Predicting outcomes for children with neuroblastoma using a multigene-expression signature: a retrospective SIOPEN/COG/GPOH study

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Summary

Background More accurate prognostic assessment of patients with neuroblastoma is required to better inform the choice of risk-related therapy. The aim of this study is to develop and validate a gene-expression signature to improve outcome prediction.

Methods 59 genes were selected using an innovative data-mining strategy, and were profiled in the largest neuroblastoma patient series (n=579) to date using real-time quantitative PCR starting from only 20 ng of RNA. A multigene-expression signature was built using 30 training samples, tested on 313 test samples, and subsequently validated in a blind study on an independent set of 236 tumours.

Findings The signature has a performance, sensitivity, and specificity of 85·4% (95% CI 77·7–93·2), 84·4% (66·5–94·1), and 86·5% (81·1–90·6), respectively, to predict patient outcome. Multivariate analysis indicates that the signature is a significant independent predictor of overall survival and progression-free survival after controlling for currently used risk factors: patients with high molecular risk have a higher risk of death from disease and higher risk of relapse or progression than patients with low molecular risk (odds ratio 19·32 [95% CI 6·50–57·43] and 3·96 [1·97–7·97] for overall survival and progression-free survival, respectively, both p<0·0001). Patients at an increased risk of an adverse outcome can also be identified in the current treatment groups, showing the potential of this signature for improved clinical management. These results were confirmed in the validation study, in which the signature was also independently statistically significant in a model adjusted for *MYCN* status, age, International Neuroblastoma Staging System stage, ploidy, International Neuroblastoma Pathology Classification grade of differentiation, and mitosis karyorrhexis index (odds ratios between 4·81 and 10·53 depending on the model for overall survival and 3·68 [95% CI 2·01–6·71] for progression-free survival).

Interpretation The 59-gene expression signature is an accurate predictor of outcome in patients with neuroblastoma. The signature is an independent risk predictor, identifying patients with an increased risk of poor outcome in the current clinical-risk groups. The method and signature is suitable for routine laboratory testing, and should be evaluated in prospective studies.

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Introduction

Few tumours have engendered as much fascination and frustration for clinicians and scientists as neuroblastoma. This tumour is one of the most frequent solid malignancies in children and, by contrast with many other paediatric malignancies, is fatal in almost half of the patients diagnosed, despite advances in multimodal anticancer therapies. Current therapeutic stratification of patients with neuroblastoma is based on risk estimation according to combinations of age, tumour stage, *MYCN* status, DNA ploidy status, and histopathology.[1] Clinical experience with this system suggests that the stratification of patients for treatment is useful, but patients with the same clinicopathological parameters, receiving the same treatment, can have markedly different clinical courses. As a consequence, patients with an intrinsic poor prognosis but who are classified as low risk or intermediate risk on the basis of the current stratification system, will receive inappropriately mild treatment, and this could lead to a

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loss of valuable time before starting the required, more intensive treatment. Alternatively, patients with an intrinsic good prognosis but recognised as high risk with the current system of stratification will undergo a toxic therapy, putting them at an unnecessary risk of long-term side-effects. Additionally, survival rates remain disappointingly low in the current high risk treatment group. Therefore, the challenge remains to identify additional tumour-specific prognostic markers for improved risk estimation at the time of diagnosis. Only then can patients receive the most appropriate therapy, be monitored more intensively if appropriate, and become eligible for new experimental therapies.

To emulate the successful identification of gene-expression signatures for other tumour types,[2–5] we sought to develop, validate, and implement a robust multigene-expression signature to accurately assess prognosis in children with neuroblastoma. By contrast with gene-expression studies in neuroblastoma published previously, we aimed for a high patient–gene ratio, testing a carefully selected small number of genes on a large panel of tumour samples. We further validated the signature in an independent set of tumours.

Methods

Patients

The initial cohort consisted of 343 patients with neuroblastoma taken from the International Society of Pediatric Oncology, European Neuroblastoma Group (SIOPEN) and from the Gesellschaft fuer Paediatrische Onkologie und Haematologie (GPOH). Patients were only included if primary untreated neuroblastoma tumour RNA (at least 60% tumour cells and confirmed histological diagnosis of neuroblastoma) was available and of sufficient quality. Almost all patients (324; 95%) were treated according to the SIOPEN protocols: HR-NBL1 (High-Risk Neuroblastoma Study, n=66); INES[14,15] (Infant Neuroblastoma European Study, n=177, consisting of five different protocols: NB99·study [resectable tumours], NB99·1 [unresectable tumours], NB99·2 [stage 4S and stage 4, no bone, lung, pleura, or CNS], NB99·3 [stage 4 with bone, pleura, lung, or CNS involvement], NB99·4 [stage 2, 3, 4, and 4S *MYCN* amplified tumours]); EUNB[16] (European Unresectable Neuroblastoma Study, n=22); or LNESG1[17] (Localized Neuroblastoma European Study Group, n=59). 33 patients from the GPOH with localised tumour treated with surgery alone were also included in the study, and were included in the LNESG1 group if older than 12 months of age at diagnosis, and in the INES group (NB99·study) if younger than 12 months of age. The remainder (n=19) were treated according to protocols similar to the above mentioned SIOPEN protocols. The median follow-up was 63 months (range 1–180 months), and was greater than 24 months for most patients (91%). At the time of analysis, 290 of 343 patients were alive (webappendix).

