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Review Article

Global genomic and RNA profiles for novel risk stratification of neuroblastoma

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Neuroblastoma is one of the most common solid tumors in children. Its clinical behavior ranges widely from spontaneous regression to life-threatening aggressive growth. The molecular etiology of neuroblastoma is still enigmatic and the overall cure rate of advanced disease is still very poor. Recent microarray-based technology provided us with important information such as comprehensive genomic alterations and gene expression profiles to help us understand the molecular characteristics of each tumor in detail. Several retrospective studies have revealed that these signatures are strongly correlated with patient prognoses and led to the construction of new risk stratification systems, some of which are considered for evaluation in upcoming clinical studies in a prospective way. Large-scale analyses using a variety of genetic tools also discovered a major familial neuroblastoma predisposition gene *ALK,* as well as new candidate susceptibility genes at 6q22 and 2q35 for sporadic neuroblastoma. Of note, *ALK* is mutated in 6–9% of sporadic cases, and is either amplified or constitutively activated through mutations mainly within the kinase domain, promoting the possibility of new therapeutic strategies using ALK inhibitors. Additional candidates for outcome predictors such as the methylation phenotype of tumor DNA and expression profiles of microRNA have also been proposed. Such variety of information will help us understand the heterogeneity of neuroblastoma biology and further, the combined use of these signatures will be beneficial in predicting prognosis with high accuracy, as well as choosing a suitable therapy for the individual patient. (*Cancer Sci* 2010; 101: 2295–2301)

Neuroblastoma is one of the most common pediatric solid tumors, which accounts for 15% of all pediatric cancer deaths. It originates from the sympathoadrenal lineage derived from the neural crest and clinical behavior is markedly heterogeneous.(1) Tumors found in patients under 1 year of age are mostly favorable and often show spontaneous differentiation or regression, whereas tumors found in patients over 1 year of age tend to grow aggressively and often have a fatal outcome.(1) Recent development of chemotherapy has dramatically increased the survival rates of many pediatric cancers; however, advanced stage neuroblastoma, especially those with genomic amplification of the *MYCN* oncogene, are frequently resistant to any therapy and the outcome for patients is still very poor.(1,2)

Table 1 shows the 10-year survival rates for patients in each stage whose tumors were clinically found in Japan and sent to Chiba Cancer Center Research Institute from 1995 to 2007. In our dataset, *MYCN*-amplified cases (n = 83) showed only a 29% long-term survival rate despite intensive multimodal therapy, while *MYCN* non-amplified cases (n = 260) displayed 65% survival. Furthermore, part of the neuroblastomas categorized in the intermediate-risk group (stage 3 or 4 tumors that possess a single copy of the *MYCN* gene) often recur after a complete response to the initial therapy. Such differences in the final outcome of patients with neuroblastoma are presumably considered attributable to differences in the genetic and biological characteristics that are reflected in the gene and protein expression profiles of the tumor.

Thus, neuroblastoma is still one of the most challenging tumors to treat. A better understanding of the molecular characteristics of neuroblastoma and a novel therapeutic strategy, which is most effective for each tumor subset, combined with precise tumor risk classification are required for improvement of the cure rate, as well as the quality of life of the patients. In this review, we summarize recent efforts to construct a novel tumor-risk stratification system for neuroblastoma based on the latest genome-wide genetic and gene expression profiling assays.

Conventional risk markers for neuroblastoma

Early studies based on cytogenetics have presented several prognostic markers such as DNA ploidy,(3) *MYCN* amplification,(4,5) and gains of chromosome arms 1q, 2p and 17q, as well as allelic losses of 1p, 3p and 11q.(6,7) Many gene expression markers have also been reported so far through comparison between neuroblastoma subsets with good and poor prognosis,(8,9) or based on neuronal functions; the genes whose mRNA expression is high in the favorable type of neuroblastoma include *TRKA*, *CD44*, *pleiotrophin*, *N-cadherin*, *H-RAS, ECEL1*, *NLRR3*, *BMCC1, NEDL1* and *ZNF423,*(10–18) and those in the unfavorable ones include *TRKB*, *TERT*, *CDC10*, *NLRR1*, *HEN2* and *LMO3*.(10,19–22) Several positional candidate genes such as *SVV/BIRC5*, *NM23-H1* and *NM23-H2* and *PPM1D* on 17q, whose expression levels are higher in advanced neuroblastomas, have also been identified.(23–26) Similarly, *TSLC1/IGSF4/CADM1,* which is mapped to 11q and known as a tumor suppressor gene for lung cancer, showed lower expression in advanced neuroblastomas.(27,28)

Gene function-based approaches identified several important signaling pathways strongly affecting tumor progression and treatment resistance. In addition to the Trk family, the PI3K/Akt pathway, Ret, HGF/c-Met pathway and Src family kinases such as Fyn and Shc family were reported to be closely associated with several prognostic factors and biological characteristics of neuroblastoma cells.(29–32) Because *MYCN* amplification is a strong poor prognostic factor, many researchers have been interested in searching for the genes transcriptionally regulated by the MYCN transcription factor to understand the molecular

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Table 1. Long-term survival rates of neuroblastoma in each international neuroblastoma staging system (INSS) stage

INSS stage	No. patients	Survival† (%)	*MYCN* amplification
1	48	100	0
2	29	92.6	3
3	60	70.4	14
4	179	33.3	64
4s	27	59.3	2
Total	343		83

†Ten-year survival rate.

characteristics in tumors with and without *MYCN* amplification.[33,34]

Thus, certain numbers of markers have been proposed; however, each single marker has still not been sufficient to be newly included in clinical practice. Therefore, current clinical studies using many divergent therapeutic strategies have adopted only limited clinical markers with a strong prognostic impact, including: patient age at diagnosis with two age cut-offs of 12 and 18 months;[35,36] disease stage defined by the international neuroblastoma staging system (INSS);[37] *MYCN* copy number and 1p loss; and sometimes add DNA ploidy and histopathological information[38] for risk stratification (US, Europe and Japan). To make an efficient tumor classification system, multiple genes that explain each character of tumor subsets need to be considered in a computational machine-learning approach that is recently sophisticated for cancer study.

