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im Dr. von Haunerschen Kinderspital,
Klinikum der Ludwig-Maximilians-Universität München
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Vessel adherent growth and molecular markers in neuroblastoma

Dissertation zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Jakob Mühling aus München
2021

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung:	18.02.2021

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geb. 26.05.1990

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Einleitende Zusammenfassung

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Abbreviations

ALK	-	Anaplastic lymphoma kinase
ATRX	-	Adenosine triphosphate dependent helicase ATRX
CT	-	Computed tomography
DNA	-	Deoxyribonucleic acid
fl-TACR1	-	Full length tachykinin receptor 1
GPOH	-	German society for pediatric oncology and hematology
HE	-	Haematoxylin and eosin
IDRF	-	Image defined risk factor
INPC	-	International neuroblastoma pathology classification
INRGSS	-	International neuroblastoma risk group stratification system
INSS	-	International neuroblastoma staging system
LDH	-	Lactate dehydrogenase
MIBG	-	Metaiodobenzylguanidine
MRI	-	Magnetic resonance imaging
MYCN	-	V-Myc myelocytomatosis viral related oncogene, neuroblastoma derived, homolog
MYCNOS	-	V-Myc myelocytomatosis viral related oncogene, neuroblastoma derived, homolog, opposite strand
NB	-	Neuroblastoma
NK1R	-	Neurokinin 1 receptor
NK1R-F	-	Neurokinin 1 receptor, full length
NK1R-T	-	Neurokinin 1 receptor, truncated
NRAS	-	Neuroblastoma rat sarcoma proto oncogene
NSE	-	Neuron specific enolase
OS	-	Overall survival
PCR	-	Polymerase chain reaction
PHOX2B	-	Paired like homeobox 2B
RNA	-	Ribonucleic acid
TACR1	-	Tachykinin receptor 1
TBP	-	TATA box binding protein
TRKA	-	Tropomyosin receptor kinase A
tr-TACR1	-	Truncated tachykinin receptor 1
UVIN	-	Unexpected vessel infiltration of neuroblastoma

1. Publications

a) Mühling J, Eberherr C, Müller Höcker J, Grote V, v. Schweinitz D, Kappler R, Fröba-Pohl A.

Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and is associated with adverse outcome

J Pediatr Surg. 2019 Nov;54(11):2336-2342. doi: 10.1016/j.jpedsurg.2019.07.012. Epub 2019 Jul 23.

Impact factor: 2.092 (2018)

b) Pohl A, Kappler R, Mühling J, v. Schweinitz D, Berger M.

Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage

Anticancer Res. 2017 Nov;37(11):6079-6085.

Impact factor: 1.935 (2018)

2. Introduction

2.1. Neuroblastoma

2.1.1 Epidemiology

Neuroblastoma (NB) is the most common, extracranial solid tumor amongst children [1, 2]. Next to hepatoblastoma, nephroblastoma and the group of primitive neuroectodermal tumors, it belongs to the category of embryonal tumors. It has a yearly incidence of approximately 1.3 / 100.000 children in Germany, which means about 140 newly diagnosed cases of NB per year. NB represents about 8-10% of all pediatric malignancies, in which a slight surplus of male patients can be found (male: female = 1.3:1.0) [1, 3].

The median age of all patients at time of diagnosis is about 17 months. Among all diagnosed children, 90% are younger than six years, and 40% have not even finished their first year of life [2, 4]. Less than 3% of all NBs are newly diagnosed during adolescence [5, 6].

Originating from mis-differentiated multipotent cells of the neural crest, NB arises from the sympathetic autonomic nervous system [7]. According to this, NB is often associated with other diseases, which have their origin in a failed development of neural cells – for example Turner syndrome, Hirschsprung's disease, Ondines disease, Noonan-Costello - syndrome or Neurofibromatosis Recklinghausen [8-11]. Besides the correlation with syndromic diseases, there is also a rare proportion of familial accumulation in NB [12].

NB is characterized by its very variable course of disease, including autonomous complete regression as well as therapy-resistant disease progress. Responsible factors for this phenomenon are not exactly known by now, although research has helped a lot to get new insights in molecular functions in NB and improved therapy within the last 30 years. Thus, the 5-year overall survival (OS) was increased from 52% up to over 74% [2]. However, despite tremendous progress in diagnostic and therapeutic measures, NB still causes about 15% of all fatalities in pediatric oncology [13].

2.1.2 Clinical and diagnostic examinations

Whereas in many cases NB is accidentally recognized during a routine sonography, its displacing growth can also lead to symptomatic suffering [14].

NB can most frequently be found in the tissues of the adrenal medulla and the paravertebral sympathetic trunk. In the majority of cases, the tumor is located in the abdominal space, followed by localizations in cervical or thoracic area, as well as in the pelvis [7, 15-17].

Metastasis occurs in skeleton, bone marrow, lymph nodes, liver and skin. An infestation of the central nervous system or the lung is rarely seen [18]. Depending on the location, excessive tumor growth can generate urinary retention, dyspnea or an affection of nervous tissue. This can result in varying neurological deficits like Horner syndrome or Opsomyoclonus syndrome [19]. Furthermore, overexpression of catecholamines can cause high blood pressure, diarrhea or hot flashes [1, 8, 15, 20].

Furthermore, frequently unspecific, general symptoms as fatigue, anorexia, diffuse pain or dystrophia can be found. A rare, but mostly misinterpreted sign are the so called “raccoon eyes”, a periorbital ecchymosis caused by a tumor infiltrating palpebral vessels [21].

Even though several studies reported about a screening method for detection of NB with urine catecholamines more than twenty years ago, there was unfortunately no therapeutic benefit.

While the frequency of diagnosed NB increased rapidly, mortality remained unaffected. This was explained by diagnosis of tumors, which would have never been clinical apparent without the urine analysis because of their spontaneous remission. Analysis of the studies showed once again the problem of the variable course of disease; thus, the screening was omitted to avoid overdiagnosis and –treatment [22-24].

However, the surplus of catecholamines and their degradation products in urine or serum can be used for diagnostic measuring.

For a full diagnostic work-up, neuron-specific enolasis (NSE), lactate dehydrogenase (LDH) and ferritin should be analyzed by blood samples as well. Additionally, imaging like sonography, MRI, CT-scan and I^{123} -metaiodobenzylguanidine (mIBG) - or skeleton-scintigraphy are implemented to visualize the extent of growth and affection of nearby

organs or tissues. As over 50 percent of NB patients show bone marrow infestation, a biopsy is recommended [25]. A biopsy of the tumor itself is necessary for histopathological analysis and identification of genetic alterations to provide an efficient staging [18].

2.1.3 Genetics

Despite intensive research, a single initiating factor for formation of NB is still not known up to now [26]. So far, many different gene alterations and –products seem to participate in the origin of NB. A complex interplay of promotion and inhibition of inter- and intracellular signaling pathways influences the biology of the tumor and is still not fully understood [4].

However, recent studies give insights in genetic characteristics of NB. So, the sporadically overexpressed *ALK* gene in combination with the *PHOX2B* gene was identified as a characteristic initiator for the hereditary form of NB, representing about 1% of all NB cases [27, 28].

Further alterations in genes like *ATRX*, *NRAS*, and *TRKA* are objects of current research as well as losses and gains of the chromosomes 1p and 17q, respectively [29-34]. Recent studies have also described a major role of telomerase activation and telomere maintenance in the progression of especially high-risk NB [35, 36].

But despite all these new insights, amplification of the *MYCN* - oncogene is still the most relevant factor for a reliable staging and prognosis of NB [37-39]. Amplification of *MYCN* starting from a tripling of the gene up to several thousand copies is detected in about 19% to 30 % of NB, depending on the investigated patient group [38, 40, 41].

MYCN functions as a tissue specific proto-oncogene, which is regularly present during the embryonal period. [37, 41-43]. Regulating the cell cycle, it is able to influence proliferation and cellular transformation as well as apoptosis and cellular transcription. It also plays a major role in metastasis of tumors with an amplification and overexpression of the gene [44-47]. As *MYCN* is also recognized to have an impact on cell adhesion, motility, migration and angiogenesis, it is known to affect the course of disease in a prognostically unfavorable way [48-52].

While *MYCN* is known as one of the most powerful genetic factor in staging and prognosis of NB, in latest studies *MYCNOS* or also called *N-CYM*, an antisense transcript of *MYCN*, containing 109 amino acids and also located on chromosome 2p23-24 , was found to be a relevant co-factor in angiogenesis, vascularization and tumorous vessel affection [13, 38, 53-60]. Due to its obligatory co-expression and its stabilizing effect on *MYCN*, it is subject to a mechanism of positive auto-regulation, triggering cells‘ survival and increasing potential for aggressive growth as well as risk for metastatic spreading by inhibiting apoptosis in case of an overexpression of *MYCN* [53, 54, 59, 61-63].

2.1.4 Histological grading and staging

Histopathological analysis is obligatory for exact diagnosis and treatment of NB. Microscopic examination of tumor sections generally shows accumulations of small, immature basophil cells. Furthermore, granules containing catecholamines and chromogranine A as well as expression of neurofilaments and S100 protein are seen. Depending on grade and quantity of differentiated cells in tumor tissue, NB can be defined into three different grades by the Hughes index [64, 65].

Histological grading is used in connotation with mitosis-karyorrhexis-index and individual factors such as age at diagnosis for the international NB pathology classification (INPC). This was developed from the Shimada classification trying to derive a prognosis from histopathological results [66].

Furthermore, there are two established clinical staging systems for NB.

The international NB staging system (INSS) regards localization and extent of tumor growth in reference to the midline, metastasis, affection of lymphatic nodes and age to divide patients in five different subgroups. Unfortunately, it also refers to extent of resection and so it is not able to provide a pre-surgical risk stratification [67].

The international NB risk group stratification system (INRGSS) is based on several criteria such as age, gene alterations, imaging and histological category of the tumor [68]. Here, image defined risk factors (IDRF) helping to evaluate an affection of nearby organs by the tumor in the diagnostic imaging are particularly noteworthy. In opposite to the INSS, the INRGSS is independent from individual surgical skills and has the advantage of a pre-therapeutic risk assessment [69].

According to these risk stratification models patients can be allotted to different risk groups, in which an age older than 18 months, metastasis and especially an amplification of *MYCN* are considered as prognostically poor criteria [68].

2.1.5 Treatment and prognosis

In their current treatment protocol (NB 2004) the German Society for pediatric oncology and hematology (GPOH) uses a risk stratification system categorizing patients into three risk groups (low, intermediate and high risk). This results in 3 different therapeutic approaches [70].

A regular observation or primary surgical resection is recommended for patients with low risk, while patients of the intermediate risk group should receive neo-adjuvant chemotherapy consisting of alternating cycles of cisplatin, etoposide, vindesine, vincristine, dacarbacin, doxorubicin, ifosfamid and cyclophosphamide, followed by surgical resection, adjuvant chemotherapy and possibly radiotherapy [71].

The therapeutic concept for the high risk group contains intensified neo-adjuvant chemotherapy with a gross-total resection and a following high-dose consolidation therapy with autologous bone marrow transplant as well as a post-consolidation therapy. Furthermore, in case of mIBG-positive tumors, a therapy with I^{123}-mIBG can be taken into consideration [71, 72].

Outcome of patients with NB is quite variable, depending on multiple factors such as age at diagnosis, grade of differentiation, genetic alterations, resectability and comorbidities.

It reaches from 30% with stage 4 patients up to over 95% with low risk patients in observation groups [68, 73, 74].

