REVIEW ARTICLE

The role of omics in neuroblastoma: Patient’s risk classification and personalised therapy

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Abstract: Neuroblastoma is an embryonic malignancy of early childhood that originates from neural crest cells and shows heterogeneous biological, morphological, genetic, and clinical characteristics. MYCN oncogene amplification has been observed in 20% of neuroblastoma cases and is one of the most reliable prognostic markers of this tumour. In the last decade, array comparative genomic hybridization (aCGH) has been widely employed to discover genome abnormalities and to evaluate patient’s risk. Several numerical and structural copy number variations including the loss of 1p, 3p, 9p, 11q, and 14q, along with the gain of 2p and 17q, was observed to be mainly associated with high-risk neuroblastoma. Extensive studies have been carried out to identify gene signatures associated with tumour progression and at least two gene signatures, a 59-gene and a 146-gene signature, can be used to significantly discriminate between low- and high-risk patients. Subsequently, the advent of next-generation sequencing (NGS) has shown that neuroblastoma is characterised by a low number of damaging somatic mutations. Mutations occurring in ALK, ATRX, and TERT genes play a crucial role in neuroblastoma development. This raises the possibility of performing an NGS signature to refine/improve patients’ risk classification. Omics data have allowed us to improve the diagnostic of neuroblastoma and to identify biological targets that are suitable for precision medicine. The present review highlights the importance of omics in neuroblastoma and updates the most recent advances in this area that are associated with personalised medicine of patients with neuroblastoma.

Keywords: genomic alterations; next-generation sequencing; neuroblastoma; omics; personalised medicine; targeted therapy


Introduction

Neuroblastoma is the most frequently diagnosed extracranial solid tumour during early childhood. It accounts for 7% of all childhood malignancies and 10% of all childhood cancer-related deaths. Neuroblastoma is a clinically and biologically heterogeneous tumour, originating from primitive sympathetic neural precursor cells. About half of all neuroblastomas arise in the adrenal medulla, whereas the remaining cases are from the paraspinal sympathetic ganglia of the chest or abdomen, or from the pelvic ganglia. Neuroblastoma shows a wide range of clinical courses, either as a localised or metastatic disease. Neuroblastoma patients are divided into five different clinical stages (1, 2, 3, 4, and 4S), with great heterogeneity in clinical presentation.

Paediatric oncology groups in the United States, Europe, and Japan have established the International Neuroblastoma Risk Group (INRG) task force, whose goal is to identify clinical and biological prognostic factors for
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patient’s risk classification and subsequently, for treatment. The INRG stratification system uses a combination of diverse prognostic factors (i.e., clinical stage, patient’s age at diagnosis, tumour histology [Shimada system] [7], grade of tumour differentiation, MYCN oncogene amplification, 11q deletion, and DNA ploidy [8]). Based on these factors, neuroblastoma patients are subdivided into very low, low, intermediate, high, and ultra-high-risk groups.

The survival rates of children with high-risk (HR) and ultra-high-risk (UHR) neuroblastoma still show only modest improvement, notwithstanding the efforts of intensive therapy. Of particular interest are metastatic tumours in infants that may show spontaneous regression without any treatment, and with complete disease remission. In contrast, the five-year overall survival rate of older children with metastatic disease remains less than 40% despite complex multimodal treatment including chemotherapy, surgery, radiotherapy, and differentiation therapy [9].

Recent discoveries concerning the genetic basis of neuroblastoma and the analyses of omics data greatly improve patients’ risk stratification. Moreover, the knowledge of the entire human genome sequence and the development of high-throughput technologies have greatly helped in understanding the behaviour of neuroblastoma tumours. The present review reports the state-of-the-art omics studies in neuroblastoma and the use of genomics information to improve the therapy for children with this type of tumour.

The genomic landscape of neuroblastoma

Neuroblastoma predisposition

Familial neuroblastoma accounts for only 1%–2% of all cases. The paired-like homeobox 2b (PHOX2B) gene, located at chromosomal region 4p12, is the first neuroblastoma predisposition gene identified [10]. PHOX2B encodes a homeobox transcription factor involved in the regulation of neural development. Two heterozygous missense mutations in the homeodomain of PHOX2B have been reported: (i) one in a familial case (R100L mutation), and (ii) another in an isolated case of neuroblastoma associated with Hirschsprung disease (R141G mutation) [10,11].

