Neuromedin U and neurotensin may promote the development of the tumour microenvironment in neuroblastoma

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Abstract
Stage 4S neuroblastoma, as defined by theInternational Neuroblastoma Staging System committee (INSS), is known to regress spontaneously and have a more favourable outcome compared with stage 4 tumours. Comparing the molecular differences between these two stages may provide insights into the progression of neuroblastoma. Our study aimed to explore the molecular differences in the tumour microenvironment (TME)

42   between INSS stage 4S and stage 4 tumours to provide an insight into the mechanisms underlying the
43   biological processes of neuroblastoma. We downloaded the datasets GSE120572 and GSE73517 from the
44   GEO database and pre-processed them using the limma package. CIBERSORT deconvolution agorithm was
45   applied to analyse the differences in 22 infiltrating immune leukocyte subsets between the two stages. We
46   used gene ontology (GO) enrichment analysis to determine the biological process (BP) annotation of
47   differentially expressed genes (DEGs) using the online WebGestalt tool. Hub genes were determined in the
48   STRING database and Cytoscape, and the expression of these genes was verified in the Oncomine database.
49   Then these critical genes were performed survival analysis in TARGET database. We further validated the hub
50   genes using a transwell assay and wound healing assay to detect the function of the genes in the
51   neuroblastoma cell line SK-N-BE(2). GO analysis revealed that the 216 DEGs were enriched in aggressive
52   biological processes. Neuromedin U (NMU) and neurotensin (NTS), which were significantly associated with
53   patients' overall survival rate, were verified to be elevated in stage 4, and to promote the proliferation and
54   invasion of theSK-N-BE(2) cell. Tumour infiltrating leukocyte analysis showed a high infiltration of regulatory T
55   cells and type 2 tumour-associated macrophages in stage 4 but not in stage 4S. Results of gene co-expression
56   correlation, and the results of previous studies, suggest that NMU and NTS may play certain roles in
57   modulating TME, thus facilitating the progression of neuroblastoma.
58

Introduction

60   Neuroblastoma arises during foetal or early post-natal life from the sympathoadrenal lineage of the neural
61   crest and presents a broad clinical spectrum characterized by enormous biological and genetic heterogeneity.
62   Neuroblastoma can spontaneously regress, mature, or demonstrate an aggressive and malignant phenotype
63   that is poorly responsive to current multimodal therapy (Castleberry, 1997; Luksch et al., 2016). In what the
64   International Neuroblastoma Staging System Committee (INSS) defines as stage 4, tumour cells metastasize
65   to distant tissues via blood and lymphatic vessels, with obviously increased lymphangiogenesis. INSS stage 4S
66   is a unique category of metastatic disease in infants with dissemination localizedin the liver and skin, with
67   minimal marrow involvement (i.e., <10% of total nucleated cells identified as malignant by bone biopsy or by
68   bone marrow aspirate) (Bethesda, 2002). Considering that stage 4S can regress spontaneously and has a
69   favourable outcome compared with stage 4, we may be able to obtain insight into the progression of
70   neuroblastoma by comparing the molecular differences between these two stages.
71

72   The tumour microenvironment (TME) is the cellular and matrix environment that includes tumour cells,
73   immune cells, stroma cells, and the extracellular matrix (McCarthy, 2014). The imbalance between the
74   effector and regulatory cell interactions in the TME are known to contribute to the major immune evasion
75   mechanisms of tumours.Tumour-infiltrating lymphocytes (TILs) play a pivotal role in regulating tumour
76   growth and anti-tumour progression. Researchers have confirmed that the infiltration of CD8^{+} T lymphocytes,
77   CD4^{+} T lymphocytes, memory T lymphocytes, and natural killer (NK) cells is linked to a favourable outcome in
78   several cancers including breast cancer, colorectal cancer, and neuroblastoma (Galon et al., 2007; Mina et al.,
79   2015). Conversely, a high level of immunosuppressive immune cells including regulatory T lymphocytes
80   (Tregs), myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages type 2 (TAM-M2)
81   may be involved in weakening the efficacy of anti-neoplasm immune responses (Carlson & Kogner, 2013)
82   leading to aggressive tumour growth and an unfavourable outcome.
83

84   The molecular and cellular mechanisms underlying the interaction between immune cells and neuroblastoma

85 have been studied. MYCN amplification has been determined to be independent high risk factor for
86 stratification in neuroblastoma study (Luksch et al. 2016), however, the existence of key genes and the
87 modulation of tumour microenvironment by these remain to be elucidated. In this study, we attempted to
88 research the overall gene alteration between stage 4S and stage 4, and to explore the impact of key genes on
89 TME processes to explain the mechanisms of neuroblastoma development.
90

Materials and methods

92

1. Data pre-processing

94 Raw data matrices of GSE120572 (Ackermann et al., 2018) and GSE73517 (Henrich et al., 2016) datasets were
95 obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120572,
96 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73517). These datasets were a natural source of
97 neuroblastoma samples without additional confounding factors. The gene expression profiling conducted by
98 array were based on the GPL16876 platform (Agilent-020382 Human Custom Microarray 44k (Feature
99 Number version)). GSE120572 contained 46 INSS stage 4S and 168 stage 4 patients, and GSE73517 contained
100 20 stage 4S and 56 stage 4 patients. In GSE120572 dataset, stage 4 included 58 MYCN amplified and 109 non-
101 amplified samples, and stage 4S included 6 amplified and 40 non-amplified samples. In GSE73517 dataset,
102 stage 4 included 26 MYCN amplified and 30 non-amplified samples, and stage 4S included 3 amplified and 17
103 non-amplified samples. The clinical background of the datasets is shown in Supplementary Table 1. We
104 obtained the official gene symbol according to the probe ID files and duplicates were merged by averaging
105 their expression. Neuroblastoma transcriptome expression data with clinical characteristics in TARGET
106 (https://ocg.cancer.gov/programs/target) database were obtained for survival analysis of critical genes. In
107 this dataset, stage 4 contained 27 MYCN amplified and 92 MYCN non-amplified samples. Stage 4S consisted
108 of 1 MYCN amplified and 19 MYCN non-amplified samples (Supplementary Table 2). All series expression
109 matrices were extracted and normalized using R 3.6.1 software, limma package and survival package. Codes
110 were uploaded to the Figshare platform [https://figshare.com/s/da446df5fa194bc24f90].
111