Although 5-year OS rates increased from 52% to over 75% during the last decades, this improvement refers primary to advanced therapy for patients at lower stages, while high risk patients still are a challenge to treat [2]. Over 50% of all high risk patients show relapse and also second malignancies during their lifetime [2, 75, 76].

2.2 Aims of the studies

The present thesis includes two studies focusing on NB.

In the first study, we analyzed a special growth form of vessel adherent NB and its clinical relevance for surgical resection and the further course of disease. Furthermore, we investigated the role of *MYCN* expression levels to serve as a potential biomarker for vessel infiltrative growth.

In a second study, we investigated the role of the neurokinin-1 receptor as a possible target for therapeutic interventions in NB.

In the following, the concept and realization of each study is shortly explained.

2.2.1 Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and is associated with adverse outcome

Despite all diagnostic and preoperative examinations, many cases of NB treated at the department of pediatric surgery of the Ludwig-Maximilians-University Munich show intraoperatively an unexpected vessel adherence impeding a complete resection.

While some tumors can easily be separated from nearby vascular structures without any damage of the vascular tissue, others show infiltrative growth into adjacent vessels. Due to this infiltrative adherence, this growth behavior seems to increase the risk for vascular damage, bleeding and intraoperative complications.

Although IDRFs define the proximity of the tumor to larger vessels in diagnostic and preoperative imaging, they are not able to specify the affection of vessels in a macroscopic and microscopic way before surgical intervention [69, 77]. Earlier research only focused on the extent of vessels' encasement by the tumor and its impact on the course of disease [77]. So up to now, there is no current study differentiating between varying forms of vessel affection or describing the observed vessel infiltrative growth of NB.

The purpose of our study was to describe and quantify the phenomenon of vessel infiltrative growth by NB and to investigate its impact on the course of disease.

As none of the current imaging studies are able to detect this vessel affection, gene expression levels of *MYCN* and *MYCNOS* were measured in tumor samples in an attempt to find predictive molecular markers for this phenomenon.

The examined cohort contained 100 patients with NB, who received surgical treatment in our department. Diagnostic, staging and therapy was performed according to international standards.

Retrospective data analysis included 15 clinical parameters such as histopathological analysis, genetic alterations, course of disease and surgical characteristics such as localization of the tumor, extent of resection and adherence to nearby structures. Here, the sort of vessel affection was especially considered.

Gene expression levels of *MYCN* and *MYCNOS* were determined in RNA extracted from homogenized tumor tissue of every single patient.

Candidate gene expression analysis was performed by real-time PCR and set in ratio with TBP (TATA-box binding protein) as a housekeeping gene. Expression values were correlated with the clinical parameters and grade of vessel affection.

Comparison of infiltrative and non-infiltrative tumors according radiologic imaging showed no possibility to differentiate between these growth behaviors only by regarding IDRF- or encasement status.

We were the first to define the phenomenon of unexpected vessel infiltration of NB (UVIN) and histopathologically proved that the tumor cells only reached into the superficial vascular wall layers without penetrating the lumen. Statistical analysis showed an appearance of UVIN in over one third of all patients as well as significant correlation of infiltrative growth with occurrence of complications, extent of resection, neoadjuvant chemotherapy, *MYCN*-amplification, IDRF positivity and vessel encasement.

Analyzing gene expression levels, our results showed that neither *MYCN* nor *MYCNOS* were appropriate markers for infiltrative growth of NB, even though the important role of *MYCN* with its strong influence on the patients' outcome was confirmed.

This study described for the first time the phenomenon of UVIN and demonstrated its relevance for the course of disease.

Considering the fact that there is no current method to detect UVIN by internistic or radiologic diagnostic, it also showed the need for an improved pre-surgical risk assessment.

Furthermore, prospective studies on larger cohorts should be used to identify marker genes and to provide deeper insights into biological and molecular functions of vessel adherent NB.

2.2.2 Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage

Treatment of NB is still a therapeutic challenge because of its variable course of disease. Current research aims for the identification of prognostic and predictive molecular markers to provide a better understanding of biological mechanisms in NB, leading ultimately to an establishment of targeted therapies.

Recent results on other embryonal tumor entities, especially on pediatric hepatoblastoma, demonstrated neurokinin-1 receptor – also known as substance P-tachykinin receptor (*TACR1*) – as a potential target for supportive therapy [78-80]. Here, growth of hepatoblastoma cells could effectively be reduced by neurokinin receptor antagonists in vitro and in vivo [81].

The neurokinin-1 receptor occurs in two naturally existing isomers, a full-length splice variant (*fl-TACR1* or *NK1R-F*) and a truncated splice variant (*tr-TACR1* or *NK1R-T*). Containing a hundred amino acids less on its C-terminal end than the full-length variant, it differs in its interaction with substance P [82-84].

The activated form of *tr-TACR1* lacks a negative feedback function, resulting in a continuous activation. Furthermore, its activated complex can also be responsible for angiogenesis in tumors and inflammatory processes [83, 85].

Latest research on targeted therapy in hepatoblastoma investigated the influence of neurokinin-1 receptor antagonists on these cellular mechanisms.

Treatment of in vitro and in vivo cell line models with aprepitant, a common *NK1R* – antagonist originally used for chemotherapy-induced nausea and vomiting, resulted in reduced tumor growth, decreased angiogenesis and a higher rate of apoptosis [86].

The purpose of this study was to investigate if the aforementioned insights in hepatoblastoma can be transferred to the therapy of NB.

The study cohort included 59 NB patients – treated according to the NB 2004 protocol of the German Society of Pediatric Oncology and Hematology – who underwent resection of NB in our department.

Clinical parameters such as age, gender, outcome, metastasis, INSS status, *MYCN* status, histology, and extent of surgical resection were analyzed retrospectively and correlated to gene- expression levels of *fl-TACR1* and *tr-TACR1*.

For that reason, RNA was extracted from every single tumor sample and gene expression levels were measured by real-time PCR in ratio to TBP (TATA-box binding protein) as a housekeeping gene.

Our study showed significantly increased gene-expression levels of *tr-TACR1* in examined NB samples. Statistical analysis revealed only *fl-TACR1* to be associated with INSS grade 4.

Correlation analysis of the other clinical parameters showed significant results neither with *tr-TACR1* nor with *fl-TACR1*.

Our study demonstrated ubiquitously increased expression *of tr-TACR1* in NB, independent of biological or individual factors. However, these results should be verified by further studies in larger cohorts.

Collectively, our study underscores the significance of *TACR1* as a promising target for further therapeutic approaches in order to improve treatment of NB.

2.3. Contribution

The postgraduate Jakob Mühling contributed to both publications by selection of patients and collection of patients' data. He processed frozen tumor tissues by slicing and HE-staining all samples for histopathological analysis.

Additionally, he extracted RNA from tumor tissue and performed real-time PCR analysis for investigation of gene-expression levels of *MYCN* and *MYCNOS*.

In the first publication *„Vessel adherent growth represents a major challenge in surgical resection of neuroblastoma and is associated with adverse outcome"* he was responsible for data evaluation and statistical analysis. Furthermore, he drafted the manuscript and designed graphics.

Aforementioned patients' data and statistical analysis were also used for the second publication *„Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage"*, where Jakob Mühling was involved in data analysis and drafting of the manuscript.

3 Summaries

3.1 Summary in English

Neuroblastoma (NB), an embryonal malignancy arising from the sympathetic nervous system, is known as the most common solid extracranial tumor amongst pediatric patients. It usually affects children under the age of six years with an incidence of about 140 newly diagnosed cases per year in Germany. Because of its highly inconstant course of disease, including spontaneous regression as well as progressive tumor growth with metastasis, therapeutic approaches reach from controlled observation to surgical intervention as well as high-dose chemotherapy with stem cell transplant and irradiation, with varying outcome.

Except for the amplification of the *MYCN* proto-oncogene and deletion of chromosome 1p, no strong predictors of outcome have been described so far.

Our research reported in the two publications was directed towards i) describing a new phenomenon that occurs intraoperatively during tumor resection and might influence the course of disease and ii) characterizing a new molecular target that might be used for future therapeutic interventions.

The first study “Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and is associated with adverse outcome“ describes a specific growth behavior of NB that we termed ”unexpected vessel infiltration of NB (UVIN)”. During operation, thus unexpectedly, these tumors present with a strong adherence to vessels that impedes complete resection without relevant damage of the affected vessels, as opposed to another type of tumors with macroscopic identical structures also surrounding, but not infiltrating nearby vessels and that are easily resectable.

Histochemistry identified that cells of UVIN tumors only infiltrate the outer vessel layers without penetrating into the lumen.

Data analysis of 100 patients treated at the Department of Pediatric Surgery of the Ludwig-Maximilians-University in Munich revealed that UVIN is detectable in over a third of all NB patients. This special growth behavior could not be identified by pre-operative diagnostic imaging and was significantly correlated with a worse course of disease. Statistical analysis showed that expression levels of the genes *MYCN* and *MYCNOS* are no appropriate markers to identify UVIN.

Our second study “Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage“ focused on possible approaches for targeted therapy in NB.

We investigated the role of the neurokinin-1 receptor, known as a potential target structure in the treatment of hepatoblastoma, in a cohort of 59 NB patients.

We showed similar expression levels of the truncated neurokinin-1 receptor, a splice variant that differs from the full-length transcript in its constant activation, in all examined samples

of NB. Accordingly, by correlating expression data with several clinical characteristics, no significant correlation with tumor biology and stage of the tumor was found. These data clearly define neurokinin-1 receptor as a generally overexpressed gene in NB independent of other biological or clinical factors, thereby advocating it as a promising target for further therapeutic approaches in NB patients.

Collectively, we were the first to define a special growth behavior of NB termed UVIN and its clinical relevance, and additionally identify *NK1R* as a target to potentially improve a successful therapy of this extraordinary tumor in the future.

3.2 Summary in German

Das Neuroblastom ist als embryonaler Tumor des sympathischen Nervensystems der am häufigsten extrakraniell auftretende solide Tumor des Kindesalters. Überwiegend sind Kinder bis zum sechsten Lebensjahr betroffen, wobei die Inzidenz in Deutschland bei etwa 140 neu diagnostizierten Fällen pro Jahr liegt. Die Therapieansätze zur Behandlung des Neuroblastoms sind ebenso variabel wie sein Verlauf, der von spontaner Regression bis hin zu fortschreitendem Tumorwachstum mit Metastasierung reicht. Abhängig vom Krankheitsstadium werden Patienten entweder lediglich beobachtet oder einer Operation zugeführt, die teilweise noch um mehrere Zyklen einer Hochdosis-Chemotherapie, eine Stammzelltransplantation oder eine Strahlentherapie ergänzt werden muss. Die Überlebensraten sind hierbei – je nach Zugehörigkeit der Patienten zu verschiedenen Risikogruppen – sehr unterschiedlich.

Als stärkste prognostische Faktoren sind bisher die Amplifikation des *MYCN* Proto-Onkogens, sowie eine Deletion auf dem 1p-Chromosom bekannt.

Unsere Forschungsarbeit, aus der die beiden vorliegenden Publikationen hervorgegangen sind, war zum einen darauf ausgerichtet, ein neues Wachstumsphänomen des Neuroblastoms, welches intraoperativ während der Resektion auffällig wird, und dessen möglichen Einfluss auf den Krankheitsverlauf zu untersuchen, zum anderen analysierten wir Neuroblastomproben hinsichtlich eines Zielmoleküls, das potentiell für zukünftige Therapieansätze genutzt werden könnte.