The second major gene predisposing individuals to neuroblastoma – the anaplastic lymphoma tyrosine kinase receptor (ALK) – was found by the linkage analysis of a collection of families with recurrent neuroblastoma [12-16]. ALK maps to chromosome 2p23 and shows several point mutations. The ALK-F1174L and ALK-R1275Q mutations are the most frequent. So far, all mutations identified are in the tyrosine kinase domain and result in constitutive autophosphorylation of the ALK protein. Recently, whole exome sequencing (WES) of germline DNA revealed GALNT14 as a novel gene that is potentially involved in neuroblastoma predisposition [17]. GALNT14 is a member of the polypeptide N-acetylgalactosaminyl transferase family and maps closely to ALK on 2p23.1, a region previously linked to neuroblastoma.

Chromosomal aberrations

Genomic alterations such as gene amplifications, gains, and deletions frequently contribute to tumourigenesis as they change the level of gene expression, hence modifying normal cellular growth and survival. Historically, one of the first aberrations discovered in neuroblastoma was the MYCN (N-myelocytomatosis; OMIM 164840) oncogene amplification [18], which is observed in 20% of neuroblastoma cases. A close relationship between MYCN amplification and tumour aggressiveness throughout all disease stages has been demonstrated [19]. In 1995, neuroblastoma was one of the first cancers for which the International Society of Paediatric Oncology European Neuroblastoma (SIOPEN) released a therapeutic trial, wherein MYCN amplification was a deciding factor for therapy.

Subsequently, genome-wide studies of neuroblastoma tumours identified several numerical and/or structural chromosomal aberrations (NCAs and SCAs, respectively) that are correlated to the disease. The loss of chromosomes 1p, 3p, 11q, and 14q and the gain of 1q, 2p, and 17q are commonly observed in advanced neuroblastoma clinical stages [20-22]. Moreover, it has been demonstrated that the loss of 3p and 11q, along with the gain of 2p and 17q, is a predictor of poor survival [23,24]. The study of these alterations has provided molecular insight into neuroblastoma tumours and has enabled the identification of reliable and robust prognostic subsets of biomarkers that can be used for treatment stratification in clinical practice [25]. Consequently, SIOPEN released the Localised Intermediate Neuroblastoma European Study (LINES) in 2012, the first European trial in which therapy in a subgroup of patients is tailored according to the presence of structural chromosomal aberrations. The study is scheduled to continue until 2026, and the estimated date for the primary completion of data collection is December 2016.

Gene expression profiling

Gene expression profiling (GEP) analyses are used in the
diagnosis of different cancers and have led to the discovery of genomic prognostic markers that have improved the risk estimation of cancer patients. Examples of how GEP analyses contributed significantly to the diagnosis of several cancers include prostate cancer, breast cancer, kidney cancer, and some childhood cancers. In recent years, multiple gene signatures have been developed to improve neuroblastoma’s risk stratification and management. Furthermore, it is expected that gene signatures could help to resolve the misclassification of a patient’s risk. Specifically, past problems could be avoided by using this method, for example: (i) patients who were classified as having low- or intermediate-risk neuroblastoma receiving an inappropriately mild treatment and exhibiting poor outcomes; and (ii) patients who were wrongly classified as HR receiving a higher dosage of drugs that induced a high degree of toxicity.

However, no gene signature has been used as a prognostic factor in neuroblastoma until recently. Based on a very elaborate data-mining strategy, De Preter et al. have developed, validated, and implemented a robust multigene expression signature of 59 genes that could be used as a more accurate assessment of prognosis in children with neuroblastoma. The 59-gene signature was tested on 313 samples and then validated on an independent set of 236 tumours. Contrary to other gene signatures, the 59-gene signature acts as an independent risk predictor to enable the identification of neuroblastoma patients with increased risk. Furthermore, this signature presents some positive characteristics: (i) it is built starting from a small quantity of biological samples; (ii) it uses real-time quantitative polymerase chain reaction (qRT-PCR) as a quantification method; and (iii) it evaluates a small number of genes. Taken together, these characteristics make the 59-gene expression signature a tool that could be rapidly translated into the clinical management of neuroblastoma.

