

Exon-level expression analyses identify MYCN and NTRK1 as major determinants of alternative exon usage and robustly predict primary neuroblastoma outcome

Alexander Schramm^{1*}, Benjamin Schowe², Kathrin Fielitz¹, Melanie Heilmann¹, Marcel Martin³, Tobias Marschall³, Johannes Köster^{1,4}, Jo Vandesompele⁵, Joelle Vermeulen⁵, Katleen de Preter⁵, Jan Koster⁶, Rogier Versteeg⁶, Rosa Noguera⁷, Frank Speleman⁵, Sven Rahmann^{3,4}, Angelika Eggert¹, Katharina Morik², and Johannes H. Schulte¹

Running title: Exon level expression analyses of neuroblastoma

Affiliations:

1 University Childrens Hospital Essen, 45122 Essen, Germany

2 Department of Computer Science, LS8, Artificial Intelligence Unit, TU Dortmund, 44227 Dortmund, Germany

3 Department of Computer Science, LS11, TU Dortmund, 44227 Dortmund, Germany

4 Department of Genome Informatics, University Hospital Essen, 45122 Essen, Germany

5 Center for Medical Genetics; Ghent University Hospital, B-9000 Ghent, Belgium

6 Department of Human Genetics, Academic Medical Center, 1105 AZ

Amsterdam, the Netherlands

7 Department of Pathology, Medical School, University of Valencia, 46100 Valencia, Spain

* Corresponding author: PD Dr. Alexander Schramm, University Childrens Hospital Essen, Hufelandstr. 55, 45122 Essen, Germany, tel: +49201723-2506, fax: -5750 alexander.schramm@uni-due.de

Total word count abstract: 197; total word count manuscript (excluding abstract, legends to figures and tables): 3985

Abstract

Background:

Using mRNA expression-derived signatures as predictors of individual patient outcome has been a goal ever since the introduction of microarrays. Here, we addressed whether analyses of tumour mRNA at the exon level can improve on the predictive power and classification accuracy of gene-based expression profiles using neuroblastoma as a model.

Methods:

In a patient cohort comprising 113 primary neuroblastoma specimens expression profiling using exon-level analyses was performed to define predictive signatures using various machine learning techniques. Alternative transcript use was calculated from relative exon expression. Validation of alternative transcripts was achieved using qPCR and cell based approaches.

Results:

Both predictors derived from the gene or the exon levels resulted in prediction accuracies >80% for both event-free and overall survival and proved as independent prognostic markers in multivariate analyses. Alternative transcript use was most prominently linked to the amplification status of the MYCN oncogene, expression of the TrkA/NTRK1 neurotrophin receptor and survival.

Conclusion:

As exon level-based prediction yields comparable, but not significantly better prediction accuracy than gene expression-based predictors, gene-based assays seem to be sufficiently precise for predicting outcome of neuroblastoma patients. However, exon-level analyses provide added knowledge by identifying alternative transcript use, which should deepen the understanding of neuroblastoma biology.

Keywords: Exon arrays, prediction, PAM, neuroblastoma, alternative transcript use

Introduction

Neuroblastoma is a childhood tumour with a wide range of clinical courses, spanning from spontaneous differentiation to rapid progression and fatal outcome. A few genetic alterations have been described to be causative for neuroblastoma including mutations of the genes encoding ALK (Chen *et al*, 2008b; George *et al*, 2008; Mosse *et al*, 2008) or PHOX2B (reviewed in (Janoueix-Lerosey *et al*, 2010; Maris, 2010)). Genomic sequencing of primary neuroblastoma recently revealed an enrichment for mutations in genes involved in neuritogenesis (Molenaar *et al*, 2012). However, the origin of the majority of spontaneous neuroblastomas remains unclear (Park *et al*, 2008). One subgroup of aggressive neuroblastomas is characterised by amplification of the *MYCN* oncogene, which occurs in about 20-25% of all primary neuroblastoma (Seeger *et al*, 1985). In the absence of the *MYCN* amplification, it remains difficult to define the individual risk of relapse and assign adequate therapy (Maris *et al*, 2007). Expression profiling has been performed in recent years to identify signatures predictive of outcome, and these signatures are still being refined for the use in clinically applicable settings (Oberthuer *et al*, 2006; Oberthuer *et al*, 2010; Ohira *et al*, 2005; Schramm *et al*, 2005b; Wei *et al*, 2004). A comparison of published microarray studies has revealed little overlap of genes within signatures identified to predict neuroblastoma outcome, although integration of data seemed possible (Chen *et al*, 2008a). Several explanations for the diversity of genes in these signatures have been suggested such as the use of different technical platforms for expression profiling, the choice of prediction algorithms and inter-laboratory variation (Irizarry *et al*, 2005). It has also been argued that genes in the same pathways are co-regulated implying a similar predictive value and redundancy of information. Still,

the fact that genes are used by one predictor and are absent in others may be a function of the probes being derived from different parts of the same gene for the different technical platforms. Methods to overcome this limitation of gene-based expression profiling include RNA sequencing or exon-level mRNA expression analyses. Here, we chose Affymetrix HuEx1.0 arrays that were designed to interrogate every known and predicted exon in the human genome. Reporter sequences that extend beyond protein coding regions to other areas of the transcribed genome are also included on these arrays. In principle, the combination of coverage and precise localization information allows in principle for analyses of regulation on the sub-gene level. This is important since a substantial proportion of protein coding genes are predicted to be alternatively spliced. In neuroblastomas, alternative splicing of the *NTRK1/TrkA* receptor has already been reported to have an impact on tumour aggressiveness (Tacconelli *et al*, 2004) suggesting that the phenomenon of differential transcripts use has a major impact on neuroblastoma biology. In this study, we wanted to explore whether analyses of mRNA expression resolved to the exon-level was capable of improving prognostic prediction of neuroblastoma patient outcome above the level currently achieved using gene expression-based predictors. We also aimed to identify factors associated with alternative exon use in neuroblastoma and sought to gain novel biological insights into neuroblastoma biology by exon-level gene expression analyses.

Materials and Methods

Patient cohort

The 113 primary neuroblastoma specimens were from tumour banks in Cologne and Essen, Germany, Ghent, Belgium and Valencia, Spain. All patients were diagnosed between 1998 and 2007 and treated according to the German neuroblastoma trials NB97, NB 2004 or the respective SIOPEX protocol. Written informed consent was obtained from patients or their parents. As many patients were identified within general neuroblastoma screening in Germany (Schilling *et al*, 2002), patients were selected to recreate a representative distribution of tumour stages. Of the 113 NB patients enclosed in our study, 57 presented with localized disease (stage 1: n= 31; stage 2: n= 11; stage 3: n= 15) and 56 with metastatic disease at the time of diagnosis. The mean and median ages of our patient cohort at diagnosis were 934 and 472 days, respectively. Approximately two third of patients were >1 yr of age at the time of diagnosis (n= 72) and the remainder (n=41) were younger than 1 yr. Amplification of the *MYCN* oncogene occurred in 21 patients. Median and mean follow-up were 2066 and 1976 days, respectively. In total, 26 patients died of their disease. Patient characteristics are summarized in Table 1.

RNA preparation

Representative areas of histologically confirmed, snap-frozen neuroblastomas were cut on dry ice. No preselection for tumour cell fraction or microenvironment was performed. Total RNA extraction of NB tumour samples was performed in by silica gel-based membrane purification methods (RNeasy Mini kit or MicroRNeasy kit, Qiagen, Hilden, Germany) according to the manufacturers' instructions. RNA

quality was controlled by capillary gel electrophoresis using either the Experion system (High-sensitivity chips, Bio-Rad) or the Bioanalyser 2100 (RNA nano kits, Agilent, Palo Alto, CA).

Array hybridizations

Fragmentation of cRNA and hybridization to HuEx1.0 microarrays (Affymetrix, Santa Clara, CA), washing, staining and scanning of the arrays in a GeneArray scanner (Agilent, Palo Alto, CA) were basically performed as previously described (Schramm *et al*, 2005a; Schramm *et al*, 2005b). Quality control of hybridizations and RMA normalization was performed using Genomics Suite (Partek Inc., St. Louis, MO) using the default settings. Data were stored in the R2 microarray analysis and visualisation platform (AMC Amsterdam, <http://r2.amc.nl>, The Netherlands) for exploration and visualisation. Microarray data have been submitted to the GEO database (Acc. no. GSE32664).

Relapse Prediction using Shrunken Centroids

A shrunken centroids PAM (prediction analyses for microarrays (Tibshirani *et al*, 2002)) model was used to predict event-free survival (EFS) or relapse as well as overall survival (OS) or death using exon- and gene expression values. Two filter steps were applied prior to running the classification algorithms. First, all features of the core probe set having a 0.3-quantile score below 5.0 were discarded. Then features that were significantly differentially expressed between two classes were identified using an F-test (significance level 0.05) to check for equal variances in each class for every exon or gene. Student's t-test was performed for exons or genes with equal class variance and Welch's t-test was used for exons or genes with non-equal class variance to calculate p-values. These steps were repeated 20

times on bootstrap-samples (drawing with replacement) from the data. Only exons and genes having p-values ≤ 0.05 in at least 95% of the rounds were kept as significantly associated to the outcome or label and these data were used to build the shrunken centroids model, which further reduced the number of features by shrinking the centroids to the relevant features. Cross-validation delivered a shrinkage parameter of 1.2 as a sensible trade-off between prediction accuracy classifier size for both gene and exon data.