Risk classification of neuroblastoma by gene expression signatures

Microarray technology enabled us to examine simultaneously enormous molecular features including gene expression and genome alterations in tumors and to construct a novel prognosis classifier by using those with high performance. One of the good practical examples is the 70 genes-based risk classification system for breast cancer, which was approved by the FDA as the first microarray-based biomarker for cancer diagnosis.[39] As for neuroblastoma, the study by Wei *et al.*[40] for the first time indicated that a gene-expression-based classifier can predict the prognosis of patients efficiently by profiling the 56 tumors using cDNA microarrays containing 42 578 cDNA clones (classification accuracy was 95% for 21 test samples).

We also conducted gene expression analysis of 136 neuroblastomas diagnosed in Japan by using a 5340 neuroblastoma-derived genes chip.[41] The top-ranked 70 prognosis-related genes were selected through machine learning with the survival information of patients and used to construct a computational algorithm for prognosis prediction. The algorithm calculates survival probabilities of each patient at 2 years or 5 years after diagnosis, which is indicated by a ''*posterior*'' value range from 0 to 1 (Fig. 1). The microarray classifier could predict patient prognosis with high efficiency (90% accuracy, 96% sensitivity and 90% specificity), and is revealed to be the only powerful predictor to classify intermediate-risk-type neuroblastoma (stage 3 or 4 without *MYCN* amplification, prediction accuracy was 86%), whereas the current clinical markers (age, stage and *MYCN*) exhibited only 64% accuracy.

Based on these results, we subsequently made a ''mini-chip'' carrying the top-ranked 200 genes for clinical use.[41] The independent test of 50 tumor samples for evaluation indicated high reproducibility as well as high efficiency (approximately 89%) for our chip system. This mini-chip test is now under clinical validation in a larger cohort in Japan. This work is the first to construct a clinically available DNA chip harboring prognosis-related genes specifically selected for prognosis prediction, which gave a highly reproducible result to those obtained by the chip for the original screening.

Oberthuer *et al.*[42] then indicated that the gene expression classifier may improve the risk estimation of current neuroblastoma trials. By using a customized oligonucleotide microarray comprising 10 163 probes, they constructed a 144-gene predictor from 77 samples, which could classify 174 patients more accurately than risk stratification of current trials from Germany, Japan and the United States ($P < 0.001$).

Asgharzadeh *et al.*[43] focused their work on 102 intermediate-risk neuroblastomas (with *MYCN* nonamplified stage 4 disease) using Affymetrix arrays containing 45 000 probes. This work describes the first gene-expression-based classifier specifically generated for metastatic neuroblastoma lacking *MYCN* amplification.

Although the precision and reproducibility of microarray analysis have been markedly improved, validation of microarray data obtained by different platforms has been necessary to evaluate selected genes using an independent technology such as quantitative real-time PCR (qPCR). Schram *et al.*[44] first applied the multiplex qPCR method to have their gene-expression-based classifier from 63 neuroblastoma patients. They showed qPCR was almost comparable, although with some exceptions, with results obtained using the Affymetrix platform, and that multiplex qPCR could provide a convenient and less expensive tool for routine application in a clinical setting.

To select reliable genes that will provide a stable prediction result to make better risk classification, meta-analysis of previously reported studies have also been conducted.[45,46] Chen *et al.*[45] compared the gene expression profiles of 42 neuroblastoma samples using both cDNA and the Affymetrix platforms and concluded that gene expression studies performed in different platforms could be integrated for prognosis analysis after removing the variation resulting from the different platforms. Quite recently, Vermeulen *et al.*[46] selected 59 genes as a prognosis classifier based on a re-analysis of seven published microarray gene expression studies combined with previously reported single-candidate prognostic genes, and conducted a large-scale retrospective study by applying qPCR to 30 training samples, 313 test samples and 236 blind validation samples. Their multigene-expression signature exhibited 85.4% accuracy, 84.4% sensitivity and 86.5% specificity for those samples.

Risk classification of neuroblastoma by genomic signatures

In the 1990s, loss of heterozygosity, fluorescence *in situ* hybridization, and comparative genomic hybridization (CGH) analyses have been used for detecting the chromosome alterations that occurred in neuroblastoma with approximately several ten megabases of resolution. From the 2000s, microarray-based technology combined with the CGH method (array CGH) enabled us to obtain genome-wide, high-resolution genomic information simultaneously (from 1 megabase to <10 kilobases). Early studies have used in-house bacterial artificial chromosome (BAC) array or cDNA microarray to identify novel cancer-related genes or crucial genome copy number alterations that determine distinct genetic subgroups for risk stratification.[47,48] Recent array CGH studies also strongly support the idea that neuroblastoma tumors can be categorized by the genomic signature into several subgroups with different alteration patterns and prognosis. To unveil DNA copy number alterations that characterize distinct subsets of neuroblastoma, we have conducted array CGH with a DNA chip carrying 2464 BAC clones (whose resolution was approximately 1 Mb) to examine genomic aberrations in 236 primary neuroblastomas in Japan (112 sporadic and 124 mass screening cases, Fig. 2).[49]

