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Differential Clinical Significance of Neurotrophin-3 Expression according to MYCN Amplification and TrkC Expression in Neuroblastoma

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ABSTRACT

Background: Neurotrophin-3 (NT-3), a member of the NT family, has only been considered an ancillary compound that provides anti-apoptotic benefits by inactivating tropomyosin receptor kinase C (TrkC)-induced apoptotic signals. However, little is known about the clinical relevance of NT-3 expression itself in neuroblastoma. The purpose of this study was to assess NT-3 expression in patients with neuroblastoma and its relevance to clinicopathologic findings and treatment outcomes.
Methods: In this study, expression of NT-3 and TrkC was analyzed using immunohistochemistry in 240 patients with newly diagnosed neuroblastoma.
Results: The results of the study revealed that NT-3 expression was associated with older age at diagnosis, localized tumors, and more differentiated tumors but was not associated with early treatment response (degree of residual tumor volume after three cycles of chemotherapy) and progression-free survival (PFS). However, when analysis was confined to patients with MYCN amplified tumors, NT-3 expression was associated with better early treatment response with borderline significance ($P = 0.092$) and higher PFS (86.9% vs. 58.2%; $P = 0.044$). In multivariate analysis in patients with MYCN amplified tumors, NT-3 was independent prognostic factor (hazard ratio, 0.246; 95% confidence interval, 0.061–0.997; $P = 0.050$). In another subgroup analysis, the early treatment response was better if NT-3 was expressed in patients without TrkC expression ($P = 0.053$) while it was poorer in patients with TrkC expression ($P = 0.023$).
Conclusion: This study suggests that NT-3 expression in neuroblastoma has its own clinical significance independent of TrkC expression, and its prognostic significance differs depending on the status of MYCN amplification and/or TrkC expression.

Keywords: Neuroblastoma; Neurotrophin-3; MYCN; TrkC

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Disclosure
The authors have no potential conflicts of interest to disclose.

Author Contributions
Conceptualization: Seo ES, Kim JS, Ma YE, Cho HW, Ju HW, Lee SH, Lee JW, Yoo KH, Sung KW, Koo HH. Data curation: Seo ES, Kim JS, Ma YE, Cho HW, Ju HW. Formal analysis: Seo ES, Kim JS, Lee JW. Investigation: Seo ES, Kim JS. Writing - original draft: Seo ES, Kim JS, Sung KW. Writing - review & editing: Seo ES, Kim JS, Yoo KH, Sung KW, Koo HH

INTRODUCTION

Neuroblastoma is the most common extracranial solid tumor in children. The clinical behavior of neuroblastoma is highly complex and heterogeneous. Some tumors either regress spontaneously or mature into a benign tumor without any treatment, whereas others progress rapidly and refractory to any treatment. These extensive and metastatic tumors frequently show high-level amplification of the MYCN locus. MYCN amplification is the genetic aberration that is most consistently and strongly correlated with poor outcome. Its association with poor outcome is not altered by otherwise favorable disease characteristics.[1,2]

Chemotherapy using cytotoxic drugs kills tumor cells by provoking apoptosis in sensitive tumors.[3,4] Early response to induction chemotherapy has prognostic significance in childhood cancer, such as acute lymphoblastic leukemia and bone tumors.[5-8] Moreover, a recent report suggested that the degree of tumor volume reduction in the early phase of induction chemotherapy was also prognostic in patients with high-risk neuroblastoma.[9] In this context, understanding the expression profile of pro-apoptotic or anti-apoptotic regulatory molecules in tumor cells might be crucial not only for evaluating drug sensitivity, but also for predicting long-term treatment outcomes.

The neurotrophins (NTs), including nerve growth factor, brain-derived neurotrophic factor, NT-3, and NT-4/5, and their preferred receptors, tropomyosin receptor kinases (TrkA, TrkB and TrkC) are anti-apoptotic and proto-oncogenic molecules.[10-14] NT/Trk signaling regulates neural development and maintenance of the neural network.[13,15] However, these molecules have also been shown to play a critical role in the tumorigenesis of neuronal or neuroendocrine origin cancers.[16,17] The first evidence for this role was reported in neuroblastoma and its association with MYCN amplification is often addressed in the literature.[18-20] Among several NT receptors, TrkC has emerged as an interesting research topic for its unusual properties of both an oncogene and a tumor suppressor. These features can be explained by the hypothesis that TrkC is a dependence receptor triggering the apoptosis signal pathway in the absence of its ligand (NT-3); it also provides a survival advantage in the presence of NT-3 by inactivating TrkC-induced apoptosis.[17,21-27] However, TrkC's preferential ligand NT-3 has never been of central interest. No study has been conducted on the clinical relevance of its expression despite some evidence of its existence and action independent of TrkC in tumors.[13,21,24,26,28,29] For these reasons, we analyzed NT-3 expression in 240 patients with neuroblastoma to investigate the clinical significance of NT-3 itself.

