Biology of Neuroblastoma

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2.1 Introduction

To date, the precise etiology of neuroblastoma is unknown, and unlike many adult malignancies, environmental factors are not thought to play a major role, although predisposing effects of prenatal exposures to potentially toxic substances warrant further investigation. However, genetic factors, both at constitutional and somatic levels, are thought to play a major role in neuroblastoma development [1].

2.2 Hereditary Genetic Factors

Several observations corroborate the hypothesis of a role of underlying hereditary genetic factors in the etiology of neuroblastoma.

First, although rare and representing less than 1% of all cases [2], familial neuroblastomas have been described. Mutations of gain of function in the tyrosine kinase domain of the ALK anaplastic lymphoma kinase gene have been detected in the majority of familial cases [3, 4]. This is thought to be associated with an autosomal-dominant pattern of inheritance with incomplete penetrance.

Second, neuroblastoma can appear in association with different clinical syndromes. Neural crest-related developmental disorders associated with an increased risk of developing neuroblastoma have been linked to inactivating mutations in the PHOX2B gene, a major regulator of neural crest development, identified as the first neuroblastoma predisposition mutation [5, 6]. While expansions of the second

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polyalanine sequence of PHOX2B are mainly observed in patients with a curse of Ondine (also called CCHS, Congenital Central Hypoventilation Syndrome) associated with a low risk of peripheral neuroblastic tumors, non-expansive mutations with Hirschsprung's disease are associated with a higher risk of developing neuroblastoma. Other associations between neuroblastic tumors and cancer susceptibility syndromes include neurofibromatosis type 1 (NF1) [7], characterized by constitutive activation of the RAS–MAPK pathway, as well as Noonan syndrome with PTPN11 gene involvement.

Third, genome-wide association studies have shown that peripheral neuroblastic tumors may occur in the context of underlying genetic factors, as it has been demonstrated that different polymorphic alleles, localized at different genome loci, influence oncogenesis [8–13]. Of a weak individual impact on the initiation of the disease (with a relative risk of 1.5–2.0 compared to the global population), these polymorphic alleles can cooperate in an individual patient to promote malignant transformation during neurological development, and some genes targeted by these polymorphic alleles play a role in the pathogenesis of neuroblastoma, including BARD1, LMO1, DUSP12, DDX4, HACE1, and LIN28B. The polymorphic alleles described show a correlation with high-risk or low-risk disease, indicating that favorable and unfavorable forms of neuroblastoma may represent distinct entities in terms of genetic events that initiate tumorigenesis.

In addition, rare cases with various aberrant constitutional karyotypes have been described in patients with neuroblastoma, including constitutional copy-number abnormalities, balanced and unbalanced translocations, and specific chromosomal deletions, including deletions of chromosome 1p [14]. In total, there are probably as yet undiscovered additional genes that predispose to neuroblastoma when they are altered in the germline. It is important to note that, to date, there are no clinically validated guidelines for determining who should be screened for germline mutations, nor how to monitor patients or families with known susceptibility alleles.

2.2.1 Somatic Genetic Alterations

2.2.1.1 Copy-Number Alterations

With only 1–2% of neuroblastomas occurring in a familial context or predisposition, over 98% of all cases occur sporadically. A large number of recurrent somatic genetic alterations have been found in neuroblastoma, the most common being quantitative genomic alterations with gains or losses in genetic material. These genetic abnormalities are related to distinct biological and clinical subgroups of the disease.

The amplification of the oncogene MYCN, located at chromosome 2p24.1, is observed in about 25% of neuroblastomas and 40% of high-risk tumors [15]. It remains one of the most important genetic alterations associated with advanced stages of disease, with an aggressive phenotype and poor survival [1]. It is the first genetic marker used in clinical practice for risk stratification and treatment adaptation [16]. Closely associated with poor survival in patients with localized disease and in infants, its prognostic impact in metastatic disease of older children with an

overall poor outcome is less clear [16, 17]. At a cytogenetic level, amplification of the MYCN oncogene occurs either as double-minute chromosomes (DM) or homogeneously staining regions (HSR), which contain between 10 and over 100 ectopic copies of the *MYCN* oncogene. The oncogenic role of MYCN has been clearly demonstrated as its ectopic expression in the neural crest is sufficient to drive neuroblastoma tumorigenesis in zebrafish and mice models [18]. Its oncogenic role is based on an enhancement of the expression of genes involved in cell proliferation, and on the repression of genes involved in differentiation and apoptosis [19].