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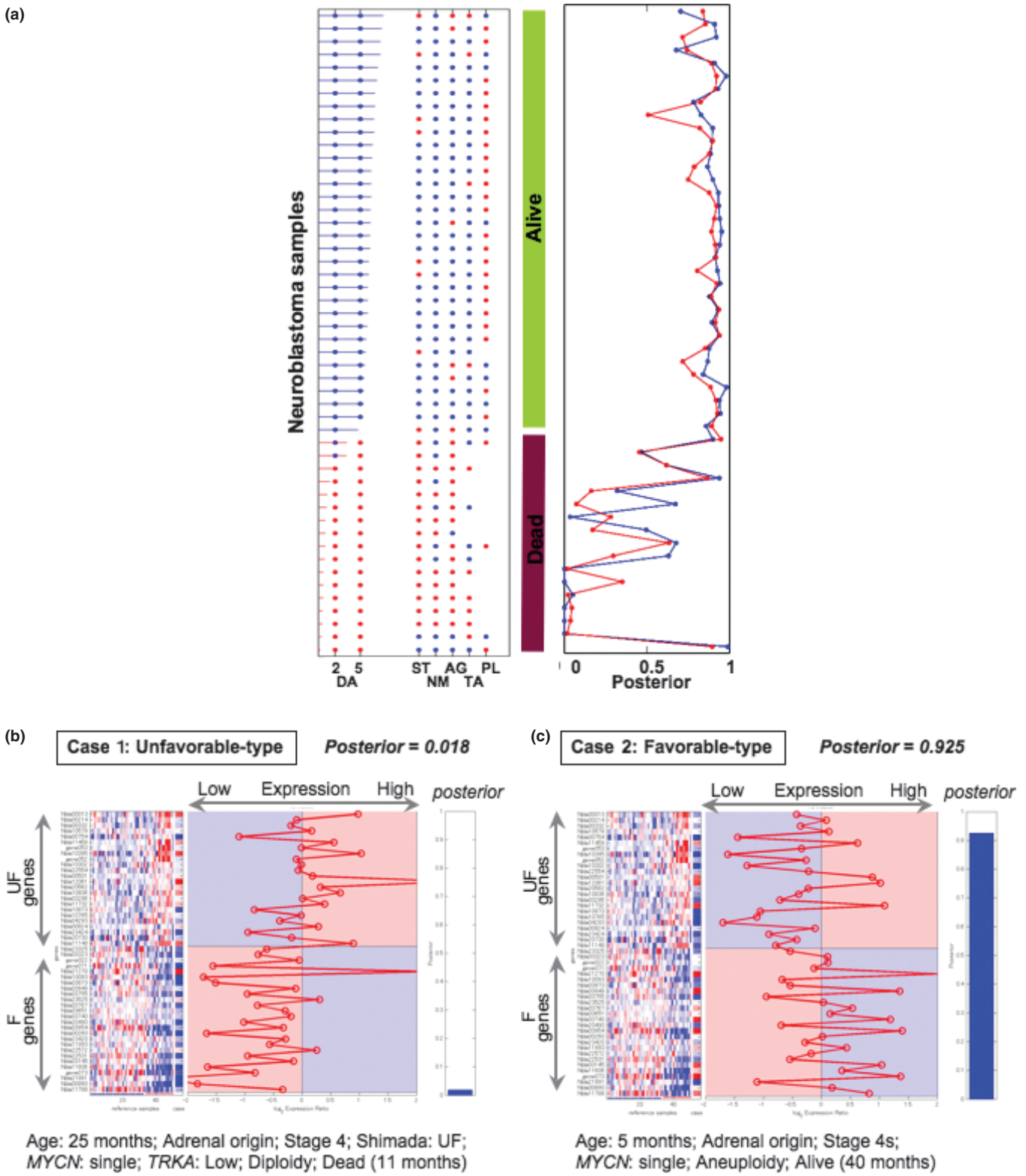

Fig. 1. Gene expression profile-based classifier to predict prognosis of the patient with neuroblastoma. (a) Posterior probability of survival at 5 years for 50 neuroblastomas measured by 200 genes-diagnostic mini-chip. Left panel: neuroblastoma samples with clinical information. DA, outcome (red, dead; blue, alive) at 2 years and 5 years after diagnosis; ST, international neuroblastoma staging system (INSS) stage (red, 3 or 4; blue, 1, 2 or 4s); NM, *MYCN* amplification (red, amplified; blue, not amplified); AG, age at diagnosis (red, ≥1 year; blue, <1 year); TA, *TRKA* expression (red, low; blue, high); PL, DNA ploidy (red, diploidy; blue, aneuploidy). A red or blue horizontal line denotes the survival period after diagnosis for a dead or alive patient, respectively. Right panel: prediction results when the supervised classifier constructed from 136 training samples is applied to the 50 independent samples (blue). Leave two out cross-validation analysis using the 50 samples (red). Higher value of posterior means a higher probability of a good prognosis. (b,c) Representative examples of the mini-chip test. Case 1 predicted as the unfavorable type. Posterior: 0.018. Case 2 predicted as the favorable type. Posterior: 0.925.

Our array CGH analysis demonstrated three major groups of genomic alterations in sporadic neuroblastomas (n = 112) that can well define the prognoses of neuroblastomas: a genetic group (GG) of silent with no obvious losses and gains except *MYCN* amplification (GG-S, n = 23; 5-year cumulative survival rate: 68%; DNA ploidy: 87% diploidy); that of partial chromosomal gains and/or losses (GG-P, n = 53; 43% survival; 77% diploidy); and that of whole chromosomal gains and/or losses (GG-W, n = 36; 80% survival; 22% diploidy). Further subcategorization of the three groups was based on signatures with strong correlation values to the prognosis (1p loss, *MYCN* amplification and 11q loss) resulting in several cohorts with highly contrasting outcomes (Fig. 2).

