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Original Research

Catecholamine excretion profiles identify clinical subgroups of neuroblastoma patients

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Abstract ***Introduction:*** Analysis of urinary catecholamine metabolites is one of the primary modalities to diagnose patients with neuroblastoma. Although catecholamine excretion patterns have been recognised in the past, their biological rationale and clinical relevance remain largely unknown. Therefore, this study was designed to identify unique catecholamine excretion patterns and elucidate their underlying biology and clinical relevance.
Patients and methods: A panel of 25 neuroblastoma cell lines was screened for catecholamine excretion. Detection of the catecholamine enzymes was performed using Western blot. Based on catecholamine enzymes presence and excreted catecholamine metabolites, excretion profiles were defined. The prevalence of these profiles was investigated *in vivo* using diagnostic urines from 301 patients with neuroblastoma and immunohistochemistry on primary tumours. The clinical relevance of the profiles was determined by linking the profiles to clinical characteristics and outcome of patients with neuroblastoma.
Results: Four excretion profiles (A-D) were identified *in vitro*, which correlated with the relative protein expression of the catecholamine enzymes. These profiles were also identified in urine samples from patients with neuroblastoma and correlated with the presence of the catecholamine enzymes in the tumour. Strikingly, in 66% of the patients, homovanillic acid and vanillylmandelic acid excretions were discordant with the catecholamine profiles. Clinical

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characteristics and outcome gradually improved from patients with profile A (predominantly high risk) towards profile D (predominantly observation), with 5-years overall survival of 35% and 93%, respectively.
Conclusions: Catecholamine profiles *in vitro* and *in vivo* reflect, to a large extent, the presence of the individual catecholamine enzymes and represent distinct subgroups of patients with neuroblastoma.

1. Introduction

Neuroblastoma, a paediatric malignancy of the developing sympathetic nervous system [1], presents in 95% of the patients with elevated levels of catecholamine metabolites, which can be used for diagnostic purposes [2–5].

Based on their successive order in the biosynthetic route, catecholamine metabolites have been classified either as immature (dopamine [DA], 3-methoxytyramine [3MT] and homovanillic acid [HVA]) or mature catecholamine metabolites (norepinephrine [NE], normetanephrine [NMN] and vanillylmandelic acid [VMA]) [6–13]. The most mature catecholamine metabolites, epinephrine [E] and metanephrine [MN], are rarely elevated in urine of patients with neuroblastoma [5]. The excretion levels of individual catecholamine metabolites have been correlated with disease stage, *MYCN* amplification (MNA) and even outcome of patients with neuroblastoma [5–9,13,14]. Although previous studies described catecholamine excretion as patterns (e.g. the dopaminergic and adrenergic patterns) and correlated them with different neuroblastoma features such as stage and MNA [6,7,11,12], their biological rationale and clinical relevance remained largely unknown. Therefore, this study was designed to identify unique catecholamine excretion patterns, elucidate their underlying biology and assess their clinical relevance.

2. Materials and methods

2.1. Catecholamine metabolites

DA (56610), 3MT (M-4251), NE (A9512), NMN (N-7127), E (E-4642) and MN (M-8625) were purchased from Sigma (Zwijndrecht, the Netherlands) and were used to test metabolite stability under culture conditions.

2.2. Catecholamine metabolites extraction and detection in culture medium

Human neuroblastoma cell lines and PC12 cells were cultured as previously described [15]. After 48 h of culturing, the culture medium was collected, centrifuged to remove cell debris and acidified to pH 2–3 using hydrochloric acid. Extraction and analysis of catecholamine metabolites in culture medium are described in supplemental document 1. Analysis of HVA and VMA was not performed because of high endogenous HVA levels in serum and the necessity for additional liver-specific metabolic steps for the synthesis of VMA [16].

2.3. Western blot

Detection of the catecholamine enzymes: tyrosine hydroxylase [TH], dopa decarboxylase [DDC], dopamine β-hydroxylase [DBH], phenylethanolamine-N-methyltransferase [PNMT], catechol-O-methyltransferase [COMT] and monoamine oxidase A [MAOA] was performed by means of immunoblots as described previously [17] All primary antibodies and the immunoblot conditions are listed in Supplemental Table 1.

2.4. Patient cohort and urine analyses

This neuroblastoma cohort (n = 301) has been described previously [5]. Urine at the time of diagnosis was collected and analysed for VMA, HVA, DA, 3MT, NE, NMN, E and MN, as described previously [5]. This study was approved by the local medical–ethical committee (reference number: W16_093#16.112).

2.5. Immunohistochemistry

Standard immunohistochemistry (IHC) was performed on primary tumours as previously described [18]. Clinical description of the patients and tumours that were used for IHC is listed in Supplemental Table 2. The primary antibodies used were directed against TH (citrate pH 6.0, 1:100,000; Santa Cruz, 25269, with DAB+), DDC (citrate pH 6.0, 1:2000; Cell Signaling, 13561, with DAB+) and DBH (Tris–EDTA pH 9.0, 1:100; Cell Signaling, 8586, with DAB+).

2.6. Statistical analyses

All statistical analyses were performed using GraphPad Prism (GraphPad Software, version 7.03). Chi-square

test was used to assess whether the frequency of the clinical characteristics was significantly different between the profiles. Event-free survival (EFS) and overall survival (OS) were estimated by using Kaplan–Meier methodology in combination with log-rank test. P-value <0.05 was regarded as significant.