Shrunken centroids analysis

Implementations of support vector machines (SVM) and PAM in Rapid Miner 5.1 (Rapid-I, Dortmund, Germany) were used to predict patient outcome and patients were divided into a training ($n = 73$) and a test set ($n = 40$). RapidMiner was applied in conjunction with the RapidMiner Feature Selection Extension. Analyses of maximum divergent courses (MDC) were carried out on a subset of 63 patients. MDC was defined by patients with localised disease without *MYCN* amplification, which had no relapse on the one hand and patients with metastatic (INSS stage 4) disease who died of their disease on the other hand. Test set data were not used in any of the validation steps until the predictor was applied.

Detection of differential gene or exon expression

The "apt-probeset-summarize" tool (Affymetrix Power Tools 1.10.0) was run to compute exon and gene expression values using the following analysis parameters on the core probe set: rma-bg (background correction as described in (Irizarry *et al*, 2003)), quant-norm (quantile normalization), pm-only (no mismatch probe adjustments) and med-polish (median polish as described in (Irizarry *et al*, 2003)). Genes and exons were discarded if their normalized, log₂ expression

count was < 5 for at least 1/3 of the patients. Relative exon expression was computed for each gene with at least two exons represented, and utilized all exons representing a given gene as $r = (e-g)/g$, where e is the normalized log₂ exon expression and g is the normalized log₂ expression of the gene. Differential expression between classes was determined by Welch's t-test, followed by false discovery correction according to Benjamini-Hochberg. Differential exon use in transcripts from the same coding gene was determined on relative exon expression levels. Genes or exons with a false discovery rate < 0.05 were reported as significantly differentially expressed. In order to estimate differential exon use, significantly differentially expressed genes were discarded and differential exon expression between groups was then re-determined.

Cell culture and *in vitro* analyses

Cultivation of the human neuroblastoma cell lines IMR32, NB69, SH-EP, WAC2 and SH-EP MYC-ER was performed as previously described (Schramm *et al*, 2005c; Schulte *et al*, 2008). Induction of MYCN in SH-EP MYC-ER was achieved using 4-hydroxy tamoxifen as described (Schulte *et al*, 2008). Propidium iodide-based FACS-analyses of DNA content was carried out as in (Eggert *et al*, 2001). Cell viability assays using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were performed as described in (Schulte *et al*, 2005). RNA was harvested from subconfluent growing cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was subsequently prepared as described (Schulte *et al*, 2005). Semiquantitative qPCR was performed using standard protocols on a ABI StepOnePlus Real-Time PCR system (Life Technologies, Carlsbad, CA) using primers spanning INCENP exons 1-2 and 10-11, respectively (Life Technologies, Carlsbad, CA).

Results

Association of known risk factors with outcome in our study cohort

We first evaluated the distribution of known risk factors (tab. 1) and their association with clinical outcome in the cohort of 113 neuroblastomas that were analysed by expression profiling in this study. The relative number of patients with *MYCN* amplification and metastatic disease at time of diagnosis resembled the situation found in the overall patient population. As expected, *MYCN* amplification, metastatic disease, age at diagnosis > 1 yr and low *NTRK1* expression were all associated with a significantly worse outcome in univariate analyses (fig. 1).

Comparison of the predictive power of gene-level or exon level-based predictors

In contrast to other studies focussing on only gene expression, we evaluated both gene-level and exon-level expression as the basis for a reliable predictor of neuroblastoma outcome. Tumour expression profiles from the 113 neuroblastoma patients (tab. 1) were obtained using Affymetrix HuExST1.0 arrays. Expression data for both the gene and exon levels were processed and normalized as described in the materials and methods section. Several methods were evaluated for prediction, including linear SVM and random trees/forests (data not shown), but PAM-based prediction was the most stable and reliable method. After filtering and enrichment for significantly differentially expressed genes, support vector machine (SVM) or PAM-based methods were used to build a predictor for EFS or OS using gene or exon expression data. SVM-based predictors were less stable when data were split into a training set (n=73) and a test set (n= 40, see also methods), which resulted in decrease in accuracy and specificity in the test set (data not shown). The PAM-based signature predicted

overall survival with an accuracy of 83.1 % (sensitivity 84.7 %, specificity 82.6 %, tab. 2). When a published PAM-based gene set was used as input for prediction (Vermeulen *et al*, 2009a), accuracy was comparable (86.1 %) but this was accompanied by a decrease in sensitivity, which dropped to 79.6 %, while specificity was higher (89.4 %). PAM prediction utilizing only the patients with maximum divergent courses only (n= 63) produced the highest prediction accuracy (89.2 %, sensitivity = 85.2 %, specificity = 91.4 %). Interestingly, both prediction accuracy and the individual classification were highly similar using either expression from the exon or gene levels and the same prediction method and data sets (tab. 2).

Gene- and exon-derived PAM signatures are independent prognostic markers of survival in multivariate analyses

As we wanted to test the robustness of our predictors and to correlate them with known prognostic markers, backward logistic regression was performed. Since the gene- and exon-derived signatures functioned similarly in predicting the individual patient outcome, we analysed their performance separately when building the regression models. Both gene- and exon-based predictors were the only significant independent predictors of overall survival in our cohort in multivariate analyses (tab. 3., $p= 0.007$, odds ratio= 9.7, for the gene based predictor; $p= 0.005$, odds ratio = 10.7, for the exon based predictor).

Identification of factors associated with differential exon usage in neuroblastoma

We analysed the impact of clinical and biological features on both differential gene and exon expression with the aim to identify master regulators governing

alternative transcript use. Using the filtering steps described in the methods section, we first determined the number of differentially expressed genes associated with either INSS stages, age at diagnosis, *MYCN* amplification, survival or *NTRK1* expression (tab. 4). Survival, *MYCN* amplification and *NTRK1* expression were associated with a massive shift of the transcriptome, reflected by significantly differential expression of >1000 genes each. This also held true when analyses were performed using expression at the exon level, which resulted in > 10.000 significantly differentially used exons for each group. Interestingly, when differential exon use was assessed for genes, whose expression was not significantly altered between groups, *NTRK1* expression and *MYCN* amplification status were strongly associated with alternative exon use. When tumours with high *NTRK1* expression were compared to tumours with low *NTRK1* expression, 471 exons were found to be differentially expressed, while *MYCN* amplification correlated with 306 differentially used exons. This finding suggests that both, *MYCN* and *NTRK1*, could be involved in governing alternative transcript use.

Validation of known and novel transcript variants in primary NB

We firstly validated a variant of the neurotrophin receptor *NTRK1*, designated TrkA-III, which has been previously associated with aggressive tumour behaviour in NB (Tacconelli *et al*, 2004), using our expression data. This variant lacks exons six, seven and nine resulting in altered *NTRK1* signalling independent of ligand binding (Tacconelli *et al*, 2004). Differential expression of exons six and seven was already visually detectable from the individual tumour profiles plotted as a heatmap (Fig 2A). *NTRK1* gene expression was highly correlated with survival, as expected, while expression of exons six or seven was not (Fig 2B, C). Notably, expression of *NTRK1* is not only correlated to prognosis, but also to patient age:

younger patients have higher *NTRK1* levels than older patients. Thus, we could validate that differential *NTRK1* variants are expressed in NB and that these variants correlate with different patient outcome for the first time in a large patient cohort.

We also wanted to gain first insights into transcripts and novel transcript variants associated with unfavourable clinical features since these could serve as novel therapeutic targets. For this reason, we focussed on genes and exons, whose expression was linked to survival and the amplification status of *MYCN* (tab. 4). Patterns of alternative exon use included different transcriptional start and stop sites as well as differential expression of isoforms from the same coding gene (Suppl. Fig. 1). To validate the *MYCN*-induced regulation of one of these transcript isoforms, we selected the gene coding for *INCENP*, a member of the chromosomal passenger complex involved in mitotic progression. Statistical analyses suggested a significant upregulation of *INCENP* exons 1-5 corresponding to the *INCENP* transcript variant, NM_020238, in patients with *MYCN*-amplified tumours (fig. 3A). This is exemplified for exon 5, whose expression was significantly associated with *MYCN* amplification ($p < 0.001$, fig. 3B), while no difference in *INCENP* exon 15 expression was observed between tumours with and without *MYCN* amplification (fig. 3C). *INCENP* was also induced upon *MYCN* activation or stable overexpression *in vitro* in two cell model systems based on the SH-EP NB cell line, which does not express detectable levels of *MYCN*. We used SH-EP derivatives engineered to stably or inducibly overexpress *MYCN* (designated WACII and SH-EP *MYCN*-ER, respectively, fig. 3D). While PCR-based assays validated *INCENP* as a *MYCN*-inducible target *in vitro*, specific isoforms were not differentially induced in assays specifically measuring both *INCENP* transcript variants compared to the longer *INCENP* transcript variant

(NM_020238_2, fig. 3D, right). Thus, INCENP could be validated as a MYCN-inducible target *in vitro*, confirming the data obtained from the primary tumours, but an isoform-specific induction of INCENP variants by MYCN remains to be confirmed.