2. Statistical methods

113 The differentially expressed genes (DEGs) located between stage 4S and stage 4 were analysed based on
114 Bayes' test using the limma package and Pvalues were adjusted using the FDR method. The screening criteria
115 of significant DEGs were set as |logFC| > 1 (FC means fold change of gene expression) and *adj.P.Val*< 0.05
116 (adjusted *P* value). Data from in vitro experiments were analysed using GraphPad Prism 8 software (San
117 Diego, USA) with the unpaired t-test method. A significant difference was determined as *P*< 0.05.
118

3. Gene ontology enrichment analysis

120 To explore the overall function of specific genes, DEGs from the two datasets were intersected to obtain
121 optimal differential genes. Gene Ontology (GO) enrichment analysis was used to explore the biological
122 process (BP) annotation using the WebGestalt online tool (http://www.webgestalt.org). The basic parameters
123 were set to include: *Homo sapiens* as the organism of interest, over-representation analysis (ORA) as the
124 method of interest, gerontology as the functional database, and gene ID type as the gene symbol. Significant
125 differences were set as FDR < 0.05.
126

4. Hub genes identification

128 The intersected genes were analysed in the STRING database (https://string-db.org/) to investigate the

129   multiple protein interactions, with a 0.7 interaction score. Cytoscape software was used to visualize the
130   networks. The intersection of the top 30 genes was ranked by degree and the hub genes were further
131   identified using the plugin CytoHubba.

132

133   The expression values of the hub genes between stage 4S and stage 4 were obtained from the Oncomine
134   database (https://www.oncomine.org/). One dataset collected 19 patients, including 4 stage 4 and 1 stage 4S
135   (Albino et al., 2008). The other one dataset collected 117 patients, including 27 stage 4 and 57 stage 4S
136   (Asgharzadeh et al., 2006). All gene expression profiles were analyzed by microarray technology. We selected
137   genes that were expressed with a significant difference (*P*< 0.01) between the two stages.

138

139   The validated genes were further analyzed the relationships between the gene expression and patients'
140   overall survival rate using survival package. Significant difference criterion was determined by a *P*-value <
141   0.05.

142

143   **5. Tumour-associated immune cell infiltration analysis**
144   Tumour infiltrating leukocytes (TILs) are an integral component of the tumour microenvironment (TME) and
145   correlate with prognosis and response to treatment. CIBERSORT (Chen et al., 2018)
146   (https://cibersort.stanford.edu) is a versatile computational method used to quantify and distinguish 22
147   leukocyte subsets from bulk tissue gene expression profiles. This database provided the gene expression
148   matrix of 22 immune cellsas a background correction file and corresponding R script. We explored the
149   differences in the infiltrating immune cells between the two stages by mapping and integrating the matrix to
150   GEO gene expression matrices by introducing the R script downloaded from CIBERSORT. Statistical
151   significance was determined by a *P* < 0.05.

152

153   **6. Validation of hub genes**
154   We obtained two significantly upregulated genes by combining the GEO database and the Oncomine
155   database, neuromedin U (NMU) and neurotensin (NTS), between stage 4S and stage 4 in neuroblastoma. To
156   validate the function of the two molecules, we performed in vitro experiment using the RNA interfering (RNAi)
157   method.

158

159   **6.1 Cell culture**
160   The human neuroblastoma cell line SK-N-BE(2) was donated by Chenjie Tang, which was purchased from
161   Zhong Qiao Xin Zhou Biotechnology Corporation (Shanghai, China). The cells were cultured in F12k & MEM
162   (v/v=1:1) medium with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, USA), 100 U/ml penicillin, and
163   100 U/ml streptomycin in 5% atmospheric CO_2 at 37˚C. Cells were cultured in 6-well plates with $2.5×10^5$
164   cells/well.

165

166   **6.2 siRNA transfection**
167   Chemosynthetic siRNAs of NMU and NTS were purchased from Ribobio Techonology Corporation
168   (Guangzhou, China). The target sequences for the NMU gene were: 5'-CAAUGUUGUAAAUGUUCAAUU-3'
169   (sense),   5'-UUGAACAUUUACAACAUUGUU-3'   (antisense),   and   for   the   NTS   gene   were: 5'-
170   GGAAGAUGACUCUGCUAAAUG-3' (sense), 5'-UUUAGCAGAGUCAUCUUCCAA-3' (antisense). Negative control
171   sequences were: 5'-GCUGUCGACUGAGUUAGAGGA-3' (sense), 5'-GUCCAUCUCAUAACACAGCUG-3' (antisense).