METHODS

Patients and tumor evaluation

A total of 240 patients who were newly diagnosed with neuroblastoma from May 1997 to November 2015 were enlisted for this study, and paraffin-embedded tissues were collected. Medical records of all patients were reviewed retrospectively. Tumor staging using the International Neuroblastoma Staging System and disease extent was evaluated by imaging studies such as computerized tomography (CT), magnetic resonance imaging (MRI), ^{131}I- or ^{123}I-metaiodobenzylguanidine (MIBG) scan, technetium^{99} bone scan, and bone marrow biopsy specimens. MYCN amplification was determined by competitive polymerase chain reaction (PCR), quantitative reverse transcription PCR, or fluorescence in situ hybridization. Tumors were classified as having favorable or unfavorable pathology according to the

International Neuroblastoma Pathology Classification. Levels of serum lactic dehydrogenase, ferritin, neuron-specific enolase, and urine vanillylmandelic acid were measured routinely at diagnosis. Risk stratification was based on age, stage, and MYCN amplification status. Stage 1, 2, and 4S tumors were stratified into the low-risk group if MYCN was not amplified, whereas stage 4 tumors in patients older than 12 months (until 2008) or 18 months (since 2009) or any tumors with amplified MYCN were classified as the high-risk group. The intermediate-risk group includes all other tumors not mentioned above.

Treatment protocol

Excisional biopsy of the primary tumor was preferred for diagnosis if the tumor was resectable. Otherwise, incisional or percutaneous needle biopsy was performed, and definitive tumor resection was delayed until after six chemotherapy cycles were completed. A combination of CEDC (cisplatin + etoposide + doxorubicin + cyclophosphamide) and ICE (ifosfamide + carboplatin + etoposide) regimens were used for induction chemotherapy in an alternating manner. Low-risk patients underwent surgery with (stage 2) or without (stage 1) six cycles of preoperative or postoperative chemotherapy. Intermediate-risk patients received nine cycles of chemotherapy followed by differentiation therapy with 13-*cis*-retinoic acid. For high-risk patients, tandem high-dose chemotherapy and autologous stem cell transplantation (HDCT/auto-SCT) with total body irradiation (until 2008) or high-dose ^{131}I-MIBG (12 or 18 mCi/kg) treatment (from 2009) followed by local radiation therapy, differentiation therapy, and immunotherapy using interleukin-2 were applied after nine cycles of induction chemotherapy.

Tumor volume measurement

Tumor volume was measured at diagnosis and after three cycles of induction chemotherapy by summing manually drawn areas on CT or MRI scans multiplied by slice thickness using computer software (Advantage Workstation, Volume Share version 2.0; GE Healthcare, Madison, WI, USA). The percent tumor volume after three cycles of chemotherapy were then calculated and compared with primary tumor volume at diagnosis. This manner of measurement was verified and used in previous reports from our centers.[9]

Real-time quantitative PCR

Total RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany). A 1ug of total RNA RT-PCR was transcribed using the QuantiTect Reverse Transcription kit (Qiagen) and performed by RT2 profiler PCR array (Qiagen) which includes NT-3′s primer, using a real-time PCR instrument (Applied Biosystems, CA, USA). The cycle threshold fluorescence values are calculated using the SDS 2.4 software (applied Biosystems). The relative expression of NT-3 was normalized to housekeeping genes. PCR conditions are used; hold for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95, and 1 minute at 60°C.

Immunohistochemistry

NT-3 stain

After fixing and embedding the tissue, samples cut with 3 μm thickness were deparaffinized and rehydrated. The samples were pretreated with envision FLEX target retrieval solution by PR Link instrument. The sections were incubated with peroxidase-blocking solution for 5 minutes and then incubated with NT-3 antibody (1:100 dilution; Abcam Inc., Cambridge, UK) overnight at 4°C. Following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies of the DAKO Envision™ detection kit (DAKO, Glostrup, Denmark) for

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50 minutes at room temperature, a chromogen reaction was done using 3,3′-diaminobenzidine (DAB), and counterstaining was performed with hematoxylin for 30 seconds.

TrkC stain

Specimens were sectioned at 3 μm and stained on positively charged glass slides. Deparaffinization, rehydration, and antigen retrieval were performed using ER1 (prediluted; pH 6.0) antigen retrieval solution on the Bond-max Leica automated slide stainer (Leica Biosystem, Melbourne, Australia) for 40 minutes at 97°C. The sections were incubated with peroxidase-blocking solution for 10 minutes and then incubated with primary Anti-NTRK3/TRKC Antibody (IHC-plus™ LS-B2176, 1:500, LifeSpan Biosciences, Seattle, USA), for 60 minutes at room temperature followed by incubation with polyclonal rabbit anti-goat immunoglobulins/HRP for 60 minutes at room temperature. The sections were developed with DAB, counterstained with hematoxylin for 30 seconds.

Interpretation

The immunostained sections were reviewed by a senior pathologist in order to determine positivity in the tumor tissue (**Fig. 1**).

Statistics

Patients were categorized into two groups according to the presence or absence of NT-3 expression. Pearson's χ^2 test and Fisher's exact test were used to compare differences in NT-3 expression with respect to known prognostic factors. For comparison of early treatment

