous, and bladder metastasis. All samples from the 47 cases with metastasis were of the metastasis lesions, while the 21 samples from the metastasis-free patients were of the primary CRC lesion. Informed consent was obtained from all patients. The patient's private identification information was deleted and the study protocol was approved by the Medical Ethics Comittee of Huashan Hospital.

### *Immunohistochemistry*

A conventional immunostaining protocol was used to assess HER-2 and NT-3 expression levels. The widest area that represented the condition of the corresponding tissue was assessed in 4-μm sections prepared from paraffin blocks using an automated staining platform (Dako Immunostainer RM2145) according to manufacturer's instructions. Tissue sections were first deparaffinized by immersion in xylene and rehydrated in a graded alcohol series. For antigen retrieval, sections were microwaved in 10 mM citric acid monohydrate for 1×5 min at 900 W and for 3×5 min at 600 W. Endogenous peroxidase activity was quenched by treatment with 0.3% $H_2O_2$. The slides were incubated overnight at 4°C with appropriate dilutions of the primary antibodies. Immunoreactions for both HER-2 and NT-3 were visualized by the Envision kit (Dako, Denmark). Sections were then subjected to the antigen retrieval procedure and then incubated with the primary rabbit antihuman HER-2 polyclonal antibody (Anti-ErbB 2 antibody ab2428, Abcam, JAPAN) for one hour at 37°C. The HER-2 immunoreactivity was visualized after a brief treatment with the Envision Plus system kit (Dako, Denmark). NT-3 polyclonal goat antibody (Anti-Neurotrophin 3 antibody ab65804, Abcam, JAPAN) immunoreactivity was visualized by brief treatment with the Envision Plus system kit (Dako, Denmark). Both positive and negative controls were included in each run.

Three experienced pathologists independently evaluated HER-2-stained and NT-3-stained slides blindly without knowledge of patient clinicopathologic information. The intensity and percentage of HER-2 immunoreactivity was scored according to the revised ToGA scoring criteria of the HercepTestTM for gastric cancer [17, 18]. In brief, no staining or membrane staining in less than 10% of invasive tumor cells was scored as 0; faint/barely perceptible membrane staining detected in 10% or more tumor cells was scored as 1+; weak to moderate complete or basolateral membranous reactivity in ≥ 10% but <30% of tumor cells was scored as 2+; strong com-