Interestingly, alternative exon usage of the first exons of the cell cycle regulating genes *CDKN2B* and *CCNB1* was significantly different between aggressive, deadly or *MYCN*-amplified tumours and patients with event-free survival or normal *MYCN* status (fig. 4 A-B). The most 5' probe sets of *CDKN2B* are expressed at very low levels, so a functional role for the observed expression difference remains to be determined. The expression of a shorter *CCNB1* isoform was verified in neuroblastoma cell lines (fig. 4C) and inhibition of Cdk1/*CCNB1* interaction using the RO3306 small molecule inhibitor resulted in an increase in the fraction of apoptotic cells (fig. 4 D). Taken together, differential expression of transcript variants suggested from the analyses of primary tumours could be validated in the *in vitro* models.

Discussion

Prediction of the individual risk and consequent adaptation of treatment is one promise of personalized cancer medicine. Using gene expression profiling for individual treatment decisions has been investigated for a decade now for many cancer types and also for neuroblastoma. While early studies published in this field suffered from data over-fitting and lack of general applicability, large studies have meanwhile provided evidence for the high sensitivity and accuracy of gene expression based classifiers using different platforms (Oberthuer *et al*, 2010; Vermeulen *et al*, 2009a; Vermeulen *et al*, 2009b). International consortia such as MAQC have defined standard operating procedures for validating microarray data (Shi *et al*, 2010). Although advances have been achieved for both technical improvements as well as standardization, concerns still remain on the clinical usability of classifiers derived from expression profiling. Here, we addressed whether the higher information density of expression profiling at the exon level rather than the gene level expression could increase the power of tumour expression-based outcome prediction. For this purpose we used a cohort of 113 neuroblastoma patients, which were profiled using Affymetrix Exon Arrays (HuEx1.0ST). Using exon-level expression achieved comparable prediction accuracy, sensitivity and specificity for both overall survival and event-free survival compared to using gene-level expression. These findings also held true, when a published PAM-derived signature of 59 genes (Vermeulen *et al*, 2009a) and the exons making up these genes were used to predict outcome in this cohort. Prediction accuracy increased when patient cohorts with maximum diversity (MDC) in clinical courses were used. For the best clinical courses, we only included patients with localized disease without *MYCN* amplification, which had never experienced a relapse. Patients with the worst clinical course had metastatic

spread at time of diagnosis (INSS stage 4) and were eventually succumbing to their disease. As previously noticed, survival prediction is possible with near perfect accuracy while prediction of relapse is more difficult. One reason for this phenomenon could be that *MYCN* amplification is a major risk factor for relapsing neuroblastoma and results in massive reprogramming of the tumour transcriptome to create this aggressive tumour phenotype. However, *MYCN* amplification alone cannot account for all aggressive, relapsing courses of the disease. Given the heterogeneity of transcriptomes from tumours with and without *MYCN* amplification, the definition of a perfect relapse signature is still an unresolved task. Nevertheless, the gene- and exon- derived signatures in our study were the only independent markers of survival in multivariate analyses, corroborating their potential usefulness for patient stratification and, in the long run, individually adapted risk assessment.

Exon-level profiling promises to yield unprecedented insights into the fine tuning of transcript use associated with aggressive or benign tumours. *MYCN* amplification has long been linked to aggressiveness in NB (Schwab *et al*, 1983; Seeger *et al*, 1985), but has only recently been associated with alternative exon use in primary NB (Guo *et al*, 2011). First mechanistic insights into MYC-driven splice site usage were provided by linking alternative splicing of the Raf kinase to differential MYC expression (Rauch *et al*, 2011). Here, we identified *NTRK1* as a second biological parameter governing gene composition, when patients with tumours strongly expressing *NTRK1* were compared to patients whose tumours weakly expressed *NTRK1*. High *NTRK1* expression, which has previously been shown to be associated with outcome in NB (Nakagawara *et al*, 1993), was also correlated with excellent outcome in our data set (Fig. 2A). Of note, expression of *NTRK1* exons 6

and 7 was even lower in advanced stage tumours (Fig. 2C), confirming previous results (Tacconelli *et al*, 2004).

We identified differential expression of > 200 predicted alternative transcripts between neuroblastomas with and without *MYCN* amplification or with high and low *NTRK1* expression. Thus, we are still at the beginning to understand the global changes induced by *MYCN* and *NTRK1* in neuroblastoma. The interplay of these factors is complicated by the fact that *MYCN* can directly bind to the *NTRK1* promoter and negatively regulate *NTRK1* expression (Iraci *et al*, 2011). Although first attempts have been made to catalog alternative splice site usage in oncogenic kinases (Druillennec *et al*, 2012), we are still far from having an overview of the functional relevance of all splicing changes intrinsic to aggressive cancer cells. Moreover, it remains to be determined if alternative transcript use rather than alternative splicing plays a role in determining the expression profiles underlying tumour aggressiveness. In neuronal development, alternative transcript use was identified as a major cause for transcriptome diversity (Pal *et al*, 2011), and similar patterns were observed in medulloblastomas (Menghi *et al*, 2011). It is evident that the altered tumour transcriptome results from aberrant transcripts due to mutations, aberrant transcript amounts from changes in regulatory networks and also from alternative transcript use, but how the latter is produced must be analysed in more tumour entities to evaluate its contribution to cancer biology.

In neuroblastoma, there are hints linking functional modules such as cell cycle regulation (Murphy *et al*, 2011) and protein biosynthesis (Boon *et al*, 2001) to *MYCN* and aggressive tumour behaviour. Moreover, gene expression of members of the chromosomal passenger complex, most prominently survivin, has been previously linked to NB. Survivin upregulation was linked to neuroblastoma aggressiveness (Islam *et al*, 2000), but this could be attributed to an unspecific

gain of chromosome 17q, which occurs than half of all neuroblastomas (Bown *et al*, 1999). Here, we provide first evidence that a second member of the chromosomal passenger complex, INCENP, is a potential MYCN target in neuroblastoma since INCENP mRNA was upregulated upon MYCN induction *in vitro* as well as in patients having tumours with amplified *MYCN*. Additionally, exon-level analyses of the INCENP coding region suggested a shorter hypothetical INCENP isoform differentially expressed between tumours with and without *MYCN* amplification. However, the existence of such an isoform, for which only a cDNA clone has previously been described, remains to be proven experimentally. *In vitro* studies corroborated MYCN-induced upregulation of INCENP exons 1-2 and 11-12. Nevertheless, it is intriguing to speculate that transcriptional reprogramming of aggressive tumour cells may not only cause differential gene expression but also alternative transcript use, which could here be exemplified for a *CCNB1* variant. In summary, expression signatures based on the gene or exon level proved to be equally powerful in predicting the clinical outcome of neuroblastoma patients, rendering gene-based assays as sufficiently precise for potential diagnostic purposes. The power of exon-based expression analyses lies in the identification of alternatively used transcripts in tumours, which can provide new insights into the aberrant circuits of the most aggressive tumour fraction, harbouring a plethora of options for therapeutic interventions.

Acknowledgements:

This work was funded by the Deutsche Forschungsgemeinschaft (DFG), Collaborative Research Effort SFB876, subprojects C1 "DimRed" (to A.S. and K.M.) and B1 "Spectra" (to S.R.) and PI10/00015 and RD06/0020/0102 (RTICC, ISCIII & ERDF) Spain (to R.N.). The authors wish to thank Ellen Mahlow and

Sabine Dreesmann for excellent technical assistance, Harald Stephan for help in preparing the figures and Kathy Astrahantseff for proof-reading the manuscript.

Disclosure Statement:

The authors declare that no conflicts of interest have to be disclosed.

References

- Boon K, Caron HN, van Asperen R, Valentijn L, Hermus MC, van Sluis P, Roobeek I, Weis I, Voute PA, Schwab M, Versteeg R (2001) N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *EMBO J* **20**(6): 1383-93
- Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman F, Nicholson J, Bernheim A, Betts DR, Vandesompele J, Van Roy N (1999) Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med* **340**(25): 1954-61
- Chen QR, Song YK, Wei JS, Bilke S, Asgharzadeh S, Seeger RC, Khan J (2008a) An integrated cross-platform prognosis study on neuroblastoma patients. *Genomics*
- Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T, Nakagawara A, Hayashi Y, Mano H, Ogawa S (2008b) Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* **455**(7215): 971-4
- Druillenec S, Dorard C, Eychene A (2012) Alternative splicing in oncogenic kinases: from physiological functions to cancer. *Journal of nucleic acids* doi: 10.1155/2012/639062
- Eggert A, Grotzer MA, Zuzak TJ, Wiewrodt BR, Ho R, Ikegaki N, Brodeur GM (2001) Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res* **61**(4): 1314-9.
- George RE, Sanda T, Hanna M, Frohling S, Luther W, 2nd, Zhang J, Ahn Y, Zhou W, London WB, McGrady P, Xue L, Zozulya S, Gregor VE, Webb TR, Gray NS, Gilliland DG, Diller L, Greulich H, Morris SW, Meyerson M, Look AT (2008) Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* **455**(7215): 975-8
- Guo X, Chen QR, Song YK, Wei JS, Khan J (2011) Exon array analysis reveals neuroblastoma tumors have distinct alternative splicing patterns according to stage and MYCN amplification status. *BMC Med Genomics* **4**: 35 doi:10.1186/1755-8794-4-35
- Iraci N, Diolaiti D, Papa A, Porro A, Valli E, Gherardi S, Herold S, Eilers M, Bernardoni R, Della Valle G, Perini G (2011) A SP1/MIZ1/MYCN repression complex recruits HDAC1 at the TRKA and p75NTR promoters and affects neuroblastoma malignancy by inhibiting the cell response to NGF. *Cancer Res* **71**(2): 404-12
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**(2): 249-64
- Irizarry RA, Warren D, Spencer F, Kim IF, Biswal S, Frank BC, Gabrielson E, Garcia JG, Geoghegan J, Germino G, Griffin C, Hilmer SC, Hoffman E, Jedlicka AE, Kawasaki E, Martinez-Murillo F, Morsberger L, Lee H, Petersen D, Quackenbush J, Scott A, Wilson M, Yang Y, Ye SQ, Yu W (2005) Multiple-laboratory comparison of microarray platforms. *Nat Methods* **2**(5): 345-50