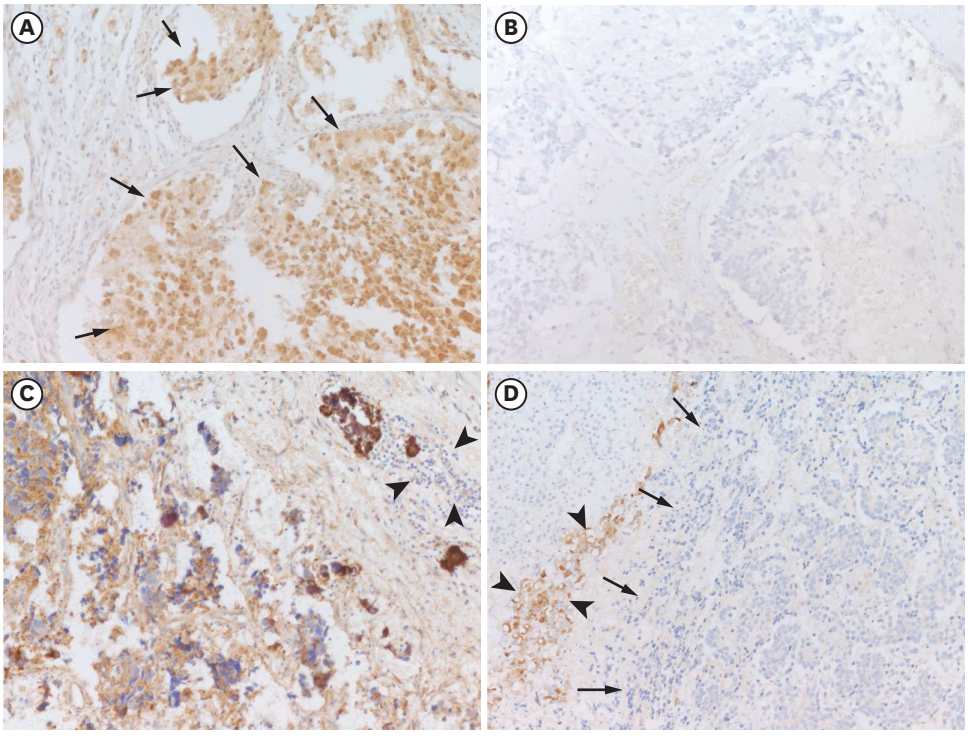

Fig. 1. Representative immunostaining of NT-3 and TrkC. (**A**) A case of neuroblastoma positive for NT-3. Neuroblasts are positive for NT-3 (arrows), in contrast to stroma cells negative for it. (**B**) A case of neuroblastoma negative for NT-3. (**C**) A case positive for TrkC. Neuroblasts are positive for TrkC, while non-neoplastic lymphocytes are negative for it (arrow heads). (**D**) A case negative for TrkC. Neuroblasts are negative for TrkC (arrows), whereas non-neoplastic chromaffin cells in adrenal medulla are positive for TrkC (arrow heads).
NT-3 = neurotrophin-3, TrkC = tropomyosin receptor kinase C.

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response, the degree of tumor volume reduction was analyzed by Mann-Whitney U test. For univariate survival analysis, overall survival (OS) and progression-free survival (PFS) rates with standard errors were estimated by Kaplan-Meier curves. The log-rank test was used to compare survival rates between NT-3 negative and positive patients. Multivariate analyses comprising known prognostic factors for progression-free survival were performed using the Cox regression. All statistical tests were 2-sided. *P* values less than 0.05 were considered statistically significant.

Ethics statement

The study was approved by the Institutional Review Board of Samsung Medical Center (2015-10-197), and informed consent was waived.

RESULTS

Patient characteristics

The median age at diagnosis of all 240 patients (135 boys and 105 girls) was 22 months (range, 0–210 months), and the most common primary site was the abdomen (185 patients). There were 72, 60, and 108 low-risk, intermediate-risk, and high-risk patients, respectively. Fifty-two (21.8%) patients had MYCN amplified tumors. The median follow-up duration among 240 patients was 75 months (range, 0–223 months) from diagnosis. The 5-year OS and PFS rates were 82.8% ± 2.5% and 81.7% ± 2.6%, respectively.

Clinical significance of NT-3 expression in all patients

Positive NT-3 expression was observed in 97 (40.4%) of 240 patients. The association between NT-3 expression and known prognostic factors are presented in **Table 1** and **Fig. 2A-D**. NT-3 expression was associated with older age (> 18 months) at diagnosis ($P < 0.001$), localized tumors ($P = 0.033$), and more differentiated histology ($P < 0.001$). However, there was no difference in MYCN amplification between patients with NT-3 expression and those without. Percent residual tumor volume after three cycles of chemotherapy was used as a surrogate marker of early treatment response. There was no difference in the degree of tumor volume reduction between patients with NT-3 expression and those without (**Fig. 2E**). Also, there was no significant difference in the 5-year OS and PFS rates between patients with NT-3 expression and those without (OS, 84.3% ± 3.7% vs. 81.8% ± 3.3%, $P = 0.752$; PFS, 86.7% ± 3.6% vs. 78.2% ± 3.6%, $P = 0.082$) (**Fig. 2F**). We also performed Q-RT-PCR in 56 patients to analyze the association between NT-3 mRNA expression and clinical variables. We classified patients into two groups (high expression above median versus low expression below median) and compared their association with known prognostic variables. The results were similar to those seen in the comparison between the immuno-histochemical NT-3 positive and negative groups. Also, there was no significant difference in early response to chemotherapy between the two groups. Lengths of follow-up were too short to compare long-term clinical outcomes in these patients because mRNA extraction was possible only in patients diagnosed recently.