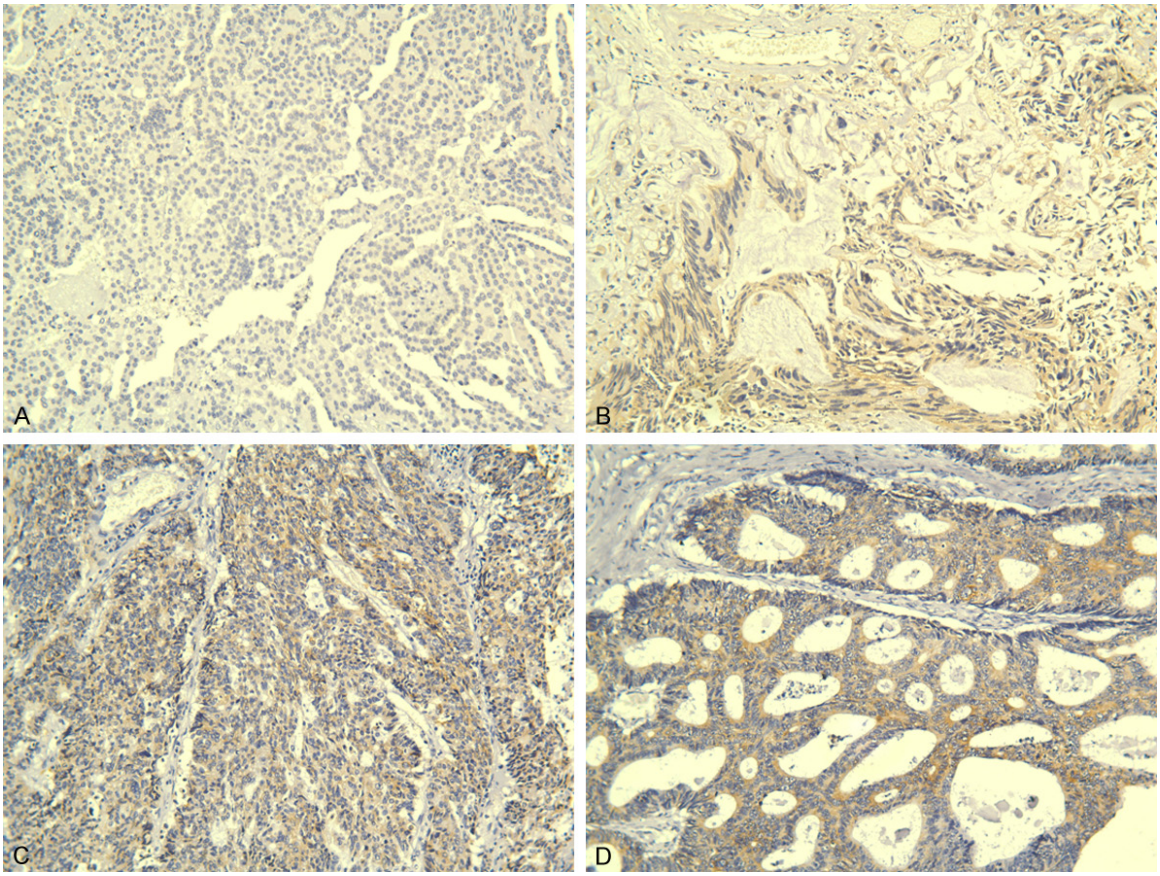


**Figure 1.** HER-2 staining scored 0 (A); HER-2 staining scored 1+ (B); HER-2 staining scored 2+ (C); HER-2 staining scored 3+ (D) Magnification 40×. IHC HER-2 expression pattern of CRC lesions.

**Table 2.** Colorectal (n = 21), BM (n = 19), liver metastasis (n = 11) and other metastasis (n = 17) HER-2 expression and immunohistochemical score

| HER-2 | Primary lesions | | BM | | Liver metastasis | | Other metastasis | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| 0 | 5 | 23.8 | 7 | 36.8 | 3 | 27.3 | 10 | 58.8 |
| 1+ | 13 | 61.9 | 5 | 26.3 | 5 | 45.4 | 7 | 41.2 |
| 2+ | 1 | 4.8 | 3 | 15.8 | 1 | 9.1 | 0 | 0 |
| 3+ | 2 | 9.5 | 4 | 21.1 | 2 | 18.2 | 0 | 0 |

plete or basolateral membranous reactivity in ≥ 30% of tumor cells was scored as 3+. A score of 0 or 1+ was considered negative while scores of 2+ and 3+ were considered positive. The intensity of NT-3 immunostaining was graded in a semi-quantitative manner [16], with a score of 0 indicating absence of stain, 1+ indicating weak staining in > 10% of cells, 2+ indicating positivity in 10%-80% of cells, and 3+ indicating strong positivity in > 80% of cells. The optical density (OD) value of the tissue measured for correlation analysis.

*Statistical analysis*

The frequencies of qualitative variables are described in percentages. Kaplan-Meier survival curves were obtained and compared by the log rank test. Proportions (%) were compared by $\chi^2$ tests and linear regression was evaluated by Pearson's correlation coefficients. For all tests, a $P<0.05$ was considered statistically significant. All analyses are performed using SPSS version 17.0 software for Windows (SPSS Inc., Chicago, IL, United States).

## Results

*Patient characteristics*

All 68 CRC patients were considered eligible and included in our analysis. Median age at time of metastasis resection or radical operation was 61 years (41-87 years). Other patient

**Table 3.** Comparison of HER-2-positive (staining score > 1+) and HER-2-negative (score < 1+) rates between BM and primary lesions or other metastasis of CRC

| Samples | Positive N (%) | Negative N (%) | *P* value |
|---|---|---|---|
| BM | 7 (36.8) | 12 (63.2) | |
| Primary lesions | 3 (14.3) | 18 (85.7) | 0.0246 |
| Liver metastasis | 3 (27.3) | 8 (72.7) | 0.0335 |
| Other metastasis | 0 (0) | 17 (100) | 0.0002 |

demographic and clinical characteristics are summarized in **Table 1**.

*Differential HER-2 expression patterns among clinical subgroups*

Typical HER-2 staining patterns are shown in **Figure 1**, the IHC expression pattern of HER-2 for each clinical subgroup in **Table 2**, and the proportions of HER-2(-) (defined as a staining score of 0 and 1+ [17, 18]) and HER-2(+) (defined as staining score of 2+ and 3+ [17, 18]) in **Table 3**. A significantly greater proportion of metastasis specimens from brain (CRC from BM) were HER-2(+) than specimens from other metastasis sites or the primary lesion (P<0.05), suggesting that HER-2 overexpression increases the risk of BM from CRC.