The validation cohort consisted of 236 patients from the Children's Oncology Group (COG-United States): 67 patients classed as low risk, 56 classed as intermediate risk, and 113 classed as high risk, with at least 24 months of follow-up for patients without an event. Treatments were unknown for most of the COG patients, since 76 (32%) of patients enrolled on a non-therapeutic or biology COG study to have their risk biomarkers determined and tumour banked. The rest of the COG patients were enrolled on 13 different therapeutic studies, and received an array of treatments depending on the COG protocol and based on their risk over that period. All laboratory analyses were performed blinded to clinical and outcome data. All patients provided consent and were enrolled on at least one COG study, and all participating institutions had institutional review board approval to take part in the COG studies (webappendix). This study was approved by the Ghent University Hospital Ethical Committee.

Procedures

We used a unique data-mining strategy to re-analyse seven published microarray gene-expression studies[6–12] containing data from nearly 690 patients with neuroblastoma to identify genes that correlate with patient outcome (webappendix). Briefly, all probes and clinical patient information were updated before re-analysis, and a uniform risk definition was applied to select training patients across the different studies. Whereas in each of the published studies a different data-mining method was used, an important step in this procedure was the use of a uniform method—prediction analysis of microarrays[13]—to generate seven new prognostic gene sets.

	p value	Odds ratio (95% CI)
Backward-selected logistic regression model for progression-free survival: entire cohort (n=257)*		
Expression signature (high risk *vs* low risk)	<0·00012	3·96 (1·97–7·97)
INSS stage (stage 4 *vs* not stage 4)	<0·00049	3·76 (1·79–7·92)
Backward-selected logistic regression model for overall survival: entire cohort (n=254)*		
Expression signature (high risk *vs* low risk)	<0·0001	19·32 (6·50–57·43)
INSS stage (stage 4 *vs* not stage 4)	0·0016	4·84 (1·82–12·91)
Backward-selected logistic regression model for progression-free survival: high-risk protocol (n=52)*		
Expression signature (high risk *vs* low-risk)	NS	NA
INSS stage (stage 4 *vs* not stage 4)	0·019	5·06 (1·30–19·72)
Backward-selected logistic regression model for overall survival: high-risk protocol (n=52)*		
Expression signature (high risk *vs* low risk)	0·0080	9·20 (1·80–47·06)
Backward-selected logistic regression model for progression-free survival: INES protocol (n=122)*		
Expression signature (high risk *vs* low risk)	0·0080	5·30 (1·54–18·23)
Backward-selected logistic regression model for overall survival: INES protocol (n=120)*		
Expression signature (high risk *vs* low risk)	0·045	7·00 (1·04–46·95)

NS=not significant. NA=not analysed. *Also tested in the model and removed for non-significance during the backward selection were age, *MYCN* status, and INSS stage.