Clinical significance of NT-3 expression according to MCYN amplification

Different types of NT receptors are expressed depending on the status of MYCN amplification.[19,30,31] Therefore, we re-analyzed the clinical relevance of NT-3 expression by dividing patients into two subgroups according to MYCN amplification. In subgroup analysis according to MYCN amplification, there was a similar association between NT-3 expression and clinicopathologic parameters except pathology, regardless of MYCN amplification

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Table 1. Characteristics of patients at diagnosis according to NT-3 expression

Parameters	NT-3 negative (n = 143)	NT-3 positive (n = 97)	*P* value
Sex, male	83 (58)	52 (54)	0.497
Age > 18 mon at diagnosis	66 (46)	68 (70)	< 0.001
Primary site			0.311
Abdomen	114 (80)	71 (73)	
Other sites	28 (20)	26 (27)	
Unknown	1	0	
Stage			0.033
1–3	64 (45)	57 (59)	
4/4S	79 (55)	40 (41)	
Pathology (INPC)			0.712
Favorable	72 (52)	51 (54)	
Unfavorable	67 (48)	43 (46)	
Unknown	4	3	
Differentiation			< 0.001
Ganglioneuroblastoma	12 (9)	33 (34)	
Differentiating	35 (25)	22 (23)	
PD/UD	92 (66)	42 (43)	
Unknown	4	1	
MYCN			0.214
Non-amplified	115 (81)	72 (74)	
Amplified	27 (19)	25 (26)	
Risk group			0.406
Low	40 (28)	32 (33)	
Intermediate	40 (28)	20 (21)	
High	63 (44)	45 (46)	
Serum LDH, IU/L	824 (176–12,160)	805 (305–15,720)	0.644
Serum ferritin, ng/mL	140 (8–16,500)	130 (3–3,284)	0.556
Serum NSE, ng/mL	44.6 (7.3–1,430.0)	40.6 (6.6–1,815.0)	0.730
24 hr urine VMA, mg/day	8.1 (0.2–205.0)	3.6 (0.1–80.0)	0.269

Values are presented as number (%) or median (range).
NT-3 = neurotrophin-3, INPC = International Neuroblastoma Pathology Classification, PD = poorly differentiated, UD = undifferentiated, LDH = lactate dehydrogenase, NSE = neuron-specific enolase, VMA = vanillylmandelic acid.

status. However, the significance of these associations was reduced in patients with MYCN amplified tumors (**Fig. 3A-C** vs. **Fig. 3F-H**). Interestingly, treatment results differed between the two groups. While the percent residual tumor volume at the early phase of induction chemotherapy had a tendency to be higher with NT-3 expression in patients with MYCN non-amplified tumors ($P = 0.114$) (**Fig. 3D**), it was lower in patients with MYCN amplified tumors with borderline significance ($P = 0.092$) (**Fig. 3I**). Furthermore, while there was no difference in PFS according to NT-3 expression in patients with MYCN non-amplified tumors (**Fig. 3E**), patients with MYCN amplified tumors showed significantly better PFS if NT-3 was expressed (86.9% ± 7.1% vs. 58.2% ± 10.3%, $P = 0.042$) (**Fig. 3J**). Multivariable Cox analysis revealed that NT-3 was an independent risk predictor of PFS with statistical significance along with older age (> 18 months) at diagnosis in patients with MYCN amplified tumors (hazard ratio, 0.246; 95% confidence interval, 0.061–0.997; $P = 0.050$, **Table 2**).

Clinical significance of NT-3 expression according to TrkC expression

Different treatment outcomes (early treatment response and PFS) according to NT-3 expression were found in patients with MYCN amplified tumors, as described above. We hypothesized that these results may be due to different NT receptor profiles between patients with or without MYCN amplification. To verify this hypothesis, expression of TrkC, the primary receptor for NT-3, was analyzed. TrkC expression showed similar associations with

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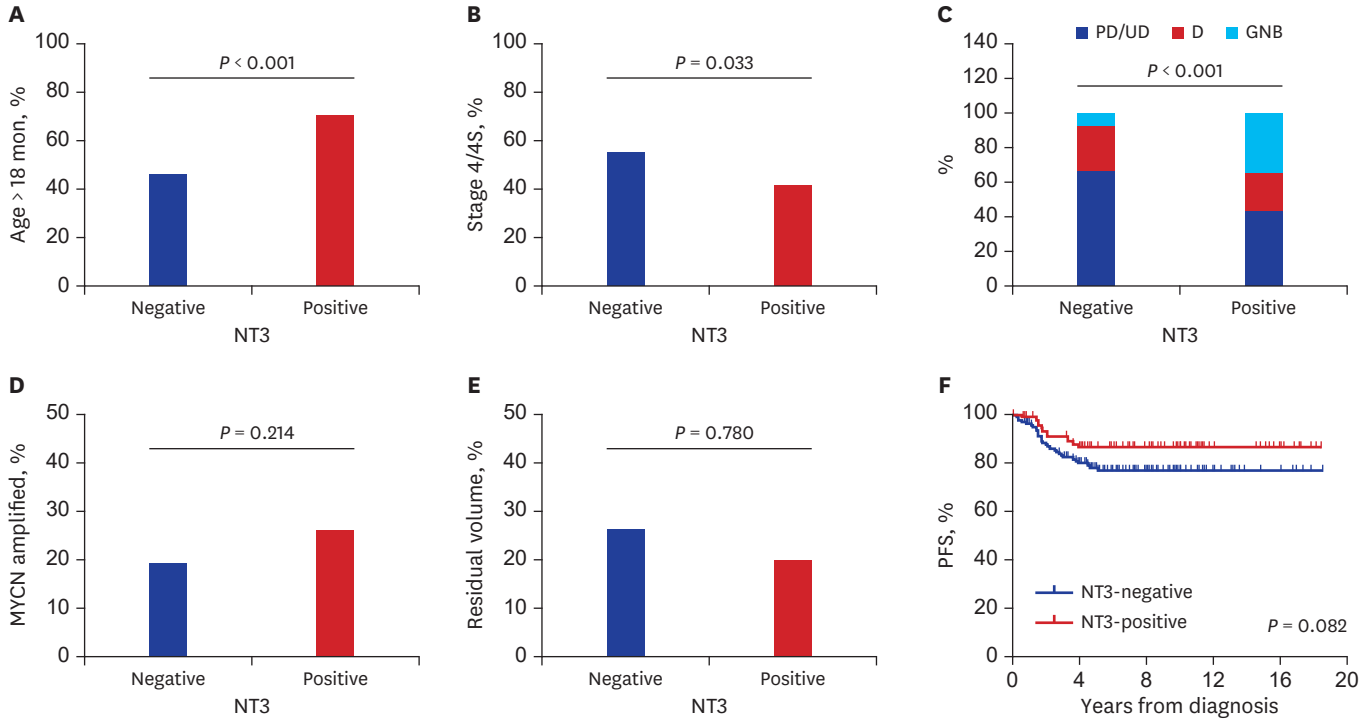