*Relationship between NT-3 and metastasis of CRC*

Typical NT-3 immunostaining patterns are shown in **Figure 2**. Proportions of NT-3(-) and NT-3(+) specimens for each clinical subgroup are shown in **Table 4**. A significantly higher proportion of BM specimens were NT-3(+) compared to specimens from liver (LM) or other tissues (P<0.05), while there was no significant difference in NT-3(+) proportion between BM and primary lesions (P > 0.05) (**Table 5**).

*Correlation between NT-3 and HER-2 staining intensity in CRC BM*

Comparison of NT-3 to HER-2 staining intensity in CRC BM specimens (as measuring by OD value) revealed a positive linear relationship (r = 0.614, P<0.05) (**Table 6**; **Figure 3**).

*Relationship between BM position and location of the primary lesion*

There was no significant relationship between location of the primary lesion (colon or rectum) and gross brain location of the metastasis (supratentorial or subtentorial) (P = 0.2710) (**Table 7**).

*Overall survival of CRC BM*

For the entire CRC BM patient subgroup, the median overall survival (OS) was 12.11 months (95% CI 9.96-14.26 months). Kaplan-Meier analysis revealed that median OS was significantly shorter in patients with HER-2(+) brain metastasis than in patients with HER-2(-) brain metastasis (9.00 months vs. 13.92 months; P = 0.06) (**Figure 4**). Similarly, OS was significantly shorter in patients with NT-3(+) brain metastasis than in patients with NT-3(-) brain metastasis (10.50 months vs. 16.60 months; P = 0.012, **Figure 5**).

## Discussion

Colorectal cancer is the third most common cancer in males and the second most common in females [19]. Brain metastases are infrequent [20] and are usually a late-stage manifestation [21]. In one study, the 5-year cumulative incidence of BM from CRC was substantially lower than the rate from lung, renal, skin, and breast cancers [22]. Nonetheless, the poor prognosis necessitates identification of pathogenic mechanisms and predictive factors for early diagnosis and treatment.

*HER-2 and BM from CRC*

HER-2 expression is a prognostic and predictive factor for CRC [23] although roles in pathogenesis and progression are still uncertain [24]. In vitro studies have found that inhibition of HER-2 can stop the proliferation of colorectal cancer cell lines [25, 26]. HER-2 gene copy number status may also influence response to clinical therapy in metastatic CRC patients [27]. It was reported that patients with HER-2(+) breast cancer had higher risk of BM than HER-2(-) cases [28]. In the present study, we examined whether HER-2 overexpression increases the risk of BM from CRC as well. Indeed, the HER-2(+) rate was 36.8% for BM from CRC, higher than the other clinical subgroups (CRC with liver, other, and no metastasis) and higher than in gastric cancer patients, but lower than in breast cancer [29] and higher than gastric cancers [30]. In light of a previous study showing high concordance between HER-2 expression in primary CRC and brain metastasis and our results showing no incidence of