Die erste Studie mit dem Titel “Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and is associated with adverse outcome“ beschreibt eine spezifische Wachstumsform des Neuroblastoms, welche wir als ”unexpected vessel infiltration of neuroblastoma (UVIN)” betitelten. Die entsprechenden Tumoren fallen hierbei intraoperativ unerwarteter Weise durch eine starke Verwachsung mit den umliegenden Gefäßen auf. Dies verhindert eine komplette Resektion des Tumors ohne einen relevanten Schaden des betroffenen Gefäßes zu provozieren. Im Gegensatz hierzu sind andere,

makroskopisch identisch erscheinende und die Gefäße ebenfalls umschließende, aber nicht infiltrierende Tumoren leicht zu resezieren.

Histopathologisch zeigte sich, dass die Zellen von UVIN-positiven Tumoren lediglich in die äußeren Schichten der Gefäßwände einwachsen ohne in das Gefäßlumen vorzudringen.

Die Analyse der von 100 Patienten, die alle in der kinderchirurgischen Klinik der Ludwig-Maximilians-Universität München behandelt wurden, erhobenen Daten zeigte, dass UVIN in über einem Drittel aller Neuroblastom-Patienten auftritt. Diese spezielle Wachstumsform konnte nicht durch die präoperative diagnostische Bildgebung dargestellt werden und war signifikant mit einem schlechteren Krankheitsverlauf assoziiert. Die statistische Analyse ergab, dass die Expressionslevel der Gene *MYCN* und *MYCNOS* nicht als geeignete Biomarker zur Identifikation von UVIN genutzt werden können.

Unsere zweite Studie mit dem Titel “Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage“ verfolgte den Ansatz, neue Möglichkeiten zur gezielten Therapie beim Neuroblastom aufzuzeigen.

Wir untersuchten hierzu an einem Kollektiv von 59 Neuroblastom-Patienten die Rolle des Neurokinin-1 Rezeptors, der bereits bei der Therapie des Hepatoblastoms als mögliche Zielstruktur bekannt geworden ist.

Die Analyse ergab in allen untersuchten Neuroblastomproben ähnlich hohe Expressionen des verkürzten Neurokinin-1 Rezeptors *tr-TACR1*. Diese Spleißvariante des Neurokinin-1 Rezeptors unterscheidet sich von der ungekürzten Form *fl-TACR1* durch seine kontinuierliche Aktivierung.

Gemäß den ubiquitären Expressionsraten zeigte sich bei deren Korrelation mit mehreren klinischen Kriterien kein signifikanter Zusammenhang mit der Tumorbiologie oder dem Tumorstadium.

Aufgrund der Tatsache, dass der Neurokinin-1 Rezeptor bei Neuroblastom-Patienten unabhängig vom Krankheitsverlauf oder biologischen Faktoren generell überexprimiert wird, ist er als vielversprechendes Zielmolekül für zukünftige Therapieansätze prädestiniert.

Zusammengefasst konnten wir durch unsere Studien erstmalig eine spezielle Wachstumsform des Neuroblastoms - genannt UVIN – und seine klinische Relevanz beschreiben und zusätzlich den Neurokinin-1 Rezeptor als mögliches Zielmolekül für eine verbesserte, erfolgreiche Therapie dieses ungewöhnlichen Tumors identifizieren.

4 Published Articles

4.1 Publication I

Mühling J, Eberherr C, Müller Höcker J, Grote V, v. Schweinitz D, Kappler R, Fröba-Pohl A.

Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and is associated with adverse outcome

J Pediatr Surg. 2019 Nov;54(11):2336-2342. doi: 10.1016/j.jpedsurg.2019.07.012. Epub 2019 Jul 23.

Journal of Pediatric Surgery 54 (2019) 2336–2342

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Vessel adherent growth represents a major challenge in the surgical resection of neuroblastoma and Is associated with adverse outcome☆

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ARTICLE INFO

Article history:
Received 28 November 2018
Received in revised form 17 July 2019
Accepted 19 July 2019

Key words:
Neuroblastoma
Vessel adherence
Adhesion
Tumor markers
Outcome
UVIN

ABSTRACT

Purpose: Neuroblastoma (NB) is the most common extracranial, solid tumor in childhood, with a peak incidence in children under 6 years of age. Due to its variable course of disease, which ranges from spontaneous regression to metastatic spread, NB still represents a significant therapeutic challenge. Strikingly, a certain number of NBs intraoperatively show vessel adhesion and/or infiltrative growth, which is often not visible in pre-operative imaging. We proposed the term unexpected vessel infiltration of NB (UVIN) to denote this phenomenon. UVIN represents a major surgical challenge.
Methods: In this study, we determined frequency and clinical relevance of UVIN in a cohort of 100 NB-patients with subsequent correlation to several unfavorable characteristics of disease. RNA expression levels of MYCN and its co-regulated antisense transcript MYCNOS to identify markers was measured by PCR.
Results: We found UVIN to be present in 34% of cases and significantly correlated with incomplete resection, MYCN amplification, complications, neoadjuvant therapy, tumor grade and MYCNOS expression levels. MYCN expression levels showed no significant results with UVIN.
Conclusion: Collectively, our data show that UVIN represents a frequent surgical problem associated with a poor outcome in NB patients. MYCN and MYCNOS seem to be no appropriate markers for UVIN.
Type of study: Prognosis study.
Level of evidence: Level III.

NB is the most frequent, extracranial solid malignancy in the pediatric population. It arises from the autonomic, sympathetic nervous system and originates from misdifferentiated progenitor cells of the neural crest. Hence, this tumor most frequently presents in the abdominal cavity, followed by the cervical, thoracic and pelvic areas [1,2]. Despite the tremendous advances in the diagnostic and therapeutic management options available for NB over the last 30 years, this tumor is still responsible for about 15% of all oncologic pediatric mortalities [3]. NB shows an extremely variable disease course, with some patients demonstrating spontaneous remission, whereas others progress and die due to the disease. Fifty percent of all NBs have already metastasized at the time of diagnosis [4].

In spite of intensive research, no clear driver for the formation of NB has been identified so far. A complex interplay of many different genetic alterations, such as *MYCN* amplification, *ALK* activations and *TERT* rearrangements, seem to influence the biology of this tumor [5–7]. Of these, amplification of *MYCN* appears to be the most important factor for staging and prognosis in NB [3,8]. *MYCN* is a proto-oncogene that is physiologically present in early stages of embryonic development and influences the regulation of cell cycle, proliferation, apoptosis and cellular transcription [9]. The frequency of *MYCN* amplification in NB is estimated to be 20%, with amplification being defined as at least three copies of the gene [1,10]. Patients with *MYCN* amplification demonstrate a significant decrease in survival [3,8,11].

The so-called *N-CYM* or *MYCNOS* gene, found at the chromosomal locus 2p23–24, on the opposite DNA strand to *MYCN*, is also thought to be involved in NB. This gene is transcribed in antisense to *MYCN*. *MYCNOS* promotes the expression of *MYCN* by interfering with other

Abbreviations: NB, Neuroblastoma; UVIN, Unexpected vessel infiltration of neuroblastoma; TBP, TATA-Box-binding-Protein; IDRF, Image defined risk factors; INRG, International Neuroblastoma Risk Group; INSS, International Neuroblastoma Staging System; TERT, Telomerase reverse transciptase; ALK, Anaplastic lymphoma receptor tyrosine kinase; DNA, Desoxyribonucleic acid; OS, Overall survival; EFS, Event free survival.

☆ Declarations of interest: none

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https://doi.org/10.1016/j.jpedsurg.2019.07.012
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factors and thus stabilizing the *MYCN* products [12,13]. In view of its obligatory co-expression with *MYCN*, a positive auto-regulation has been anticipated [14,15]. Concomitant overexpression of *MYCN* and *MYCNOS* increases not only cell survival and aggressive growth but also the risk for metastasis due to inhibition of apoptosis [12,16].

Owing to its molecular genetic variety, NB represents an extraordinary therapeutic challenge, with surgical intervention playing a major role [17,18]. Close contact of the tumor with vital structures, particularly blood vessels, can be a significant problem during attempts at surgical resection and can potentially result in incomplete resections. Important blood vessels have often been found to be affected in locally advanced or metastatic NB and the impact this has on the clinical course of the disease has been well documented [19]. As a consequence of these issues, assessment of image-defined risk factors (IDRF) is one of the first diagnostic steps in NB evaluation including the assessment of vessel involvement. This information then influences the preoperative INRGSS staging of the NB. Despite this however, surgical complications are still regularly encountered. This is because of the presence of strong adhesion of some of the tumors to the vessel walls, a phenomenon which is independent from the extent of encasement of those walls as determined by the preoperative imaging. [20].

The aim of this study was to document this invasive growth phenomenon in a systematic way and investigate its clinical relevance for the course of disease. Furthermore, we evaluated the expression of *MYCN* and *MYCNOS* as putative molecular indicators for this invasive growth pattern of NB.

1. Material and methods

1.1. Patients

One hundred patients of Caucasian origin with NB were treated between 2009 and 2016 in the department for pediatric surgery at the Dr. von Hauner children's hospital, Munich, Germany. All tumors were resected or assisted and documented for the occurrence of vessel adhesive growth by the same surgeon (D.v.S.). Diagnosis, therapy and staging were performed according to current guidelines and international standards (grading: INRG; staging: INSS). Patients were treated according to the standard protocol (NB 2004, German Society of Pediatric Oncology and Hematology, GPOH). Written informed consent was obtained from the patients' parents or the patient itself and the study protocol was approved by the Committee of Ethics (Ludwig-Maximilians-University of Munich).

Patients' charts were reviewed retrospectively for age, sex, INSS stage, *MYCN* amplification, metastasis, extent of resection, histology, complications, recurrence, neoadjuvant therapy, event-free survival (EFS) and outcome (overall survival, OS). INSS stages 1, 2 and 4S were defined as low grade, 3 and 4 as high grade disease. Death or recurrence was defined as events in EFS. (see Table 1, column 1 & 2). Tumor tissue from each patient was frozen in liquid nitrogen for further analysis.

1.2. Categorizing the type of vessel involvement

Three categories of vessel involvement were defined. Firstly, preoperative imaging studies at the time of diagnosis were reviewed for the occurrence of IDRF (concerning vessel encasement) as described by Monclair et al. [21]. The tumors were thus identified as IDRF-positive or IDRF-negative.

Secondly, tumors were differentiated based on the degree of "in situ" vessel involvement as NB frequently presents intraoperatively as a mass closely associated with bigger vessels, such as the caval or renal vein. Envelopments of more than 50% of a vessel in situ were classified as encasement of the affected vessel. The tumors were thus identified as "encasing" or "not encasing" [19].

Table 1
Clinical characteristics and correlation with UVIN. Apart from different kinds of vessel involvement, 13 additional parameters were analyzed in 100 patients. Grade 1, 2A, 2B and 4S are classified as low grade, 3 and 4 as high grade (columns 1&2). The analysis for correlation of UVIN and all clinical characteristics was performed with GraphPad Prism in Chi-Square tests at a *p*-value lower than 0.05. Frequency is given in number of patients and percentage in parentheses. Significant results (bold printed) were found in correlation with complications, MYCN-amplification, extent of resection, IDRF vessel status, encasement, neoadjuvant chemotherapy and grade of disease. Other clinical parameters showed no significance.