Fig. 2. Clinical significance of NT-3 expression. NT-3 expression was associated with (**A**) older age at diagnosis, (**B**) localized tumors, and (**C**) more differentiated histology. (**D**) However, there was no difference in the proportion of MYCN amplification according to NT-3 expression. (**E**, **F**) There was no difference in degree of tumor volume reduction or PFS between patients with NT-3 expression and those without.
NT-3 = neurotrophin-3, GNB = ganglioneuroblastoma, D = differentiated, PD = poorly differentiated, UD = undifferentiated, PFS = progression-free survival.

known prognostic factors as those observed with NT-3 expression (**Table 3** and **Fig. 4A-C**). However, MYCN amplification was rarely found in patients with TrkC expression (**Table 3** and **Fig. 4D**). In addition, TrkC expression was associated with a poorer early treatment response (**Fig. 4E**), although there was no difference in PFS (**Fig. 4F**). Next the clinical relevance of NT-3 expression was compared for patients with and without TrkC expression. NT-3 expression showed similar associations with known prognostic factors irrespective of TrkC expression although the significance of these associations was somewhat reduced in patients without TrkC expression (**Fig. 5A-C** vs. **Fig. 5F-H**). However, there was opposite early treatment response according to NT-3 expression between patients with and without TrkC expression. Percent residual tumor volume at the early phase of induction chemotherapy was lower if NT-3 was expressed in patients without TrkC expression with borderline significance ($P = 0.053$) (**Fig. 5D**). Conversely, it was higher in patients with TrkC expression ($P = 0.023$) (**Fig. 5I**). However, unlike the early treatment response result, there was no difference in PFS according to NT-3 expression irrespective of TrkC expression (**Fig. 5E and J**).

DISCUSSION

This study investigated NT-3 expression and its association with clinical features at diagnosis and treatment response/outcomes in neuroblastoma. It suggests that NT-3 expression in neuroblastoma has its own clinical significance independent of TrkC expression, and its prognostic significance differs depending on the status of MYCN amplification and/or TrkC expression.

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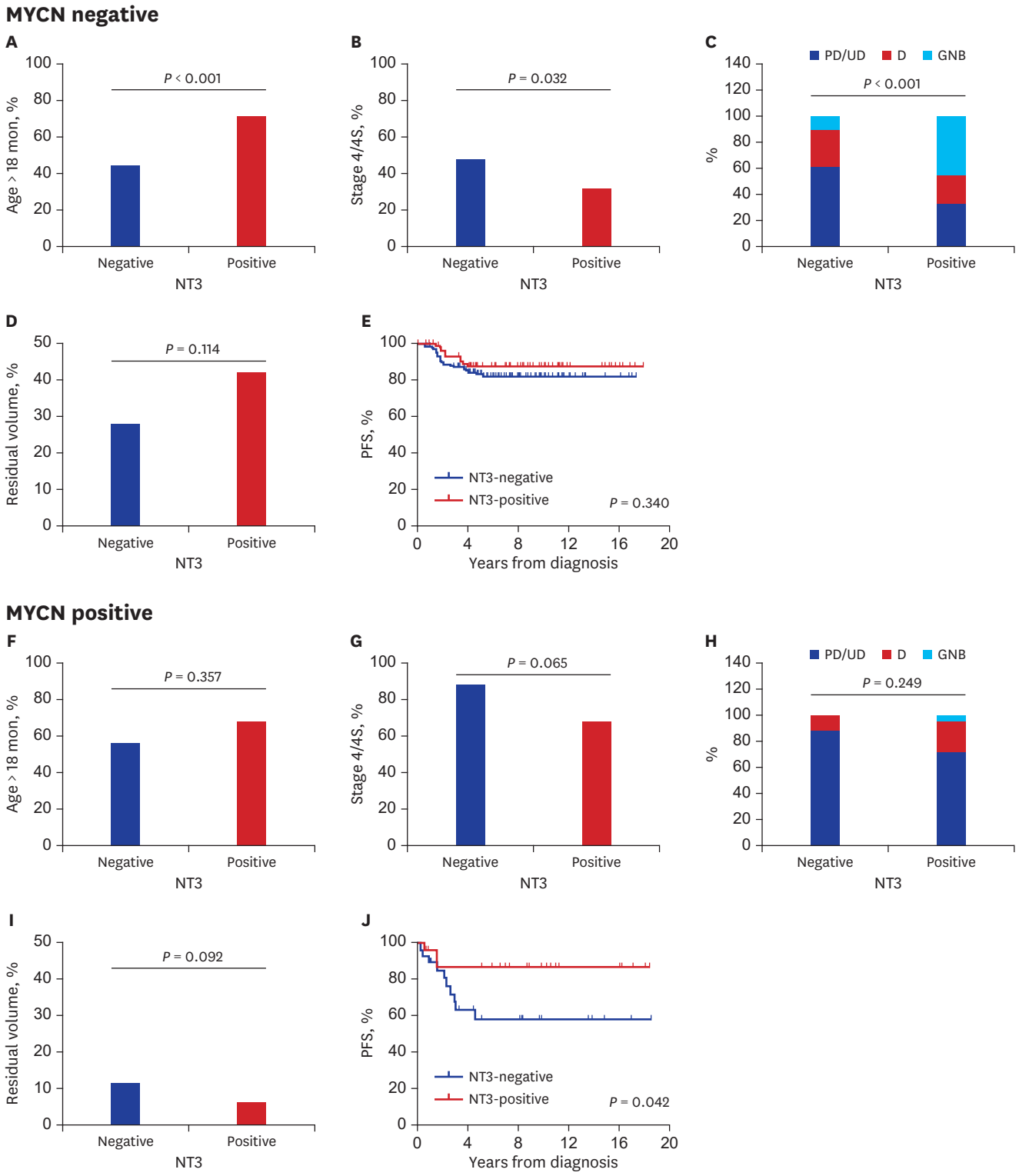