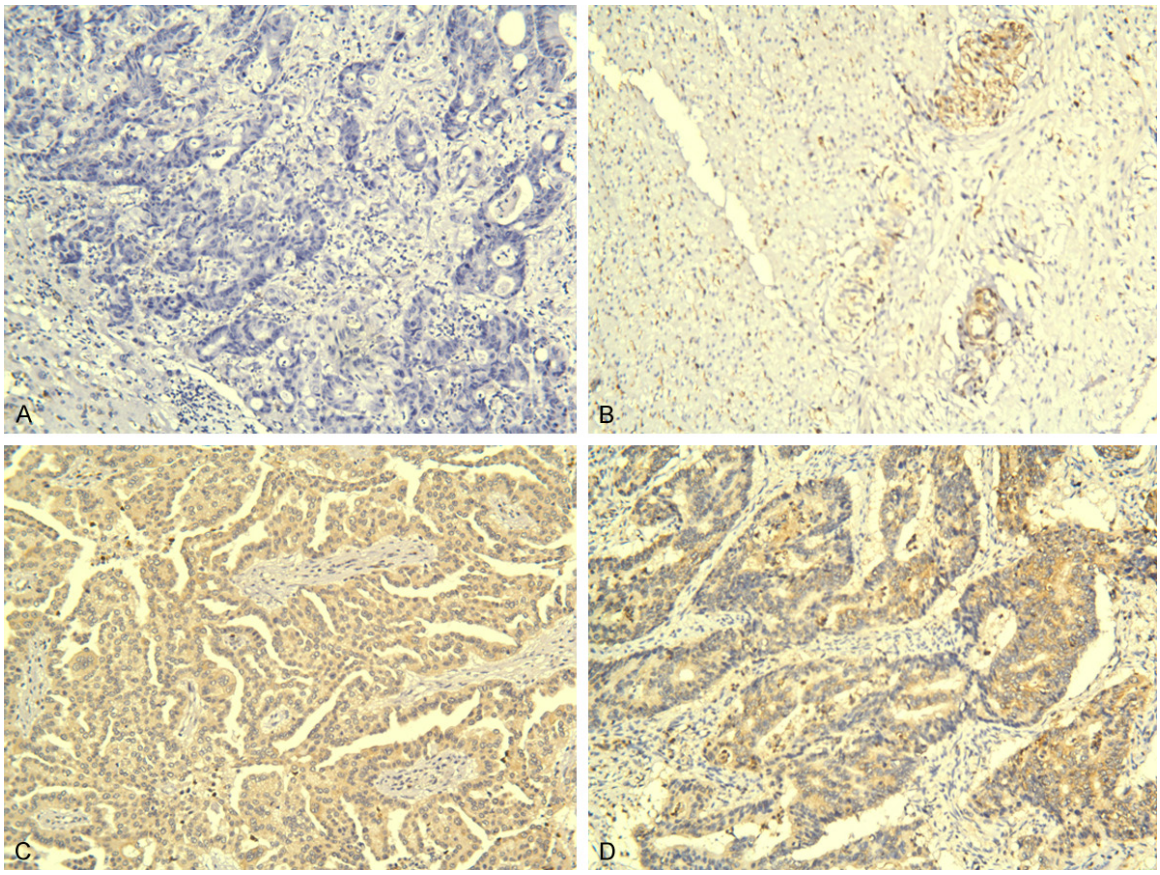


**Figure 2.** NT-3 staining scored 0 (A); NT-3 staining scored 1+ (B); NT-3 staining scored 2+ (C); NT-3 staining scored 3+ (D) Magnification 40×. IHC NT-3 expression pattern of CRC lesions.

**Table 4.** Colorectal (n = 21), BM (n = 19), liver metastasis (n = 11) and other metastasis (n = 17) NT-3 expression and immunohistochemical score

| NT-3 | Primary lesions | | BM | | Liver metastasis | | Other metastasis | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| 0 | 1 | 4.8 | 0 | 0 | 2 | 18.2 | 1 | 5.9 |
| 1+ | 5 | 23.8 | 5 | 26.3 | 6 | 54.5 | 9 | 52.9 |
| 2+ | 15 | 71.4 | 13 | 68.4 | 3 | 27.3 | 7 | 41.2 |
| 3+ | 0 | 0 | 1 | 5.3 | 0 | 0 | 0 | 0 |

**Table 5.** Comparison of NT-3-positive and NT-3-negative rates between BM and primary lesions or other metastasis of CRC

| Samples | Positive N (%) | Negative N (%) | *P* value |
|---|---|---|---|
| BM | 14 (73.7) | 5 (26.3) | |
| Primary lesions | 15 (71.4) | 6 (28.6) | 0.2887 |
| Liver metastasis | 3 (27.3) | 8 (72.7) | 0.0179 |
| Other metastasis | 7 (41.2) | 10 (58.8) | 0.0352 |

HER-2(+) primary lesions in the no-metastasis subgroup, we suggest that HER-2 overexpression may increase the risk of metastasis, particularly to liver and brain. However, there were great differences between the results of the rate of HER-2-positive cases of CRC BM and primary lesions or other metastasis (**Table 3**, P<0.05). CRC BM got the highest rate (BM 36.8%, primary lesions 14.3%, liver metastasis 27.3%, other metastasis 0%). As a result, HER-2 was supported to be the specific mark of CRC BM [31].