172

173 siRNAs were diluted and transiently transfected with lipofectamine 2000 in cells (Invitrogen, Thermo Fisher
174 Scientific, USA) in 6-well plate (Corning, USA) for a final concentration of 100 nM per well once the cell
175 monolayer reached 80%-90% in thebase of each well.Cells were harvested after 48 hours of transfection for
176 Transwell assay and wound-healing assay. Experiments were repeated in triplicate.
177
178 **6.3 Western blot**
179 To validate the interfering effect, we used a Western blot assay to detect the expression of NMU and NTS
180 before and after transfection of siRNA. Rabbit polyclonal anti-NMU (20 kDa, Lot#: ab229782) antibody and
181 rabbit polyclonal anti-NTS (20 kDa, Lot#: ab233107) antibody were purchased from Abcam (USA); GAPDH
182 rabbit monoclonal antibody(36 kDa, Lot#: AF1186) and HRP-labeled goat anti-rabbit IgG (H+L, Lot#: A0208)
183 were purchased from Beyotime (Shanghai, China). The optimal dilutions of the primary antibodies and
184 secondary antibody were determined by anti-NMU and anti-NTS as 1:1,000, anti-GAPDH as 1:3,000, and the
185 secondary antibody as 1:500, respectively.
186
187 Cells were trypsinized and washed twice with phosphate buffer saline (PBS, Hyclone, USA). Protein lysate was
188 obtained by RIPA lysis buffer (Beyotime, Shanghai) according to the manufacturer's instructions. Protein
189 lysates (50 μg in each well) of identical quality were loaded and separated by polyacrylamide gel
190 electrophoresis and transferred to the polyvinylidene fluoride membrane(Bio-Rad, USA). Primary antibodies
191 were incubated in 4°C for 16 hours, and secondary antibodies were incubated in room temperature for 1
192 hour. Finally, ECL chemiluminescence reagent (Millipore, USA) was used to detect the expression of protein.
193
194 **6.4 Transwell assay**
195 Cells were harvested and added to a 24-well Transwell plate (Corning, USA) with 1×10^4 cells per well after 48
196 hours of transfection. Cells were cultured for 24 hours and then stained with 1% crystal violet solution to
197 detect the invading cell numbers under the microscope. Experiments were repeated twice, and ten fields of
198 photos taken under microscope were taken for each group.
199
200 **6.5 Wound-healing assay**
201 The cell monolayer in the 6-well plate was scraped by 10 μl pipette tip, then washed twice using PBS (Hyclone,
202 USA). Three fields of photos were taken for each group at 200x resolution by microscope during the initial (0
203 hour) gap width.Three addition fields of photos were taken for each group after 48 hours of cell cultivation to
204 assessthe residual gap width. The width counts were calculated by imagePro Plus software (Media
205 Cybernetics Inc., USA). The change of gap width was determined by $| \text{width}_{0h}\text{-width}_{48h} |$; three fields of
206 photos under microscope were adopted for each group.
207
208 **7. Co-expression relationships of hub genes and immune-related genes**
209 Pearson test was used to analyze the relationships of hub genes expression and infiltrated immune cells,
210 thereby to detect the potential function of these critical genes in neuroblastoma development. Correlation
211 coefficient was determined by a r > 0.5 and $P < 0.05$.
212
213 # Results
214
215 **1. Gene ontology analysis revealed that genes were enriched in aggressive biological**

216 **progression**
217 We obtained 309 and 1,103 DEGs from the GSE120572 and GSE73517 datasets, respectively, when
218 comparing INSS stage 4S and stage 4. Of these, 216 intersected DEGs were further screened from the two
219 datasets (Figure 1A).
220
221 Consequently, GO analysis revealed that BP terms of the 216 DEGs were enriched in positive regulation of the
222 development process, cell adhesion, signal transduction, and negative regulation of cell death, suggesting
223 that these genes are involved in aggressive tumour development (Figure 1B, Supplementary Table 3).
224
225 **2. NMU and NTS were identified as elevated hub genes in stage 4**
226 We constructed protein-protein interactions (PPI) networks in the STRING database to learn more about the
227 function of the identified genes. Their networks were subsequently visualized by Cytoscape (Figure 2A,
228 Supplementary Figure 1). The top 25 hub genes were screened out with the Degree method using CytoHubba
229 (Figure 2B, Supplementary Table 4).
230
231 We then extracted expression values of the 25 genes in stage 4S and stage 4 from the Oncomine database,
232 respectively, and compared the differences between the two stages. Results showed NMU and NTS were
233 significantly elevated in stage 4, without obvious differences being found between the two stages for the
234 remaining 23 genes (Figure 2C, 2D).
235
236 Combining data from TARGET database, we found that patients' overall survival rate in high NMU expression
237 group was significantly lower than that in low expression group (P = 2.25e-06). NTS presented similar action
238 (P = 0.019) (Figure 2E, 2F).
239
240 **3. Significant tumour-associated lymphocytes infiltration occurred in stage 4**
241 By integrating the 22 immune cells basic gene expression matrix, which as a background gene expression file,
242 to GSE120572 and GSE73517, we found there was no significant ($P > 0.05$) immune cell infiltration in INSS
243 stage 4S for any of the two datasets. Conversely, obvious infiltration of tumour-associated macrophages type
244 2 (TAM-M2) and regulatory T cells (Tregs) was determined in stage 4, for both datasets (Figure 3A-3D).
245
246 **4. NMU and NTS promote cell proliferation and invasion of SK-N-BE(2)**
247 The results of the Western blot assay showed obvious interference of the target genes (Figure 4A, 4B). The
248 results of the transwell assay showed that the invaded cell number in si-NMU and si-NTS group were
249 significantly less than the corresponding negative control ($P < 0.0001$, t=12.81; $P < 0.0001$, t=8.714),
250 respectively (Figure 4C-4E, 4F-4H). The results of the wound-healing assay revealed the interference of NMU
251 and NTS led to a significant migration distance less than the negative control ($P = 0.0003$, t=11.69; $P = 0.0002$,
252 t=13.04), respectively (Figure 4I-4J, 4K-4L, 4M). Our results suggested that NMU and NTS could facilitate the
253 invasion and migration of SK-N-BE(2).
254
255 **5. NMU and NTS were significantly correlated with immune-related genes**
256 Results of genes co-expression analysis showed that expression of NMU was positively associated with CD4,
257 CD25, FOXP3, NFAT, IL-4 and IL-13 (Figure 5A-5F, 5M-5R). NTS presented positive relationships with CD163,
258 CD206, IL-10, TGF-β, STAT3 and STAT4, both in GSE120572 and GSE73517 datasets (Figure 5G-5L, 5S-5X).
259

Discussion

261 The tumour microenvironment is the cellular environment comprising tumour cells, immune cells, stromal
262 cells, and the extracellular matrix. The TME plays three important roles in stimulating the development of
263 tumours by providing a growing environment for tumours, disabling the efficacy of anti-tumour drugs, and
264 assisting tumour cell evasion from immune surveillance (Kerkar & Restifo, 2012; Patel et al., 2018; Potter,
265 2007). The mechanisms underlying the interaction between immune cells and neuroblastoma cells have been
266 studied, but many questions remain unanswered, including the identification of key genes and how they to
267 modulate TME. INSS stage 4S neuroblastoma is a special category of metastatic disease that may regress
268 spontaneously. It has been shown to have a favourable outcome when compared to INSS stage 4 (Nickerson
269 et al., 2000; Schleiermacher et al., 2003); thus, comparing the molecular differences between the two stages
270 may provide some explanation for tumour progression. We demonstrated the significant infiltration of Tregs
271 and TAM-M2 in stage 4 cases from two datasets, without obvious immune cell infiltration in stage 4S.