Table 1: **Multivariate logistic regression analysis in the SIOPEN/GPOH cohort**

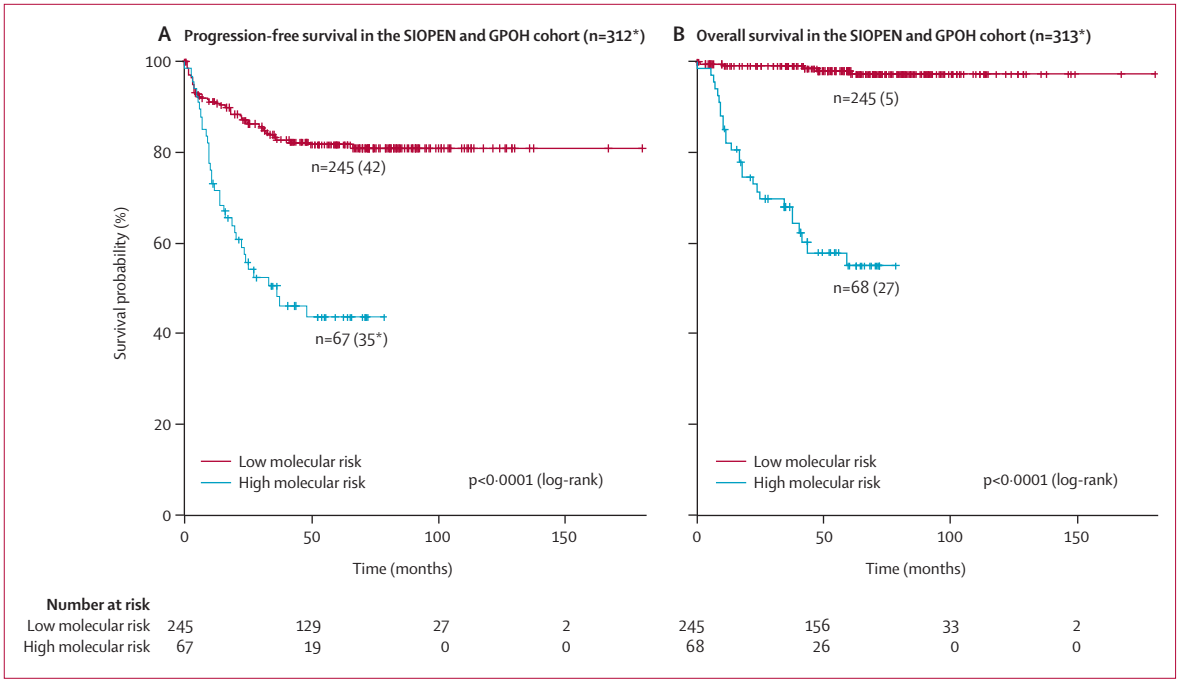

Figure 1: **Kaplan–Meier and log-rank analysis for progression-free (A) and overall (B) survival of the entire SIOPEN and GPOH cohort**
Number of patients at low and high molecular risk as predicted by the 59-gene signature. Numbers in parentheses refer to number of patients who experienced an event. *Missing relapse date for one high-molecular-risk case.

We extended this study by extensive screening of previously published reports for single candidate prognostic genes (793 abstracts). We composed a list of 59 prognostic genes that were independently identified in at least two of the seven prognostic gene sets or list of genes published previously (webappendix).

Total RNA extraction from primary neuroblastoma tumour samples was done in the individual collaborating laboratories using three different methods, and starting from 20 ng of total RNA, a sample pre-amplification method was applied (WT-Ovation; NuGEN, Bemmel, Netherlands) (webappendix). Based on the assessment of RNA purity and integrity as described in the webappendix, about 80% of the samples were of an acceptable quality and were retained[18] (RNA Quality Index ≥5 as determined by Experion [software version 3.0; Bio-Rad, Nazareth Eke, Belgium] and the absence of enzymatic inhibitors[19]).

A real-time quantitative PCR (qPCR) assay was designed for each of the 59 prognostic genes and five reference genes by PrimerDesign and validated through an extensive in-silico analysis pipeline.[20] PCR plates were prepared using a 96-well head pipetting robot (Sciclone ALH3000; Calliper Life Sciences, Teralfene, Belgium), qPCR was done on a high-throughput 384-well plate instrument (LC480; Roche Diagnostics, Vilvoorde, Belgium), and data analysed with qBasePlus software version 1.1. To detect and correct for inter-run variation and enable data to be compared with other laboratories, we used external standards (Biolegio; Nijmegen, Netherlands) run in parallel with patient samples. Further details on gene-expression analysis and on data pre-processing are available in the webappendix.

Statistical analysis

The multigene-expression signature was built using 30 training samples randomly selected from the SIOPEN cohort, tested on the remaining SIOPEN and GPOH samples, and validated in a blind manner using COG samples (webappendix).

For the SIOPEN and GPOH cohort, the R-language for statistical computing (version 2.6.2) was used to train and test the prognostic signature, to evaluate its performance by receiver operating characteristic (ROC) curve and area under the curve (AUC) analyses, and for Kaplan–Meier survival analyses. Multivariate logistic regression analyses were done using SPSS (version 16). Currently used risk factors such as age at diagnosis (≥12 months *vs* <12 months), International Neuroblastoma Staging System (INSS) stage (stage 4 *vs* not stage 4), and *MYCN* status (amplified *vs* not amplified) were tested, and variables with p values less than 0·05 were retained in the model. Since an interaction between the signature and risk factors was not expected to occur, interaction terms were not included in the models. For ROC and multivariate analyses, only patients with an event and patients with sufficient follow-up time (≥36 months) if no event occurred were included, since 95% of events in neuroblastoma are expected to occur within the first 36 months after diagnosis.