Fig. 3. Clinical significance of NT-3 expression in subgroup analysis according to MYCN amplification.
(**A**-**C** vs. **F**-**H**) There was similar association between NT-3 expression and clinicopathologic parameters, such as age at diagnosis and stage regardless of MYCN amplification status. However, treatment results differed between the two groups. (**D**, **I**) While percent residual tumor volume at early phase of induction chemotherapy tended to be higher if NT-3 was expressed in patients with MYCN non-amplified tumors, it was lower in patients with MYCN amplified tumors with borderline significance. (**E**, **J**) Furthermore, while there was no difference in PFS according to NT-3 expression in patients with MYCN non-amplified tumors, patients with MYCN amplified tumors showed better PFS if NT-3 was expressed.
NT-3 = neurotrophin-3, GNB = ganglioneuroblastoma, D = differentiated, PD = poorly differentiated, UD = undifferentiated, PFS = progression-free survival.

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Table 2. Multivariate analysis of risk factors for progression in MYCN amplified neuroblastoma

Characteristics	Progression (n = 13 cases)		
	HR	95% CI	*P* value
Age			
< 18 mon at diagnosis	1		
≥ 18 mon at diagnosis	5.236	1.106–24.779	0.037
Stage			
1–3	1		
4/4S	1.963	0.217–17.768	0.548
NT-3 expression			
Negative	1		
Positive	0.246	0.061–0.997	0.050

Cox proportional hazards regression analysis is used. Age and NT-3 expression were independent prognostic factors with a statistical significance and stage was not an independent prognostic factor in patients with MYCN amplified tumor.
HR = hazard ratio, CI = confidence interval.

Table 3. Characteristics of patients at diagnosis according to TrkC expression

Parameters	TrkC negative (n = 106)	TrkC positive (n = 63)	*P* value
Sex, male	56 (53)	36 (57)	0.586
Age > 18 mon at diagnosis	42 (40)	48 (76)	< 0.001
Primary site			0.151
Abdomen	83 (79)	42 (67)	
Other sites	22 (21)	21 (33)	
Unknown	1	0	
Stage			0.037
1–3	55 (52)	43 (68)	
4/4S	51 (48)	20 (32)	
Pathology (INPC)			0.798
Favorable	54 (52)	34 (54)	
Unfavorable	50 (48)	29 (46)	
Unknown	2	1	
Differentiation			< 0.001
Ganglioneuroblastoma	8 (8)	31 (49)	
Differentiating	24 (24)	15 (24)	
PD/UD	72 (69)	17 (27)	
Unknown	4	1	
MYCN			0.001
Non-amplified	82 (78)	61 (97)	
Amplified	23 (22)	2 (3)	
Risk group			0.329
Low	42 (40)	25 (40)	
Intermediate	24 (23)	20 (32)	
High	40 (38)	18 (29)	
NT-3 expression			0.001
Negative	80 (75)	32 (51)	
Positive	26 (25)	31 (49)	
Serum LDH, IU/L	914 (305–14,435)	702 (377–9,583)	0.005
Serum ferritin, ng/mL	179 (3–16,500)	130 (8–1,491)	0.027
Serum NSE, ng/mL	59.3 (7.4–1,850.0)	27.4 (7.4–722.5)	0.016
24 hr urine VMA, mg/day	10.0 (0.2–93.7)	7.4 (1.8–74.4)	0.061

Values are presented as number (%) or median (range).
TrkC = tropomyosin receptor kinase C, INPC = International Neuroblastoma Pathology Classification, PD = poorly differentiated, UD = undifferentiated, NT-3 = neurotrophin-3, LDH = lactate dehydrogenase, NSE = neuron-specific enolase, VMA = vanillylmandelic acid.