3. Results

3.1. Catecholamine excretion in vitro

In order to assess the stability of catecholamine metabolites, to allow measurement of catecholamine excretion *in vitro,* purified catecholamine metabolites (10 μM) were kept in culture medium (37 °C and pH 7.4). After 24 h, the concentrations of DA, NE and E were reduced by more than 90%, whereas the concentrations of 3MT, NMN and MN remained stable even after 48 h (Fig. 1A). Therefore, only 3MT, NMN and MN were analysed in subsequent *in vitro* experiments.

Analysis of culture media of 25 different neuroblastoma cell lines identified four different excretion profiles (Fig. 1B): in profile A, only 3MT was excreted (n = 4); in profile B, equal amounts of 3MT and NMN were present (n = 4); in profile C, the concentration of NMN was two-fold higher than that of 3MT (n = 1) and in profile D, no catecholamine metabolites excretion was detected (n = 16). MN was not excreted by any of the tested cell lines.

Normal catecholamine biosynthesis involves all four biosynthetic enzymes (TH, DDC, DBH and PNMT) and would result in excretion of 3MT, NMN and MN

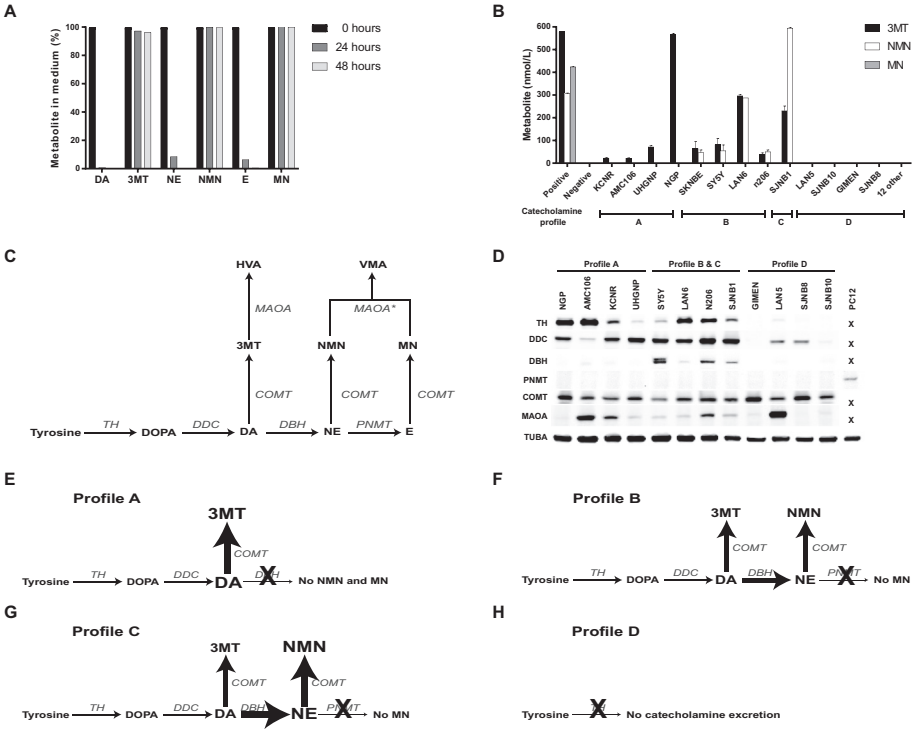

Fig. 1. Catecholamines profiles *in vitro*. (A) Stability of catecholamine metabolites under normal culture conditions (37°C and pH 7.4) in a plain culture medium was tested for a period of 0, 24 and 48 hours. (B) Screening for 3MT, NMN and MN excretion in culture medium of neuroblastoma cell line (n=25); the positive and negative controls were urine from a neuroblastoma patient and culture medium without cells, respectively. Cells were divided into catecholamine profiles based on their excretion: only 3MT = profile A (n=4), both 3MT and NMN = profile B (n=4), twice as much NMN compared with 3MT = profile C (n=1) and non-excreting = profile D (n=16). 12 other cell lines = LAN1, IMR32, NMB, SKNAS, SKNSH, SJNB6, SHEP, ACN, SJNB12, TR14, SKNFI, CHP134. (C) Simplified catecholamines metabolites biosynthesis and degradation under normal conditions. (D) Protein expression of the catecholamine enzymes TH, DDC, DBH, PNMT, COMT and MAOA. PC12, a rat pheochromocytoma cell line, was included as positive control for PNMT but was not tested for the remaining enzymes (x). (E) model for cell lines with profile A, DBH blockage. (F) Model for cell lines with profile B, PNMT blockage. (G) Model for cell lines with profile C, PNMT blockage with relatively more active DBH compared with profile B. (H) Model for cell lines with profile D, TH blockage. The X indicates which enzyme is affected and consequently causes the catecholamine profile. Relative pathway activity is indicated by arrows thickness and relative metabolite levels by letter size. DA = dopamine, 3MT = 3-methoxytyramine, NE = norepinephrine, NMN = normetanephrine, E = epinephrine, MN = metanephrine, HVA = homovanillic acid, VMA = vanillylmandelic acid, TH = tyrosine hydroxylase, DDC = dopa decarboxylase, DBH = dopamine β-hydroxylase, PNMT = phenylethanolamine-N-methyltransferase, COMT = catechol-O-methyltransferase, MAOA = monoamine oxidase A, TUBA = α-tubulin, * = metabolism toward VMA requires further enzymatic steps in the liver.