**Genomics of neuroblastoma**

Genomic studies have greatly improved owing to next-generation sequencing (NGS) techniques. The advent of NGS technologies such as gene-targeted sequencing, whole-genome sequencing (WGS), WES, and whole-transcriptome sequencing (RNA-seq) has notably improved the discovery of genomic alterations and molecular characterisation of diseases. Over time, the performance of these technologies has improved as a result of reductions in sample preparation time, data production, and cost. To date, WES and gene-targeted sequencing have been widely used for diagnostic purposes; both techniques are used to search for alterations (i.e., synonymous, missense, nonsense, and frameshift mutations, deletions, and insertions) in gene-coding sequences for Mendelian disease and cancers. Unfortunately, owing to the huge amounts of sequencing data and the time-consuming nature of data processing, bioinformatics analyses still remain the most problematic aspect in generating reliable results. Indeed, sequencing data are filtered using bioinformatics software and scripts to obtain a large number of single nucleotide variants (SNV). Particularly for cancer studies, it is necessary to take into consideration that genomic alterations are usually somatic, show low frequency, and are differently distributed in cellular subclones.

Neuroblastoma shows a mixture of malignant and non-malignant cells, thus making the analysis of somatic mutations difficult at times. To overcome the difficulties related to tumour tissue heterogeneity, it is necessary to perform NGS sequencing at a high coverage depth (i.e., how often a single base is covered by the reads of sequencing probes). Unfortunately, experiments using high coverage involve high costs. The use of NGS methods in neuroblastoma has enabled a deeper molecular characterisation of the tumour. This approach has allowed researchers to identify genomic alterations responsible for the molecular pathogenesis of neuroblastoma and resolve copy number alterations at high resolution.

To date, several large-scale sequencing studies have been performed in order to discover genomic alterations underlying the diverse clinical phenotypes of neuroblastoma. The analysis of 370 neuroblastoma cases at all clinical stages by WES and WGS revealed structural alterations of PTPRD, ODZ3, TRIO, DCL1, CSMD1, ATRX, ARID1A, and ARID1A genes, and a few high-frequency recurrent somatic variants in the ALK, CHD9, PTK2, NAV3, NAV1, FZD1, ARID1A, ARID1B, TIAM1, SOS1, ARHGAP10, PTPN11, OR5T1, PDE6G, MYCN, and NRAS genes (Table 1). Interestingly, neuroblastoma tumours have been reported to harbour a somatic coding mutation count of only 12 mutations per tumour, which is drastically lower than the count reported for adult cancers. Furthermore, recurrent somatic mutations in tumours of patients with neuroblastoma correlate with patients’ age at diagnosis and telomere length. Cheung et al. discovered that ATRX loss-of-function mutations and deletions are associated with neuroblastoma and are predominantly observed in adolescent and young adult patients. Recently, two WGS studies showed that chromosomal rearrangements are involved in the pathogenesis of neuroblastoma.
In particular, Peifer et al. demonstrated that telomerase reverse transcriptase (TERT) rearrangements are associated with poor outcome in a subgroup of HR neuroblastoma patients. Due to the genomic rearrangements occurring in TERT super enhancers, the biological effects of these rearrangements are the increase of TERT transcription. Moreover, another effect induced by these rearrangements is a massive chromatin remodelling and DNA methylation of the affected region. It has been found that genomic remodelling switches off the transcriptional silencing of TERT in HR neuroblastoma patients, inducing telomerase activation that increases tumour cell lifespan. Subsequently, Valentijn et al. screened 108 neuroblastoma cases at all clinical stages in