273 Tregs are immune cells characterized by immunosuppression and hypoergia (Campbell, 2015) and can
274 restrain the function of effector cells and attenuate the body's immune response, accelerating tumour
275 angiogenesis and development (Sakaguchi et al., 2020). With single-cell sequencing analysis, Zheng et al.
276 (2017) proved that non-functional CD8^{+} T cells and Tregs were enriched in liver cancer tissues, suggesting that
277 Tregs may inhibit CD8^{+} T cell activity to promote liver cancer progression. Further, tumour-associated
278 macrophages (TAMs) participate in the process to facilitate tumour immune escape through polarization
279 (Hao et al., 2012). Studies have shown that tumour-derived IL-4, IL-5, IL-6, IL-10, and IL-13 can stimulate TAM
280 polarization towards TAM-M2 (Martinez et al., 2008). Further, cytokines including IL-4, TGF-β, IL-17, VEGF,
281 PDGF, M-CSF, and MMPs secreted by TAM-M2 (Coffelt et al., 2009) are involved in tumour invasion,
282 metastasis, angiogenesis, and lymphangiogenesis. IL-10 derived from TAM-M2 has been implicated in the
283 suppression of NK cells and cytotoxic T lymphocytes (Benner et al., 2019), thereby reducing the tumour cells
284 to be killed. The accumulation of Tregs and TAM-M2 is a significant event in tumour development.

286 It is difficult to determine the underlying causes of the changes in TME between INSS stage 4S and stage 4.
287 We sought to determine the molecular alterations influencing TME development and found that neuromedin
288 U (NMU) and neurotensin (NTS) are hub genes elevated in stage 4 compared to stage 4S. In addition, we
289 compared the gene expression differences between MYCN amplified and MYCN non-amplified groups in
290 these stages, however, NMU and NTS were not found in the differentially expressed genes. This suggested
291 that NMU and NTS may not be driven by MYCN amplification (The results were not presented). Studies that
292 have linked NMU to cancer indicate that NMU is significantly upregulated in most malignancies, including
293 neuroblastoma (Martinez et al., 2017; Przygodzka et al., 2019). NMU upregulation in the tissues and cell lines
294 of malignancies correlated with increased invasiveness and metastatic potential of tumour cells and
295 advanced stages, such as head and neck carcinoma, pancreatic tumour and lung cancer (Tokumaru et al.
296 2004; Wang et al. 2016; Yang et al. 2018). Lebedev et al. (2019) demonstrated that the increased expression
297 of NMU was associated with poor prognosis in neuroblastoma. Similarly, the oncogenic action of NTS has
298 been described in different cancers with effects at each step of progression (Ouyang et al., 2017). For
299 example, NTS promotes glioma cell invasiveness through NTSR1 in vitro and in vivo (Ouyang et al. 2015).
300 NMU and NTS are known to exert functions by binding to their relevant receptors and activating the
301 corresponding signalling pathways to directly or indirectly accelerate tumours into malignant characteristics.

303   The influence of NMU and NTS on the tumour microenvironment has not been reported. NMU and NTS
304   receptors, which belong to G-protein-coupled receptor (GPCR) families, were found to be expressed on the
305   membrane of immune cells including T cells, B cells, macrophages, dendritic cells, mast cells, and on tumour
306   cells (Hedrick et al., 2000; Kim et al., 2006; Magazin et al., 2004; Saada et al., 2012). Johnson et al. (2004)
307   confirmed that the binding of NMU to NMUR increased the synthesis and secretion of IL-4, 5, 6, 10, and 13 in
308   CD4^{+} T cells, and may be mediated as follows: activated phospholipase C (PLC) induced by G protein
309   hydrolysed phosphoinositide 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5 Tris-
310   phosphate (IP3); IP3 subsequently elicited Ca^{2+} release from the endoplasmic reticulum, which resulted in
311   calmodulin binding, stimulation of calcineurin, and dephosphorylation of NFAT family members, thereby
312   facilitating the production of ILs (Johnson et al., 2004; Park et al., 2012; Smith-Garvin et al., 2009). The
313   binding of IL-4 and IL-13 to their receptors is linked to macrophage polarization to the M2 type via activation
314   of the JAK-STAT pathways (Martinez et al., 2008; Mosser & Edwards, 2008). Kim et al. (2006) suggested that
315   NTS immediately mediated the stimulation of the JAK-STAT1 pathway in macrophages. The JAK-STAT
316   pathway is reported to be pivotal for TAM polarization to the M2 type (Wilson, 2014). IL-10 and TGF-β are
317   powerful stimulants secreted by TAM-M2 and can transform CD4^{+} T cells into Tregs(Liu et al., 2013; Shen et
318   al., 2015; Zhou et al., 2017).
319
320   In this study, we found NMU was significantly correlated with the expression of CD4, CD25, FOXP3, NFAT, IL-4
321   and IL-13, suggesting NMU may perform positive action in Tregs activation and engage in TAM-M2
322   polarization related cytokines release from CD4+ T cells. Meanwhile, NTS presented positive relationships
323   with CD163 and CD206, which are the molecular biomarkers of TAM-M2, reminding us that NTS may involve
324   in polarization of tumour infiltrating macrophages to M2 subtype. Co-expression of IL-10, TGF-β, STAT3 and
325   STAT4 partially demonstrated NTS may facilitate M2 cells to release cytokines, thereby in return to
326   strengthen CD4+ T cells activation. Moreover, results showed NMU and NTS were inversely correlated with
327   neuroblastoma patients' overall survival rate. These further provided us an evidence that NMU and NTS may
328   influence neuroblastoma development by interfering its microenvironment.
329
330   Our data, and the results of previous studies, suggest that NMU and NTS may mediate the interaction of
331   cancer cells and immune cells and may influence the progressionof neuroblastoma (Figure 6), however
332   further in vitro and in vivo experiments should be undertaken to confirm this speculation.
333