Most studies that examined the role of NT/NT receptors in neuroblastoma focused on the clinical significance of NT receptor expression, considered one of prognostic factors.[12,22] Among several NT receptors, TrkC has been covered in numerous studies as its distinctive features unlike other NT receptors. TrkC is a tyrosine kinase receptor and it was expected to behave as a proto-oncogene. However, TrkC acts as a tumor suppressor in various

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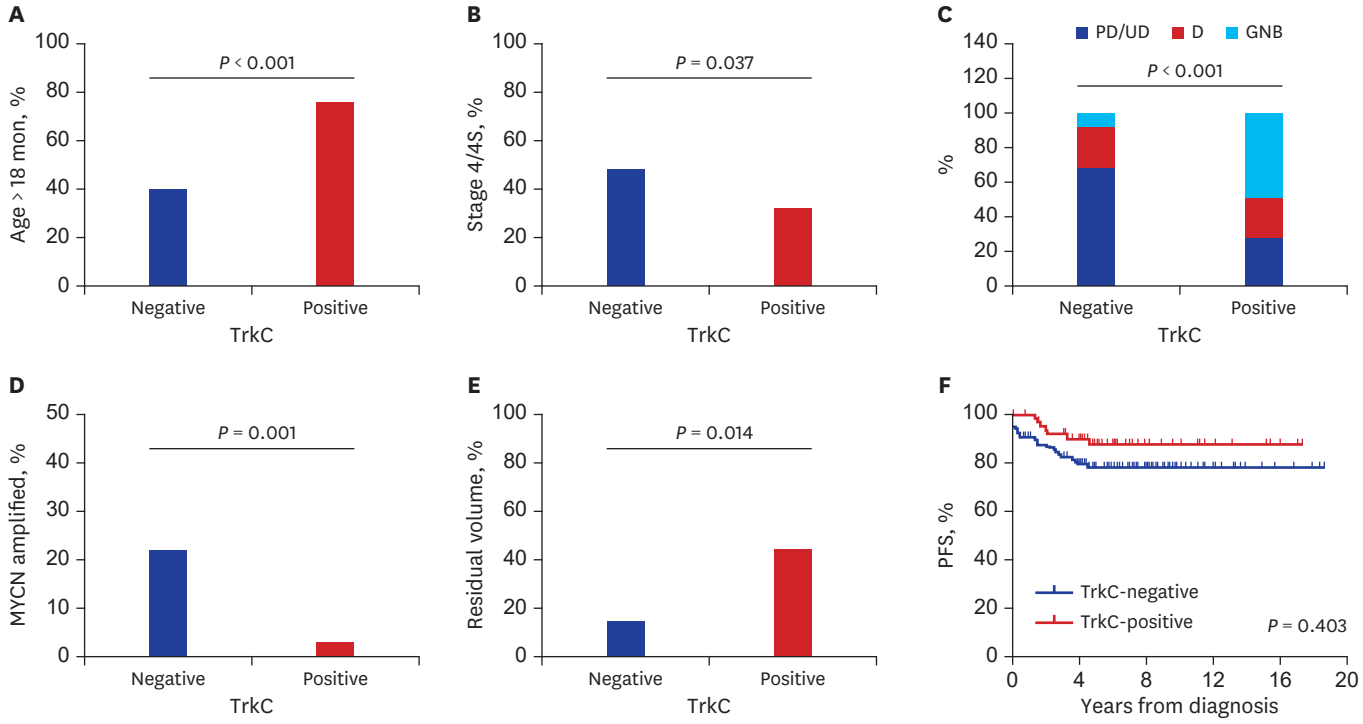

Fig. 4. Clinical significance of TrkC expression. (**A**-**C**) TrkC expression showed similar associations with known prognostic factors as those observed in NT-3 expression. (**D**) However, MYCN amplification was rarely detected in tumors with TrkC expression. (**E**, **F**) In addition, TrkC expression was associated with poorer early treatment response, although there was no difference in PFS.
TrkC = tropomyosin receptor kinase C, GNB = ganglioneuroblastoma, D = differentiated, PD = poorly differentiated, UD = undifferentiated, PFS = progression-free survival, NT-3 = neurotrophin-3.

cancers, including neuroblastoma, medulloblastoma, and colon cancer and its expression was reported to be associated with good prognosis.[17,18,21,22,25,26,32] This paradox has been explained by the proposal that TrkC is a dependence receptor that triggers apoptosis in the absence of a ligand.[21,24] Our results support the clinically dependent nature of TrkC because the early response to induction chemotherapy was poorer in patients with TrkC/NT-3-co-expressing neuroblastoma than in patients with only TrkC-expressing neuroblastoma.

Meanwhile, our study suggests that NT-3 has its own clinical significance independent of TrkC. The relevance of NT-3 expression with clinicopathologic variables was not altered whether TrkC was expressed or not, although the degree of significance varied. More importantly, NT-3 expression was suspected to be a good prognostic marker in patents with MYCN amplified neuroblastoma, and these results were not explained by the absence of TrkC expression with MYCN amplification only. A possible explanation is that NT-3 might develop pro-apoptotic signals mediated through other NT receptors because NT-3 can bind and activate other receptors such as TrkA, TrkB and $p75^{NTR}$.[13,28,29,33-35] Specifically, $p75^{NTR}$ might be a candidate receptor to elucidate our results. The $p75^{NTR}$ receptor creates apoptosis signals in the absence of Trk receptors while apoptosis is inhibited when it is co-expressed with Trk receptors.[13,30,31]

Targeting of NTs or Trk receptors is considered novel adjuvant chemotherapy. There is some evidence that selective or pan-Trk inhibitors such as lestaurtinib enhance the antitumor efficacy of chemotherapy, and clinical trials of some inhibitors are underway.[36,37] NT-3 interference also has evidence of antitumor effect in both in vitro and in vivo studies.[24]

