

# Molecular Markers in Neuroblastoma

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## Abstract

Neuroblastoma, one of the common malignant childhood tumors, arises from neuroblast cells derived from the neural crest and destined for the adrenal medulla and the sympathetic nervous system and shows remarkable biological heterogeneity, resulting in favorable or unfavorable outcomes. Some tumors make rapid progress with a fatal outcome. In other instances, the tumors regress spontaneously in infants or to differentiate into a benign ganglioneuroma in older patients. This heterogeneity within neuroblastoma depends on the molecular characteristics of tumor cells. Several distinct genomic alterations have been found in neuroblastoma, including MYCN amplification, DNA ploidy, deletion of the short arm of chromosome 1, gain of chromosome 17q, and deletion of 11q. The difference of expression was also found in genes related to cellular growth, differentiation, and apoptosis of neural network including signaling by NTRK1 or ALK receptor tyrosine kinases, and telomerase activity. And this presentation discusses diagnostic and prognostic molecular markers for extensive heterogeneity of neuroblastoma. This should lead to more risk-adapted therapies according to the genetic markers by which individual neuroblastomas are biologically characterized.

**Keywords:** Neuroblastoma, Prognosis, MYCN, Ploidy, Chromosome, Telomerase, Telomere, Apoptosis.

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## Introduction

Neuroblastoma, which is derived from neuroblast in neural crest, is the most common malignant solid tumor in children. The incidence of neuroblastoma is about <sup>1</sup> case per 7,000 born babies a year.<sup>1</sup> More than 90% of children with neuroblastoma are diagnosed within the first 5 years of age. The tumors exhibit two distinct patterns of clinical behavior: life-threatening progression (unfavorable) and spontaneous regression or maturation to ganglioneuroma (favorable). Many patients who are diagnosed at more than one year of age have advanced neuroblastomas with metastasis usually showed poor outcome despite multimodal therapies. On the other hand, the majority of infant tumors showed good outcome and some of them undergo complete regression without any treatment.<sup>2-4</sup> Since more than 80% of neuroblastomas produce catecholamine, their metabolites (vanillylmandelic acid and homovanillic acid) are

detectable in the urine and mass-screening projects to detect earlier stages of neuroblastoma have been carried out in some countries including Japan. Recent report revealed that the incidence of this disease increased more two fold whereas the incidence of advanced neuroblastoma in older patients reduced and the cumulative mortality rate reduced significantly, indicating that a large number of neuroblastomas occurred in infants without detection clinically and spontaneously regress or mature behind the scenes but some of them may progress into malignant phenotypes.<sup>5</sup> These projects also gave us a lot for solving the biological problem in neuroblastoma.<sup>6-8</sup> Transition from favorable type to unfavorable type has been clearly suggested,<sup>9</sup> indicating the mechanism of malignant alteration of this tumor should be elucidated.

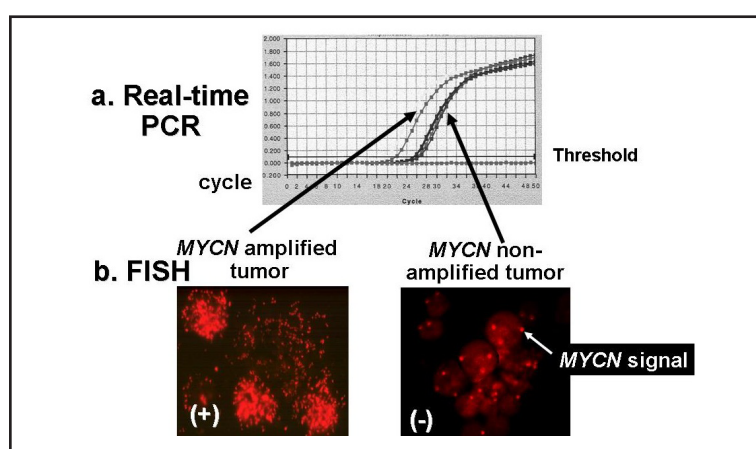
Clinically, to distinguish progressive tumors from favorable tumors is needed because multimodal