Conclusions

335   Our results indicate that NMU and NTS may play roles in the deterioration of the neuroblastoma
336   microenvironment and may provide evidence for future treatments.
337

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Figure 1

Gene Ontology enrichment analysis for biological processes (GO_BP) of significant differentially expressed genes (DEGs), which were compared between stage 4 and 4S. .

(A) 216 DEGs were intersected from GSE120572 and GSE73517 datasets. **(B)** GO enrichment analysis showed aggressive characteristics of these DEGs that implicating in tumor biological processes.
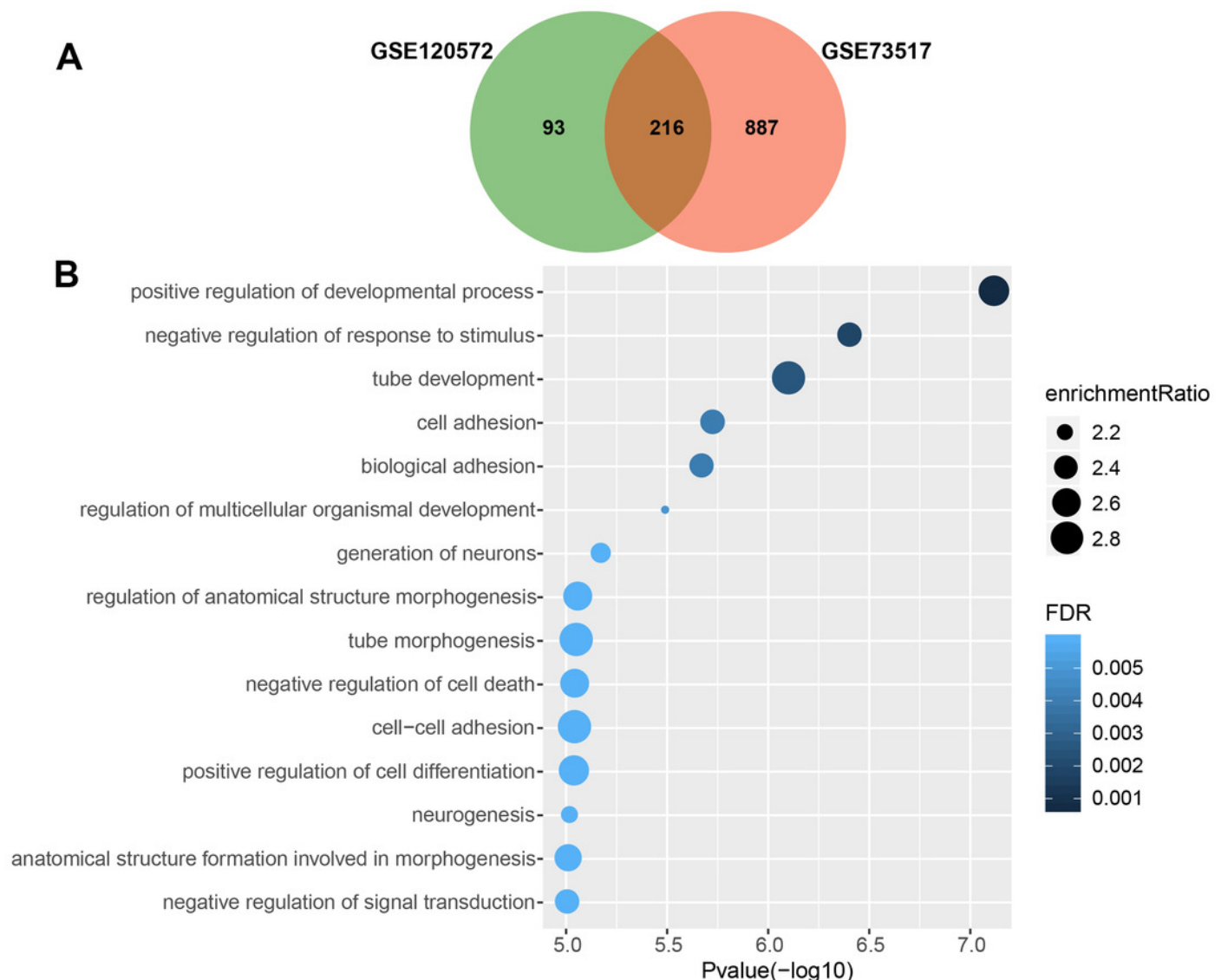
Figure 2

Screening and validation of hub genes.