Statistical analyses of our two classifiers constructed by array CGH (GG-S, GG-P and GG-W) and gene expression profiling (*posterior* value)[41] in sporadic neuroblastomas showed that, in addition to the gene expression signature, the genomic signature is also a significant prognostic indicator for all and/or the intermediate risk-type of neuroblastoma (Table 2). More importantly, multivariate analysis indicated that these two signatures were mutually independent prognostic indicators (Table 2, bottom), especially among the patients with intermediate-risk-type tumors.[49] To validate this risk classification in a new, independent sample set, we started additional array CGH analysis by applying another genome platform (Agilent oligo-microarray and Affymetrix SNP chip) (Ohira *et al.*, manuscript in preparation).

Similarly, Janoueix-Lerosey *et al.*[50] examined 493 French patients by array CGH and indicated that analysis of the overall genomic pattern is essential to predict relapse in neuroblastoma patients. Their subclassification uses the structural alterations of ''segmental'' and ''numerical'' in addition to *MYCN* amplification. The resulting five subgroups have clearly differing outcomes. Tumors presenting exclusively numerical were associated with excellent survival, whereas the presence of segmental alterations with or without *MYCN* amplification was the strongest predictor of relapse.

In contrast to the gene expression microarray analysis, array CGH technology needs smaller amount of tumor materials and provides highly reproducible data even if different kinds or a lot of platforms are used. Considering that the gene expression signature and the genome alteration signature were independent risk predictors,[49] prospective studies using both gene expression and array CGH signatures will really be necessary to validate each potential in risk stratification for neuroblastoma.

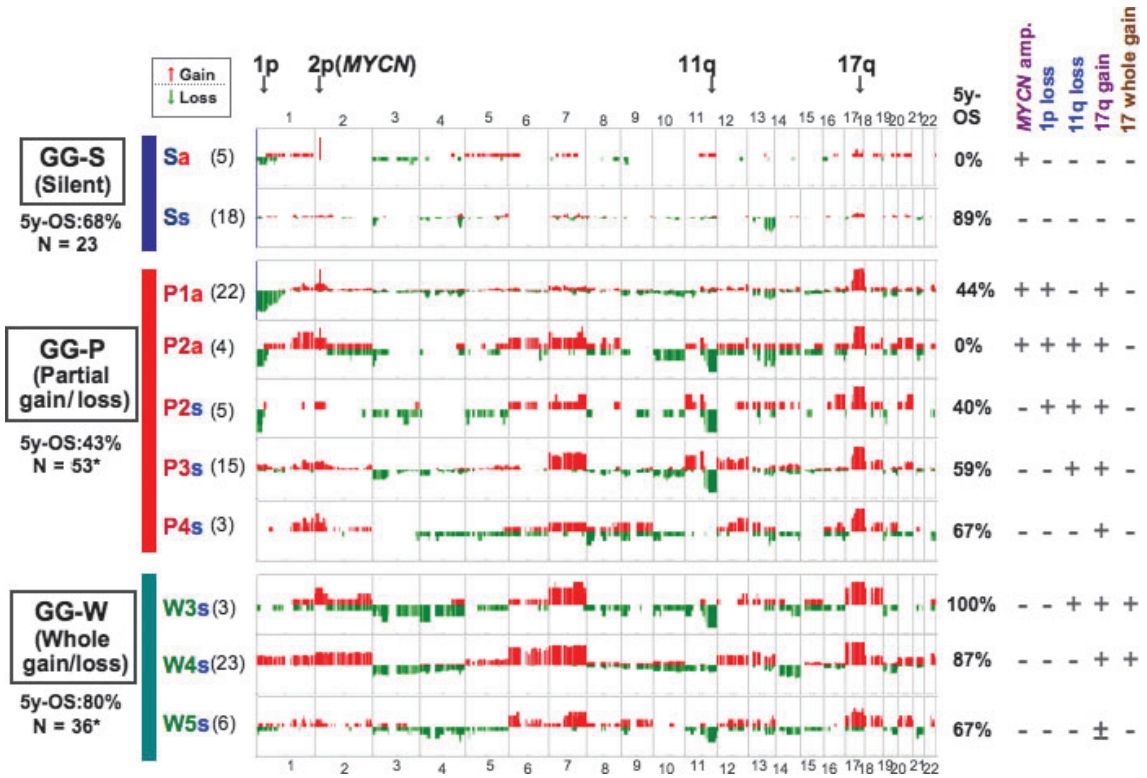

Fig. 2. Genome alteration-based risk classification of neuroblastoma. Representative genomic signatures of 112 sporadic neuroblastomas detected by array comparative genomic hybridization (CGH). In this figure, only the major subgroups (sample number in genomic subgroups ≥3) are shown. The panel in the middle shows 20 Mb-averaged frequencies of gains (upper panel, shown by red lines) and losses (lower panel, shown by green lines) at chromosome locations complementary to bacterial artificial chromosome (BAC) clones in each genomic subgroup. The right panel shows the 5-year overall survival rates (5y-OS), as well as the important features of chromosomal events including *MYCN* amplification, deletions of chromosomes 1p and 11q and gains of chromosome 17q or whole chromosome 17. Genomic groups (GG-S, GG-P, and GG-W) and subgroups (Sa, Ss, etc.) with the sample number involved in each group (*N*, total sample number in each genomic group; *note that only representative groups are shown) and subgroup (in parenthesis) are also indicated on the left. a, *MYCN* amplified; s, *MYCN* non-amplified. Note that GG-S, GG-P and GG-W corresponded well with the pattern of chromosome 17 abnormalities, namely, no gain of either chromosome 17 or 17q, gain of chromosome 17q and gain of whole chromosome 17, respectively. Subgroup 1 harbors 1p loss but not partial 11q loss. Subgroup 2 has both 1p and 11q losses. Subgroup 3 has partial 11q loss but not 1p loss. Subgroup 4 has no partial 1p and 11q loss. Subgroup GG-W5 is the exception and has whole chromosomal gains and losses in several chromosomes, but not whole chromosome 17 gain. GG-W subgroups showed a favorable prognosis, except for two cases with *MYCN* amplification. Sa is very poor (5y-OS: 0%), whereas Ss showed favorable prognosis (5y-OS: 89%). Patients with GG-P tumors exhibited various survival rates but a lower range, from 0% to 67%; the P2s subgroup (5y-OS: 40%) had a worse survival rate than P3s (5y-OS: 59%). It may be that 1p and 11q loss may have a similar impact on patient survival and work additively to the prognosis. Only the P1a subgroup showed a better prognosis among Pa tumors (5y-OS: 44%). For more detail, see reference 49.