For the **qBasePlus software** see http://www.qbaseplus.com

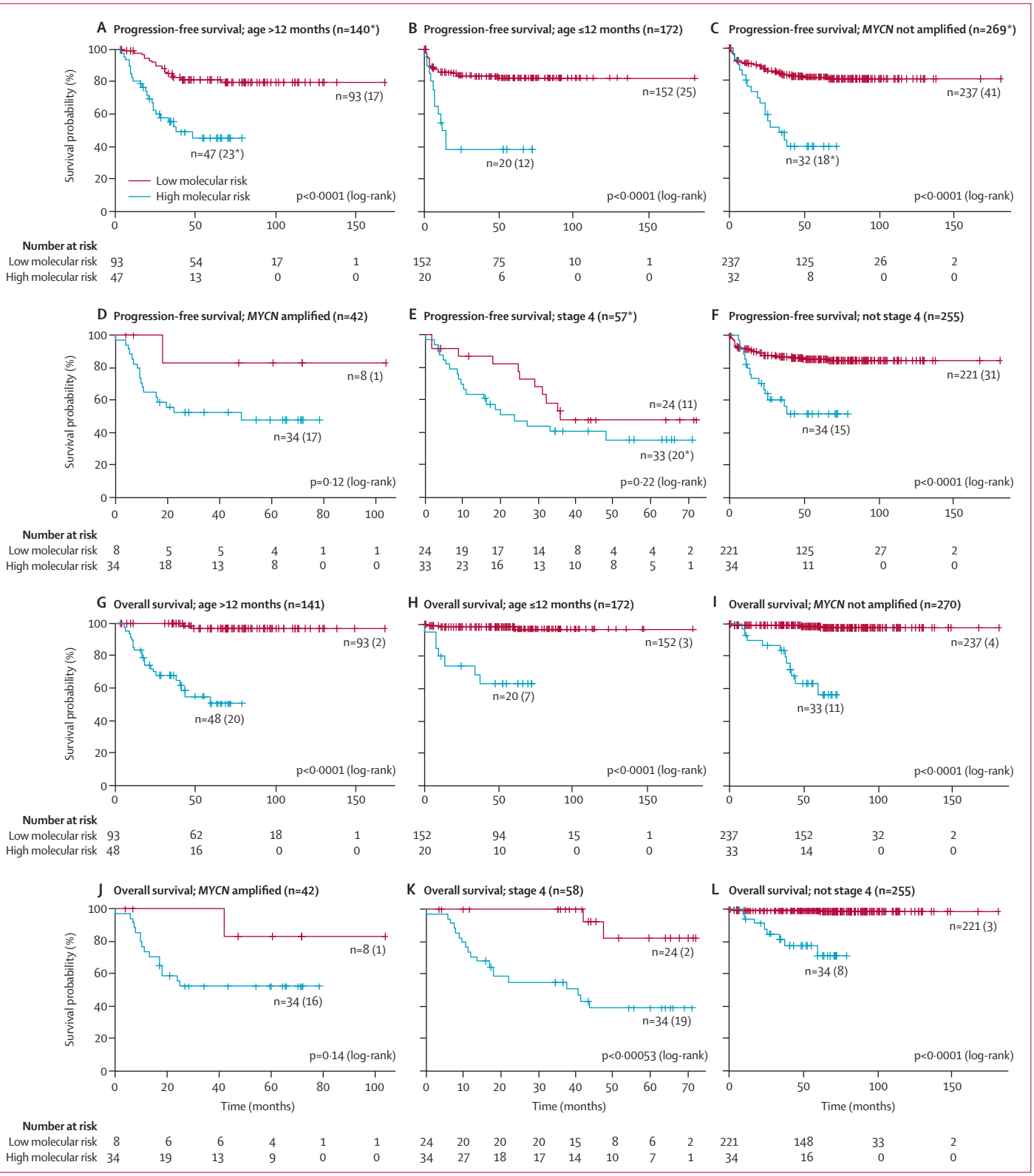

Figure 2: **Kaplan–Meier and log-rank analysis for progression-free (A–F) and overall (G–L) survival of the stratified SIOPEN and GPOH cohort according to currently used risk factors**
Number of patients at low and high molecular risk as predicted by the 59-gene signature. Numbers in parentheses refer to number of patients that experienced an event. Missing *MYCN* status for one high-molecular-risk case. *Missing relapse date for one high-molecular-risk case.