Islam A, Kageyama H, Takada N, Kawamoto T, Takayasu H, Isogai E, Ohira M, Hashizume K, Kobayashi H, Kaneko Y, Nakagawara A (2000) High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene* **19**(5): 617-23

Janoueix-Lerosey I, Schleiermacher G, Delattre O (2010) Molecular pathogenesis of peripheral neuroblastic tumors. *Oncogene* **29**(11): 1566-79

Maris JM (2010) Recent advances in neuroblastoma. *N Engl J Med* **362**(23): 2202-11

Maris JM, Hogarty MD, Bagatell R, Cohn SL (2007) Neuroblastoma. *Lancet* **369**(9579): 2106-20

Menghi F, Jacques TS, Barenco M, Schwalbe EC, Clifford SC, Hubank M, Ham J (2011) Genome-wide analysis of alternative splicing in medulloblastoma identifies splicing patterns characteristic of normal cerebellar development. *Cancer Res* **71**(6): 2045-55

Molenaar JJ, Koster J, Zwijnenburg DA, van Sluis P, Valentijn LJ, van der Ploeg I, Hamdi M, van Nes J, Westerman BA, van Arkel J, et al. (2012) Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. *Nature* doi: 10.1038/nature10910.

Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkamani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM (2008) Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* **455**(7215): 930-5

Murphy DM, Buckley PG, Bryan K, Watters KM, Koster J, van Sluis P, Molenaar J, Versteeg R, Stallings RL (2011) Dissection of the oncogenic MYCN transcriptional network reveals a large set of clinically relevant cell cycle genes as drivers of neuroblastoma tumorigenesis. *Molecular carcinogenesis* **50**(6): 403-11

Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM (1993) Association between high levels of expression of the Trk gene and favorable outcome in human neuroblastomas. *N Engl J Med* **328**: 847-854

Oberthuer A, Berthold F, Warnat P, Hero B, Kahlert Y, Spitz R, Ernestus K, Konig R, Haas S, Eils R, Schwab M, Brors B, Westermann F, Fischer M (2006) Customized oligonucleotide microarray gene expression-based classification of neuroblastoma patients outperforms current clinical risk stratification. *J Clin Oncol* **24**(31): 5070-8

Oberthuer A, Hero B, Berthold F, Juraeva D, Faldum A, Kahlert Y, Asgharzadeh S, Seeger R, Scaruffi P, Tonini GP, et al. (2010) Prognostic impact of gene expression-based classification for neuroblastoma. *J Clin Oncol* **28**(21): 3506-15

Ohira M, Oba S, Nakamura Y, Isogai E, Kaneko S, Nakagawa A, Hirata T, Kubo H, Goto T, Yamada S, Yoshida Y, Fuchioka M, Ishii S, Nakagawara A (2005) Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas. *Cancer Cell* **7**(4): 337-50.

Pal S, Gupta R, Kim H, Wickramasinghe P, Baubet V, Showe LC, Dahmane N, Davuluri RV (2011) Alternative transcription exceeds alternative splicing in generating the transcriptome diversity of cerebellar development. *Genome research* **21**(8): 1260-72

Park JR, Eggert A, Caron H (2008) Neuroblastoma: biology, prognosis, and treatment. *Pediatr Clin North Am* **55**(1): 97-120

Rauch J, Moran-Jones K, Albrecht V, Schwarzl T, Hunter K, Gires O, Kolch W (2011) c-Myc regulates RNA splicing of the A-Raf kinase and its activation of the ERK pathway. *Cancer Res* **71**(13): 4664-74

Schilling FH, Spix C, Berthold F, Erttmann R, Fehse N, Hero B, Klein G, Sander J, Schwarz K, Treuner J, Zorn U, Michaelis J (2002) Neuroblastoma screening at one year of age. *N Engl J Med* **346**(14): 1047-53

Schramm A, Schulte JH, Astrahantseff K, Apostolov O, Limpt V, Sieverts H, Kuhfittig-Kulle S, Pfeiffer P, Versteeg R, Eggert A (2005a) Biological effects of TrkA and TrkB receptor signaling in neuroblastoma. *Cancer Lett* **228**(1-2): 143-53

Schramm A, Schulte JH, Klein-Hitpass L, Havers W, Sieverts H, Berwanger B, Christiansen H, Warnat P, Brors B, Eils J, Eils R, Eggert A (2005b) Prediction of clinical outcome and biological characterization of neuroblastoma by expression profiling. *Oncogene* **24**(53): 7902-12

Schramm A, von Schuetz V, Christiansen H, Havers W, Papoutsi M, Wilting J, Schweigerer L (2005c) High activin A-expression in human neuroblastoma: suppression of malignant potential and correlation with favourable clinical outcome. *Oncogene* **24**(4): 680-7

Schulte JH, Horn S, Otto T, Samans B, Heukamp LC, Eilers UC, Krause M, Astrahantseff K, Klein-Hitpass L, Buettner R, Schramm A, Christiansen H, Eilers M, Eggert A, Berwanger B (2008) MYCN regulates oncogenic MicroRNAs in neuroblastoma. *Int J Cancer* **122**(3): 699-704

Schulte JH, Schramm A, Klein-Hitpass L, Klenk M, Wessels H, Hauffa BP, Eils J, Eils R, Brodeur GM, Schweigerer L, Havers W, Eggert A (2005) Microarray analysis reveals differential gene expression patterns and regulation of single target genes contributing to the opposing phenotype of TrkA- and TrkB-expressing neuroblastomas. *Oncogene* **24**(1): 165-77.

Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Godstein M, Trent J (1983) Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and neuroblastoma tumors. *Nature* **305**: 245-248

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D (1985) Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med* **313**(18): 1111-6.

Shi L, Campbell G, Jones WD, Campagne F, Wen Z, Walker SJ, Su Z, Chu TM, Goodsaid FM, Puztai L, et al. *Nat Biotechnol* **28**(8): 827-38

Tacconelli A, Farina AR, Cappabianca L, Desantis G, Tessitore A, Vetuschi A, Sferra R, Rucci N, Argenti B, Screpanti I, Gulino A, Mackay AR (2004) TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. *Cancer Cell* **6**(4): 347-60.

Tibshirani R, Hastie T, Narasimhan B, Chu G (2002) Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A* **99**(10): 6567-72

Vermeulen J, De Preter K, Laureys G, Speleman F, Vandesompele J (2009a) 59-gene prognostic signature sub-stratifies high-risk neuroblastoma patients. *Lancet Oncol* **10**(11): 1030

Vermeulen J, De Preter K, Naranjo A, Vercruyse L, Van Roy N, Hellemans J, Swerts K, Bravo S, Scaruffi P, Tonini GP, et al. (2009b) Predicting outcomes for children with neuroblastoma using a multigene-expression signature: a retrospective SIOPEN/COG/GPOH study. *Lancet Oncol* **10**(7): 663-71

Wei JS, Greer BT, Westermann F, Steinberg SM, Son CG, Chen QR, Whiteford CC, Bilke S, Krasnoselsky AL, Cenacchi N, Catchpoole D, Berthold F, Schwab M, Khan J (2004) Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma. *Cancer Res* **64**(19): 6883-91

Table captions and legends to figures

Table 1: Clinical and biological characteristics of the 113 patients with primary NB included in this study.

Table 2: Precision of outcome prediction using PAM analysis of exon or gene level- derived signatures.

Values are presented as the fraction of the total for accuracy, sensitivity and specificity. The signature was derived from the PAM feature selection process described in the methods sections. A published gene set consisting of 59 genes was used to predict patient outcome in PAM59 using either the gene- or exon-based expression data (Vermeulen *et al*, 2009a). Only patients with maximum divergent courses were included in the PAM-MDC analysis to test the signature derived from PAM.

Table 3: Multivariate analyses identified gene- and exon-based signatures as independent prognostic markers for overall survival.

Predictive power of mRNA based signatures were compared to age at diagnosis (Age, < or > 12 months at diagnosis), *MYCN* status (MNA, *MYCN* amplified vs. *MYCN* single copy), INSS stage (INSS, stage IV vs. non-stage IV), or *NTRK1* expression (TrkA, *NTRK1* expression < or > median). Odds ratios and 95% confidence intervals are presented.

Table 4: Association of clinical and biological features with differential gene and exon expression.