Other recurrent amplifications concern the *ALK* gene on chromosome 2p23, as well as amplicons of chromosome 12q13–14 encompassing, among others, the MDM2 and CDK4 genes [20]. Recent data indicate that NB with focal amplifications other than MYCN might present with atypical clinical features and a poorer outcome [21].

Other recurrent structural alterations recurrently observed in neuroblastoma concern segmental chromosome alterations (SCA) corresponding to unbalanced chromosome translocations, including deletions of chromosome arms 1p, 3p, 4p, and 11q, and gains of 1q, 2p, or 17q. Although several recurrently altered chromosome regions have been identified, chromosome breakpoints are not recurrent but lie scattered over wide genomic regions. Deletion of 1p36 is observed in 20–35% of cases and is associated with poor survival in multivariate analyses as well as with aggressive disease markers [22, 23]. Deletions of 11q in a consensus region at 11q23 occur in approximately 40% of cases and are inversely correlated with *MYCN* amplification, identifying a molecularly distinct high-risk patient subset, characterized by advanced stage, older age, as well as a higher genomic instability with a higher number of chromosome breakpoints [24, 25]. Gains of chromosome 17q21-qter represent the most frequent genetic alteration in neuroblastoma, occurring in 70% of tumors. Numerous studies have reported that 17q gain is significantly associated with advanced stage of disease, increased patient age, *MYCN* amplification, as well as other unfavorable genetic parameters [26].

Although intense decade-long research has focused on the identification of hypothetical tumor-suppressor genes or oncogenes in recurrently altered regions of chromosome loss or gain, the smallest regions of overlap remain quite extensive and to date do not point to single-gene candidates as tumor suppressors or oncogenes, suggesting that an overall imbalance of copy-number regions is of importance in neuroblastoma oncogenesis.

Although the individual segmental chromosome alterations have been shown to correlate with outcome, importantly, the overall genomic profile has been shown to be of prognostic impact in neuroblastoma [27–30]. Whereas an overall genomic copy-number profile characterized by numerical chromosome alterations, consisting of gains or losses of whole chromosomes, is associated with a favorable outcome, segmental chromosome alterations of any chromosome region, without or with numerical chromosome alterations, are associated with advanced stage of disease, with an increased age at diagnosis and, importantly, a higher risk of relapse in multivariate analyses [27, 28, 31]. In addition to the determination of MYCN amplifications status, the overall genomic copy-number profile determined by array

comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) array is now considered part of routine work up in particular in low- and intermediate-risk neuroblastoma and might be used for treatment stratification within prospective clinical protocols [1].

Higher-resolution copy-number analyses have also revealed smaller recurrent interstitial events. Indeed, SNP arrays have identified alterations on chromosome 9p, with homo- or hemizygous deletions encompassing the *CDKN2A* gene [32]. Other sporadic copy-number alterations include focal TERT gains [33] and micro-deletions encompassing the *PTPRD* gene [34].

More complex rearrangements resulting from chromothripsis, corresponding to massive genomic rearrangements acquired in a single catastrophic event, have been described in neuroblastoma, but their association with other genomic and clinical subtypes remains to be determined [35, 36].

2.2.1.2 Single-Gene Mutations

Recent next-generation sequencing approaches have indicated that most neuroblastomas harbor only few mutations, with an average of 10–20 predicted non-synonymous variations in coding regions per genome, indicating an exonic mutation frequency of 0.2–0.4 per Mb. [35, 37–39] The frequency of somatic events strongly correlates with tumor stage, higher-stage tumors harboring a higher number of mutations.

The most frequent recurrent somatic mutation in neuroblastoma concerns the gene *ALK* (anaplastic lymphoma kinase), with mutations activating the tyrosine kinase domain in approximately 10% of all cases at diagnosis. [3, 4] The somatic ALK-1174 mutation appears to contribute to a more aggressive phenotype, but unlike ALK-1275 mutations, these specific mutations are not found in familial neuroblastoma, suggesting that they are not tolerated in the germline [40, 41]. The oncogenic role of activating ALK mutations in neuroblastoma has been demonstrated in vitro and in vivo in both zebrafish and mouse models, with coexpression of ALK-F1174L and MYCN producing a synergistic effect for neuroblastoma tumorigenesis in mice. New-generation small-molecule inhibitors targeting the activated kinase domain of ALK are now available, making this a promising target for molecular therapy, possibly in combination therapies, but still requiring more specific development [40, 42, 43].