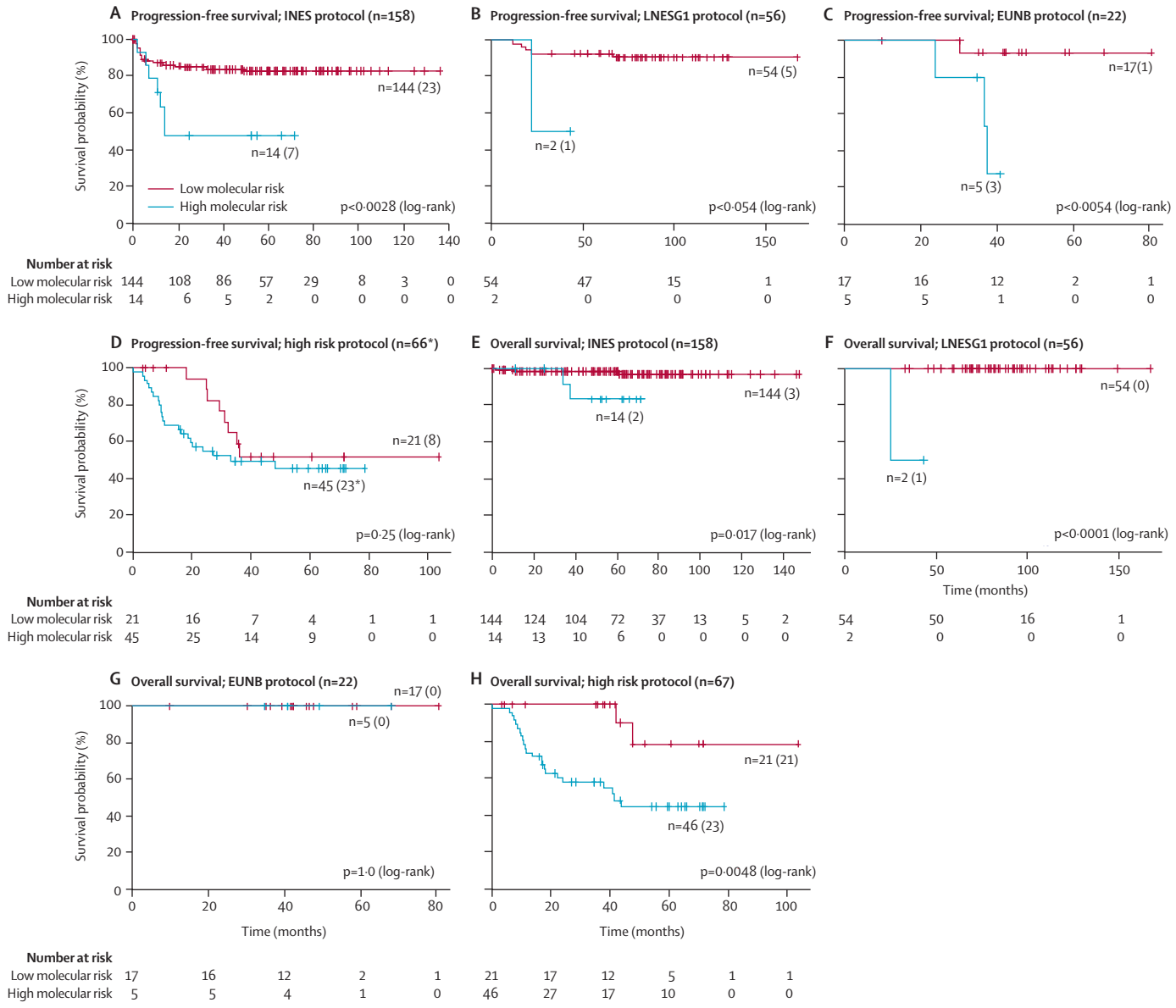

***Figure 3*: Kaplan–Meier and log-rank analysis for progression-free (A–D) and overall (E–H) survival of the SIOPEN cohort stratified according to treatment group**
Number of patients at low and high risk as predicted by the 59-gene signature. Numbers in parentheses refer to number of patients who experienced an event. *Missing relapse date for one high-risk case.

A case–control study was set up to validate the signature in the COG cohort (webappendix). This was done to ensure there was a sufficient number of events in each risk group—ie, to increase the power from what would have resulted from a random sample. Failure (cases) was defined as relapse, progression, or death from disease (progression-free survival), and death (overall survival) within a 2-year follow-up period, and control defined as non-failure in the first 2 years of follow-up. Controls and cases with complete data were selected in a 2:1 ratio to increase the sample size and power. Multivariate logistic regression analyses were done to determine whether the signature was a significant independent predictor after controlling for known risk factors. Statistical analyses were done with SAS (version 9).

Role of the funding source

The funding source had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication. All authors had access to the raw data as they are publicly available (webappendix). The corresponding author had full access to all of the data and the final responsibility to submit the manuscript for publication.