INSS stage (**Stage**, stage IV vs. non-stage IV), age at diagnosis (**Age**, < or > 12 months at diagnosis), *MYCN* status (**MNA**, *MYCN* amplified vs. *MYCN* single copy), outcome (**Survival** or **Relapse** refer to overall survival and relapse-free survival) or *NTRK1* expression (**NTRK1/TrkA** expression < or > median) were analysed using Welch's t-test for significant association with gene (#diff expr genes) or exon (#diff expr exons) expression. To identify alternative exon use only genes without significant expression changes between the group analysed were considered and then exons were identified that were significantly differentially expressed (#alt used exons).

Fig. 1: Significant association of clinical and biological features with outcome (overall survival) as visualised by Kaplan-Meier analyses. 1A: *MYCN* status (*MYCN* sc = *MYCN* single copy; MNA= *MYCN*-amplified); 1B: metastatic spread (local vs. metastatic); 1C: Age at diagnosis (Age < or > 1yr at diagnosis); 1D: TrkA expression (TrkA expression < or > median).

Fig. 2: Association of alternative exon usage in *NTRK1* with outcome. **A:** The heat map shows *NTRK1* exon-level expression (rows, exon 1 on top) in our patient cohort. Exon 6/7 depicts the exons, for which the respective probes on the array are located. Red indicates exon upregulation and green indicates downregulation of an exon as compared to the median expression of the respective exon across all patients in our study. High *NTRK1* expression was also correlated with young (<1yr) patient age. Age= patient age at diagnosis; green > 1 yr; red < 1yr. **B:** Differential association of *NTRK1* exon expression with event-free survival (EFS)

or relapse. Left: NTRK1 exon 2 expression is high in EFS and low in patients with relapse ($p = 9 \times 10^{-15}$). Right: correlation between NTRK1 exon 6 expression in NB patients with EFS is less pronounced although still significant ($p = 0.002$). **C**: Real-time RT-PCR of NTRK1 exon 6 shows preferential expression in patients with EFS when compared to patients who experienced relapse (M= marker, - and + depict the negative and positive controls, respectively).

Fig. 3: Expression pattern of the INCENP gene in primary NB and in *in vitro* NB models. **A**: A shorter INCENP isoform comprising only exons 1-5 (NM_020238) was preferentially expressed in MYCN-amplified patients, while expression differences were less pronounced for the longer INCENP transcript NM_020238_2. Data are presented as log₂ of mean expression in each group +/- standard error of the mean. **B**: Expression of INCENP exon 5 was significantly elevated in MYCN-amplified patients (MNA) when compared to patients with normal MYCN (MYCNsc), while expression of exon 15 was not (**C**). **D**: Semiquantitative real-time PCR revealed that expression of INCENP exons 1-2 and 10-11 was upregulated *in vitro* in a MYCN-negative NB cell lines, SH-EP, upon MYCN overexpression (WAC2) or MYCN induction (SH-EP MYCN+).

Fig. 4: Differential transcript expression of the cell-cycle associated genes, p15INK4B (CDKN2B) and cyclin B1 (CCNB1). **A**: CDKN2B isoforms are differentially regulated between tumours with and without MYCN amplification, but not between tumours from patients with event-free survival (EFS) and non-survivors (DoD). **B**: A short isoform of CCNB1 (red line) is preferentially expressed in tumours from non-survivors (DoD). For both, A and B, data are presented as log₂ of mean expression in each group +/- standard error of the mean. **C**: Higher

expression of this shorter CCNB1 isoform was also detected in NB cell lines. **D:** Inhibition of the CCNB1/Cdk1 interaction by a small molecule inhibitor induced apoptosis in these cell lines.

Figure 1

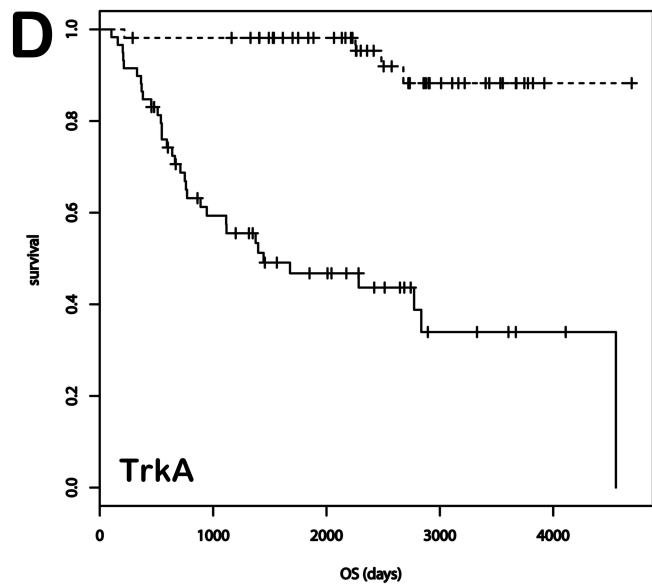
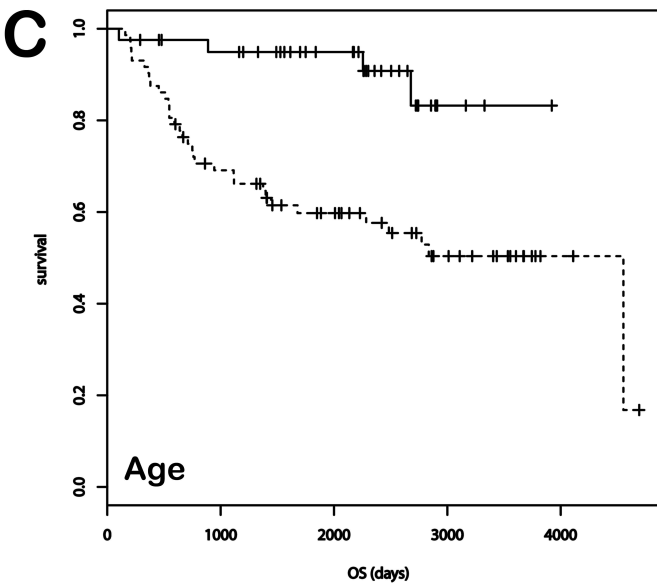
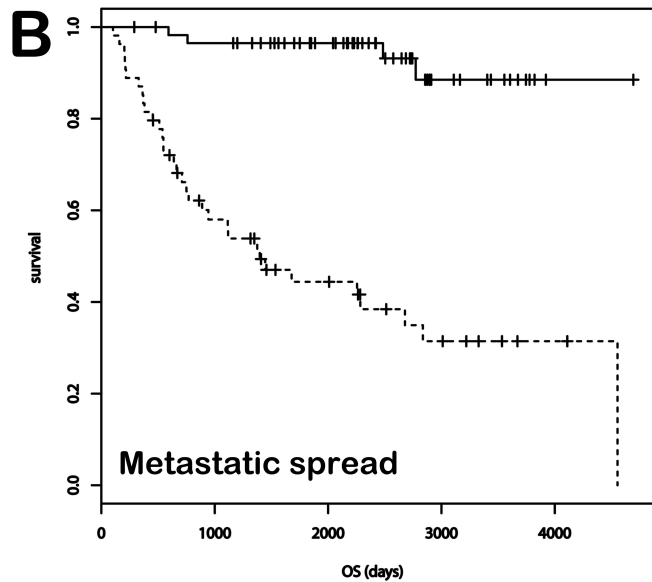
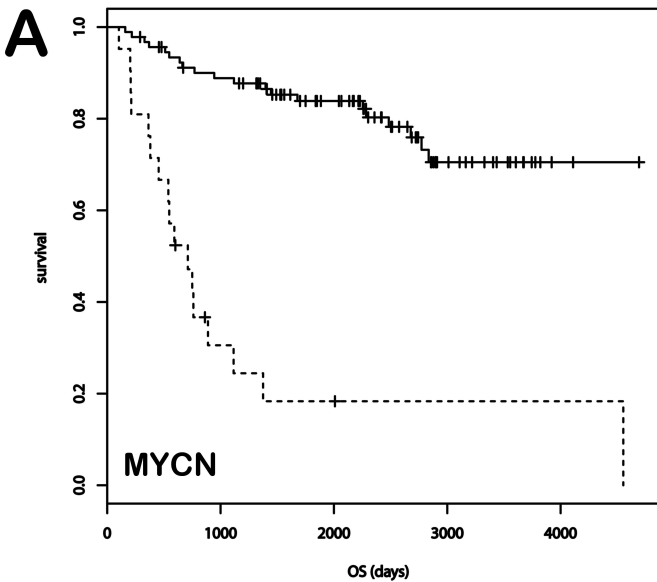