(Fig. 1C). To explain the *in vitro* observed profiles, protein expression levels of the different catecholamine enzymes were analysed in a panel of cell lines. In cell lines with profile A, TH and DDC protein expression but no or hardly any DBH protein expression was observed (Fig. 1D). TH, DDC and DBH protein expression was demonstrated in cell lines with profiles B and C (Fig. 1D). All cell lines with profile D lacked TH protein expression, with the exception of LAN5, which weakly expressed this enzyme (Fig. 1D). PNMT was not present in any neuroblastoma cell line but could be detected in PC12, a pheochromocytoma cell line (Fig. 1D). COMT was detected in all cell lines and most of them also expressed MAOA; however, MAOA expression level was variable among the cell lines (Fig. 1D).

Based on the *in vitro* catecholamine excretion and the presence of the biosynthetic enzymes, four profiles A-D were identified. Cell lines with profile A excreted 3MT and no NMN because of absent/very low DBH expression (Fig. 1E). Cell lines with profile B and C excreted both 3MT and NMN but not MN due to the absence of PNMT (Fig. 1F and G). However, in profile C, NMN levels were twice as high as 3MT levels, suggesting that DBH might be relatively more active in profile C cell lines compared to profile B cell lines (Fig. 1G). Finally, cell lines with profile D do not excrete catecholamine metabolites at all, because of absence/very low expression of TH protein (Fig. 1H). Taken together, it can be concluded that the catecholamine profiles, as observed *in vitro,* are the result of the relative expression of the biosynthetic catecholamine enzymes.

3.2. Catecholamine excretion profiles in vivo

Diagnostic urines from 301 patients with neuroblastoma were classified according to the *in vitro* established profiles. Patients with profile A had more than 10-fold elevation of DA and 3MT, whereas NMN was negligibly elevated (n = 26, Fig. 2A). Patients with profile B had more than 10-fold elevation of DA and NMN, which was accompanied by 8-fold elevation of 3MT (n = 135, Fig. 2B). Profile C was characterised by more than 20-fold elevation of NMN accompanied by 3-fold elevation of DA, 3MT and NE (n = 124, Fig. 2C). Finally, profile D had no elevation of any of the six catecholamine metabolites (n = 16, Fig. 2D).

As mentioned previously, measurements of HVA and VMA *in vitro* could not be performed; however,

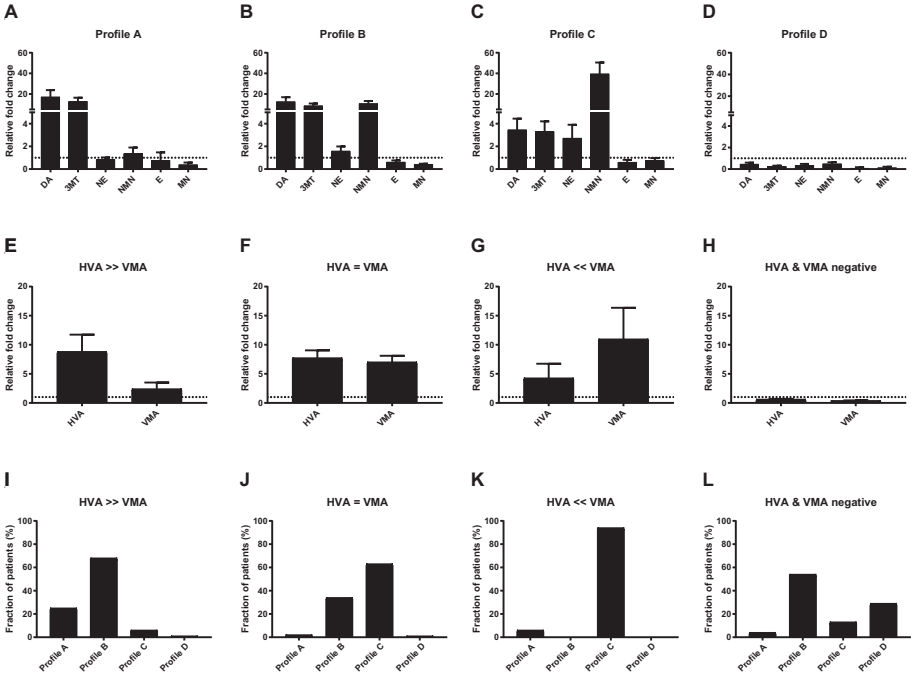

Fig. 2. Urinary catecholamines profiles in patients with neuroblastoma. Patients with neuroblastoma of all stages (n = 301) were divided as per the four *in vitro* identified catecholamine profiles (profiles A-D). (A) = profile A (n = 26), (B) profile B (n = 135), (C) profile C (n = 124), (D) profile D (n = 16). Based on the *in vitro* established catecholamine profiles, corresponding HVA-VMA groups were defined. (E) VMA/HVA < 0.5 (n = 81), (F) 0.5 ≤ VMA/HVA ≤ 2 (n = 156), (G) VMA/HVA > 2 (n = 16), (H) negative for both HVA and VMA (n = 48). Bars depict the average fold-change per metabolite with its 95% confidence interval. The dashed line indicates a fold-change of 1 and every value above it was regarded as elevated. (I)–(L) Concordance between the HVA-VMA groups and the catecholamine profiles. DA = dopamine, 3MT = 3-methoxytyramine, NE = norepinephrine, NMN = normetanephrine, E = epinephrine, MN = metanephrine, HVA = homovanillic acid, VMA = vanillylmandelic acid.