appropriate therapies are necessary to improve the prognosis of the patients with progressive tumors and aggressive therapy should be avoided in the patients with favorable tumors to reduce side effects, late complications and medical expense. To identify the molecular and biological heterogeneity of neuroblastoma clearly, numerous multilateral approaches has been performed and several distinct alterations have been found including *MYCN* amplification, DNA ploidy, chromosomal loss and gain, expression of *NTRKA*, telomerase activity, and others. In this review, molecular evaluation of heterogeneity of neuroblastoma is summarized and clinical application of these data is presented for adequate treatments. This should lead to more risk-adapted therapies according to the genetic markers by which individual neuroblastomas are biologically characterized. This approach will lead to find the molecular marker to distinct progressive tumors at diagnosis as well as the molecular targets to treat these tumors. In addition, it will provide insights into mechanisms of malignant transformation, progression, spontaneous regression and maturation in neuroblastoma.

## Biological and molecular markers

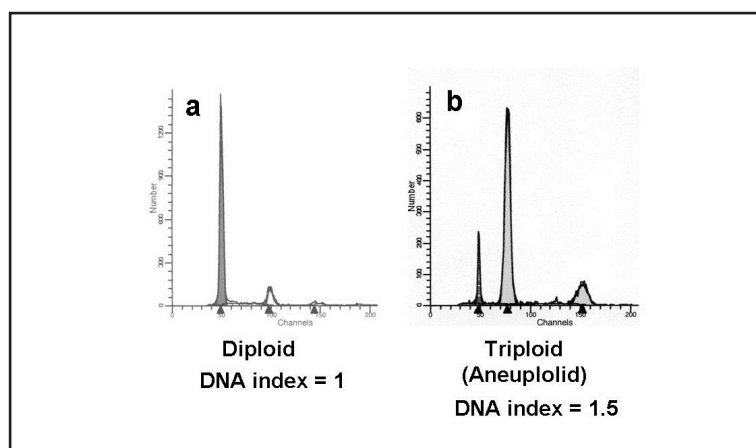
### *MYCN* amplification

Amplified *MYCN* was observed in 30%-50% of advanced neuroblastomas, but was rarely detected in early stages of tumors.<sup>10,11</sup> In patients who have the non-amplified *MYCN* tumors without metastasis, overall survival was approximately 90% over a 5-year period, but less than 30% of patients survived a 2-year period when *MYCN* was amplified at more than ten copies.<sup>9,12</sup> Thus, amplified *MYCN* is established as a powerful clinical marker of high-risk neuroblastoma and the only tumor genetic marker has been used as a basis for treatment stratification in neuroblastoma clinical trials.<sup>13-16</sup> Recently, FISH (fluorescence in situ hybridization) analysis of primary tumors for detection at the single cell level revealed that individual cells from *MYCN*-amplified tumors typically stray widely from the copy numbers estimated by molecular analyses.<sup>17,18</sup> Thus, recent evaluation for *MYCN* amplification was performed by FISH and quantitative PCR for DNA copies (figure 1). More recently, using serum DNA-based real-time quantitative PCR with a single-copy



**Figure 1.** Detection of *MYCN* amplification in neuroblastoma

Copy numbers of the *MYCN* gene are usually determined by quantitative real time PCR (TaqMan®) (a) and fluorescence in situ hybridization (FISH)(b).



**Figure 2.** DNA ploidy by flow cytometric analysis

*a:* Diploid: the G0/G1 showed a read large peak and G2 showed a small blue peak.

*b:* Aneuploid: The read peak is diploid of the cells contained in the tumor specimen. The yellow large peak is G0/G1 tumor cells and yellow small one is G2 tumor cells. The ratio of tumor G0/G1 peak / normal G0/G1 peak was near 1.5. Thus, this tumor was defined as triploid.

reference gene, *MYCN* amplification in serum DNA is a valuable diagnostic tool to discriminate the patients with *MYCN* amplified tumors from other patients.<sup>19</sup> This method might become a powerful diagnostic tool as well as a promising indicator of therapeutic efficacy and relapse in the follow-up of patients with *MYCN* amplified tumors.