			UVIN	
Clinical parameters	Number of patients (100)	34 Positive (%)	66 Negative (%)	Significance
Recurrence				$p = 0.4545$
Yes	22	9 (40.9)	13 (59.1)	
No	78	25 (32.1)	53 (67.9)	
Complications				$\mathbf{p = 0.0344}$
Yes	49	22 (44.9)	27 (55.1)	
No	51	12 (23.5)	39 (76.5)	
Metastasis				$p = 0.0588$
Yes	48	21 (43.8)	27 (56.2)	
No	52	13 (25.0)	39 (75.0)	
MYCN-amplification				$\mathbf{p = 0.0091}$
Yes	13	9 (69.2)	4 (30.8)	
No	87	25 (28.7)	62 (71.3)	
Sex				$p = 0.2951$
Male	52	15 (28.8)	37 (71.2)	
Female	48	19 (39.6)	29 (60.4)	
Extent of resection				$\mathbf{p = 0.0045}$
Incomplete	63	28 (44.4)	35 (55.6)	
Complete	37	6 (16.2)	31 (83.8)	
Neoadjuvant therapy				$\mathbf{p = 0.0002}$
Yes	81	34 (42,0)	47 (58.0)	
No	19	0 (0.00)	19 (100)	
IDRF vessel				$\mathbf{p < 0.0001}$
Positive	60	30 (50.0)	30 (50.0)	
Negative	40	4 (10.0)	36 (90.0)	
Encasement				$\mathbf{p < 0.0001}$
Positive	64	34 (53.1)	30 (46.9)	
Negative	36	0 (0,0)	36 (100.0)	
Age at first diagnosis				$p = 0.8283$
> 18 months	62	22 (35.5)	40 (64.5)	
< 18 months	38	12 (31.6)	26 (68.4)	
Outcome				$p = 0.0683$
Dead	21	11 (52.4)	10 (47.6)	
Alive	79	23 (29.1)	56 (70.9)	
Course of disease				$p = 0.0734$
With event	32	15 (44.4)	17 (55.6)	
Event free	68	19 (16.2)	49 (83.8)	
Grade				$\mathbf{p = 0.0014}$
High grade	80	33 (41.3)	47 (58.7)	
Low grade	20	1 (5.0)	19 (95.0)	
Primary localization				$p = 0.7126$
Thoracic	8	2 (25,0)	6 (75,0)	
Abdominal	92	32 (34,8)	60 (65,2)	
Histology				$p = 0.9406$
GN	8	3 (37,5)	5 (62,5)	
GNB	34	11 (32,4)	23 (67,6)	
Diff. NB	29	11 (37,9)	18 (62,1)	
Undiff. NB	29	9 (31,0)	20 (69,0)	

Thirdly, in the category of encasing tumors, we differentiated tumors into two subsets. The first of these had macroscopically visible vessel infiltration and were difficult to remove from the affected vessels. The second of these could be quite easily separated from the vessel surface without causing any damage. To denote this phenomenon, we propose the term "unexpected vessel infiltration of

neuroblastoma" (UVIN). The encasing tumors were therefore either UVIN positive or UVIN negative.

1.3. Histopathological analysis

Histological examination of each tumor was performed on hematoxylin–eosin stained sections of formalin-fixed paraffin-embedded tissues by a trained pathologist (J.M.-H.). Immunohistochemical staining of neuron-specific enolase (NSE) was performed on formalin-fixed paraffin-embedded tissue sections using a pretreatment of 15′ min in citrate buffer in the microwave, followed by an incubation with an antibody against NSE (DakoCytomation, Hamburg, Germany; 1:50) and alkaline phosphatase anti-alkaline phosphatase detection as previously described [22]. Special microscopic imaging was done in one of three most representative tumors, which unfortunately led to the resection of affected organs as kidney and adrenal gland.

1.4. RNA isolation and cDNA synthesis

RNA was isolated from fresh frozen tumor tissue of every single patient using TRI reagent (Sigma Aldrich International, St. Gallen, Switzerland) according to the manufacturer. cDNA was synthesized from 2 μg RNA using random hexameres (Roche Diagnostics, Mannheim, Germany) and SuperScript II (Invitrogen) as recommended by the supplier.

1.5. Real-time PCR

We quantified gene-expression levels of *MYCN*, *MYCNOS* and *TBP* (TATA box binding protein) using TaqMan assays (HS 00232074 m1, HS01040745m1, HS00427620m1 from Life Technologies, Carlsbad, CA, USA). PCR reactions were set up with TaqMan Master Mix (Life Technologies) and run in duplicates on a Mastercycler Realplex2 cycler (Eppendorf, Hamburg, Germany).

Amplification of the housekeeping gene TATA-Box-binding-Protein (*TBP*) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the ΔΔct method as previously described [23].

1.6. Statistical analysis

Non-parametric Spearman correlation and association studies of expression data with clinical parameters using the Fisher's exact test were performed in GraphPad Prism. Estimates for survival were generated using the Kaplan–Meier method, and curves were compared by using log-rank test. A level of $p < 0.05$ was considered to be significant.

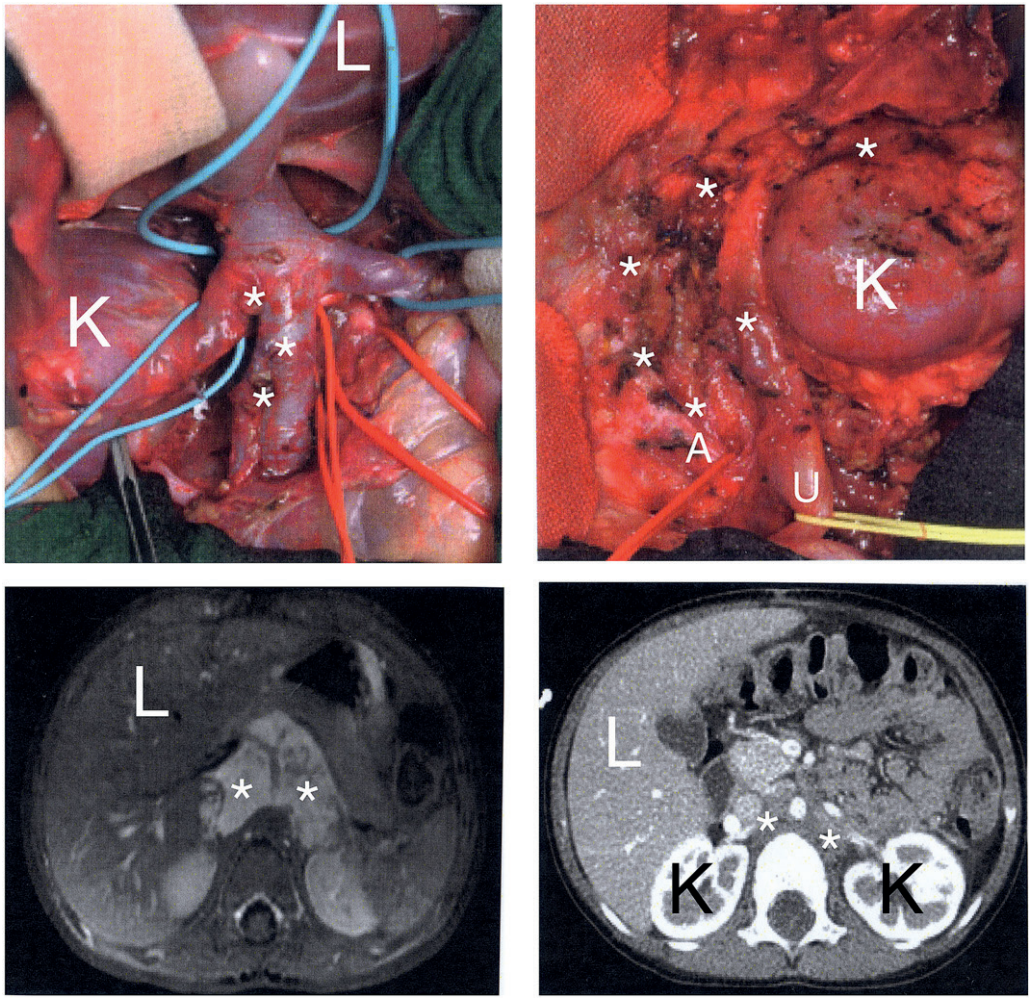

Fig. 1. Different forms of vessel involvement and their appearance "in situ" and in diagnostic imaging. Intraoperative pictures above show tumors with encasement (left) and infiltrative growth (right). While tumor tissue (marked with stars) could be easily separated from the encased vessel (left side: renal veins and caval vein), the resection of the infiltrative tumor endangered the integrity of the affected vessel (right side: inferior mesenteric artery, marked with A, U shows the ureter). As visible on CT scans of the same patients (below), the quality of vessel contact in affected areas is not predictable. Both were defined as "IDRF vessel" positive. For better orientation, organs are marked with L = liver and K = kidney.

2. Results

2.1. IDRF-positive tumors and encasement

First, diagnostic imaging was reviewed for the occurrence of IDRF (vessel encasement) as described by Monclair et al. [21]. Imaging showed IDRF-positive tumors in 60% (60 patients) of all analyzed cases. Correlation analyses with clinical parameters (Suppl. Table 1) revealed a significant association of IDRF-positivity with complications ($p = 0.0023$), extent of resection ($p = 0.0353$), INSS stage ($p = 0.0001$), encasement ($p < 0.0001$), UVIN ($p < 0{,}0001$) and neoadjuvant chemotherapy ($p < 0.0001$). However, OS and EFS showed no statistical significant difference between IDRF-positive and –negative cases (Suppl. Fig. 1).

Next, intraoperatively observed in situ encasement of the vessels was determined and revealed this characteristic in 64% (64 patients) of all analyzed cases. Correlation analyses of encasement with clinical parameters showed significant associations with complications ($p = 0.0228$), extent of resection ($p = 0.0182$), and neoadjuvant chemotherapy ($p < 0.0001$). Strong associations were found with higher grades of disease, UVIN and IDRF-positivity (each $p < 0.0001$) (Suppl. Table 2).

2.2. Unexpected vessel infiltration of neuroblastoma (UVIN)

In the course of performing surgical resections on advanced NB patients we observed that the degree of vessel encasement was not always consistent with the difficulty of tumor removal. Some tumors, independent of degree of vessel envelopment, adhered more to the vessel wall than others, thereby impeding surgical resection (Fig. 1). The presence of this phenomenon cannot be reliably identified in advance by preoperative imaging.

Evaluation of the documented cases operated on in our department revealed a frequency of occurrence of unexpected vessel infiltration of neuroblastoma (UVIN) of 34% (34 patients). 5 tumors (14.7%) showed a sole infiltration of veins, and 9 tumors (26.5%) a sole infiltration of arteries. In 20 UVIN-positive tumors (58.8%) the infiltration was detected in both vessel categories. Upon histological staining we found that the tumor/vessel-interface of UVIN-positive cases showed tumor cell infiltration within the outer vascular wall layers of large vessels, but without intima and intraluminous involvement (Fig. 2). Immunohistopathological staining of NSE confirmed the existence of agglomerations of NB cells in the vessel wall.