### Table 1. Genomic alterations associated to neuroblastoma

<table>
<thead>
<tr>
<th>Genes</th>
<th>OMIM</th>
<th>Chromosome Location</th>
<th>Abnormality</th>
<th>Sequencing method</th>
<th>Sample</th>
<th>References</th>
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<td>WGS</td>
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<td>[37]</td>
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<td>Primary tumour</td>
<td>[37]</td>
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<td>[37]</td>
</tr>
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<td>ATRX</td>
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<td>Xq21.1</td>
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<td>WGS</td>
<td>Primary tumour</td>
<td>[37,39,40,42]</td>
</tr>
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<td>603024</td>
<td>1p36.11</td>
<td>Structural variant/Single Nucleotide Variant</td>
<td>Targeted capture/WGS</td>
<td>Primary tumour</td>
<td>[38]</td>
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<tr>
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<td>614556</td>
<td>6q25.3</td>
<td>Structural variant/Single Nucleotide Variant</td>
<td>Targeted capture/WGS</td>
<td>Primary tumour</td>
<td>[38]</td>
</tr>
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<td>2p23.2-2p23.1</td>
<td>Single Nucleotide Variant/structural variant</td>
<td>WES</td>
<td>Primary tumour/relapse tumour</td>
<td>[40,52]</td>
</tr>
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<td>WES</td>
<td>Primary tumour/relapse tumour</td>
<td>[40,52]</td>
</tr>
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<td>WES</td>
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<tr>
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<td>Single Nucleotide Variant</td>
<td>WES</td>
<td>Primary tumour/relapse tumour</td>
<td>[40,52]</td>
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<td>Rearrangements</td>
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<td>WGS</td>
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<td>[52]</td>
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<td>[52]</td>
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<td>Relapse tumour</td>
<td>[52,53]</td>
</tr>
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<td>WGS</td>
<td>Relapse tumour</td>
<td>[52]</td>
</tr>
<tr>
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<td>[52,53]</td>
</tr>
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<td>[53]</td>
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<td>WES</td>
<td>Primary tumour</td>
<td>[39]</td>
</tr>
</tbody>
</table>

WGS: Whole Genome Sequencing; WES: Whole Exome Sequencing; N.A.: not available

References:

2. Valentijn EP, et al. (2016) Screened 108 neuroblastoma cases at all clinical stages in...
order to find new TERT structural rearrangements. The authors confirmed TERT overexpression in the presence of TERT rearrangements, which causes a significant increase of telomere length in TERT-rearranged cases compared to stage 4 tumours without TERT rearrangements. These two reports describe TERT rearrangements as the second most frequent genetic defect in neuroblastoma. This insight could be useful in developing new drugs that act on TERT activity in order to tailor therapeutic approaches for neuroblastoma HR patients.

The genetic heterogeneity of neuroblastoma indicates the presence of cellular subclones in which genomic changes could drive the expansion of a dominant subclone towards an appreciable cancer mass. This supports the idea that the presence of cellular subclone(s) in primary tumours at the time of diagnosis could be responsible for drug resistance and disease relapse. A recent study in neuroblastoma focused on the heterogeneity of primary tumour biopsies to investigate the occurrence of ALK mutations at a subclonal level by using deep sequencing techniques. Bellini et al. detected F1174 and R1275 mutations at diagnosis in 10% of their cases, with subclonal events in more than half of these cases. The mutations were not detected by the Sanger sequencing methods because they occurred at a low frequency. These findings are of clinical importance for clonal tumour evolution and disease relapse. Gathering information regarding the clonal evolution of ALK would help to tailor pharmacological treatment against ALK mutations with ALK inhibitors, an approach that has been used for other diseases such as melanoma. At the moment, a targeted therapy that takes into account tumour subclonal events has not been adopted in clinical practice for neuroblastoma. To date, there is little available information about the mutational portrait of neuroblastoma after chemotherapy. In 1983, Feder and Gilbert demonstrated the heterogeneity of neuroblastoma for the first time by studying the presence of subclonal lineages in primary tumour and metastatic samples in a patient with neuroblastoma. Subsequently, Gotoh et al. revealed intra-tumoural clonal heterogeneity using cytfluorometry and fluorescent in situ hybridisation analyses. Mora et al. studied 1p allele distribution and DNA ploidy to explain the development of malignant cells during the clinical progression of neuroblastoma. It has also been observed that MYCN amplification is strongly correlated with poor prognosis and advanced tumour stage. Moreover, tumours with MYCN amplification are often resistant to therapy. This suggests the presence of intra-tumoural subclones with MYCN amplification, hence explaining why MYCN amplification correlates with poor prognosis and resistance to therapy. However, the mechanisms involved in tumour progression subsequent to treatment remain unknown. Recently, thanks to the NGS technology, neuroblastoma heterogeneity has been explored in detail. Mutational analyses of tumour biopsies at relapse and at diagnosis have demonstrated that tumour biopsies collected at relapse generally contain more genomic mutations and SCAs than those observed in primary tumours. Eleveld et al. found that 78% of tumours at relapse harboured mutations predicted to hyperactivate the RAS-MAPK signalling pathway (Table 1).