*NT-3 and BM of CRC*

Neurtrophin-3 belongs to the mammalian neurotrophin (NT) family that regulates the survival, development, and differentiation of specific neuronal populations [32, 33]. However, NT-3 production may also be a survival advantage for tumor cells, resulting in growth and metastasis [15, 34]. A recent study suggested that NT-3 may promote the growth and survival of

**Table 6.** The relationship between NT-3 and HER-2 in CRC BM

| Model | R | R Square | Adjusted R Square | | |
|---|---|---|---|---|---|
| NT-3/HER-2 | 0.614 | 0.378 | 0.314 | | |
| Model | Sum of Squares | df | Mean Square | F | *P* value |
| Regression | 31.236 | 1 | 31.236 | 10.311 | 0.005 |
| Residual | 51.501 | 17 | 3.029 | | |
| Total | 2584.000 | 19 | | | |

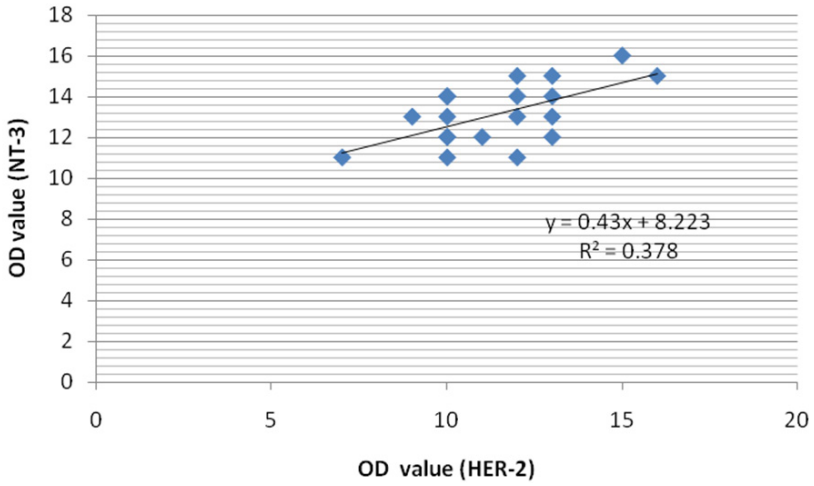


**Figure 3.** The linear regression diagram of NT-3 and HER-2 of CRC BM.

**Table 7.** The relationship between BM positions and the locations of cancer

| Samples | Supratentorial BM N (%) | Subtentorial BM N (%) | *P* value |
|---|---|---|---|
| Colon cancer | 8 (72.73) | 3 (27.27) | 0.2710 |
| Rectal cancer | 4 (50.00) | 4 (50.00) | |

metastasized breast cancer cells in brain by re-epithelialization and suppression of microglial attack [16]. As in the case of HER-2, a higher proportion of BM specimens was NT-3(+) compared to metastases from other tissues. We speculate that NT-3(+) of CRC gains higher incidence than NT-3(-) ones in BM, but not specific in other sites, possibly by enhancing survival in the new environment [16]. Long-term follow-up of patients with and without NT-3(+) primary lesions is required to address this issue.

*The underlying regulation between NT-3 and HER-2*

The mechanisms through which NT-3 and HER-2 overexpression enhance the risk of metastasis are currently unclear. As mentioned, NT-3 may promote breast cancer cell survival [16] by increasing HER-2 and E-cadherin expression levels at the cell-cell junction and reducing the expression of EMT-inducing transcription factor, thereby promoting the mesenchymal-epithelial transition (MET) and the capacity for proliferation in the brain. The same report also suggested that overexpression of NT-3 resulted in upregulation of HER-2 but not TrkC [16], which is the high-affinity TKR for NT-3 [35, 36]. In our study, the expression levels of NT-3 and HER-2 in BM specimens were positively and linearly correlated, suggesting that NT-3 overexpression may upregulate HER-2, although further research is needed to clarify the mechanism. For instance, inhibitors of HER-2 signaling, such as AG879 and Lapatinib, and/or inhibitors of NT-3 could be used to assess the influences of each factor alone and together in preclinical models.

*Position of CRC BM*

While there was a significant relationship between HER-2 expression and metastasis, especially BM, there was no apparent relationship between the position of the primary lesion (colon or rectum) and that of the metastasis. One study suggested that BM from colon cancer always occurred in the supratentorial brain, while that from rectal cancer occurred in the subtentorial part of the brain [37]. In the current study, colon cancer BM was supratentorial more often than rectal cancer BM (72.73% vs. 50.00%) (**Table 7**), consistent with this previous result, although the difference did not reach significance due to the small sample size.