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Table 2. Clinical impact of genomic and gene expression signatures, as well as other conventional prognostic factors in sporadic neuroblastomas

	Sporadic neuroblastomas (*n* = 112)			
	n	*P*-value	HR	CI
Univariate analysis				
Array CGH signature (P *versus* W + S)	53 *vs* 59	0.003	2.6	(1.4, 4.9)
Gene exp. signature (Posterior <0.5 *vs* ≥0.5)	22 *vs* 18	<0.001	11.2	(2.5, 49.4)
Age at diagnosis (≥1 year old *versus* <1 year old)	74 *vs* 38	0.006	2.7	(1.2, 5.8)
INSS stage (3, 4 *vs* 1, 2, 4s)	73 *vs* 38	<0.001	4.9	(1.9, 12.5)
MYCN (Amplification *versus* Single copy)	36 *vs* 75	<0.001	4.0	(2.2, 7.4)
Multivariate analysis				
Array CGH signature (P *versus* W + S)	15 *vs* 25	0.045	2.9	(1.0, 8.3)
Gene exp. signature (Posterior <0.5 *vs* ≥0.5)	22 *vs* 18	0.002	7.5	(1.7, 33.4)

CGH, comparative genomic hybridization; CI, confidence interval; HR, hazard ratio; INSS, international neuroblastoma staging system; *n*, sample number; P, *P*-value.

ALK gain-of-function mutations in familial and sporadic neuroblastoma cases

A familial history of neuroblastoma is identified in 1–2% of cases. A genome-wide scan in neuroblastoma pedigrees and subsequent resequencing of candidate loci have identified a major familial neuroblastoma predisposition gene *Anaplastic lymphoma kinase* (*ALK*).[51] Somatic *ALK* mutations or amplifications were also identified in 6–9% of sporadic cases.[52–65] ALK is a receptor tyrosine kinase predominantly expressed in the developing nervous system. Most of the ALK mutations reported so far are localized within the kinase domain and are assumed to produce constitutively activated proteins. A number of neuroblastoma cell lines were also shown to harbor activating ALK mutations. According to the recent success of small molecule tyrosine kinase inhibitors in a certain subset of cancers, such as gefitinib in non-small-cell lung carcinoma with epidermal growth factor receptor (EGFR) mutations, a similar therapeutic approach based on inhibition of ALK-mediated signaling will be expected to target oncogenic ALK mutations in neuroblastoma. Several studies using the existing ALK inhibitor compounds have shown that neuroblastoma cell lines harboring different activating ALK mutations appear to respond differently to the agent. The reasons behind this differential response to the inhibitor are currently unclear, but may indicate differences in the genetic background, or reflect complex genetic predispositions acquired by these cells.[56]

Under the current protocols, patients with *ALK* mutation or amplification appear to have a worse prognosis. However, ALK abnormalities seem to highly correlate with *MYCN* amplification, so whether *ALK* mutation is an independent risk factor for poor prognosis or not needs to be further investigated in a large cohort.[52]

Loss-of-function germ line mutations have been detected in *PHOX2B*, a homeobox gene functioning as an important regulator of normal autonomic nervous system development, implicating this pathway in neuroblastoma initiation in a certain subset of cases.[57–59] However, subsequent studies for this gene have indicated that *PHOX2B* mutations explain only a small subset of hereditary neuroblastoma and are rare in sporadic neuroblastomas.

Genome-wide association study: risk alleles associated with malignant neuroblastoma

Since the majority of neuroblastomas arise without a family history of the disease, the hypothesis that multiple common DNA variations cooperate to increase the risk for neuroblastic malignant transformation was considered. By using a large number of samples from more than 1700 neuroblastoma patients, as well as over 4000 controls, Maris *et al.*[60,61] conducted a genome-wide association study (GWAS) of 550 000 SNP genotypes and identified common SNP alleles within the putative *FLJ22536* and *FLJ44180* genes at 6p22 and within the introns of the *BARD1* gene at 2q35, which are associated with sporadic neuroblastoma with malignant phenotypes. BARD1 is known to heterodimerize with the familial breast cancer gene product BRCA1 and is considered to be essential for the tumor suppressive function of BRCA1.

Copy number variations (CNV) have been shown to significantly influence mRNA expression levels and recent studies have described associations of CNV with some of the common diseases. Subsequent CNV-based GWAS indicated that a common CNV at 1q21.1 likewise contributes to neuroblastoma susceptibility, and that this CNV leads to an altered expression of *NBPF23*, a new member of the neuroblastoma breakpoint family (NBPF) genes.[62]

Ongoing studies are now focused on understanding the biological consequences of these common DNA variations in the developing sympathetic neuroblast and how these influence malignant transformation.