in vivo catecholamine metabolites can be converted to HVA and VMA. HVA and VMA are routinely measured in urine of patients with neuroblastoma, and the VMA/HVA ratio <0.5 has been linked to neuroblastoma biology including disease stage and *MYCN* status [5,19]. For this reason, four additional groups were defined, according to the VMA/HVA ratio, reflecting the four catecholamine profiles described above. Profile A corresponds with VMA/HVA<0.5 (n = 81, Fig. 2E), profile B with 0.5 ≤ VMA/HVA≤2 (n = 156, Fig. 2F), profile C with VMA/HVA>2 (n = 16, Fig. 2G) and profile D with HVA and VMA negative (n = 48, Fig. 2H). Subsequently, concordance between the HVA-VMA groups and profiles A-D was also studied. The majority of the patients had discordance between their HVA-VMA groups and their biochemically corresponding catecholamine profile (Fig. 2I,J,L), with exception of patients with VMA/HVA>2 (Fig. 2K). The discordance is best illustrated in the HVA and VMA negative group, as 71% of these patients clearly excrete catecholamines (profile A-C, Fig. 2L), suggesting that HVA and VMA, even though they are the distal end-metabolites, they do not necessarily reflect the levels of the proximal catecholamine metabolites. Therefore, the HVA-VMA groups were not discussed further in this study.

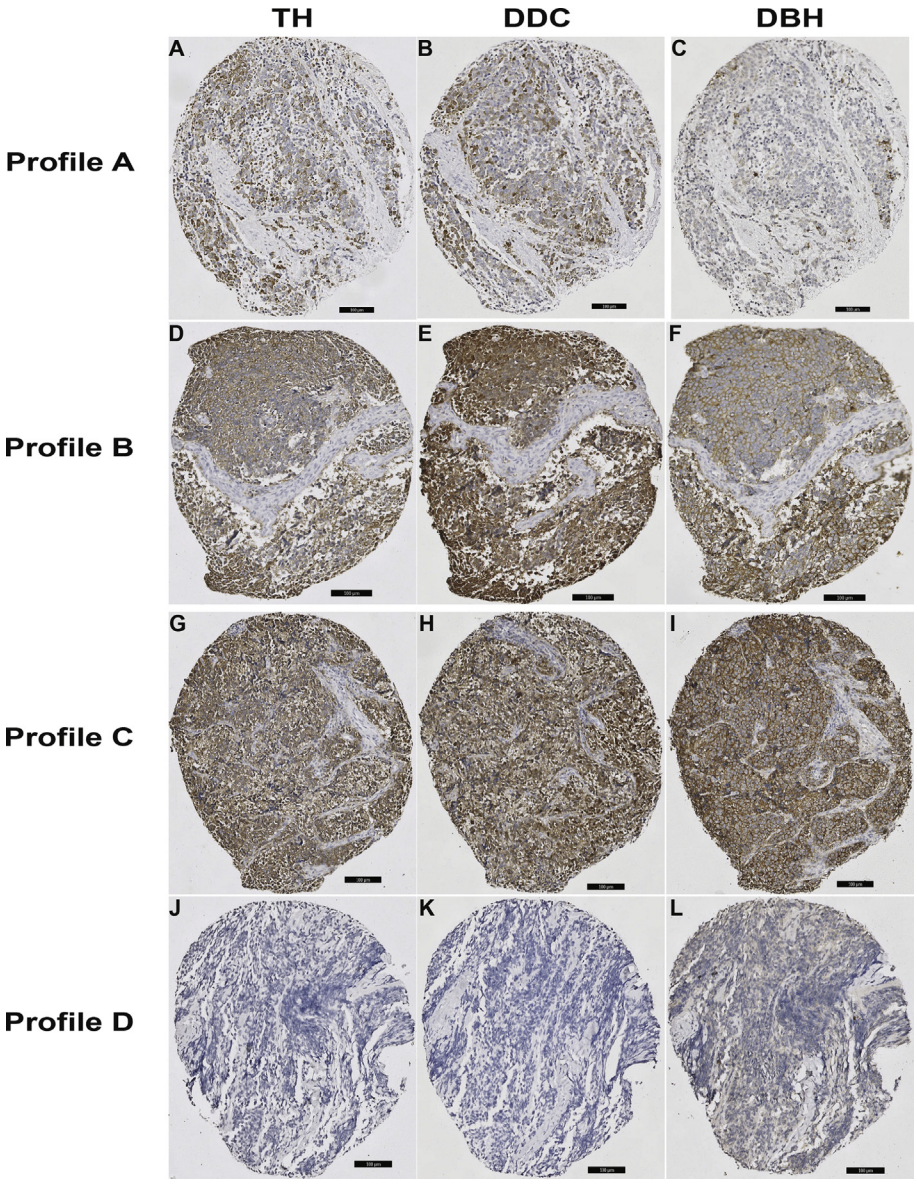

Fig. 3. Immunohistochemistry and catecholamine profiles. Catecholamine biosynthetic enzymes were stained in primary tumours from patients with neuroblastoma with profile A (A)–(C), profile B (D)–(F), profile C (G)–(I) and profile D (J-L). The complete cohort (n=29) and the score per staining are listed in Table 1. Photos were obtained at magnification of 10x, and the black bar indicates 100 μm length. TH = tyrosine hydroxylase, DDC = dopa decarboxylase, DBH = dopamine β-hydroxylase.