2.3. UVIN affects the clinical course of disease

Comparison of clinical characteristics of UVIN-positive and –negative cases revealed a significant association of UVIN with complications ($p = 0.0344$), *MYCN* amplification ($p = 0.0091$), the extent of resection ($p = 0.0045$), grade ($p = 0.0014$), IDRF ($p < 0.0001$), encasement ($p < 0.0001$) and neoadjuvant chemotherapy ($p = 0.0002$). In a Pearson's chi-square test with metastasis and relapse, UVIN only showed a significant result with metastasis ($p = 0.048$). The other analyzed clinical characteristics showed no statistical significance (Table 1). Kaplan–Meier estimates showed a trend towards a significant difference of NB with or without UVIN in EFS ($p = 0.0616$) and

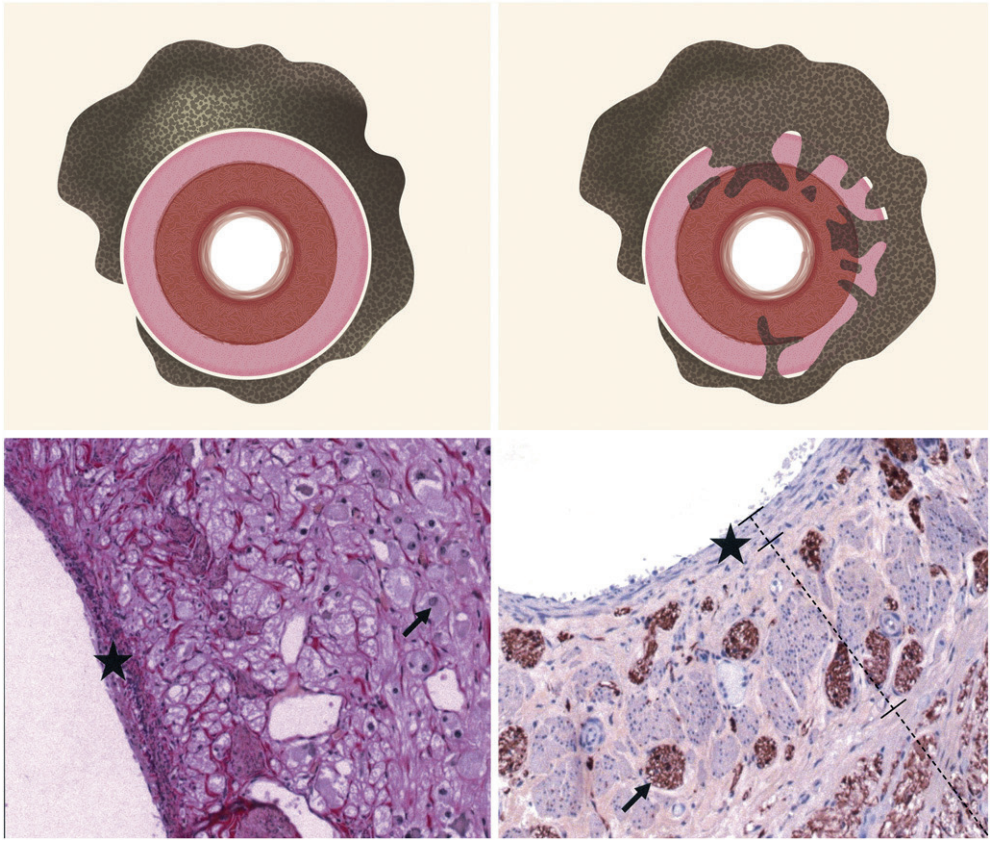

Fig. 2. Detailed analysis of vessel infiltrating tumors. a) Schematical graphics (above) demonstrate the essential difference between tumors with vessel encasement (left) and vessel infiltrating growth (UVIN, right). The vessel itself is not infiltrated in encasing tumors while the UVIN shows an expansion of tumor tissue through outer vessel layers. It is important to note that the intima remains unaffected. b) This was proven in the histopathological examination. Below a microscopic partial view on a HE – stained (left) and NSE – stained (right) section of the renal vein, affected by a representative UVIN tumor diagnosed as a Hughes III neuroblastoma; darker agglomerations of NB cells (black arrows) show an infiltration of the outer vascular walls of vena renalis, but without intraluminous or vessel intima (black star) involvement. Scaling was added for better differentiation of the single vessel wall layers.

overall survival ($p = 0.0634$). Kaplan–Meier estimates with MYCN stratification showed no significant results in EFS, but significant results as well in UVIN positive ($p = 0.0262$) as UVIN negative ($p = 0.0198$) tumors in OS (Fig. 3).

2.4. MYCN and MYCNOS gene expression are unable to predict UVIN

Analysis of *MYCN* and *MYCNOS* gene-expression showed a significant increase of transcript levels in tumor samples, which were initially classified as *MYCN* amplified ($p < 0.0001$ and $p < 0.0006$). Furthermore, we found significant associations of *MYCN* expression with recurrence ($p = 0.0371$) and outcome ($p = 0.0112$) and *MYCNOS* expression with outcome ($p = 0.0068$) (Table 2). Correlation analysis of *MYCN* gene expression level with UVIN did not show a statistically significant result.

Interestingly, in contrast to *MYCN*, *MYCNOS* gene expression levels showed a significant correlation with UVIN ($p = 0.0055$) (Fig. 4). However, in 24% of the tumor samples gene expression levels of *MYCNOS* were under the detection limit and thus precluded from statistical analysis.

3. Discussion

Over the last few decades, the appropriate extent of NB surgery has been an ongoing topic of debate with recommendations from studies diverging over the need for complete versus incomplete resection [24]. A recent study suggests improvement of outcome with complete resection in localized NB. However, for metastatic NB, improvement is only seen in *MYCN* amplified tumors and complete macroscopical resection [25].

One major rationale for incomplete resection is tumor encasement of the vessels and sometimes even invasive growth into vessel walls or adjacent organs. However, investigation into this phenomenon, which we observed in more than a third of our patients, has so far been limited. Therefore, the purpose of this study was to identify the clinical and prognostic relevance of this type of invasive tumor growth form.

IDRF are defined as surgical risk factors. Consistent with recent publications, IDRF-status in our study population was also significantly associated with surgical complications, extent of resection and INSS stage [26]. Furthermore, IDRF diagnosed in pretherapeutic imaging studies were also significantly associated with intraoperative observation of vessel encasement and also infiltrative vessel growth. Nevertheless, even though elicitation of IDRF status helps with preparation of surgery, it still is not a reliable factor for prediction of invasive growth. Despite the fact, that IDRF status was significantly associated with encasement- and UVIN-status, differentiation between UVIN-positive and -negative patients and encasement-positive and –negative patients was not reliably possible using this measure. This may be because pretherapeutic imaging, as described by Monclair et al. for compilation of

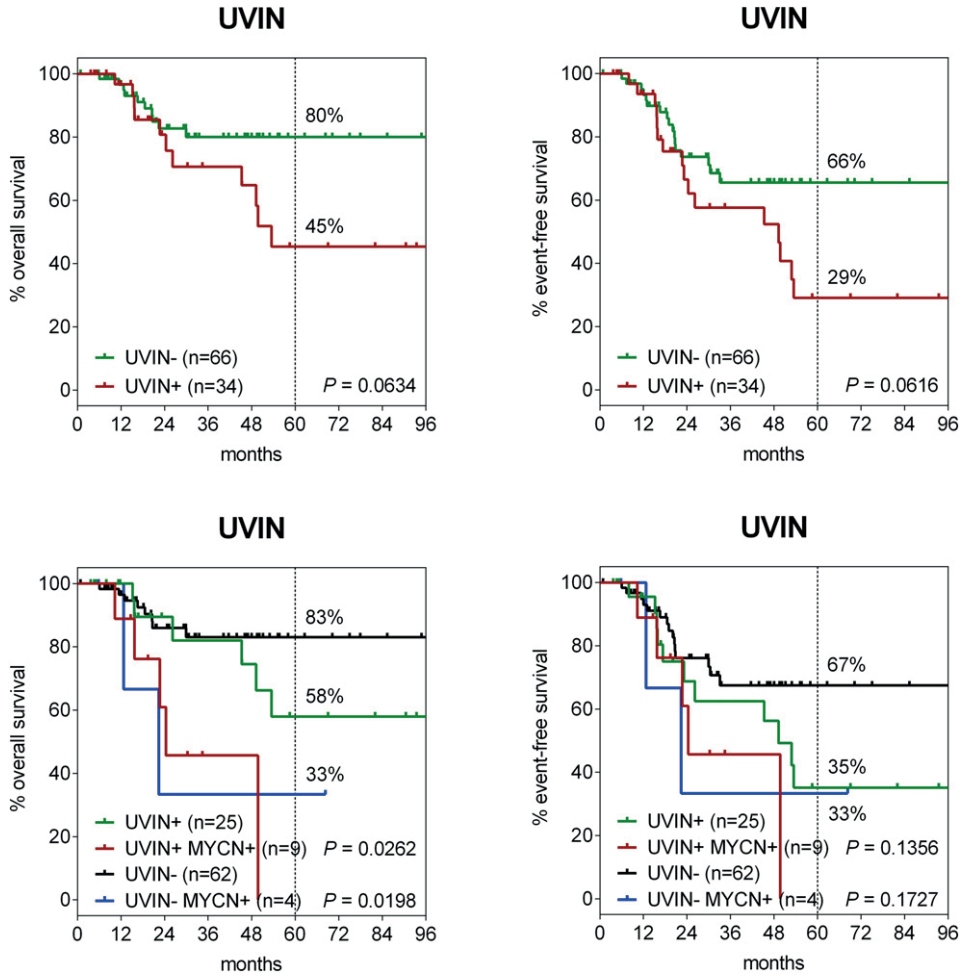

Fig. 3. Kaplan–Meier analysis of OS (left) and EFS (right) of UVIN without (above) and with MYCN stratification (below). OS (left) was analyzed over 96 months. Tumors with and without UVIN were compared by log-rank-test at a *p*-value lower than 0.05. EFS (right) was also analyzed over 96 months. Recurrence or death was defined as events. Tumors with and without UVIN were compared by log-rank-test at a *p*-value lower than 0.05. In each analysis a strong trend to a worse outcome in UVIN positive tumors was detectable. With MYCN stratification (figures below) significant results could be detected as well in UVIN positive as in UVIN negative tumors at the OS analysis. EFS showed no significant results.

Table 2
Correlation of MYCN/MYCNOS gene expression rates with clinical characteristics and UVIN Gene expression levels were analyzed with real time qPCR and normalized to TBP housekeeping gene expression. Correlation with given parameters was analyzed with GraphPad Prism Mann–Whitney test at a *p*-value lower than 0.05. Significant results (bold printed) were found in MYCN with recurrence, MYCN-amplification status and outcome, in MYCNOS with UVIN, MYCN-amplification and outcome.

	MYCN	MYCNOS
UVIN	0.242	**0.0055**
Recurrence	**0.0371**	0.1171
Complications	0.9658	0.9999
Metastasis	0.8623	0.1406
MYCN – amplification	<**0.0001**	<**0.0006**
Sex	0.9658	0.5653
Grade	0.4396	0.1706
Extent of resection	0.9391	0.644
Age at first diagnosis	0.7852	0.231
Outcome	**0.0112**	**0.0068**

Significant results are now marked as bold-printed.

IDRF-status is performed too early (takes place in advanced NB prior to administration of several cycles of chemotherapy) to allow truly accurate surgical planning and differentiation between these groups. More likely however it is a result of the limitations of the imaging studies which are unable to assess the histological degree of tumor invasion and are therefore an inaccurate tool for prediction of vessel status [27]. Thus although our results (in a patient cohort that was comparable to previous studies) further supported the use of the IDRF status in the planning of surgery in NB patients by demonstrating the strong correlation of an IDRF vessel-positive status with the presence of encasement and also UVIN, they also highlighted the limitations of this imaging system in terms of truly accurate prediction of vessel invasion.