For the **raw data** see http://medgen.ugent.be/jvermeulen

	p value	Odds ratio (95% CI)
Backward-selected logistic regression model for progression-free survival (n=209)*		
Expression signature (high risk *vs* low risk)	<0·0001	3·68 (2·01–6·71)
Logistic regression models for overall survival with separate forced adjustment for variables (n=111)†		
Expression signature (high risk *vs* low risk)	<0·0001	10·53 (4·00–27·78)
Age (≥18 months *vs* <18 months)	NS	NA
Expression signature (high risk *vs* low-risk)	<0·0001	7·14 (2·70–18·87)
INSS stage (stage 4 *vs* not stage 4)	0·015	3·52 (1·28–9·71)
Expression signature (high risk *vs* low-risk)	0·0048	4·81 (1·62–14·29)
MYCN status (amplified *vs* not amplified)	0·0084	4·93 (1·51–16·13)
Expression signature (high risk *vs* low risk)	<0·0001	7·19 (2·71–18·87)
Ploidy (diploid *vs* hyperdiploid)	0·014	3·37 (1·28–8·85)

NA=not analysed. NS=not significant. *Also tested in the model and removed for non-significance during the backward selection were age, *MYCN* status, ploidy, MKI, INSS stage, and INPC grade of differentiation, in the order of removal from the model. †Insufficient number of deaths with MKI and INPC grade of differentiation data.

Table 2: **Multivariate logistic regression analysis in the COG cohort**

Results

A set of 59 genes with prognostic power in at least two independent studies was selected based on a re-analysis of seven published microarray gene-expression studies[6–12] combined with an extensive screening of previously published reports for single candidate prognostic genes (table 1, webappendix). A prognostic multigene signature was subsequently built based on the expression of the 59 genes using 15 deceased high-risk and 15 low-risk patients with a long progression-free survival time. Patients with low-risk disease or high-risk disease based on their expression of the 59-gene panel were defined as having a low or high molecular risk.

This multigene-expression signature significantly distinguished the remaining 313 patients (missing relapse date for one high molecular risk case) in the SIOPEN and GPOH cohort with respect to progression-free survival and overall survival (p<0·0001; figure 1). Progression-free survival 5 years from the date of diagnosis was 81·2% (95% CI 76·8–87·0) for the group of patients at low molecular risk, compared with 43·6% (32·4–58·6) for the group of patients at high molecular risk. The 5-year overall survival was 98·0% (96·1–100) and 55·0% (43·1–70·1) in the low and high molecular risk groups, respectively. Patients with an increased risk for both a shorter progression-free and overall survival could also be identified after stratification using current European risk factors such as age, *MYCN* status, and INSS stage (figure 2).

Subsequently, we tested the signature within each SIOPEN treatment protocol. In the group of patients treated according to the INES (NB99·study, NB99·1, NB99·2, and NB99·3), LNEGS1, and HR-NBL1 (with inclusion of patients sharing the same high-risk features as described in the webappendix and treated according to similar protocols) protocols, patients with an increased risk for death could be identified (p=0·017, p<0·0001, and p=0·0048, respectively). While the signature was able to identifying patients at risk of disease progression or relapse amongst patients treated according to the INES, LNESG1, and EUNB protocols (p=0·0028, p=0·054, and p=0·0054, respectively), there was no difference in progression-free survival between patients at high and low molecular risk treated according to the HR-NBL1 protocol (figure 3).

Multivariate logistic regression analysis of the SIOPEN and GPOH patients was performed within a subset of the overall SIOPEN and GPOH cohort, as described in the Methods section. Table 1 shows that the signature and INSS stage were the only significant independent predictors of overall and progression-free survival, respectively (odds ratio [OR] 19·32 [95% CI 6·50–57·43] and 3·96 [1·97–7·97] in case of an adverse outcome signature). Furthermore, within the INES and HR-NBL1 protocols, multivariate logistic regression analysis showed that the signature was the only significant independent predictor for overall survival (OR 7·00 [95% CI 1·04–46·95] and 9·20 [1·80–47·06], respectively).

The probability that a patient will be correctly classified by the signature based on a ROC-curve analysis (AUC) was 85·4% (95% CI 77·7–93·2) and 66·9% (59·2–74·6) for overall survival and progression-free survival, respectively, outperforming current risk factors such as age (62·3% [52·2–72·4] and 53·5% [45·8–61·2]), INSS stage (77·0% [66·8–87·1] and 65·4% [57·6–73·2]), and *MYCN* status (72·7% [61·7–83·8] and 57·2% [49·3–65·2]). The signature predicted overall survival with a sensitivity of 84·4% (27/32; 95% CI 66·5–94·1) and a specificity of 86·5% (192/222; 81·1–90·6).