Because *in vitro* the profiles were a reflection of the relative presence of the catecholamines biosynthetic enzymes, we wondered whether urinary catecholamine profiles reflect the intratumoural relative presence of the catecholamines biosynthetic enzymes. IHC for TH, DDC and DBH on primary tumours showed that two of the three tumours from patients with profile A were positive for both TH and DDC while being almost negative for DBH (Fig. 3A–C, Table 1). The third patient with profile A was negative for TH and DDC while being weakly positive for DBH (Table 1). All tumours from patients with profile B (n = 12) and profile C (n = 10) were strongly positive for TH, DDC and DBH (Fig. 3D–I, respectively). All tumours from patients with profile D (n = 4) were negative for all three enzymes (Fig. 3J–L, Table 1). These results show that the urinary catecholamine profiles also reflect the presence of the catecholamine enzymes in the tumour.

3.3. Catecholamine excretion profiles represent different clinical subgroups

To investigate whether the four catecholamine profiles represent different clinical subgroups, the clinical characteristics of each profile were examined. Patients with profile A presented predominantly (>70%) with stage 4 disease, older age (>18 months) and their tumours usually (>60%) had genetic aberration such as MNA and LOH1p (Fig. 4A–D). Compared to profile A, profile B contained less patients with stage 4 disease (63%), more patients with age <18 months and much less (30%) patients with MNA and LOH1p (Fig. 4A–D). Patients with profile C had in 49% of the cases stage 4 disease, but the majority were younger than 18 months and only a few (<20%) had MNA and LOH1p (Fig. 4A–D). Finally, patients with profile D had the complete opposite characteristics of patients with profile A, since the majority of them had low stage disease (75%), were younger than 18 months and rarely (10%) had aberration such as MNA and LOH1p (Fig. 4A–D). Ganglioneuroblastoma was significantly ($p < 0.001$) more prevalent in patients with profile D compared to the other profiles (Fig. 4E), while ^{123}I-metaiodobenzylguanidine (MIBG) non-avid tumours where also more prevalent among patients with profile D, but not significantly more compared to patients with profile B (Fig. 4F). Primary tumours arising in the adrenal gland and thoracic cavity were more prevalent in profile A and profile D, respectively (Fig. 4G). Finally, when the profiles were stratified based on risk groups, profile A consisted predominantly of high-risk patients (81%), whereas profile D comprised mainly of observation patients (81%, Fig. 4H).

Since the profiles defined different clinical subgroups, we wondered whether these profiles were also associated with different clinical outcome. Patients with profile A had a dismal prognosis with 5-years event-free survival (EFS) and overall survival (OS) of 31% and 35%, respectively (Fig. 5A and B). On the other hand, patients with profile D had excellent outcome (5-years EFS and OS of 88% and 93%, respectively, Fig. 5A-B). Patients with profile B and C had similar 5-years EFS (53% and 60%, respectively, Fig. 5A); however, the 5-years OS of patients with profile B was significantly worse than that of patients with profile C (62% and 74%, respectively, p = 0.018, Fig. 5B). Because we previously described elevation of 3MT (>2.9-fold) as an independent risk factor for poor outcome in neuroblastoma [14], we also investigated the combination of the latter with the four profiles. Patients with >2.9-fold elevation of 3MT had a poor outcome regardless of their profiles (Fig. 5C and D). The remaining patients, with the exception of patients with profile A (n = 4, 5-years OS = 50%), had a 5-years OS >80% (Fig. 5C and D). Thus, the profiles represent clinical subgroups with unique clinical characteristics and outcome, but the elevation of 3MT above the prognostic cut-off appeared to be more relevant for clinical outcome.

Table 1
Immunohistochemistry scores in primary tumours from patients with different catecholamine profiles.

		TH		DDC		DBH	
	Patient	Fraction	Score	Fraction	Score	Fraction	Score
Profile A	246	90%	+++	60%	++	<5%	+
	430	NR	–	NR	–	80%	+
	555	90%	++	50%	++	<5%	+
Profile B	62	>95%	++	>95%	+++	>95%	+++
	166	>95%	+++	>95%	+++	>95%	+++
	168	90%	++	70%	+	90%	++
	179	>95%	+++	>95%	+++	>95%	+++
	203	70%	++	>95%	+++	>95%	+++
	417	50%	+++	50%	++	50%	+++
	425	>95%	+++	>95%	+++	>95%	+++
	469	>95%	+++	>95%	+++	>95%	+++
	482	>95%	++	>95%	+++	>95%	+++
	489	>95%	++	>95%	+++	>95%	++
	490	>95%	+++	>95%	+++	>95%	+++
	509	>95%	++	>95%	+++	>95%	+++
Profile C	82	80%	++	>95%	+++	>95%	+++
	127	>95%	+++	>95%	+++	>95%	+++
	170	>95%	++	>95%	+++	>95%	+++
	226	>95%	+++	>95%	+++	>95%	+++
	406	80%	++	>95%	+++	>95%	+++
	409	>95%	++	>95%	+++	>95%	+++
	444	50%	+	>95%	+++	>95%	+++
	457	>95%	+++	>95%	+++	>95%	+++
	527	>95%	+++	>95%	+++	>95%	+++
	545	80%	+	>95%	+++	>95%	+++
Profile D	540	NR	–	NR	–	NR	–
	590	NR	–	NR	–	NR	–
	646	NR	–	NR	–	NR	–
	801	NR	–	NR	–	NR	–

Tumours from patients with profile A (n = 3), profile B (n = 12), profile C (n = 10) and profile D (n = 4) were stained for tyrosine hydroxylase (TH), dopa decarboxylase (DDC) and dopamine β-hydroxylase (DBH). Staining was negative (–), weak (+), moderate (++) or strong (+++). Fraction indicates the part of the tumour that was positive for the staining. In case the tumour was negative for the staining, the positive fraction was not relevant (NR).