Figure 2

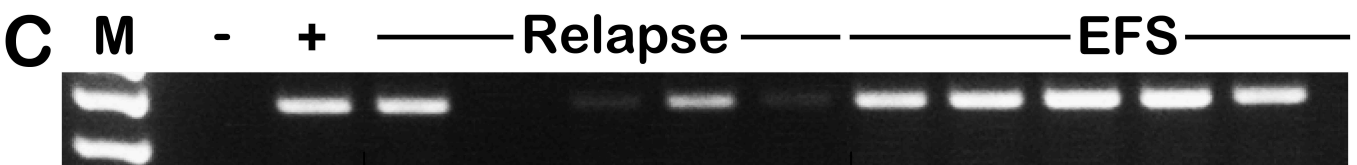
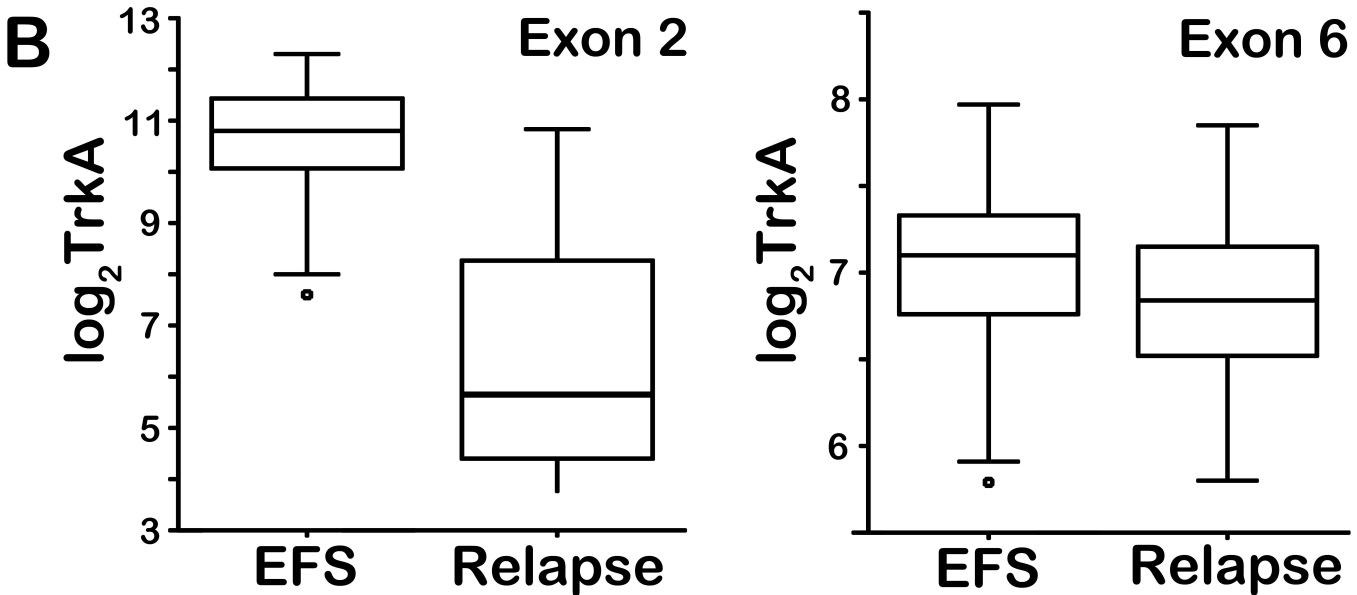
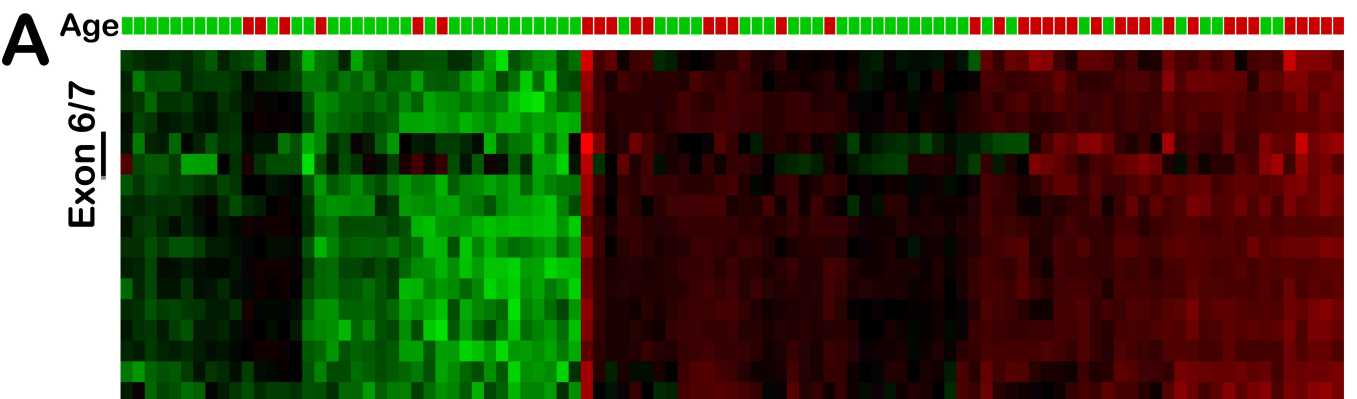
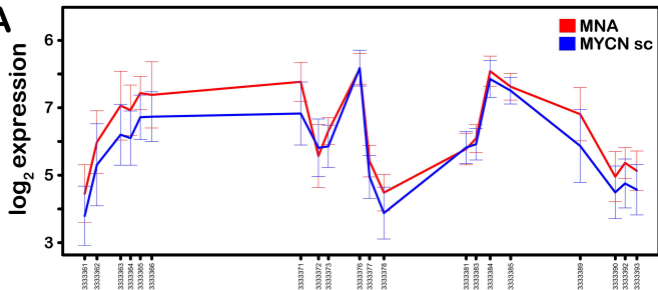
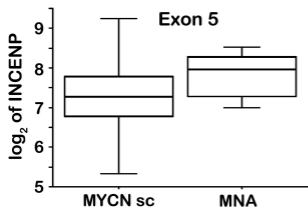


Figure 3

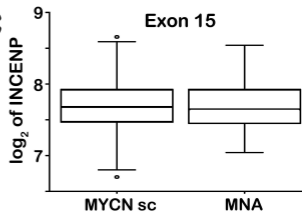
A



B



C



D

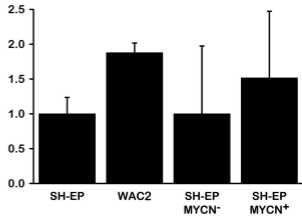
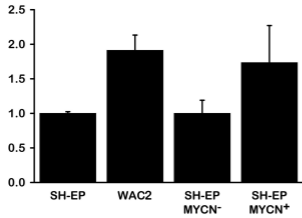
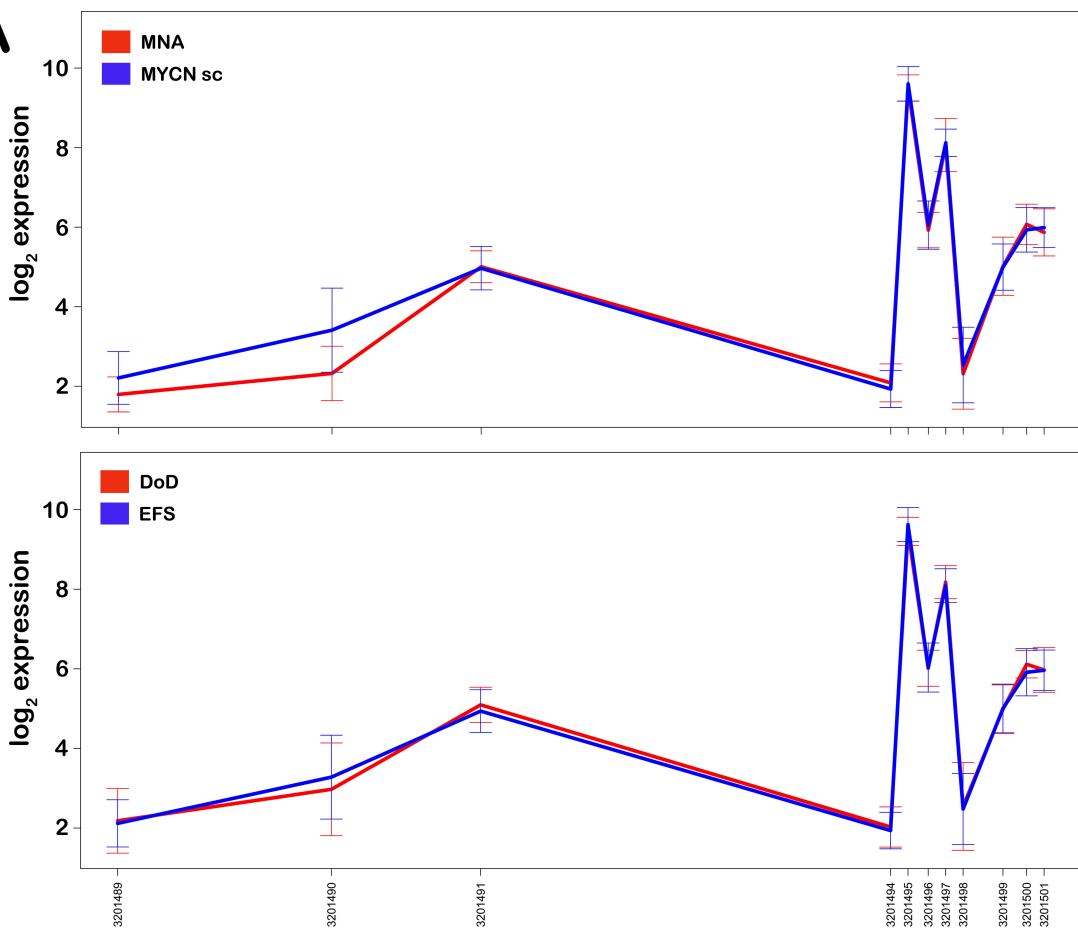
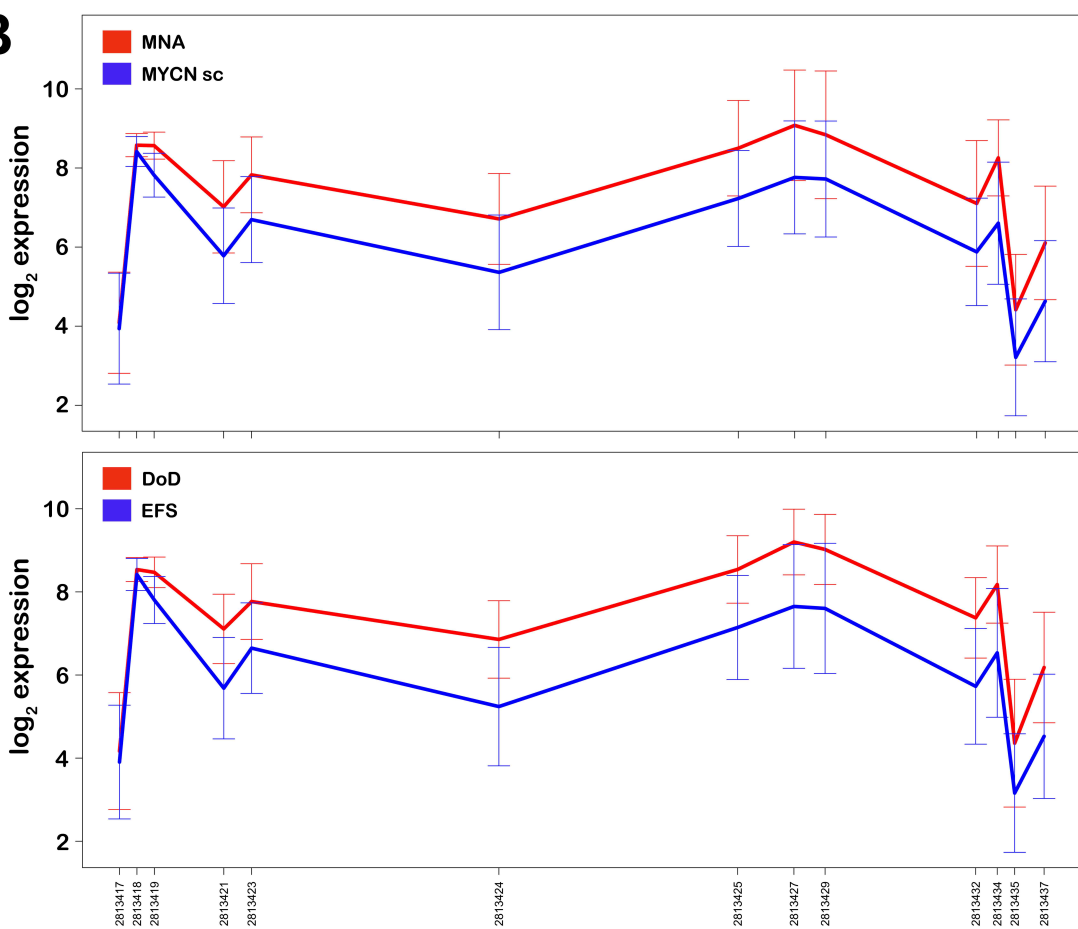