Due to this and the fact, that IDRF, encasement and UVIN all showed strong correlations with the treatment of neoadjuvant chemotherapy, we have to assume that there is no proper way for a pre-operative risk stratification for UVIN so far.

Correlation of *MYCN* or *MYCNOS* gene expression with invasion, migration and vessel adhesion of tumor cells is also a popular area for current research [12,28–30]. Therefore, we analyzed if gene expression levels correlate with vessel infiltration status.

Our results indicate a relationship between *MYCN* gene expression and *MYCN* amplification, as well as a significant correlation of the gene expression levels of *MYCNOS* with the status of *MYCN* amplification. These results reflect the previous insights of the obligatory co-expression and overexpression of *MYCN* and *MYCNOS* and the multiple

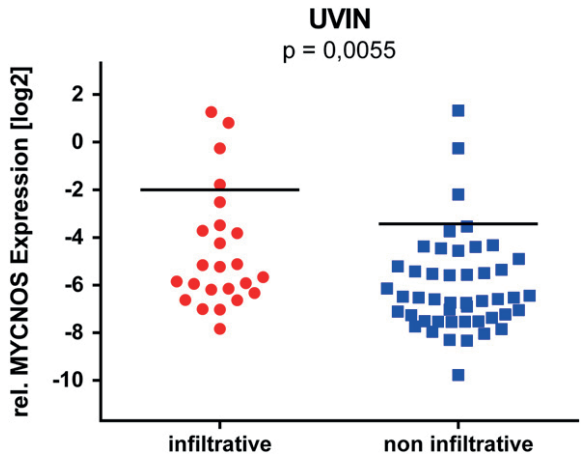

Fig. 4. MYCNOS gene expression in UVIN-positive and -negative NB. MYCNOS gene expression levels were quantified by real time PCR relative to TBP (TATA – box – binding protein) housekeeping gene. Statistical analysis of MYCNOS gene expression levels with GraphPad Prism showed a significant difference between UVIN-positive and –negative NB in Mann–Whitney test at a *p*-value lower than 0.05.

described cooperative auto-regulation pathways of the two genes [14,15,31–33].

However, even though *MYCN* amplification status was correlated with vessel infiltrative growth, similar results could not be confirmed for *MYCN* gene expression level. Significant correlations with clinical criteria like recurrence and outcome confirmed once again the importance of *MYCN* amplification as an unfavorable prognostic marker. However, the question as to whether *MYCN* gene expression has the same importance for the clinical course as *MYCN* amplification is still not solved.

Analysis of *MYCNOS* gene-expression showed a significant correlation with *MYCN* amplification status, as well as the overexpression of *MYCN* gene expression. It also showed a significant correlation between *MYCNOS* gene expression and the vessel adhesive growth of NB. However, the results of our *MYCNOS* gene expression analysis should be interpreted with caution because a number of the samples collected had to be excluded from statistical analysis due to undetectable mRNA transcript levels (24% of the total samples taken). This could be a potential source of analytical bias and should be taken into account when considering the results obtained. It might for example be the cause of our results showing an apparent correlation between *MYCNOS* gene expression and vessel adhesive growth in NB even though a correlation between *MYCN* gene expression and vessel adhesive growth was not found. Of note, this was one of the only two areas in which *MYCN* and *MYCNOS* results did not concur in our study.

Alternatively, these results could reflect a real phenomenon such as the *MYCNOS* antisense transcript interacting with other molecular factors that are involved in the vascular adhesion process. This has indeed been found to be the case in other studies involving NB and bladder cancer. In these studies, *MYCNOS* was found to act as a co-factor involved in increasing tumor growth, aggressiveness and vascularization [12,16,34]. The study by Kaneko et al. provides more information on the complex interplay between *MYCN*, *MYCNOS* and other factors which induce the transformation of substrate adherent S-cells in NB [16]. The way in which this, or any other molecular pathways, may be involved in triggering UVIN is an important area for future research.

Analysis of patients' data demonstrated that UVIN was associated with a poor clinical course of disease. Although these results were statistically not significant, at least a trend towards significance for both EFS and OS was seen. Regarding the *MYCN* stratified analysis, which showed significant results as well in UVIN positive as in UVIN negative tumors, one more time the strong impact of *MYCN* on the course of disease was shown. Furthermore, the trend of UVIN positive tumors can be visualized in Fig. 3 where the decreased OS und EFS compared to UVIN negative patients with time is clearly visible.

Of note, with regards to the apparent link between UVIN and a poorer prognosis are the following factors: Firstly, the overall number of study participants was low thus making the demonstration of statistical significance more difficult to achieve. Secondly, patient treatment was not uniform across the cohort and thus external factors (eg. number of chemotherapy cycles received and their potential effect on the invasiveness of NB) may be playing a confounding role in the results obtained. Thirdly, it is not clear whether the UVIN is the cause of the poorer prognosis or whether it is merely an indicator of another, as yet unknown, underlying molecular factor that is actually responsible for driving the progression of the disease.

4. Conclusion

Our study demonstrates the value of further risk stratification in the assessment of NB, above and beyond the existing IDRF status method. We propose that NB tumors should also be classified according to the degree of in situ vessel encasement and according to the presence or absence of UVIN. Given that these factors represent not only a major surgical problem, but also affect the clinical course of the disease, we suggest the regular identification of the UVIN and encasement status

in every NB by the operating surgeon. Also if the definition may depend on the surgeon's experience, a postoperative characterization – combining the surgeon's evaluation and the histopathological analysis – would be desirable to collect data for further studies on this phenomenon. This additional classification process has prognostic implications and may therefore allow the application of more tumor-specific therapeutic management programs in the future. Unfortunately our results do not support a role for gene expression levels of *MYCN* and *MYCNOS* as a marker for UVIN at this present time. Further larger prospective studies are needed to understand the cause and implications of UVIN and the role that *MYCN* and *MYCNOS* or other genes, which have yet to be identified, play in this process.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpedsurg.2019.07.012.

Acknowledgments

This work was supported by grants of the Muenchener Medizinische Wochenschrift, Munich, Germany and the Friedrich Baur foundation, Munich, Germany (to A.F-P.). We are grateful to Fatemeh Promoli for technical assistance, Christina Busch for illustration, and Rebecca Maxwell for critically reviewing the manuscript.

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4.2 Publication II

Pohl A, Kappler R, Mühling J, v. Schweinitz D, Berger M.

Expression of Truncated Neurokinin-1 Receptor in childhood neuroblastoma is independent of tumor biology and stage

Anticancer Res. 2017 Nov;37(11):6079-6085.

ANTICANCER RESEARCH *37*: 6079-6085 (2017)
doi:10.21873/anticanres.12056

Expression of Truncated Neurokinin-1 Receptor in Childhood Neuroblastoma is Independent of Tumor Biology and Stage

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Abstract. *Background: Neuroblastoma is an embryonal malignancy arising from the aberrant growth of neural crest progenitor cells of the sympathetic nervous system. The tachykinin receptor 1 (TACR1) – substance P complex is associated with tumoral angiogenesis and cell proliferation in a variety of cancer types. Inhibition of TACR1 was recently described to impede growth of NB cell lines. However, the relevance of TACR1 in clinical settings is unknown. Patients and Methods: We investigated gene expression levels of full-length and truncated TACR1 in 59 neuroblastomas and correlated these data with the patients' clinical parameters such as outcome, metastasis, International Neuroblastoma Staging System (INSS) status, MYCN proto-oncogene, bHLH transcription factor (MYCN) status, gender and age. Results: Our results indicated that TACR1 is ubiquitously expressed in neuroblastoma but expression levels are independent of clinical parameters. Conclusion: Our data suggest that TACR1 might serve as a potent anticancer target in a large variety of patients with neuroblastoma, independent of tumor biology and clinical stage.*

Neuroblastoma (NB), an embryonal malignancy arising from the aberrant growth of neural crest progenitor cells of the sympathetic nervous system, is the most common solid extracranial tumor found in infancy and childhood (1-3). NB is characterized by its clinical heterogeneity: whereas very young children often demonstrate spontaneous tumor regression, older children frequently suffer from progressive disease with poor outcome. Although recent therapeutic approaches in multimodal therapies demonstrated better overall survival, more than 50% of children with high-risk disease do not respond to modern chemotherapy regimens, resulting in progressive disease. Overall, NB accounts for 12% of all pediatric oncological deaths (1, 3-6).

On the molecular level, several mutations have been discovered for NB. For this and other reasons, NB has served as a paradigm for biological risk assessment and treatment assignment. For example, amplification of MYCN proto-oncogene, bHLH transcription factor (*MYCN*) and hemizygous deletion of chromosomes 1p and 11q are found in up to 30% of patients with NB and are known to correlate with worse outcome and poor prognosis(2). However, the exact mechanisms of tumor formation and progression are still incompletely understood and other oncogenic drivers of tumorigenesis are still to be discovered (2).

Substance P (SP)–tachykinin receptor 1 (TACR1) (also named neurokinin-1 receptor) complex, which acts as a neuronal transmitter, has been linked to inflammation and cell migration (7). Furthermore, several studies have reported that activation of TACR1 (through binding of SP) is associated with tumoral angiogenesis and cell proliferation (8). Among the three subtypes of tachykinin receptors, TACR1 has the highest affinity for the ligand SP (9).

Two splice variants of *TACR1* are known: a full-length variant (fl-*TACR1*) and a truncated variant (*tr-TACR1*). The latter lacks 100 amino acids at its C-terminal end, which serves as a substrate for G protein receptor kinases and has been reported to differ in its biological function in interaction with SP (10). As a consequence of splicing, the truncated variant lacks sensitivity for negative feedback inhibition, leading to constant activation of the receptor complex(11). Several studies revealed the importance of tr-*TACR1* in cancer (10-13).

Importantly, the SP–TACR1 receptor system was recently described to be a potent anticancer target in NB (14). In the present study, we investigated the role and clinical relevance of fl-*TACR1* and tr-*TACR1* in tumor samples from children with NB.

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Key Words: Neurokinin-1 receptor, *TACR1*, tachykinin receptor 1, neuroblastoma, cancer, outcome.

Patients and Methods

Patients and tumor tissues. Between 2009 and 2014, 59 patients with NB underwent either biopsy or surgical resection in our Department. Patients were all treated according to the protocol of the German Society of Pediatric Oncology and Hematology (GPOH, study protocol NB2004). For this study, the patients' charts were reviewed retrospectively regarding clinical information: age, gender, International Neuroblastoma Staging System (INSS) status, outcome, metastasis, *MYCN* status, histology, and extent of surgical resection were obtained and correlated with gene-expression data.

Extent of surgical resection was noted as biopsy only when less than 50% of the tumor mass was removed. An incomplete resection was defined as when more than 50% but less than 89% of the tumor was macroscopically removed. Resection of more than 90% of tumor but still with visible tumor remnants (90-99%) was considered a near-complete resection. Removal of 100% macroscopical tumor mass was considered complete resection (15).

This study was approved by the Institutional Ethics Committee of the University Hospital (LMU Munich; N. 431-11). Written consent was given by the patients' parents for collection of data and laboratory analysis.

RNA extraction and reverse transcription. In essence, samples were treated with TRIzol® reagent for isolation of RNA, according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was dissolved in RNase-free water. Reverse transcription of RNA-samples (2 μg each) was performed utilizing SuperScriptTM II reverse transcriptase (Invitrogen Life Technologies), as recommended by the supplier.