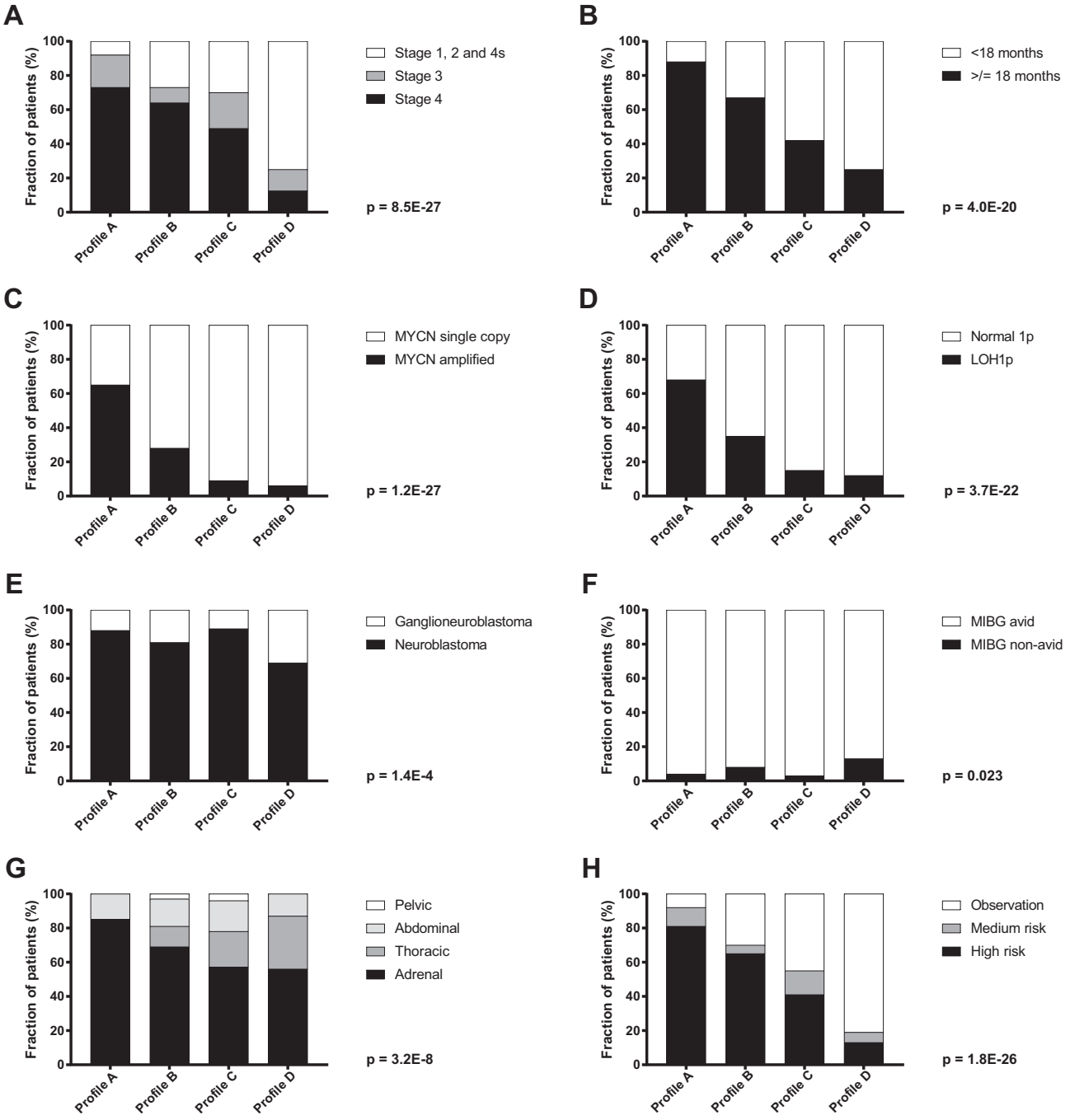

Fig. 4. Catecholamine profiles and their relation to clinical characteristics of patients with neuroblastoma. The frequency of the different INSS stages (A, n = 301), age at diagnosis (B, n = 301), *MYCN* status (C, n = 291), chromosome 1p (D, n = 257), histopathology (E, n = 301), ^{123}I-metaiodobenzylguanidine (MIBG) avidity (F, n = 298), location of the primary tumour (G, n = 298) and risk group as per GPOH NBL2004/DCOG2009 (H, n = 301) were analysed for every profile separately. Missing data were omitted from the analyses. Chi-square test was used to assess whether the frequencies of the clinical characteristics was significantly different between the profiles. LOH1p = loss of heterozygosity for 1p.

4. Discussion

In this study, we identified four different catecholamine excretion profiles *in vitro*, explained their biochemical rationale and confirmed their existence in patients with neuroblastoma. Furthermore, we showed that each profile represents a different subgroup of patients with neuroblastoma that has its own unique clinical characteristics and outcome.