(A) Workflow of hub genes screening. **(B)** Visualized network of Hub genes by Cytoscape, 25 hub genes were included by Degree method. **(C-D)** NMU and NTS were further determined to be significanly elevated in Oncomine database. (**E-F**) NMU and NTS were inversely correlated with neuroblastoma patients' overall survival rate.
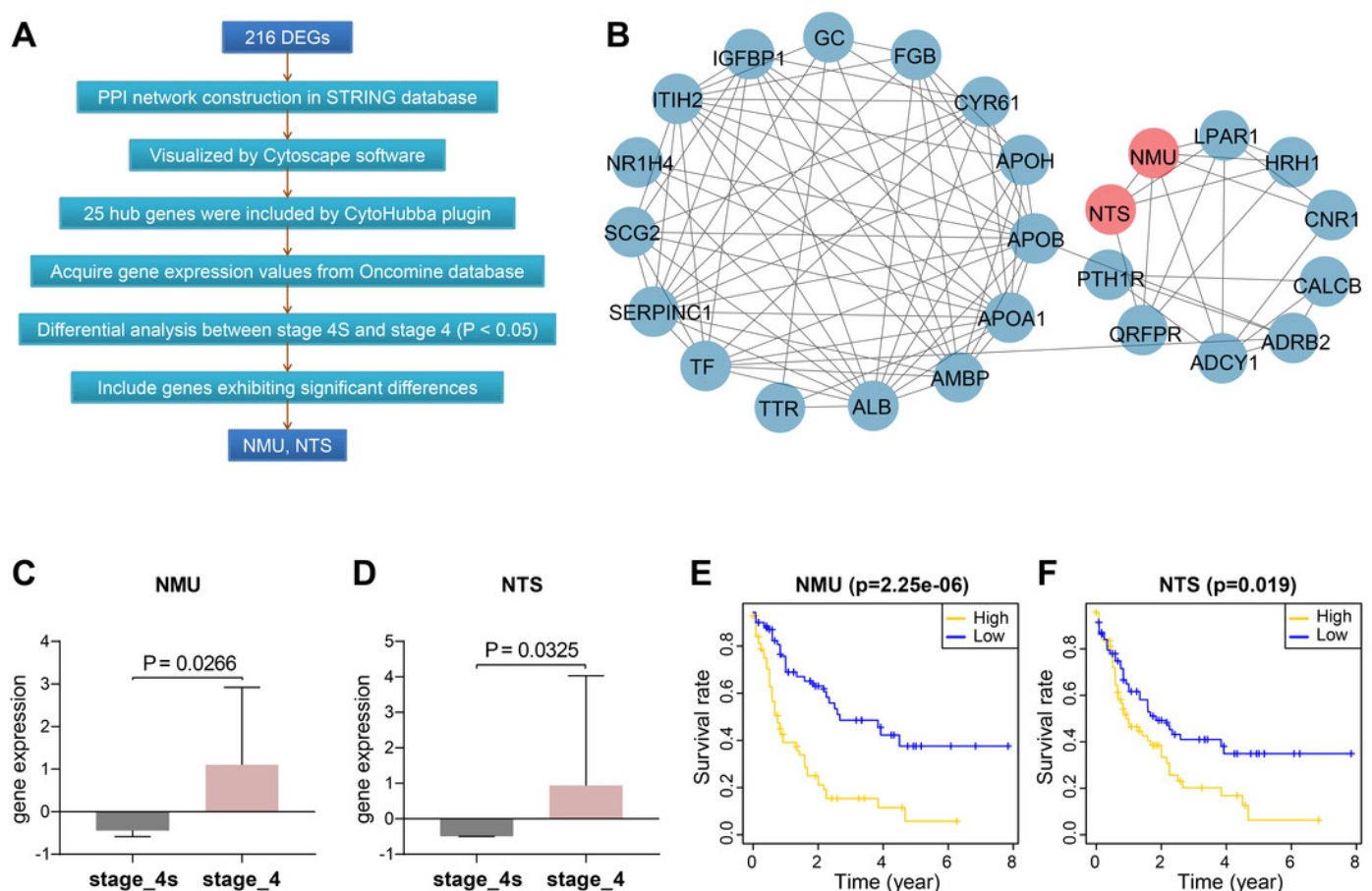
Figure 3

Immune cells infiltrating analysis.

(A-B) The proportions of 22 infiltrating immune cells in stage 4 of neuroblastoma of GSE120572 dataset displayed by box plot and heatmap. **(C-D)** The proportions of 22 infiltrating immune cells in stage 4 of neuroblastoma of GSE73517 dataset displayed by box plot and heatmap.
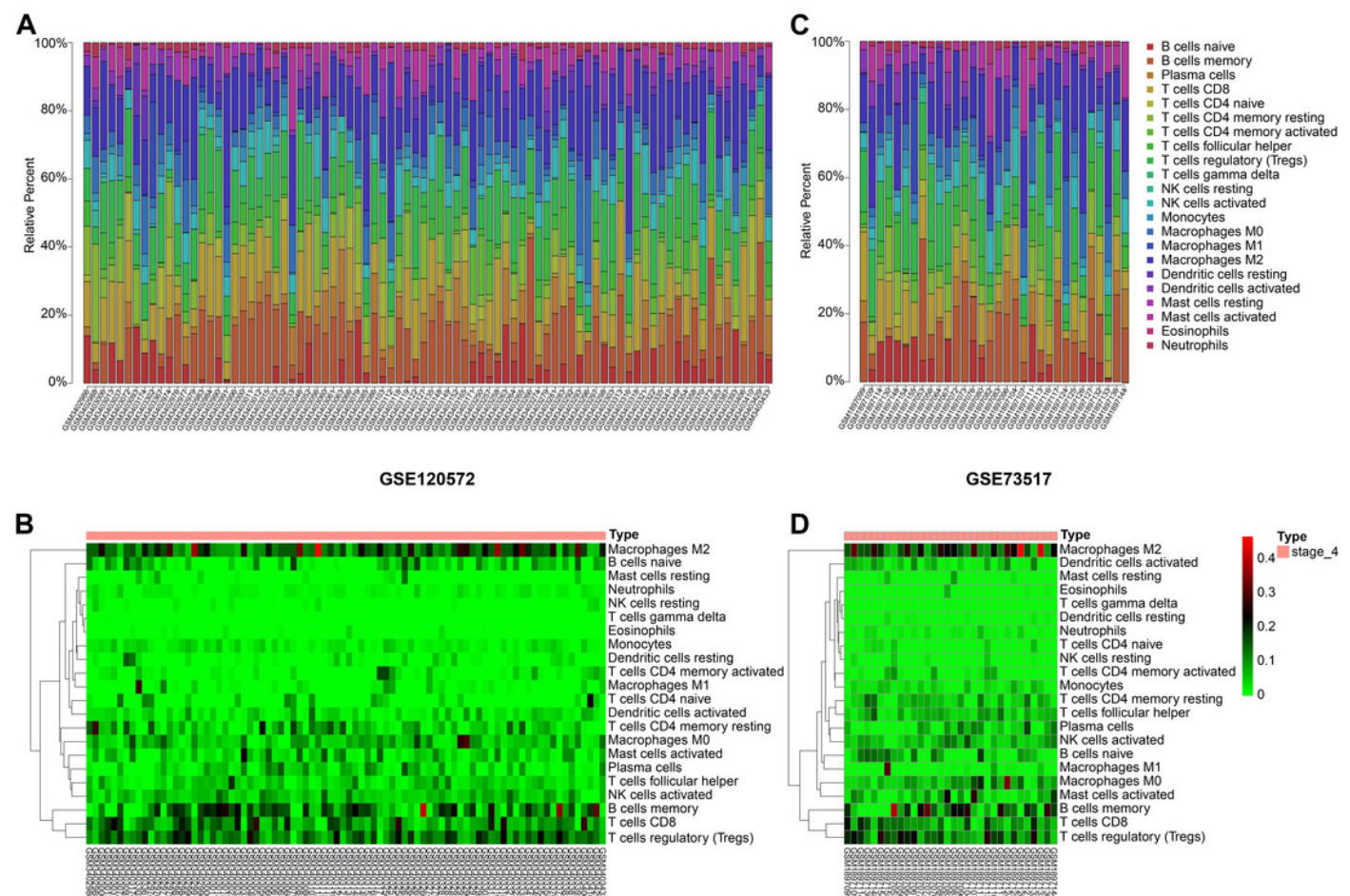
Figure 4