To validate the multigene-expression signature in a completely independent patient cohort, 236 COG tumours were tested in a blind manner. The same signature as used for the SIOPEN and GPOH cohort identified COG patients who were at greater risk for progression, relapse, or death. Multivariate logistic regression analysis showed that the signature was independently statistically significant in a model adjusted for MYCN status, age, INSS stage, ploidy, International Neuroblastoma Pathology Classification (INPC) grade of differentiation, and mitosis karyorrhexis index (MKI). The signature was the only independent significant predictor for progression-free survival, with complete data for 139 controls and 70 cases. Patients at high molecular risk had a greater risk for relapse or progression (OR 3·68, 95% CI 2·01–6·71). In terms of overall survival, there were not enough deaths to power the fit of a logistic regression model with forced inclusion of all factors. Therefore, separate models testing the signature with adjustment for one risk factor at a time were fitted, with complete data for 74 controls and 37 cases. In each model comparing the signature to a given risk factor, the expression signature was always a more significant predictor than any other variable (table 2).

Discussion

Identification of more specific and sensitive markers for outcome prediction and response to therapy is required to further improve the choice of risk-related therapy for children with neuroblastoma. Using a carefully selected set of 59 prognostic genes based on an innovative data-mining strategy, we did a gene-expression study on the largest neuroblastoma patient series to date, covering 579 patients in total. Our robust prognostic multigene expression signature was tested on a large set of SIOPEN and GPOH tumours from uniformly treated patients and validated on an independent set of COG tumours. The signature is a strong independent risk predictor, able to identify patients with increased risk in the current risk groups.

Our study is unique in that a carefully selected set of only 59 genes was tested on a large panel of 579 tumour samples, thus increasing statistical power and robustness through this high patient–gene ratio. Several previous studies have attempted to identify prognostic signatures in neuroblastoma based on genome-wide mRNA-expression profiles. However, an important limitation of most published gene-expression studies is their lack of statistical power due to extremely low patient–gene ratios. As such, there are inherent but often overlooked statistical issues, such as data over-fitting, unstable gene lists, and lack of study power.[21] Consequently, for any small set of tumours, a gene classifier can be easily established, which is of little or no utility if it is not validated on an independent patient cohort.

After establishing and successfully testing our prognostic signature on the total patient cohort, we went on to assess the value of the signature in relation to the currently used risk factors, using multivariate logistic regression analysis and survival analysis after stratifying patients based on the currently used risk factors. The signature significantly discriminates between patients in most of the clinical risk subgroups. Possible reasons for the absence of discrimination in some subgroups might be the relatively low number of patients in these subgroups or insufficient follow-up times. Most importantly, the multivariate analysis attributes independent significant value to the signature. Based on this signature, patients with a higher risk of death by disease can be identified (OR 19·32; 95% CI 6·50–57·43), indicating that our gene signature clearly outperforms the other risk factors. This demonstrates the potential of this gene-expression signature to improve the clinical management of neuroblastoma patients.

Of further interest is the fact that survival analyses within the groups of patients treated according to the current European treatment protocols clearly show that the signature enables the discrimination of patients with different disease outcomes. This is an important finding, especially within the current high-risk category of patients treated according to the HR-NBL1 protocol, as there is currently no information on genomic aberrations or other factors available to identify a group of patients with worse outcomes in this subgroup of patients. All but one patient who achieved second complete response in this subgroup were classified as having low molecular risk and had experienced late relapses (mean 32·2 months, median 31·6 months) compared with the group of patients who did not achieve second complete response, of whom all but two were classified as having high molecular risk and had experienced early relapses (mean 13·6 months, median 10·0 months). Furthermore, death from disease also occurred earlier in the group of patients with high molecular risk (mean 12·3 months, median 9·6 months) compared with patients with low molecular risk (mean 27·1 months, median 27·1 months). Consequently, patients with high molecular risk within this subgroup could be candidates for new and hopefully more effective targeted therapies, whereas some of those with a low molecular risk might be spared haematopoietic stem cell transplantation. By the same token, patients who have a high molecular risk and who are currently treated with surgery alone or mild chemotherapy might benefit from more appropriate therapies such as those used in the current HR-NBL1 protocol.

An essential step in the validation procedure of our signature is its performance assessment on an independent set of COG tumours, whereby all analyses were performed blinded to clinical and outcome data. Similar performance of the expression signature was seen, indicating that the signature can yield reproducible results in independent patient cohorts. Moreover, irrespective of possible confounding factors related to patient ethnicity, treatment with other drugs, and RNA extraction with different standard operating procedures, the success of this validation study also confirms the robustness of the signature. Here, for the first time to our knowledge, the added value of the signature compared with currently used risk-stratification systems has been confirmed on a totally independent set of tumours in a blind study.