Previously, dopaminergic and adrenergic excretion patterns have been recognised, and it was suggested that the former might be caused by a DBH deficiency in the

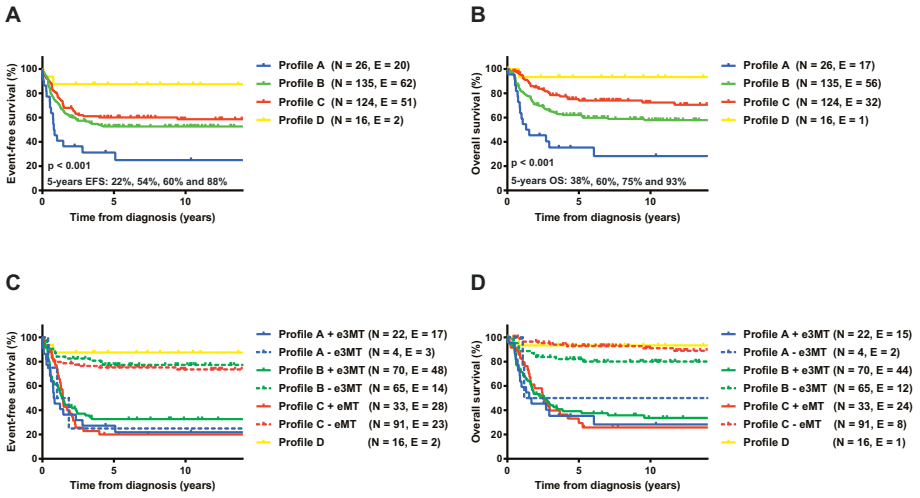

Fig. 5. Catecholamine profiles and clinical outcome. Using a cohort of 301 confirmed patients with neuroblastoma, event-free survival (EFS) and overall survival (OS) were calculated for every catecholamine profile (A and B, respectively). EFS and OS (C and D, respectively) were also calculated for the combination of the profiles with (+, continuous lines) and without (-, dashed lines) 3-methoxytyramine elevation (e3MT) above the prognostic cut-off (14). N = number of patients in the group, E = number of event/deaths in the group.

tumour [6,7,12,13,20]. In our study, we identified profile A and profiles B-C, which are comparable with the previously described dopaminergic and adrenergic pattern, respectively. Furthermore, based on our *in vitro* and *in vivo* data, we showed that profile A was generally associated with lack of DBH expression, whereas profile B-C always expressed DBH, supporting the DBH deficiency hypothesis. In line with previous suggestions [21,22], we showed here that neuroblastoma cell lines do not express PNMT and consequently do not excrete E and MN. Finally, we showed both in cell lines and in patients that absence of catecholamine excretion (profile D) was caused by lack of TH expression in the tumour cells. In contrast to the situation *in vivo*, profile D was the most common profile *in vitro* (64%). This might partly be explained by that fact that cell lines can lose TH expression under culture conditions, but regained TH expression in xenografts [23,24]. Interestingly, profile D was only observed in 29% of HVA and VMA negative patients, suggesting that one of the final conversion steps (e.g. oxidation by MAOA) is not occurring. This hypothesis was suggested in previous studies [6,7,25] and is supported by the fact that cell lines, such as NGP, excrete 3MT but had very low MAOA expression, which could prevent the final conversion of 3MT to HVA. Whether or not a low tumoural MAOA activity is present in HVA and VMA negative patients remains to be established.

The dopaminergic pattern was frequently seen in patients with stage 4 disease and patients with MNA [6,7,11] and was also associated with poor outcome [10]. In our study, patients with profile A had similar clinical characteristics and poor outcome, strengthening the possibility that profile A might represent the previously described dopaminergic pattern. Furthermore, in this study, we also described three additional profiles, each with its own unique clinical characteristics and outcome, which tend to improve from mainly high risk (profile A) towards mainly observation patients (profile D). However, differences in outcome between patients with profiles A-D are probably the reflection of the fraction of patients (85%, 52%, 26% and 0%, respectively) with 3MT levels above the prognostic cut-off [14]. Therefore, concerning clinical outcome, elevated 3MT proved to be more relevant for future risk assessment, as demonstrated previously [14]. Interestingly, patients with profile D represent a group of patients with excellent outcome, even though their tumour might be less differentiated since it lacks TH [26]. Furlan *et al.* showed that normal neural crest cells increase their TH expression during differentiation [27], which might suggest that neuroblastoma in patients with profile D arises from an earlier development stage compared to neuroblastoma in patients with one of the other three profiles. However, because histopathology was not studied in-depth in this study, further studies are required to test this hypothesis.

Taken together, catecholamine excretion profiles reflect catecholamine biochemistry in neuroblastoma. These profiles can be linked to prognostic relevant factors such as stage and MNA; however, their correlation to histopathology remains understudied. Therefore, further studies integrating catecholamine profiles and histopathology are warranted.

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Conflict of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.01.014.

References

[1] Park JR, Eggert A, Caron H. Neuroblastoma: biology, prognosis, and treatment. Hematol Oncol Clin N Am 2010;24(1):65–86.

[2] Hoehner JC, Gestblom C, Hedborg F, Sandstedt B, Olsen L, Pahlman S. A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. Lab Invest 1996;75(5):659–75.

[3] LaBrosse EH, Comoy E, Bohuon C, Zucker JM, Schweisguth O. Catecholamine metabolism in neuroblastoma. J Natl Cancer Inst 1976;57(3):633–8.