The functional examination of NMU and NTS in SK-N-BE(2) cell.

(A-B) Western blot suggested that siRNA of NMU and NTS could significantly decreased protein expression compared to negtive control group. **(C-E)** Transwell assay showed downregulated NMU reduced invaded cell number compared to negative control. **(F-H)** Transwell assay showed downregulated NTS reduced invaded cell number compared to negative control. **(I-L)** Compared to negative group, si-NMU group or si-NTS showed a slower migration speed from 0 hour to 48 hours. **(M)** Wound healing assay revealed that downregulated NMU or NTS reduced tumour cell migration speed compared to negative control.

A

	si-NC	si-NMU	
NMU			- 20 kDa
GAPDH			- 36 kDa

B

	si-NC	si-NTS	
NTS			- 20 kDa
GAPDH			- 36 kDa

C

si-NMU

D

si-NC

E

Invaded cell number

P <0.001

si-NMU   si-NC

F

si-NTS

G

si-NC

H

Invaded cell number

P <0.001

si-NTS   si-NC

I

si-NMU (0h)   si-NMU (48h)

J

si-NC (0h)   si-NC (48h)

K

si-NTS (0h)   si-NTS (48h)

L

si-NC (0h)   si-NC (48h)

M

The width of wound

***   ***

si-NMU si-NC   si-NTS si-NC
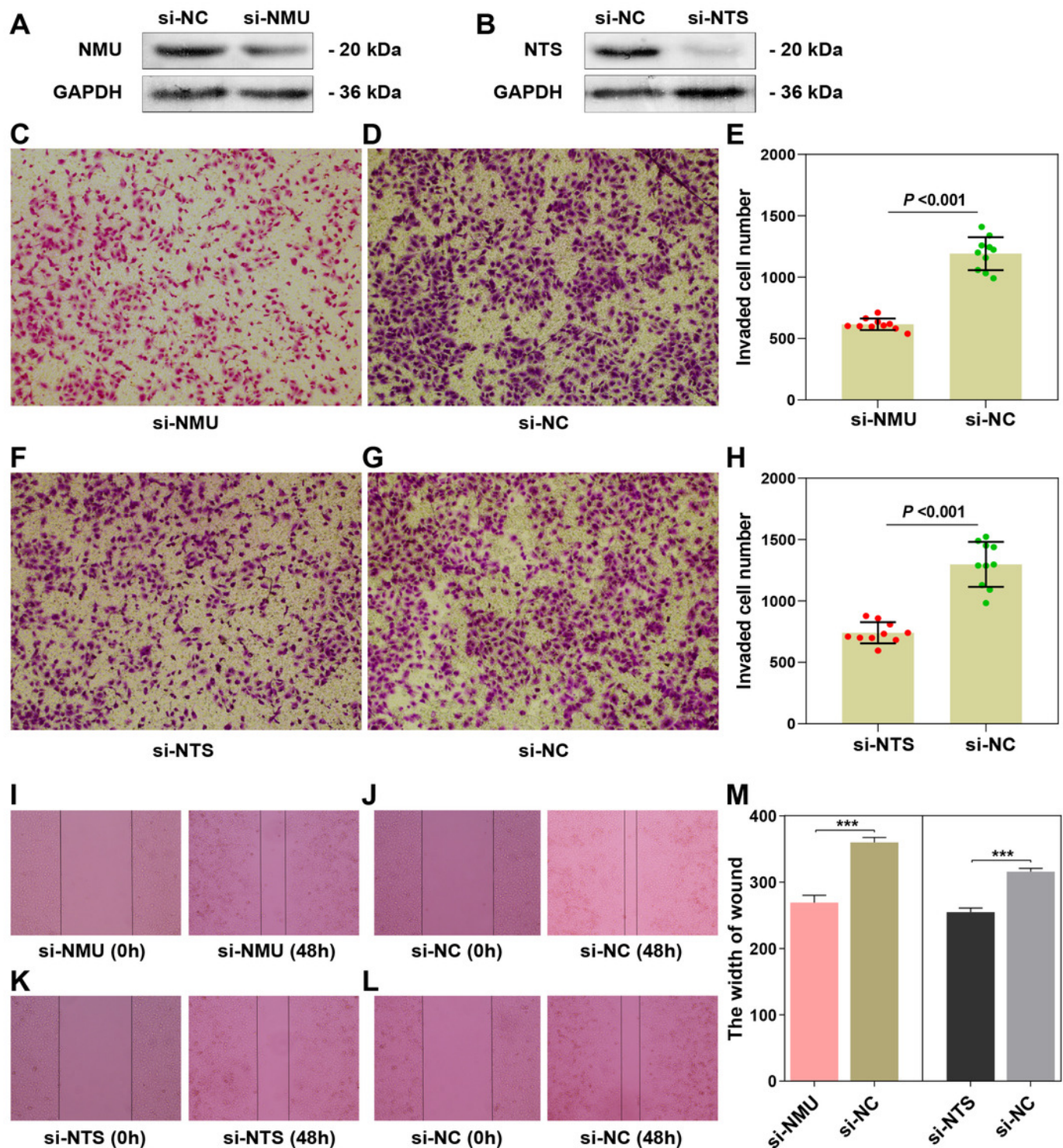
Figure 5

The genes co-expression correlation analysis for NMU and NTS in GSE120572 and GSE73517 datasets.

(**5A-5F, 5M-5R**) NMU was positively associated with CD4, CD25, FOXP3, NFAT, IL-4 and IL-13. (**5G-5L, 5S-5X**) NTS presented positive relationships with CD163, CD206, IL-10, TGF-β, STAT3 and STAT4, both in GSE120572 and GSE73517 datasets.
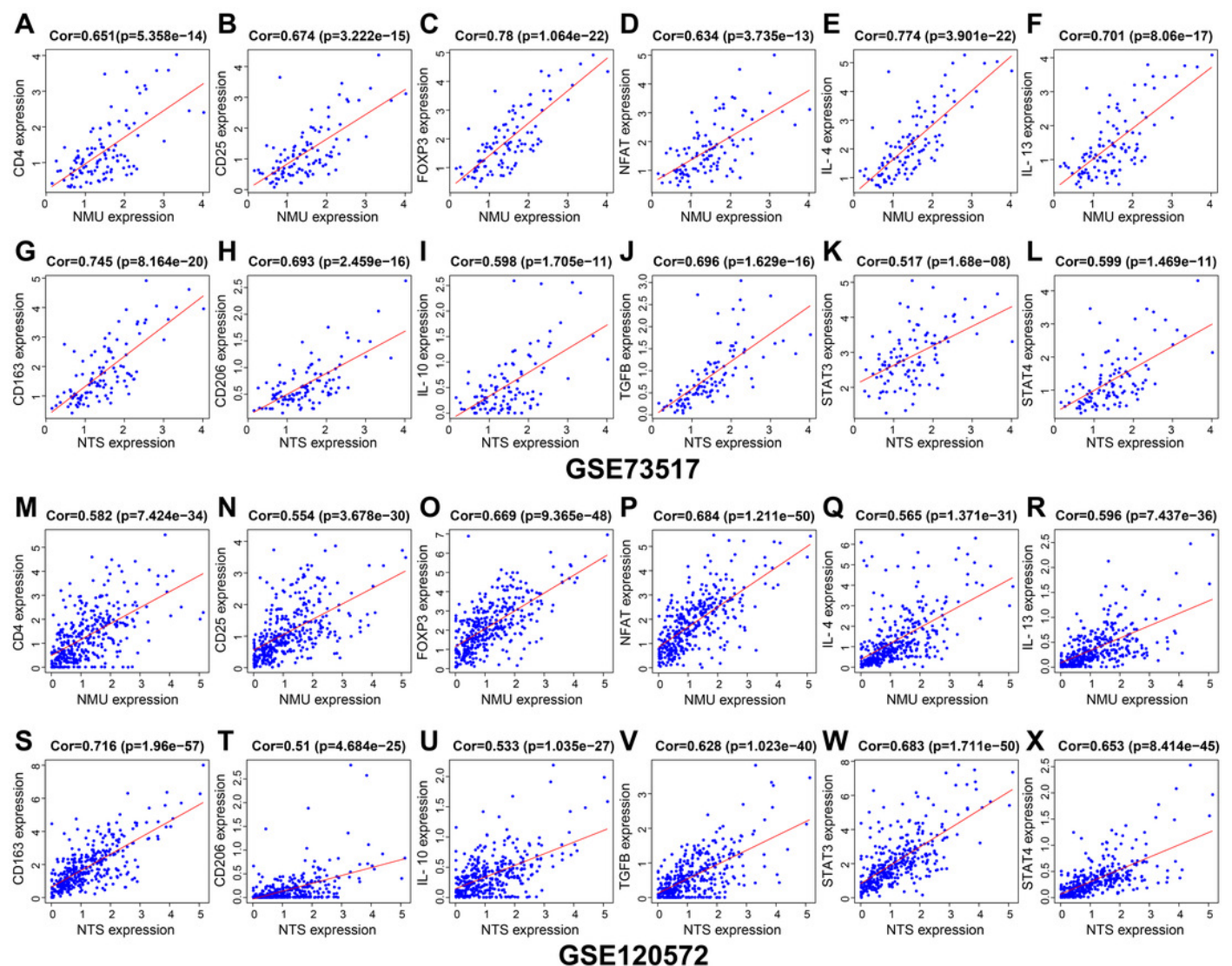
Figure 6

The possible mechanism of interactions between tumor cells and tumor-associated leukocytes

Neuroblastoma cells may synthesize and secret NMU to influence the generation of IL-4 and IL-13 by CD4^{+}T cells. Meanwhile, NTS, which is also secreted by tumor cells, combine with IL-4 and IL-13 to stimulate the polarization of TAM to M2. In addition, IL-10 and TGF-β secreted by excessively activated M2 could stimulate CD4^{+}T cells to transform into Tregs. These complicated interaction networks thus in return to accelerate neuroblastoma development.