Other recurrent mutations in neuroblastoma target distinct cellular pathways and include PTPN11 mutations (in 3% of cases), as well as genes involved in cytoskeleton maintenance, neuritogenesis, and other regulators of the RAC/Rho pathway [35].

Interestingly, genes involved in chromatin remodeling have been found to be targeted in a significant number of cases, either by mutations or by structural variations, including mutations in the ARID1A/ARID1B genes [38]. Somatic alterations of ATRX, consisting either of mutations or small interstitial deletions, are associated with an increase in telomere length and with an absence of the ATRX protein in the nucleus. ATRX alterations appear to be more frequent in older children and occur in mutually exclusive fashion with MYCN amplifications [39]. ATRX mutations are associated with activation of a telomere maintenance mechanism termed

alternate lengthening of telomeres (ALT), which may be associated with primary chemotherapy resistance.

Recurrent genomic rearrangements of the promoter region of the telomerase reverse transcriptase (TERT) gene on chromosome 5p15.33 have been described in >10% of neuroblastoma cases, with structural rearrangements of TERT resulting from chromothripsis in some cases [44, 45]. These rearrangements, which are associated with increased TERT expression, target regions immediately up- and downstream of TERT, and position the TERT coding sequence to strong enhancer elements, resulting in massive chromatin remodeling and DNA methylation of the affected region. [44] Occurring in mutually exclusive fashion with *MYCN* amplification and *ATRX* mutations, these rearrangements define a further subgroup of high-risk disease, with TERT rearrangements (23%), ATRX deletions (11%), and MYCN amplifications (37%) identifying three almost non-overlapping groups of high-stage neuroblastoma, each associated with very poor prognosis.

Thus, a large number of high-risk neuroblastomas are affected by genetic alterations of either *MYCN*, *TERT*, or *ATRX*, all of which converge to an activation of telomere-lengthening mechanisms either by direct activation or by ALT, leading to a capacity of near-infinite cell proliferation. [46] Advances in the development of inhibitors of these pathways and their evaluation in clinical trials will lead to new treatment opportunities.

Future studies will determine if the association between these major players in neuroblastoma oncogenesis with distinct genetic profiles and mutational patterns might serve for the definition of different risk groups in particular in high-risk neuroblastoma.

Overall, large-scale sequencing efforts have highlighted distinct mutational signatures which are thought to reflect distinct biochemical cellular processes [47]. In neuroblastoma, in some cases a predominance of C > T transitions, termed mutational signature 1, are observed, with an association with age. Other mutational signatures observed in neuroblastoma, although rarer, concern the canonical double-stranded break signature linked to mutations in *BRCA1* or *BRCA2* or to a "BRCAness" phenotype [48]. Altogether, these studies underline the heterogeneity of somatic genetic alterations in neuroblastoma and highlight the importance to pursue efforts of molecular characterization.

2.2.1.3 Expression Profiles

In addition to genetic changes, neuroblastoma can also be characterized by specific expression profiles. Indeed, to date, a large number of studies have focused on the analysis of differential expression patterns in NB, seeking to identify expression patterns that might be enable to distinguish patients with different clinical courses and thus define different prognostic groups in high-risk disease, and to potentially identify new therapeutic targets.

Thus, different expression signatures, based on 144-gene or 59-gene signatures, reliably distinguished patients with distinct clinical courses, with the strongest difference observed in non-high-risk disease [49, 50]. Using real-time PCR expression data, an expression signature based on only three genes (*CHD5*, *PAFAH1B1*, and

NME1) has been able to discriminate patients with different outcomes [51]. Among high-risk patients, an expression profile based on 55 genes defined patient populations with divergent outcome [52]. Differential expression signature depending on *MYCN* has led to a definition of a 157-gene signature which identified NB patients with poor prognosis independent of the genomic *MYCN* status, those without *MYCN* amplification presenting stabilization of MYCN at the protein level [53].

More recently, based on the hypothesis that tumor-associated inflammatory cells might contribute to the differences in age-dependent outcome of patients with metastatic NB, expression of genes representing tumor-associated macrophages, such as *CD33/CD16/IL6R/IL10/FCGR3*, contributed to a novel 14-gene tumor classification score. Progression-free survival was 47% versus 12% for patients with a low-versus a high-risk score, indicating that interactions between tumor and inflammatory cells may contribute to an aggressive metastatic NB phenotype [54].