Epigenetic alterations

Recently, epigenetic alterations have been reported to be strongly related to the patient prognosis with neuroblastoma. Methylation of CpG islands (CGI), which is deeply involved in embryonic development and tissue differentiation, is one of the epigenetic alterations in cancer. According to early studies, promoters such as *RASSF1A*, *CASP8*, *BLU* and *DCR2* genes, were frequently hypermethylated in primary neuroblastomas and neuroblastoma cell lines.[63]

Abe *et al.*[64–66] first proposed that CpG island methylator phenotype (CIMP) in the tumor genome, which is defined sensitively by five CGI such as the *Protocadherin beta* family, is strongly associated with poor survival of patients with neuroblastoma with extremely high hazard ratios, reaching as high as 22 and 9.5 in Japanese and German cases, respectively. Strikingly, the CIMP was also a significant and strong prognostic marker among the cases without *MYCN* amplification.[65,66]

Very recently, a sensitive method to detect methylation of *RASSF1A* and *DCR2* promoters in circulating tumor-originated genomic DNA in serum has been developed.[67,68] Hypermethylation of these genes is strongly correlated with poor prognosis. This technique will also be available for other systems like genome copy number changes in the near future, so the diagnosis and risk classification of neuroblastoma will be quicker and less stressful for patients.

Expression profiles of non-coding RNA also correlate the neuroblastoma subtypes

MicroRNA (miRNA) is a class of small non-coding RNA that regulates gene expression at a post-transcriptional level.[69,70] Its deregulation has recently been implicated in the pathogenesis of neuroblastoma. For instance, miR-34a on chromosome 1p acts as a suppressor of oncogene in neuroblastoma,[71–73] whereas some miRNA, such as miR-17-5p, OncomiR-1 and miR-9, behave like oncogenes or metastasis-promoting genes through direct up-regulation by MYCN.[74–76] Expression profiling studies of miRNA have identified a set of miRNA that are differentially expressed between favorable and unfavorable tumor subtypes of neuroblastoma[74,76–78] and these expression profiles were also predicative of the clinical outcome, highlighting the potential for miRNA-mediated diagnostics and therapeutics. Bray and co-workers analyzed 145 neuroblastomas for miRNA expression (430 loci) by stem-loop RT qPCR.[79] They developed a signature from 15 miRNA that was predictive of overall survival with 73% sensitivity and 87% specificity in the 49 validation set of tumors.

The large non-coding RNA are another class of non-coding RNA that are usually produced by RNA polymerase II and lack a significant open-reading frame.[70] We have identified *ncRAN*, a novel large non-coding RNA transcript whose higher expression significantly correlated with a poor prognosis of patients ($P = 0.0002$), and was recognized as an independent prognostic factor from age, disease stage and *MYCN* expression.[80] Because a certain number of such long non-coding cDNA have been observed in our neuroblastoma cDNA library,[8,9,41] further analysis of such clones will be necessary to examine the role of long non-coding RNA in neuroblastoma biology.

Future directions

The era of personalized medicine is likely to see an escalation in the use of biomarkers to ensure cancer patients receive optimal treatment. Microarray contents are still getting more variations with higher resolution (genomes, SNP, transcripts, miRNA and proteins). In addition, new generation DNA sequencing technologies have been developed rapidly. In the next few years, we will have a lot of information about mutations of oncogenes and tumor suppressors, as well as detailed mRNA expression profiles to be integrated into future personalized medicine for neuroblastoma. Data obtained by these technologies will facilitate our understanding for the mechanism of heterogeneity in the clinical phenotype. The integration of multiple molecular profiles after sufficient validation by multivariate-statistical analyses will make tumor risk classification for neuroblastoma much more feasible.