Figure 4

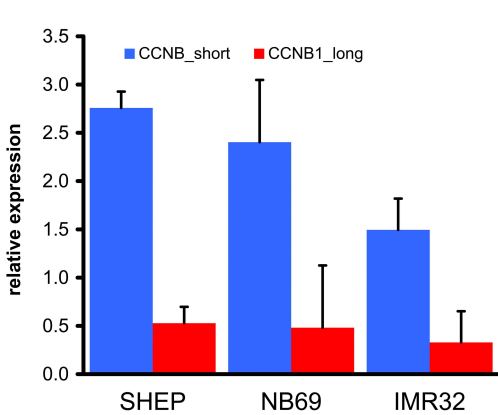
A



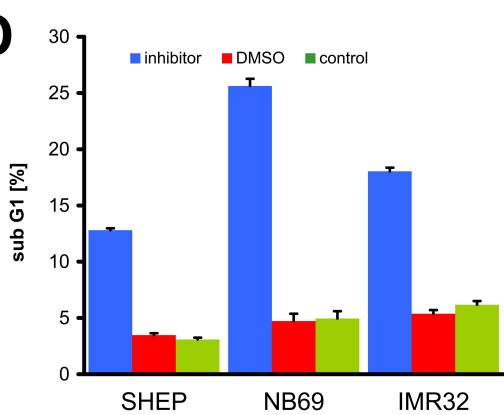
B



C



D



		Number	% of total
Age (year)	<1	41	36.2
	>1	72	63.8
INSS stage	1	31	27.4
	2	11	9.8
	3	15	13.2
	4	39	34.5
	4s	17	15.1
MYCN status	<i>normal</i>	92	81.4
	<i>amplified</i>	21	18.6
Event-free survival	<i>yes</i>	75	66.3
	<i>no</i>	38	33.7
Overall survival	<i>yes</i>	87	77
	<i>no</i>	26	23

Table01

Prediction of OS

	Gene-based			Exon-based		
	Prediction accuracy	Sensitivity	Specificity	Prediction accuracy	Sensitivity	Specificity
PAM	83.1%	84.7%	82.6%	83.6%	86.5%	82.8%
PAM59	86.1%	79.6%	89.4%	84.9%	80.8%	86.9%
PAM-MDC	89.2%	85.2%	91.4%	88.8%	85.2%	90.7%

Table02

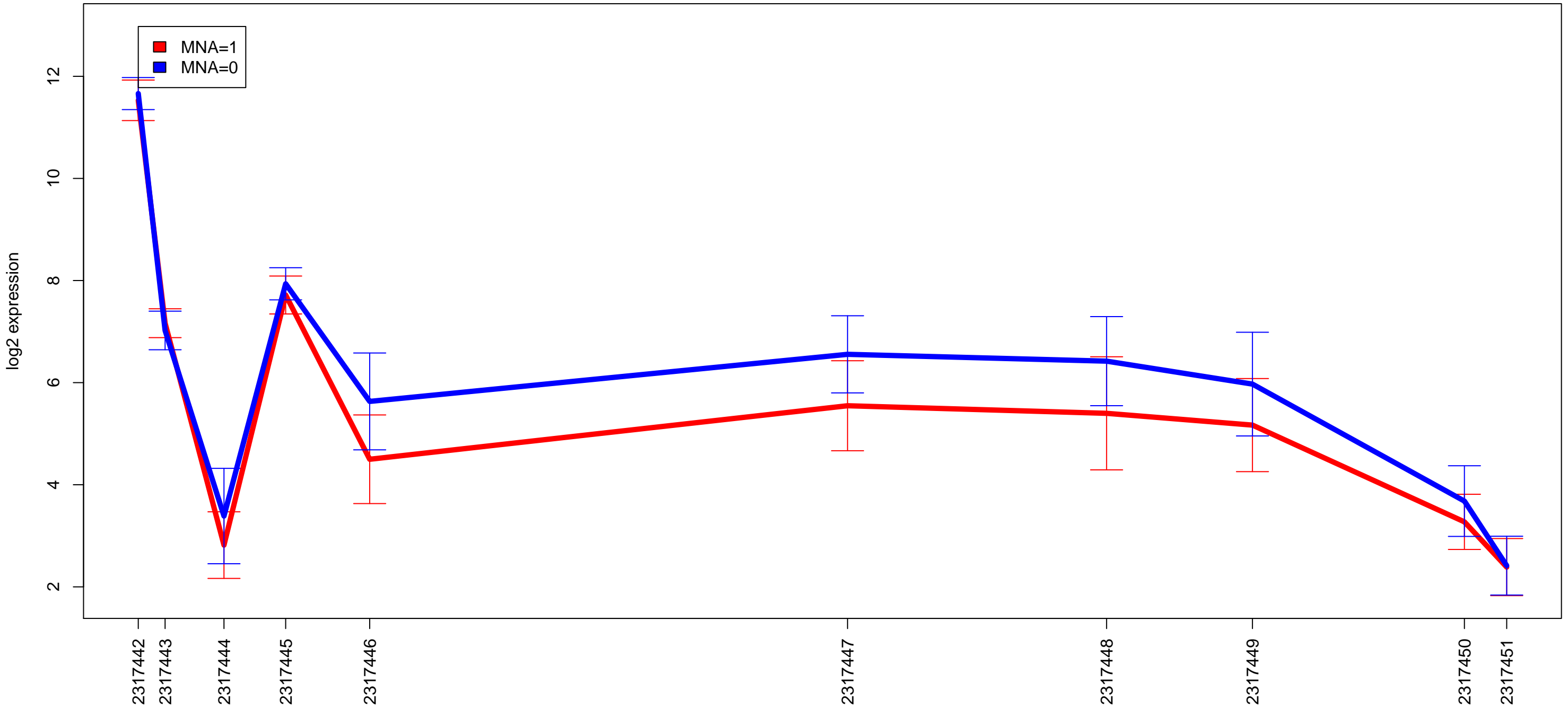
	p-value	odds ratio	95% confidence interval	
Age	0.42	2.2	0.3	18.9
MNA	0.36	1.9	0.5	8.1
TrkA	0.42	2.8	0.3	64.8
INSS Stage IV	0.10	3.3	0.8	14.4
Exon signature	0.006	10.7	2.2	71.0
Age	0.33	2.6	0.4	21.8
MNA	0.31	2.1	0.5	8.5
TrkA	0.39	2.9	0.3	66.9
INSS Stage IV	0.09	3.4	0.9	14.8
Gene signature	0.007	9.7	2.1	61.3