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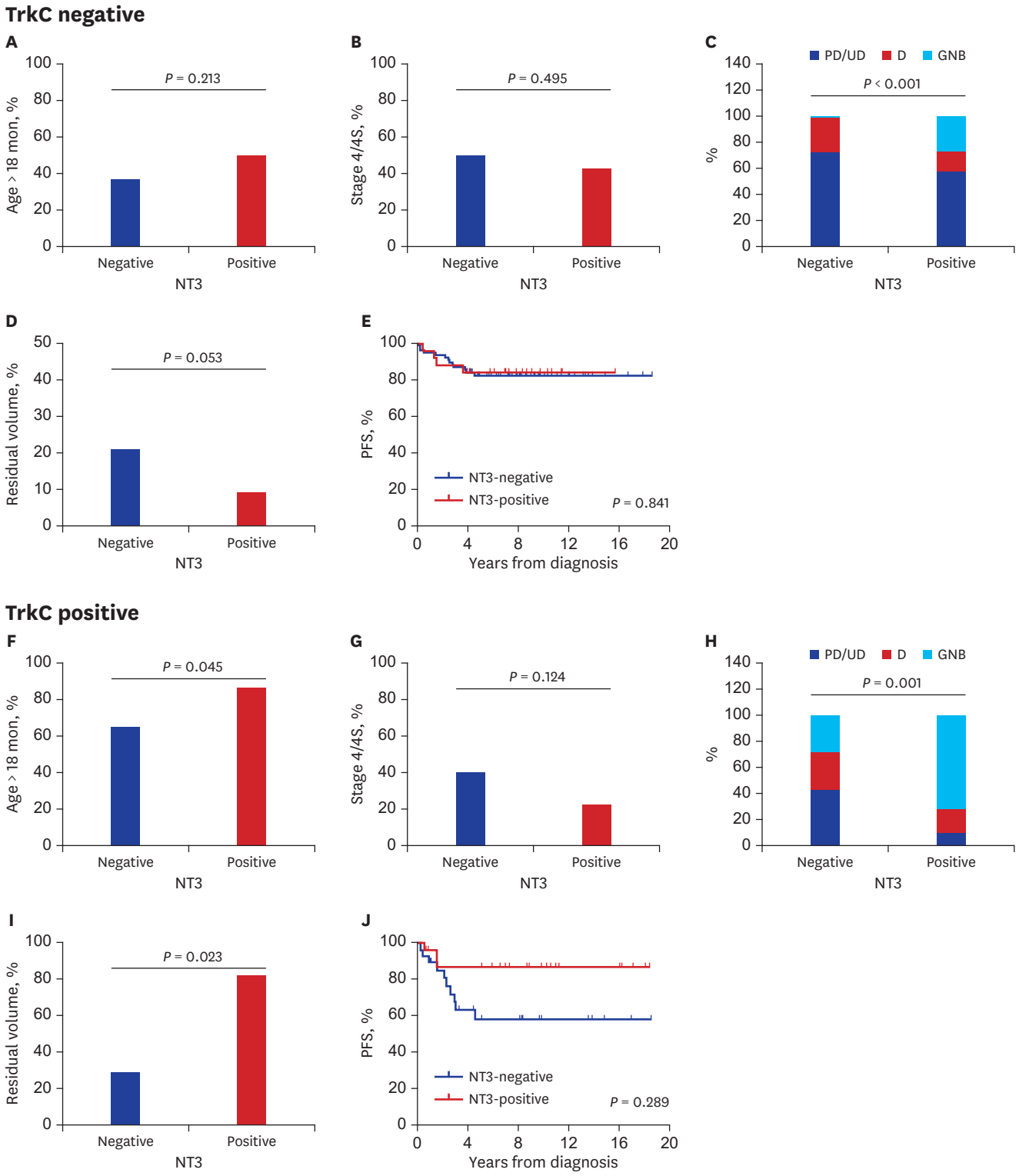

Fig. 5. Clinical significance of NT-3 expression in subgroup analysis according to TrkC expression. (**A**-**C** vs. **F**-**H**) NT-3 expression showed similar associations with known prognostic factors irrespective of TrkC expression. However, there was opposite early treatment response according to NT-3 expression between patients with TrkC expression and those without. (**D**, **I**) While the percent residual tumor volume at early phase of induction chemotherapy was lower if NT-3 was expressed in patients without TrkC expression with borderline significance, it was higher in patients with TrkC expression. (**E**, **J**) However, there was no difference in PFS according to NT-3 expression irrespective of TrkC expression.
TrkC = tropomyosin receptor kinase C, NT-3 = neurotrophin-3, GNB = ganglioneuroblastoma, D = differentiated, PD = poorly differentiated, UD = undifferentiated, PFS = progression-free survival.

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However, according to our results, those treatments should be applied selectively because they may be either therapeutic or pathogenic under different circumstances. There should be clear demonstration and explanation of interaction between each NT and various NT receptors in neuroblastoma. The role of MYCN amplification in expression and interaction of NT/NT receptors should also be explored.

Our study has some limitations. First, this is an observational study without functional studies on molecular biological mechanisms to explain how these unexpected results may be observed. Second, we evaluated gene expression mostly in protein level based on immunohistochemistry because mRNAs should be extracted from fresh tissue, which was possible only in patients who were diagnosed recently. Third, we evaluated only NT-3 and TrkC expression in this study. It is necessary to explore the clinical relevance of various types of NTs and NT receptors in the future study. Nonetheless, our study showed some unexpected and important findings.

In summary, we demonstrated that NT-3 expression has its own clinical significance in neuroblastoma independent of its receptor, TrkC. NT-3 expression is associated with older age at diagnosis, localized tumors, more differentiated histology, better (poorer) early treatment response in the absence (presence) of its primary receptor TrkC, and significantly better PFS in MYCN amplified neuroblastoma. Therefore, NT-3 expression should be carefully appreciated before interpreting its clinical meaning. Furthermore, the status of MYCN amplification and NT receptor profile should be verified when NT-3 is considered a therapeutic target.

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