References

1 Brodeur GM. Neuroblastoma: biological insight into a clinical enigma. *Nat Rev Cancer* 2003; **3**: 203–16.
2 Maris JM. The biologic basis for neuroblastoma heterogeneity and risk stratification. *Curr Opin Pediatr* 2005; **17**: 7–13.
3 Look AT, Hayes FA, Nitschke R, McWilliams NB, Green AA. Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. *N Engl J Med* 1984; **311**: 231–5.
4 Schwab M, Alitalo K, Klempnauer KH *et al.* Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 1983; **305**: 245–8.
5 Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984; **224**: 1121–4.
6 Bown N, Cotterill S, Lastowska M *et al.* Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med* 1999; **340**: 1954–61.
7 Attiyeh EF, London WB, Mosse YP *et al.* Chromosome 1p and 11q deletions and outcome in neuroblastoma. *N Engl J Med* 2005; **353**: 2243–53.
8 Ohira M, Morohashi A, Inuzuka H *et al.* Expression profiling and characterization of 4200 genes cloned from primary neuroblastomas: identification of 305 genes differentially expressed between favorable and unfavorable subsets. *Oncogene* 2003; **22**: 5525–36.
9 Ohira M, Morohashi A, Nakamura Y *et al.* Neuroblastoma oligo-capping cDNA project: toward the understanding of the genesis and biology of neuroblastoma. *Cancer Lett* 2003; **197**: 63–8.
10 Nakagawara A, Arima M, Azar CG, Scavarda NJ, Brodeur GM. Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. *Cancer Res* 1992; **52**: 1364–8.
11 Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993; **328**: 847–54.
12 Nakagawara A, Milbrandt J, Muramatsu T *et al.* Differential expression of pleiotrophin and midkine in advanced neuroblastomas. *Cancer Res* 1995; **55**: 1792–7.
13 Tanaka T, Slamon DJ, Shimoda H *et al.* Expression of Ha-ras oncogene products in human neuroblastomas and the significant correlation with a patient's prognosis. *Cancer Res* 1988; **48**: 1030–4.
14 Kawamoto T, Ohira M, Hamano S, Hori T, Nakagawara A. High expression of the novel endothelin-converting enzyme genes, Nbla03145/ECEL1alpha and beta, is associated with favorable prognosis in human neuroblastomas. *Int J Oncol* 2003; **22**: 815–22.
15 Hamano S, Ohira M, Isogai E, Nakada K, Nakagawara A. Identification of novel human neuronal leucine-rich repeat (hNLRR) family genes and inverse association of expression of Nbla10449/hNLRR-1 and Nbla10677/hNLRR-3 with the prognosis of primary neuroblastomas. *Int J Oncol* 2004; **24**: 1457–66.
16 Machida T, Fujita T, Oo ML *et al.* Increased expression of proapoptotic BMCC1, a novel gene with the BNIP2 and Cdc42GAP homology (BCH) domain, is associated with favorable prognosis in human neuroblastomas. *Oncogene* 2006; **25**: 1931–42.
17 Li Y, Ozaki T, Kikuchi H, Yamamoto H, Ohira M, Nakagawara A. A novel HECT-type E3 ubiquitin protein ligase NEDL1 enhances the p53-mediated apoptotic cell death in its catalytic activity-independent manner. *Oncogene* 2008; **27**: 3700–9.
18 Huang S, Laoukili J, Epping MT *et al.* ZNF423 is critically required for retinoic acid-induced differentiation and is a marker of neuroblastoma outcome. *Cancer Cell* 2009; **15**: 328–40.
19 Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. *Mol Cell Biol* 1994; **14**: 759–67.
20 Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, Shay JW. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* 1995; **1**: 249–55.
21 Hossain MS, Ozaki T, Wang H *et al.* N-MYC promotes cell proliferation through a direct transactivation of neuronal leucine-rich repeat protein-1 (NLRR1) gene in neuroblastoma. *Oncogene* 2008; **27**: 6075–82.
22 Aoyama M, Ozaki T, Inuzuka H *et al.* LMO3 interacts with neuronal transcription factor, HEN2, and acts as an oncogene in neuroblastoma. *Cancer Res* 2005; **65**: 4587–97.
23 Islam A, Kageyama H, Takada N *et al.* High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. *Oncogene* 2000; **19**: 617–23.
24 Godfried MB, Veenstra M, v Sluis P *et al.* The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. *Oncogene* 2002; **21**: 2097–101.
25 Okabe-Kado J, Kasukabe T, Honma Y, Hanada R, Nakagawara A, Kaneko Y. Clinical significance of serum NM23-H1 protein in neuroblastoma. *Cancer Sci* 2005; **96**: 653–60.
26 Saito-Ohara F, Imoto I, Inoue J *et al.* PPM1D is a potential target for 17q gain in neuroblastoma. *Cancer Res* 2003; **63**: 1876–83.
27 Ando K, Ohira M, Ozaki T *et al.* Expression of TSLC1, a candidate tumorsuppressor gene mapped to chromosome 11q23, is downregulated in unfavorable neuroblastoma without promoter hypermethylation. *Int J Cancer* 2008; **123**: 2087–94.
28 Nowacki S, Skowron M, Oberthuer A *et al.* Expression of the tumour suppressor gene CADM1 is associated with favourable outcome and inhibits cell survival in neuroblastoma. *Oncogene* 2008; **27**: 3329–38.
29 Berwanger B, Hartmann O, Bergmann E *et al.* Loss of a FYN-regulated differentiation and growth arrest pathway in advanced stage neuroblastoma. *Cancer Cell* 2002; **2**: 377–86.