Interestingly, cell lines containing similar mutations showed sensitivity to the inhibition of MEK, a downstream node in the canonical growth-promoting pathway. A similar study was performed by Schramm et al. to identify molecular changes acquired during tumour progression, with potential relevance for targeted treatment at tumour recurrence. Using a combination of WES, array comparative genomic hybridization (aCGH), DNA methylation analysis, and GEP, they showed that the amount of mutations in biopsies at relapse is higher than the amount of mutations in primary tumours. Of particular interest, the authors identified Hippo-YAP signalling activation as the unique event that is significantly associated with tumours in relapsing patients. Therefore, these studies underline the necessity to deeply investigate both primary tumours and tumours at relapse in order to develop tailored therapeutic strategies for the refractory disease. Finally, functional studies in vitro and in vivo have to be implemented in order to evaluate whether the discovered gene mutations are involved in tumour cell fate and behaviour.

Towards establishing the NGS signature to improve the diagnostics of neuroblastoma

Genomic sequencing approaches are useful to derive a more precise risk stratification of neuroblastoma patients compared to conventional approaches. Risk stratification of neuroblastoma patients and tailored therapy play an important role in the modern treatment of neuroblastoma. The INRG task force has developed a set of consensual guidelines for the molecular diagnostics of neuroblastoma; the detection of minimal disease in bone marrow, blood, and stem-cell preparations, and imaging as well as staging. Nowadays, copy number aberration (CNA) is used as a prediction factor of a patient’s risk, whereas a patient’s gene expression profile has
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not yet been introduced as a prognostic marker in neuroblastoma. In March 2015, a new NGS neuroblastoma signature associated with tumours of HR patients was discussed during the SIOPEN Genomics Meeting in London. During the meeting, a panel of mutated genes discovered by exome sequencing was proposed for the development of an NGS signature tailored for neuroblastoma. The introduction of NGS sequencing for molecular characterisation of neuroblastoma is the first step in addressing therapeutic approaches towards personalised medicine (Figure 1). In recent years, data obtained through omics technologies have provided a great hope that we are coming closer to personalised medicine, which will enable the treatment of patients according to their distinct molecular profile. However, we believe that the huge amount of molecular and biological data produced by omics technologies need to be integrated and managed by a team of clinical and non-clinical experts. This means that specialists including oncologists, pathologists, geneticists, bioinformaticians, biologists, and biotechnologists must combine their knowledge in order to improve diagnosis and to get the right drug for the right person at the right dose, moving from traditional medicine to personalised medicine.

Figure 1. From bench to bed to bench again. The figure represents a route to achieving precision medicine. After the collection of tumour biopsy, nucleic acids and proteins are extracted. DNA and RNA are used to perform genomic and transcriptomic profiling, respectively; and proteins are used to perform proteomics and metabolomics studies. Data from the described omics studies are analysed via bioinformatics algorithms and tools. The huge amount of molecular and biological data produced from the omics technologies is managed by a team of experts to derive the right diagnosis and to obtain personalised medicine.

Ethics statement
The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

Author contributions
M.R. Esposito, S. Aveic, and A. Seydel contributed to the design and preparation of the manuscript. G.P. Tonini contributed to the content. M.R. Esposito and G.P. Tonini were also involved in drafting and revising the manuscript.

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