To reduce the gene set to a smaller, more robust gene subset, we used several methods including Spearman rank correlation clustering and selection of one or two genes in each gene cluster, top ranking univariate Cox and logistic regression analyses and the rank product method. Although similar classification performance could be obtained, the 59-gene list always slightly outperformed the reduced lists.

Inspection of the genes included in the signature reveals seven genes that have either previously been linked with neuroblastoma biology (*MYCN, NTRK1,* and *ODC1*[22]) or have been proposed as positional candidate genes, including *CAMTA1*[23] and *CHD5*[24] on 1p, *BIRC5*[25,26] on 17q, and *CADM1 (IGSF4)*[27,28] on 11q. Gene ontology analysis of the signature and a comparison of the gene list with the super *PCNA* gene (proliferation signature; V Detours, IRIBHM, Université Libre de Bruxelles, Brussels, Belgium; unpublished data) showed that only

very few genes are involved in cell-cycle regulation and proliferation. This is by contrast with signatures in many other cancer entities (eg, breast cancer[29]), in which typically more than two-thirds of the genes are implicated in proliferation. In line with this, there seem to be very few genes involved in inflammation, also typically seen in other cancers.[30] Additional gene ontology analysis of the prognostic gene list showed that genes implicated in neuronal differentiation such as *PTN*, *NRCAM*, *DPYSL3*, *SCG2*, *DDC*, *FYN*, *NTRK1*, *MAPT*, *PMP22*, *CHD5*, and *MTSS1*, are enriched among the genes that are expressed in low-risk tumours. Further scrutinising of the prognostic gene list and functional analyses might identify genes that have a role in neuroblastoma pathogenesis, and could therefore serve as potential therapeutic targets.

Important features of the applied real-time qPCR quantification strategy for marker-gene-expression analysis are speed, accuracy, cost-effectiveness, applicability in routine laboratories, and minimal required amounts of RNA. As the tumour sample size is often very limited, the applied RNA amplification procedure can make material for diagnostic and prognostic work-up more accessible. Another key to the success of our strategy is the possibility of using universally applicable, quantifiable external standards. These synthetic standards not only allow careful monitoring and correction of inter-run variation, but also enable the exchange of data between different laboratories, irrespective of the use of a different PCR instrument or reagents. Further validation of this strategy will enable large multicentre studies to be undertaken at different sites (JV; unpublished data). A crucial issue for all gene-expression studies is RNA quality. The accuracy of gene-expression profiling might be influenced by this metric depending on the quantification method used, the number of genes included in the classifier, expression differences, intra-group variability, and expression levels of the marker genes. The effect of RNA quality on gene expression has been extensively discussed in the literature, and conflicting data exist. To enable firm conclusions to be drawn from our study, we used stringent RNA quality and purity requirements and excluded around 20% of the samples. Moving forward, further studies should evaluate the effect of RNA quality on classification performance, and establish a cut-off designating sufficient quality for reliable class prediction. At the same time, standard operation procedures should be introduced to maximise the extraction and storage of high-quality RNA.

In conclusion, we established and validated a robust prognostic multigene-expression signature in the largest neuroblastoma population to date. The signature can act as an independent risk predictor enabling the identification of patients with increased risk in the current treatment groups. Our signature has important advantages compared with previously published gene-expression classifiers, such as the smaller amounts of starting material required, the lower number of genes, the higher cost efficiency and speed of the quantification method, and the suitability of the procedure for cross-laboratory comparison. This study should form the basis for future investigations such as large well-defined prospective studies with international collaboration. A further challenge is the performance of an integrated analysis for determining the prognostic performance of combining this expression signature with other genomic features of the tumour including microRNA and gene copy number profiling, and epigenetic markers, along with the currently used clinicobiological factors for risk stratification.

Contributors

JV, KDP, GL, FS, and JVDS contributed to the conception and design of the study, the analysis and interpretation of the data, and the drafting of the article. JV, JH, and LV had substantial contributions to the development and validation of the technical procedures. KDP, AN, PMG, and WBL did the statistical analysis. NVR, KS, SB, PS, GPT, RN, MP, IJL, OD, VCo, PFA, KB, JB, BM, and MDH managed tissue banking, *MYCN* copy number assessment, and provision of the samples. MF, AO, JM, GS, BDB, HR, AC, VCa, JK, UP, RL, and MDH managed the patients, databases, and the provision of clinical information. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declared no conflicts of interest.

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