*HER-2, NT-3, and survival of CRC BM*

Park and colleagues reported worse prognosis in HER-2(+) CRC patients [38], but other have

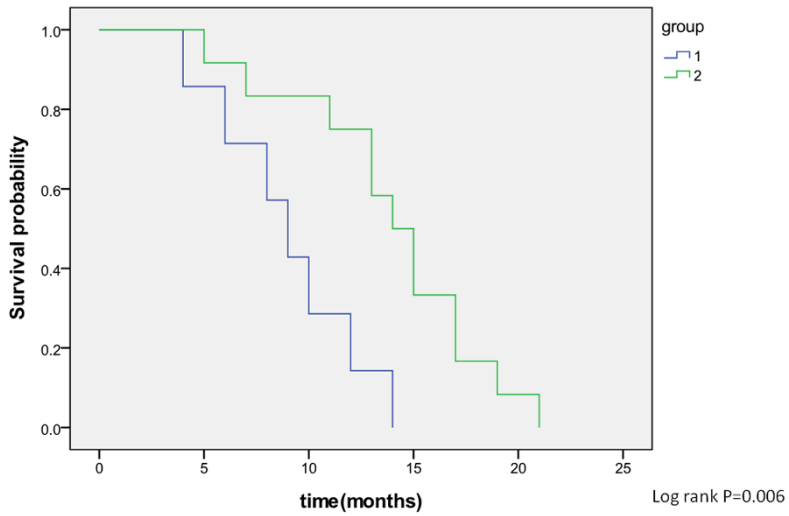


**Figure 4.** Overall survival of the CRC BM population: BM HER-2(+) (group 1: score 2+/3+) vs. BM HER-2(-) (group 2: score 0/1+).

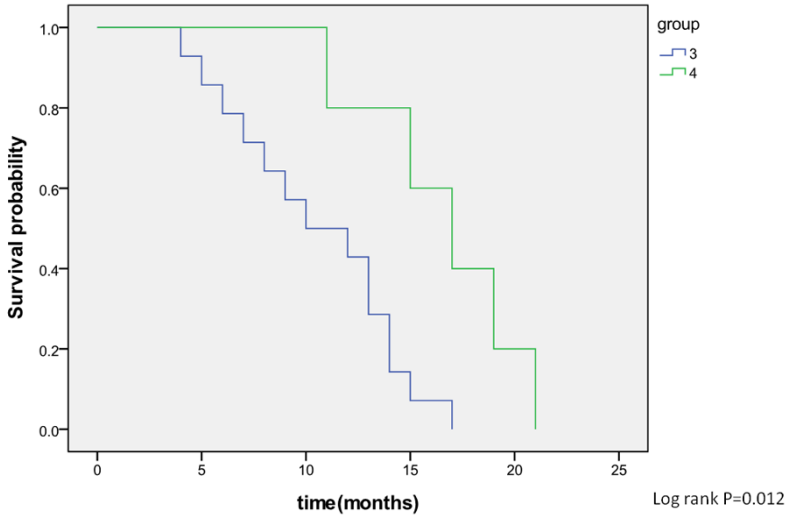


**Figure 5.** Overall survival of the CRC BM population: BM NT-3(+) (group 3: score 2+/3+) vs. BM NT-3(-) (group 4: score 0/1+).

found no difference in OS between HER-2(+) and HER-2(-) cases [39, 40]. Further, this issue has not been addressed specifically in CRC with BM patients. In the current study, patients with HER-2(-) BM from CRC had better prognosis as evidenced by significantly longer mean OS (**Figure 4**). Similarly, NT-3(-) cases showed better prognosis, consistent with the proposed interaction of HER-2 with NT-3 in promoting the survival and growth of BM [16]. Taken together, our data suggest that dual HER-2/NT-3 overexpression or amplification increases the risk of metastasis from CRC, particularly to the brain.

## Conclusions

The incidence of BM from CRC has increased due to improved treatment of the systemic disease and concomitant prolonged survival, but little is known about the underlying mechanisms [41]. This is the first study reporting NT-3 status and the relationship between NT-3 and HER-2 expression in CRC with brain metastasis. Brain metastases from CRC showed higher rates of HER-2 and NT-3 overexpression (36.8% and 73.7%, respectively) than other CRC metastases and primary CRC lesions. Our results highlight the therapeutic potential of targeting HER-2 signaling for reducing the risk of brain metastasis from CRC. Overexpression of HER-2 and NT-3 lead to poor prognosis of patients with BM from CRC. However, our study has several limitations. The cohort of patients was relatively small and final clinical outcomes have not yet been determined. Further analysis on larger populations is warranted to confirm these results and provide a sufficient sample for ROC analysis of the sensitivity and specificity of NT-3 or HER-2 overexpression for predicting brain metastasis from CRC. Much further study is also required to elucidate the underlying mechanisms for promotion of metastasis by NT-3 and HER-2. Such insights could lead to new clinical therapies for CRC patients with BM.

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## Disclosure of conflict of interest

None.

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