Reverse transcription polymerase chain reaction (RT-PCR). Two microliters of cDNA sample were utilized in each PCR reaction with the following specific primers for fl-*TACR1* and tr-*TACR1* as well as TATA-box-binding-protein (*TBP*) housekeeping gene: For fl-*TACR1*: Forward, 5'-AACCCCATCATCTACTGCTGC-3' and reverse, 5'-ATTTCCAGCCCCTCATAGTCG-3' (NM_001058.3); for tr-*TACR1* forward, 5'-GGGCCACAAGACCATCTACA-3' and reverse, 5'-AAGTTAGCTGCAGTCCCCAC-3' (NM_015727.2); and for *TBP*: forward, 5'-GCCCGAAACGCCGAATAT-3' and reverse, 5'-CCGTGGTTCGTGGCTCTCT-3'. Samples for amplification reactions had a final volume of 20 μl. iTaq SYBR-green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for amplification reactions. Samples were incubated at 95˚C for 7 min, followed by 40 cycles of 95˚C for 30 s, 60˚C for 30 s and 72˚C for 30 s, with the final extension cycle performed at 72˚C for 7 min. The transcript numbers were normalized according to the expression of the housekeeping gene. Relative quantification of gene expression was performed using the 2 -ΔΔCt method as described by Pfaffl (16).

Statistical analysis. Results are given as the mean±standard error of the mean. Gene-expression levels of tumor samples are displayed as dot plots for each group. Correlation analysis was performed using a Mann–Whitney *U*-test and a standard *t*-test. Differences with a p-value of less than 0.05 were considered as statistically significant. Statistical calculations were performed using biostatics software from GraphPAD Prism® (La Jolla, CA, USA).

Kaplan–Meier curves were used for demonstration of overall survival (OS), and two-sided log-rank test was utilized for comparison of survival curves.

Table I. *Patient characteristics*.

	Patients (n)	%
Gender		
Female	26	44.1
Male	33	55.9
Histology		
Ganglioneuroma (GN)	5	8.5
Ganglioneuroblastoma (G1)	19	32.2
Differentiated neuroblastoma (G2)	14	23.7
Undifferentiated neuroblastoma (G3)	21	35.6
Outcome		
Alive	46	77.9
Died from disease	13	22.1
Extent of surgery		
Biopsy only	2	3.4
Incomplete resection (<50%-90%)	6	10.2
Nearly complete resection (>90-99%)	24	40.7
Complete resection (100%)	27	45.7
MYCN status		
Amplified	6	10.2
Not amplified	53	89.8
Metastasis		
Yes	26	44.1
No	33	55.9
International Neuroblastoma Staging System		
1	6	10.2
2a	6	10.2
2b	1	1.7
3	20	33.9
4	25	42.3
4s	1	1.7

MYCN: MYCN proto-oncogene, bHLH transcription factor.

Results

TACR1 shows high expression in human NB. Gene-expression profiles of fl-*TACR1* and tr-*TACR1* were investigated in human NB samples. In general, fl-*TACR1* showed a low expression profile, whereas tr-*TACR1* demonstrated much higher expression levels in NB samples (Figure 1a). This difference was statistically significant ($p<0.0001$). However, fl-*TACR1* and tr-*TACR1* expression did not demonstrate a significant correlation ($r=0.2201$, $p=0.0969$) (Figure1b). Furthermore, we performed an analysis of the association between expression of each splice variant (with cutoff as the overall mean expression) and overall prognosis. There was no statistically significant difference for either splice variant (Figure 1c).

Clinical characteristics. The mean patient age at diagnosis was 40.5 months, with a range from 0 months to 19.2 years. Twenty-six patients (44.1%) had metastases at diagnosis. For further patient characteristics, see Table I.

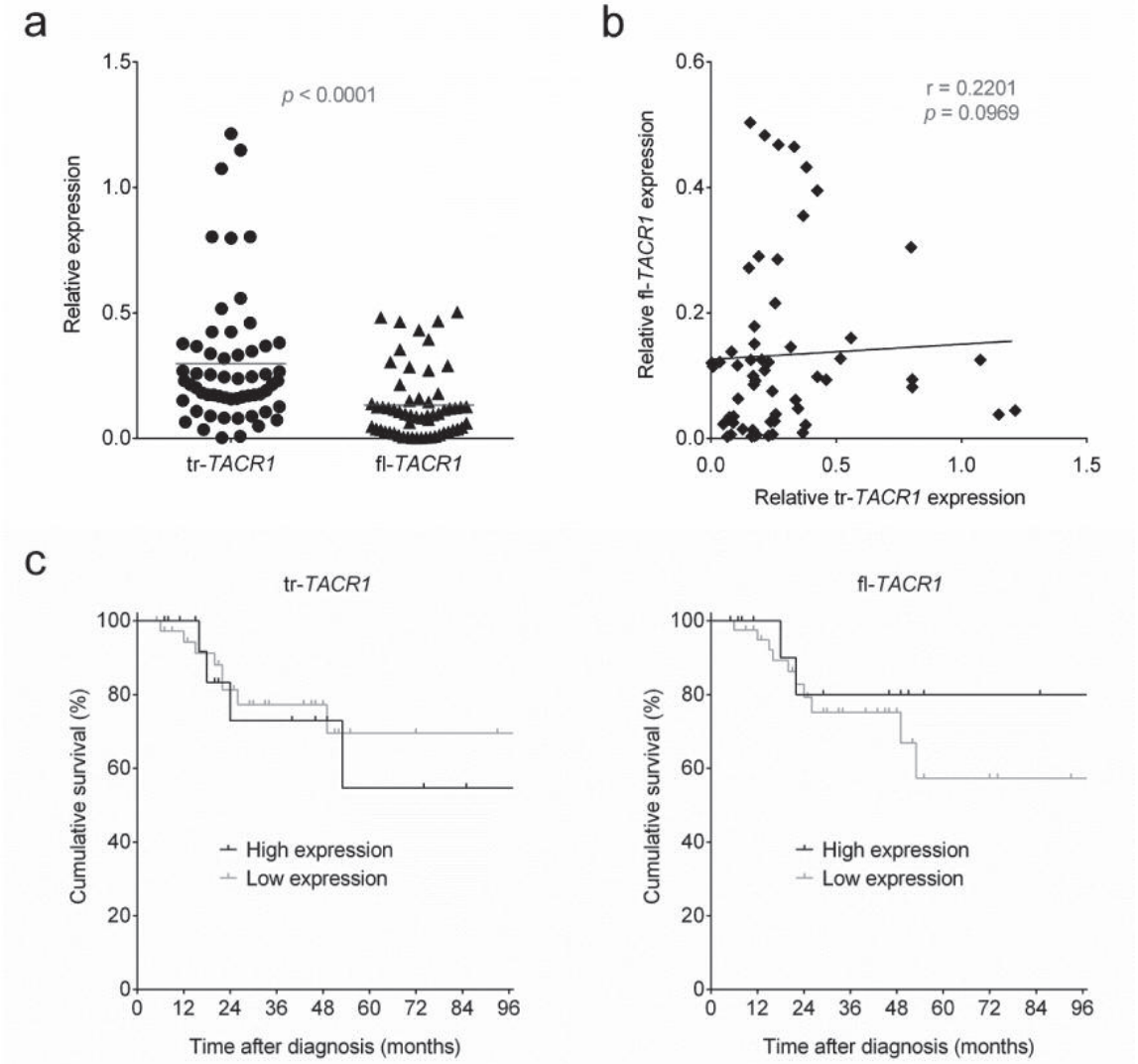

Figure 1. *a: Gene-expression of full-length tachykinin receptor 1 (fl)-TACR1 and truncated (tr)-TACR1 in human neuroblastoma samples. b: Correlation between fl-TACR1 and tr-TACR1. c: Association between expression of each splice variant (with cut-off as the overall mean expression) and overall prognosis.*

Gene-expression levels of fl-TACR1 and tr-TACR1. Both fl-*TACR1* and tr-*TACR1* variants as well as their ratio were analyzed for association with clinical and biological findings of NB samples. Neither fl-*TACR1* nor tr-*TACR1* were significantly associated with histology, age or gender (Figure 2). Clinical parameters such as *MYCN* status and metastasis, which are linked with aggressive disease, also demonstrated no significant association with tr-*TACR1* nor fl-*TACR1*. Only fl-*TACR1* was significantly higher in INSS stage 4 (p=0.0164, Figure 3), but there was no association for tr-*TACR1* nor their ratio. As was the case for the other clinical parameters mentioned, the analysis of gene-expression levels did not show any significant association with outcome (Figure 3).

Discussion

An abundance of studies have proven the importance of the SP–TACR1 complex in cancer and disease progression (7, 10). Moreover, during the past decade, *TACR1* and its high expression in a variety of cancer types has become the focus of attention for targeted therapy. Tachykinin-receptor antagonists such as aprepitant, currently used as a clinical drug for the treatment of chemotherapy-related nausea and vomiting, in several reports demonstrated *in vitro* and *in vivo* efficacy against TACR1, resulting in a robust anticancer effect (12, 14, 17). Similar effects have been observed for other TACR1 antagonists.

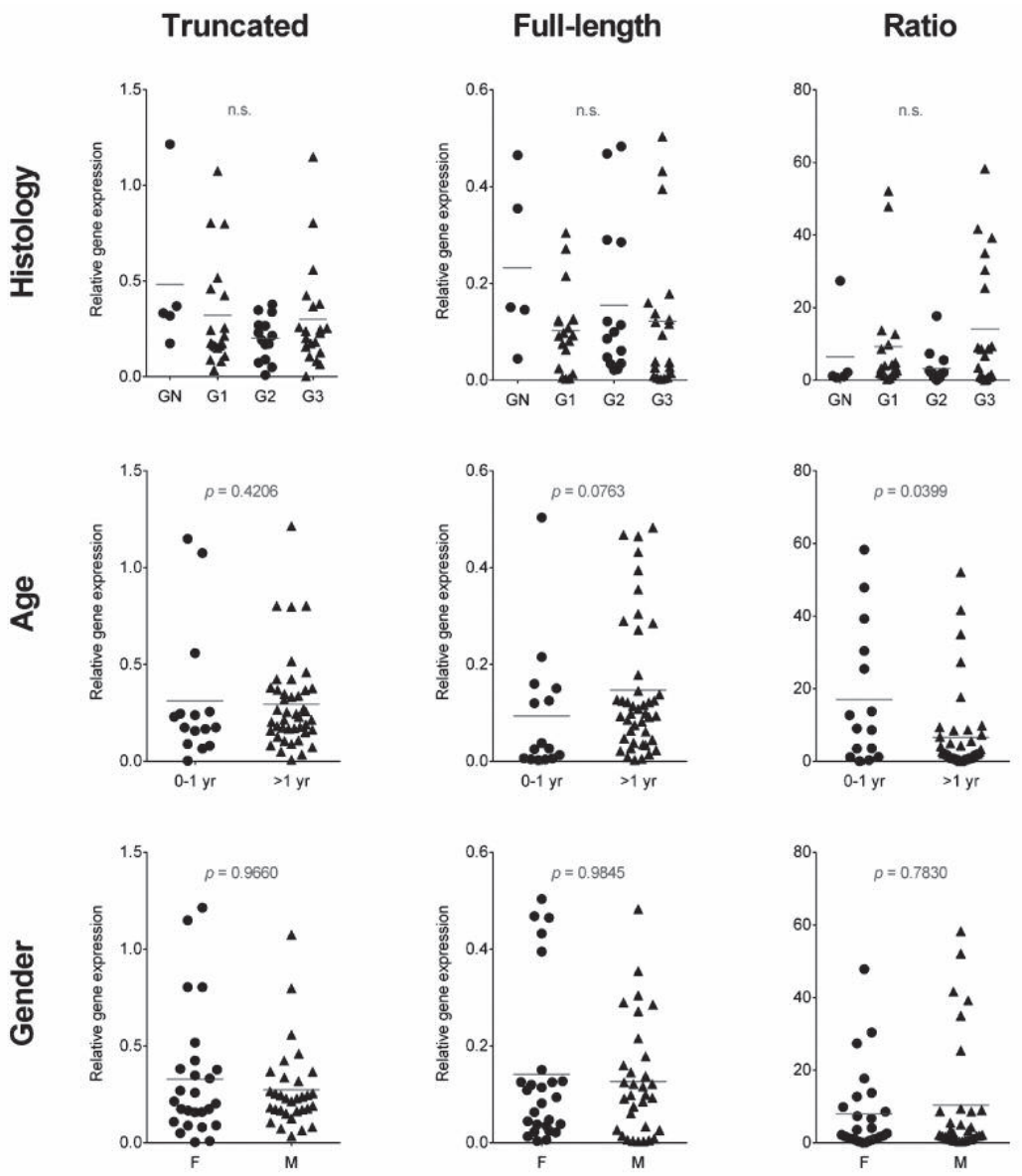

Figure 2. *Gene-expression of full-length tachykinin receptor 1 (fl)-TACR1 and truncated (tr)-TACR1, and their ratio in human neuroblastoma samples according to histology, age and gender. GN: Ganglioneuroma, G1: gangioneuroblastoma, G2: differentiated neuroblastoma, G3: undifferentiated neuroblastoma, yr: year, F: female, M: male.*