In addition to messenger RNA, expression levels of non-coding RNAs are also highly variable. Micro-RNAs function as regulators of gene expression at the posttranscriptional level in diverse cellular processes and constitute the most widely studied non-coding RNA molecules in NB. *MYCN* modulates the expression of several classes of non-coding RNAs, especially some micro-RNAs, and it can also regulate the expression of long non-coding RNAs such as T-UCRs (Transcribed UltraConserved Regions) and non-coding RNA, whereas other long non-coding RNAs remain to be characterized in NB. The landscape of T-UCRs in NB has been studied recently and has revealed a correlation with the *MYCN* status, and preliminary studies have suggested that T-UCR-based expression signatures might distinguish short-from long-term survivors in high risk NB [55].

The miRNA expression pattern can also be used to classify NB patients according to survival [56, 57]. An advantage of the study of miRNA rather than mRNA expression signatures is linked to the greatest stability of miRNAs, and thus to an analytical feasibility even with formalin-fixed, paraffin-embedded (FFPE) samples as opposed to frozen samples [57]. The miRNA expression pattern can also be used to classify NB patients according to survival [56, 57].

The paucity of recurrent genetic mutations as compared to adult tumors indicates that additional mechanisms such as epigenetic alterations may play an important role in the molecular pathogenesis of these developmental tumors. Alterations in DNA methylation represent one of the most common molecular events in neoplasia, and CpG-island hypermethylation of gene promoters is a frequent mechanism for functional inactivation of relevant tumor-associated genes in neuroblastoma. Promoter methylation patterns which are associated with patient subgroups and distinct clinical features have been identified [58]. Further studies are now necessary to determine whether these genome-wide methylation patterns correlate with outcome and other prognostic molecular markers in NB patients.

Taken together, to date, many studies have demonstrated the feasibility of expression profiling of mRNA, miRNA, other non-coding RNAs, or epigenetic modifiers in order to determine different prognostic subgroups among NB patients. However, there is little, if any, overlap between the genes of the different signatures rendering cross-study comparisons unfeasible. Furthermore, although most expression signatures clearly distinguish prognostic groups in the overall population, differences in

survival among high-risk patients are frequently not very marked. The routine setup of real-time determination of expression profiles in a prospective clinical trial setting, and their interpretation, remains a clear challenge.

2.2.1.4 Spatial and Temporal Heterogeneity

Neuroblastoma presents important spatial and temporal heterogeneity. Spatial heterogeneity has been recorded for several recurrent somatic genetic alterations in neuroblastoma. Indeed, MYCN alterations might occur only in a subset of neuroblastic cells of a given tumor [59]. Segmental chromosome alterations might also vary between different neuroblastic cell populations [60, 61]. Furthermore, mutations can also be observed at a heterogeneous level. In some tumors, low-level mutated allele fractions for ALK driver mutations have been observed [62].

In neuroblastoma, temporal heterogeneity can also occur. Indeed, genetic alterations may evolve over time and clonal evolution is common, leading to the acquisition of somatic alterations in known oncogenic pathways, some of which may be targeted. ALK-activating mutations, in some instance present in a minor subclone at diagnosis, might emerge at relapse [63]. Furthermore, activation of the MAPK pathway and other signaling pathways for epithelial–mesenchymal transition (EMT) processes may appear during a relapse and represent promising targets for targeted molecular treatment approaches [64, 65]. In total, spatial and temporal genetic heterogeneity plays an important role in neuroblastoma.

However, multi-site tumor biopsies, or sequential biopsies from the same tumor, can only rarely be realized, and recently liquid biopsies have emerged as a very promising tool to explore somatic genetic alterations with regards to both spatial and temporal heterogeneity. Indeed, circulating tumor DNA, a fraction of cell-free DNA, can readily be extracted from plasma of neuroblastoma patients. This can serve for the detection of MYCN amplification [66]. Copy-number alterations or mutations such as ALK can also be detected in ctDNA. [67–69] More recently application of whole-exome sequencing techniques to sequential ctDNA samples from NB patients has provided further evidence of the importance of clonal evolution in the progression of neuroblastoma, enabling the description of resistant clones emerging at the time of relapse [70].

Altogether, as neuroblastoma is in general associated with a low mutational burden and only few recurrently occurring mutations, it might be considered a copy-number disease, with large chromosome segments contributing to oncogenesis by gene dosage effects. Further ongoing efforts will enable to determine whether epigenetic changes also play a role in neuroblastoma oncogenesis.

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