doi: 10.1111/j.1349-7006.2010.01681.x

30 Miyake I, Hakomori Y, Shinohara A *et al.* Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines. *Oncogene* 2002; **21**: 5823–34.
31 Miyake I, Ohira M, Nakagawara A, Sakai R. Distinct role of ShcC dockingprotein in the differentiation of neuroblastoma. *Oncogene* 2009; **28**: 662–73.
32 Terui E, Matsunaga T, Yoshida H *et al.* Shc family expression in neuroblastoma: high expression of shcC is associated with a poor prognosis in advanced neuroblastoma. *Clin Cancer Res* 2005; **11**: 3280–7.
33 Boon K, Caron HN, van Asperen R *et al.* N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. *EMBO J* 2001; **20**: 1383–93.
34 Alaminos M, Mora J, Cheung NK *et al.* Genome-wide analysis of gene expression associated with MYCN in human neuroblastoma. *Cancer Res* 2003; **63**: 4538–46.
35 Evans AE, D'Angio GJ, Randolph J. A proposed staging for children with neuroblastoma. Children's cancer study group A. *Cancer* 1971; **27**: 374–8.
36 London WB, Castleberry RP, Matthay KK *et al.* Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. *J Clin Oncol* 2005; **23**: 6459–65.
37 Brodeur GM, Pritchard J, Berthold F *et al.* Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993; **11**: 1466–77.
38 Shimada H, Ambros IM, Dehner LP *et al.* The International Neuroblastoma Pathology Classification the Shimada system. *Cancer* 1999; **86**: 364–72.
39 van't Veer LJ, Dai H, van de Vijver MJ *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; **415**: 530–6.
40 Wei JS, Greer BT, Westermann F *et al.* Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma. *Cancer Res* 2004; **64**: 6883–91.
41 Ohira M, Oba S, Nakamura Y *et al.* Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas. *Cancer Cell* 2005; **7**: 337–50.
42 Oberthuer A, Berthold F, Warnat P *et al.* Customized oligonucleotide microarray gene expression-based classification of neuroblastoma patients outperforms current clinical risk stratification. *J Clin Oncol* 2006; **24**: 5070–8.
43 Asgharzadeh S, Pique-Regi R, Sposto R *et al.* Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification. *J Natl Cancer Inst* 2006; **98**: 1193–203.
44 Schramm A, Vandesompele J, Schulte JH *et al.* Translating expression profiling into a clinically feasible test to predict neuroblastoma outcome. *Clin Cancer Res* 2007; **13**: 1459–65.
45 Chen QR, Song YK, Wei JS *et al.* An integrated cross-platform prognosis study on neuroblastoma patients. *Genomics* 2008; **92**: 195–203.
46 Vermeulen J, De Preter K, Naranjo A *et al.* Predicting outcomes for children with neuroblastoma using a multigene-expression signature: a retrospective SIOPEN/COG/GPOH study. *Lancet Oncol* 2009; **10**: 663–71.
47 Mosse YP, Greshock J, Margolin A *et al.* High-resolution detection and mapping of genomic DNA alterations in neuroblastoma. *Genes Chromosomes Cancer* 2005; **43**: 390–403.
48 Wang Q, Diskin S, Rappaport E *et al.* Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. *Cancer Res* 2006; **66**: 6050–62.
49 Tomioka N, Oba S, Ohira M *et al.* Novel risk stratification of patients with neuroblastoma by genomic signature, which is independent of molecular signature. *Oncogene* 2008; **27**: 441–9.
50 Janoueix-Lerosey I, Schleiermacher G, Michels E *et al.* Overall genomic pattern is a predictor of outcome in neuroblastoma. *J Clin Oncol* 2009; **27**: 1026–33.
51 Mosse YP, Laudenslager M, Longo L *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008; **455**: 930–5.
52 Chen Y, Takita J, Choi YL *et al.* Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008; **455**: 971–4.
53 Janoueix-Lerosey I, Lequin D, Brugieres L *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008; **455**: 967–70.
54 George RE, Sanda T, Hanna M *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008; **455**: 975–8.
55 Osajima-Hakomori Y, Miyake I, Ohira M, Nakagawara A, Nakagawa A, Sakai R. Biological role of anaplastic lymphoma kinase in neuroblastoma. *Am J Pathol* 2005; **167**: 213–22.
56 Rongshi L, Morris SW. Development of anaplastic lymphoma kinase (ALK) small-molecule inhibitors for cancer therapy. *Med Res Rev* 2008; **28**: 372–412.
57 Amiel J, Laudier B, Attie-Bitach T *et al.* Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat Genet* 2003; **33**: 459–61.
58 Mosse YP, Laudenslager M, Khazi D *et al.* Germline PHOX2B mutation in hereditary neuroblastoma. *Am J Hum Genet* 2004; **75**: 727–30.
59 Trochet D, Bourdeaut F, Janoueix-Lerosey I *et al.* Germline mutations of the paired-like homeobox 2B PHOX2B; gene in neuroblastoma. *Am J Hum Genet* 2004; **74**: 761–4.
60 Maris JM, Mosse YP, Bradfield JP *et al.* Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. *N Engl J Med* 2008; **358**: 2585–93.
61 Capasso M, Devoto M, Hou C *et al.* Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. *Nat Genet* 2009; **41**: 718–23.
62 Diskin SJ, Hou C, Glessner JT *et al.* CNV Copy number variation at 1q21.1 associated with neuroblastoma. *Nature* 2009; **459**: 987–91.
63 Yang Q, Kiernan CM, Tian Y *et al.* Methylation of CASP8, DCR2, and HIN-1 in neuroblastoma is associated with poor outcome. *Clin Cancer Res* 2007; **13**: 3191–7.
64 Abe M, Ohira M, Kaneda A *et al.* CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res* 2005; **65**: 828–34.
65 Abe M, Westermann F, Nakagawara A, Takato T, Schwab M, Ushijima T. Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett* 2007; **247**: 253–8.
66 Abe M, Watanabe N, McDonell N *et al.* Identification of genes targeted by CpG island methylator phenotype in neuroblastomas, and their possible integrative involvement in poor prognosis. *Oncology* 2008; **74**: 50–60.
67 Yagyu S, Gotoh T, Iehara T *et al.* Circulating methylated-DCR2 gene in serum as an indicator of prognosis and therapeutic efficacy in patients with MYCN nonamplified neuroblastoma. *Clin Cancer Res* 2008; **14**: 7011–19.
68 Misawa A, Tanaka S, Yagyu S *et al.* RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: a prognostic marker. *Br J Cancer* 2009; **100**: 399–404.
69 Mattick JS, Makunin IV. Non-coding RNA. *Hum Mol Genet* 2006; **15**: R17–19.
70 Prasanth KV, Spector DL. Eukaryotic regulatory RNAs: an answer to the 'genome complexity' conundrum. *Genes Dev* 2007; **21**: 11–42.
71 Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene* 2007; **26**: 5017–22.
72 Cole KA, Attiyeh EF, Mosse YP *et al.* A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene. *Mol Cancer Res* 2008; **6**: 735–42.
73 Wei JS, Song YK, Durinck S *et al.* The MYCN oncogene is a direct target of miR-34a. *Oncogene* 2008; **27**: 5204–13.
74 Fontana L, Fiori ME, Albini S *et al.* Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS ONE* 2008; **3**: e2236.
75 Ma L, Young J, Prabhala H *et al.* miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* 2010; **12**: 247–58.
76 Chen Y, Stallings RL. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res* 2007; **67**: 976–83.
77 Schulte JH, Horn S, Otto T *et al.* MYCN regulates oncogenic microRNAs in neuroblastoma. *Int J Cancer* 2008; **122**: 699–704.
78 Buckley PG, Alcock L, Bryan K *et al.* Chromosomal and microRNA expression patterns reveal biologically distinct subgroups of 11q-neuroblastoma. *Clin Cancer Res* 2010; **16**: 2971–8.
79 Bray I, Bryan K, Prenter S *et al.* Widespread dysregulation of miRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: association of miRNA expression with survival. *PLoS ONE* 2009; **4**: e7850.
80 Yu M, Ohira M, Li Y *et al.* High expression of ncRAN, a novel non-coding RNA mapped to chromosome 17q25.1, is associated with poor prognosis in neuroblastoma. *Int J Oncol* 2009; **34**: 931–8.