[4] Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993;11(8):1466–77.

[5] Verly IR, van Kuilenburg AB, Abeling NG, Goorden SM, Fiocco M, Vaz FM, et al. Catecholamines profiles at diagnosis: increased diagnostic sensitivity and correlation with biological and clinical features in neuroblastoma patients. Eur J Cancer 2017;72:235–43.

[6] Laug WE, Siegel SE, Shaw KN, Landing B, Baptista J, Gutenstein M. Initial urinary catecholamine metabolite concentrations and prognosis in neuroblastoma. Pediatrics 1978;62(1):77–83.

[7] Zambrano E, Reyes-Mugica M. Hormonal activity may predict aggressive behavior in neuroblastoma. Pediatr Dev Pathol 2002;5(2):190–9.

[8] Abeling NG, van Gennip AH, Overmars H, Voute PA. Simultaneous determination of catecholamines and metanephrines in urine by HPLC with fluorometric detection. Clin Chim Acta 1984;137(2):211–26.

[9] Izbicki T, Bozek J, Perek D, Wozniak W. Urinary dopamine/noradrenaline and dopamine/vanillylmandelic acid ratios as a reflection of different biology of adrenergic clones in children's neuroblastic tumors. J Pediatr Surg 1991;26(10):1230–4.

[10] Nakagawara A, Ikeda K, Tasaka H. Dopaminergic neuroblastoma as a poor prognostic subgroup. J Pediatr Surg 1988;23(4):346–9.

[11] Nakagawara A, Ikeda K, Higashi K, Sasazuki T. Inverse correlation between N-myc amplification and catecholamine metabolism in children with advanced neuroblastoma. Surgery 1990;107(1):43–9.

[12] Abeling NG, van Gennip AH, Overmars H, Voute PA. Biochemical monitoring of children with neuroblastoma. Radiother Oncol 1986;7(1):27–35.

[13] Izbicki T, Izbicka E, Mazur J. Prognostic significance of biochemical heterogeneity of catecholaminergic clones in neuroblastoma. J Pediatr Surg 2006;41(9):1506–12.

[14] Verly IRN, van Kuilenburg ABP, Abeling N, Goorden SMI, Fiocco M, Vaz FM, et al. 3-Methoxytyramine: an independent prognostic biomarker that associates with high-risk disease and poor clinical outcome in neuroblastoma patients. Eur J Cancer 2018;90:102–10.

[15] Cuperus R, Leen R, Tytgat GA, Caron HN, van Kuilenburg AB. Fenretinide induces mitochondrial ROS and inhibits the mitochondrial respiratory chain in neuroblastoma. Cell Mol Life Sci 2010;67(5):807–16.

[16] Blombery PA, Kopin IJ, Gordon EK, Markey SP, Ebert MH. Conversion of MHPG to vanillylmandelic acid. Implications for the importance of urinary MHPG. Arch Gen Psychiatry 1980;37(10):1095–8.

[17] van Kuilenburg AB, Meijer J, Maurer D, Dobritzsch D, Meinsma R, Los M, et al. Severe fluoropyrimidine toxicity due to novel and rare DPYD missense mutations, deletion and genomic amplification affecting DPD activity and mRNA splicing. Biochim Biophys Acta 2017;1863(3):721–30.

[18] van Groningen T, Koster J, Valentijn LJ, Zwijnenburg DA, Akogul N, Hasselt NE, et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. Nat Genet 2017;49(8):1261–6.

[19] Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3(3):203–16.

[20] Candito M, Thyss A, Albertini M, Deville A, Politano S, Mariani R, et al. Methylated catecholamine metabolites for diagnosis of neuroblastoma. Med Pediatr Oncol 1992;20(3):215–20.

[21] Franscini LC, Vazquez-Montes M, Buclin T, Perera R, Dunand M, Grouzmann E, et al. Pediatric reference intervals for plasma free and total metanephrines established with a parametric approach: relevance to the diagnosis of neuroblastoma. Pediatr Blood Cancer 2015;62(4):587–93.

[22] Grouzmann E, Lamine F. Determination of catecholamines in plasma and urine. Best Pract Res Clin Endocrinol Metabol 2013;27(5):713–23.

[23] Reynolds CP, Biedler JL, Spengler BA, Reynolds DA, Ross RA, Frenkel EP, et al. Characterization of human neuroblastoma cell lines established before and after therapy. J Natl Cancer Inst 1986;76(3):375–87.

[24] Tomayko MM, Triche TJ, Reynolds CP. Human neuroblastoma cell lines regain catecholamine fluorescence when xenografted into athymic (nude) mice. Int J Dev Neurosci 1996;14(6):771–7.

[25] Graham-Pole J, Salmi T, Anton AH, Abramowsky C, Gross S. Tumor and urine catecholamines (CATs) in neurogenic tumors. Correlations with other prognostic factors and survival. Cancer 1983;51(5):834–9.

[26] Bourdeaut F, Janoueix-Lerosey I, Lucchesi C, Paris R, Ribeiro A, de Pontual L, et al. Cholinergic switch associated with morphological differentiation in neuroblastoma. J Pathol 2009;219(4):463–72.

[27] Furlan A, Dyachuk V, Kastriti ME, Calvo-Enrique L, Abdo H, Hadjab S, et al. Multipotent peripheral glial cells generate neuroendocrine cells of the adrenal medulla. Science 2017;357(6346).