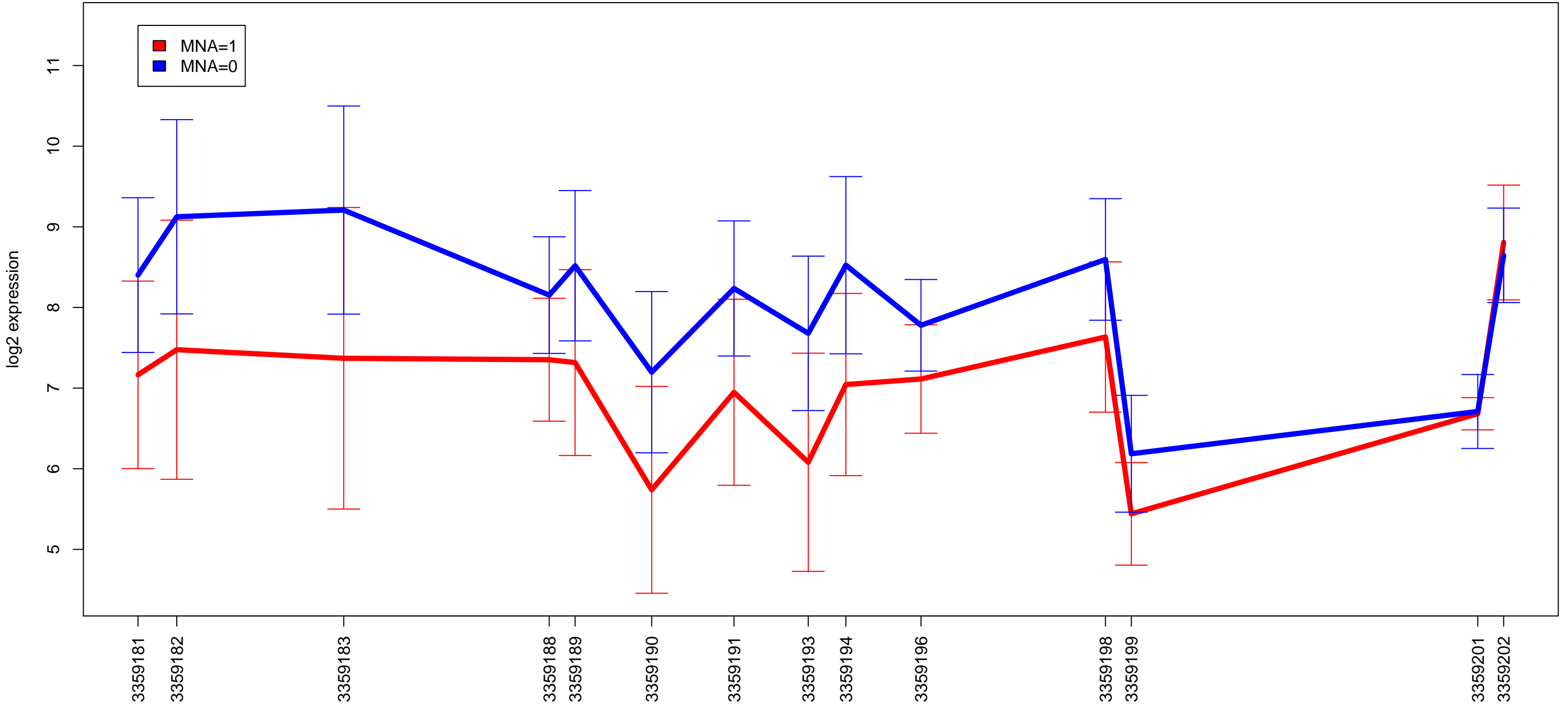
Table03

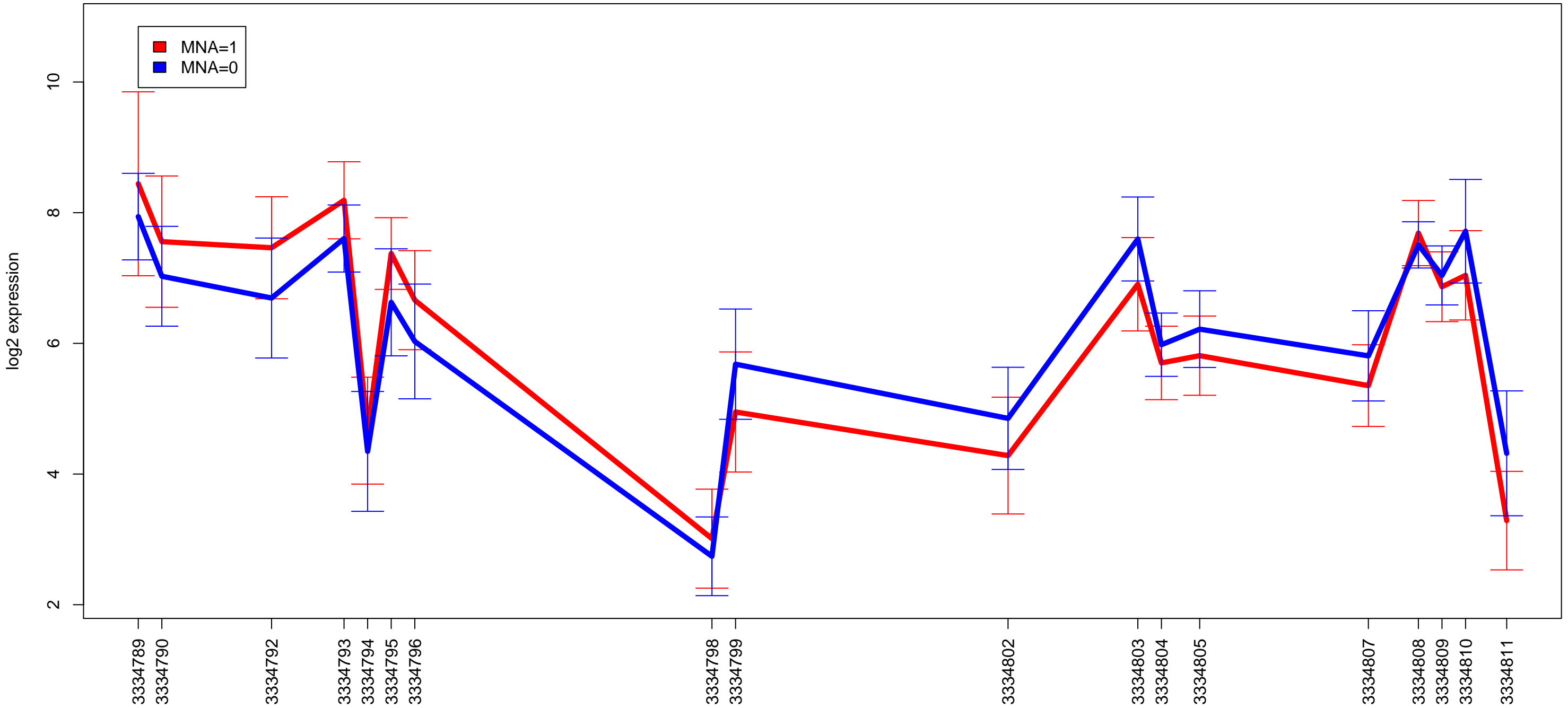
Class	# differentially expressed genes	# differentially expressed exons	# alternatively used exons
Stage	1277	11805	41
Age	472	5230	0
MNA	2278	23382	256
Survival	3936	34475	306
Relapse	1343	13165	31
TrkA	2748	32681	471

Table04

TPRG1L







Legend to supplementary figure 1

Representative examples of alternative transcript use of genes associated with MYCN amplification in primary NB. Blue lines represent log₂ of mean expression in MYCN single copy tumors, while red lines represent log₂ of mean expression in MYCN amplified tumors. Probes are plotted relative to their position in the coding sequence according to Hg18. The minimum distance of two probes was set to 2% of the total gene length for the sake of readability of the individual probe designations.

A: The most 5' coding exons of the gene coding for TRPGL1 are equally expressed between MYCN-amplified and MYCN non-amplified tumors, while the 3' coding exons are significantly different between the two groups. **B:** The 5' coding exons of the gene coding for tyrosine hydroxylase (TH) are down-regulated in MYCN-amplified compared to MYCN non-amplified tumors, while the 3' coding exons are equally expressed between the two groups. **C:** The 5' coding exons of the gene coding for sorting nexin 15 (SNX15) are down-regulated in MYCN single copy tumors compared to MYCN non-amplified tumors, while the 3' coding exons are vice versa up-regulated in MYCN single copy tumors compared to MYCN non-amplified tumors.