Little is known about the role of *TACR1* in oncogenesis and tumor progression in NB. Nowicki *et al*. investigated the cell immunophenotype of metastatic and primary tumors in NB and found, among other nervous tissue markers, high expression of SP in both groups (18). In 2005, Munoz *et al*. reported *TACR1* expression in two NB cell lines (19). Perhaps the most sophisticated study regarding this topic so far was recently published by Henssen *et al*. The authors examined tachykinin receptors in a panel of NB cell lines. Their results suggest *TACR1* expression in all tested cell lines, even though expression levels varied. Our previous studies in hepatoblastoma are consistent with this finding (12, 14). In a second step, growth inhibition was induced with targeting NB cells *in vivo* and *in vitro* (mouse xenograft model) with fosaprepitant, a tachykinin receptor antagonist similar to aprepitant. The Authors concluded that gene expression of *TACR1* is increased in NB cell lines resulting in expression of this receptor, making it an attractive target for targeted therapy with TACR1 antagonists (14). Interestingly, NB is currently the only solid cancer in

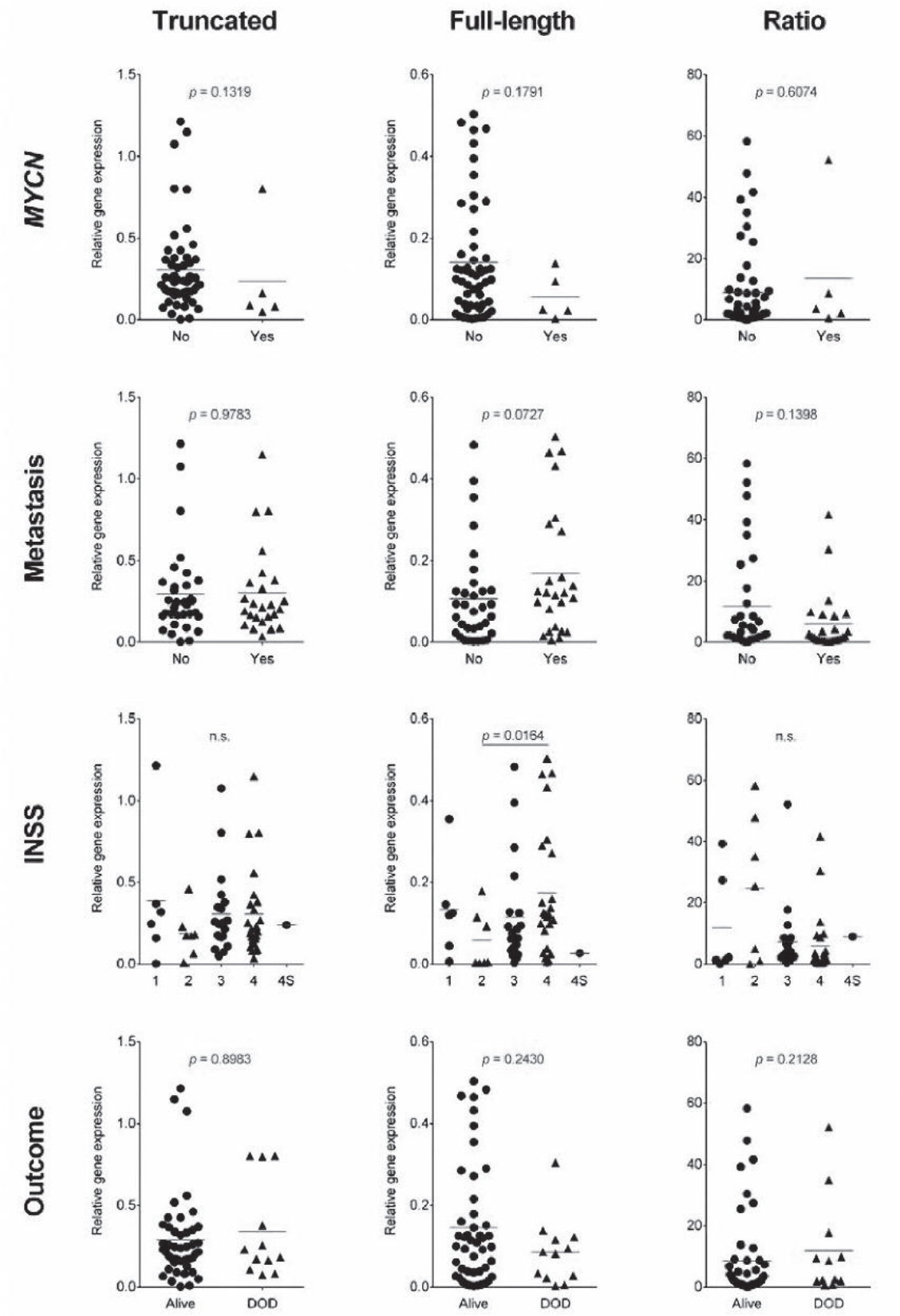

Figure 3. *Gene-expression of full-length tachykinin receptor 1 (fl)-TACR1 and truncated (tr)-TACR1, and their ratio in human neuroblastoma samples according to MYCN proto-oncogene, bHLH transcription factor (MYCN) status, presence of metastasis, International Neuroblastoma Staging System (INSS), and outcome. DOD: Died of disease.*

childhood for which a positive therapeutic effect has been proven for targeted therapy (*via* GD2). This understanding raises hope that there could potentially be other targets *via* which a similarly promising effect can be achieved.

In the present study, we demonstrated ubiquitous gene expression of fl-*TACR1* and tr-*TACR1* in a cohort of children with NB. Our results suggest increased gene expression of tr-*TACR1* and low expression of fl-*TACR1* in NB tumor tissue. Interestingly, gene expression levels were independent of clinical markers such as INSS stage, *MYCN* status and histology. These findings are in accordance with what has been found for other tumor types. As was briefly mentioned above, it is generally understood that it is the expression of the truncated splice variant that is dominantly involved in cancer formation (20, 21). This finding strongly correlates with our own data published recently for the expression profile of the SP–TACR1 receptor system in hepatoblastoma, another aggressive childhood cancer (11, 12). Compared to normal liver tissue, we found that gene expression levels of fl-*TACR1* and tr-*TACR1* were both increased, whereas the level of tr-*TACR1* was significantly increased compared to fl-*TACR1* (p=0.0301) (11). Similar to the results presented here for NB, in hepatoblastoma, we found no correlation between the expression profile of *TACR1* and clinical behavior or disease stage. Yet TACR1 antagonists had a remarkable therapeutic effect on hepatoblastoma growth both *in vitro* and *in vivo*.

Our findings presented in this study are furthermore in accordance with those of other studies. Chen *et al*. examined expression of SP and *TACR1* in tissue microarrays of colorectal cancer and adjacent healthy tissue with immunohistochemistry. Expression of SP and *TACR1* were both significantly increased in samples of patients with lymph node metastasis; furthermore, high *TACR1* expression was also correlated with TNM stage III and IV. Besides these significant correlations, expression of SP and *TACR1* was not associated with clinical parameters such as age, sex, gender, distant metastasis and pathological grading (7).

Somewhat different results were found in a recent study in breast cancer (10). The different role of fl- and tr-*TACR1* in breast cancer cell lines and breast cancer tumor samples were investigated. The authors found high gene-expression levels of tr-*TACR1* in cells of advanced malignancy, whereas fl-*TACR1* demonstrated mainly high gene-expression levels in normal breast and mesenchymal cells; in breast cancer cells, gene expression of fl-*TACR1* was markedly reduced (10). Moreover, results suggest that fl-*TACR1* was inversely correlated with invasiveness, proliferation and metastasis, whereas high expression of tr-*TACR1* seemed to affect progressive disease and metastasis. Interestingly, we found similar results when investigating hepatoblastoma (12), however, as mentioned above, we did not find any correlation between disease stage or prognosis for the data presented here in NB nor for hepatoblastoma presented previously.

As all scientific studies, ours has several flaws, potentially biasing our data. Firstly, we only investigated gene-expression levels not protein-expression levels. Just as there are variations in the splice products prior to expression, there could be mRNA abrogation or other mechanisms that alter the expression profile that we did not account for. The solution to this would be immunohistochemical staining of our samples in order to correlate the protein-expression profile with the mRNA profile. Although such staining is generally feasible, it is increasingly challenging given the unavailability of appropriate antibodies against the tr-*TACR1* (20). Thirdly, NB is a very heterogeneous tumor. By taking one sample per tumor, the examined areas might not be representative of the whole specimen. Furthermore, we lacked a control group with healthy tissue to compare gene-expression levels simply due to the nature of the tumor type we investigated.

Nevertheless, despite these limitations, we feel that our findings support evidence that NB tissue, similarly to other cancer types, ubiquitously expresses *TACR1*. This understanding could hold tremendous clinical relevance and make *TACR1* an attractive therapeutic target for a large variety of clinical and biological NB subsets. Further research is needed to clarify the exact clinical and biological role of *TACR1* in the oncogenesis of childhood NB.

Acknowledgements

The Authors are grateful to Fatemeh Promoli for technical assistance.

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Received August 6, 2017
Revised September 15, 2017
Accepted September 19, 2017

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6. Acknowledgements

I would like to thank Prof. Dr. med. D. von Schweinitz for the possibility to realize this present thesis and research at his pediatric surgery department of the Ludwig-Maximilians-University Munich as well as for his immense expert's report on surgical matters.

My deepest thanks also go to Professor Dr. rer. nat. R. Kappler and Dr. A. Fröba-Pohl for their support and patience during conceptioning and realization of this project. Their advice has always been enriching and inspiring.

I would also like to thank my colleagues in the laboratory for their help, their teaching and a perfect atmosphere of professionality and friendship during the last years.

Last but not least, my greatest thanks to my parents and my fiancée, who always believed in me when I did not.

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