The copy numbers of amplified *MYCN* gene has been considered to be consistent within a tumor; not only at different tumor sites, but also at different times in vivo.<sup>20</sup> However, there were some reports to identify that the *MYCN* gene was amplified during progression of neuroblastoma.<sup>21,22</sup> Epidemiological analysis revealed that advanced tumors decreased in the large cohort that underwent mass-screening and increased incidence of infant favorable tumors without *MYCN* amplification.<sup>5</sup> In the majority of cases amplified *MYCN*, 1p deletion and 17q gain coexist in the same tumors, while amplified *MYCN* rarely, if ever, occurs without either 1p deletion or 17q gain or both. These phenomena imply that *MYCN* amplification is a later event in the sequence of genetic aberrations underlying neuroblastoma progression.<sup>23</sup>

The size of amplicon with amplified DNA

encompassing *MYCN* ranges from 100 kb to more than 1 Mb.<sup>24</sup> Recently, Alk (anaplastic lymphoma kinase) gene located near *MYCN* gene is activated in neuroblastoma by amplification as well as mutations in the tyrosine kinase domain.<sup>25</sup> The germline mutation of Alk gene is considered as a major familial neuroblastoma predisposition gene.<sup>26</sup> Thus, the amplification of the 2p 23-24 loci is critical events in distinguishing neuroblastoma biologies.

## DNA Ploidy

Cytogenetic and flow cytometric analyses have been used for evaluating DNA ploidy in neuroblastoma. Flow cytometric analysis revealed that hyperdiploidy, mostly the near-triploidy, is mainly observed in favorable tumors of younger patients, whereas diploid is usually detected in advanced tumors with unfavorable outcomes (figure 2).<sup>27</sup> In addition, in children 12-24 months of age, diploidy predicted resistance for chemotherapy, whereas half of the patients with hyperdiploidy achieved long-term disease-free survival. The DNA ploidy did not have its prognostic significance for patients over 2 years of age.<sup>28</sup>

Cytogenetic analyses classified neuroblastomas

into four ploidy patterns: near-diploid, near-triploid, near-tetraploid, and near-pentaploid tumors.<sup>29</sup> The near-diploid and near-tetraploid tumors were usually detected in children older than 1 year and frequently had genetic abnormalities involving 1p and *MYCN* amplification. On the other hand, near-triploid and near-pentaploid tumors were predominantly detected in infants with favorable outcome and rarely showed genetic abnormalities. Near-diploidy and near-tetraploidy have been identified as one of the most useful markers for poor prognosis.<sup>30</sup>

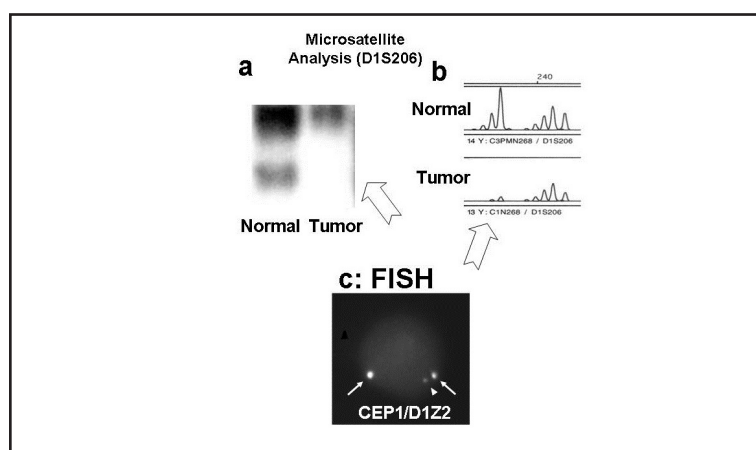
### Chromosome loss and gain

Chromosome loss and gain in neuroblastoma was reported in various chromosome regions, most frequently chromosome 1p (figure 3), followed by 11q, and 17q. More recently CGH and microarray analyses have substantially contributed to the identification of unbalanced 17q gain in primary neuroblastoma.<sup>31-34</sup>

Several reports using microsatellite markers (figure 3) identified that chromosome 1p deletion occurs in approximately 35% of all neuroblastomas,<sup>35,36</sup> and a smallest region of overlapping deletion (SRO) was refined to a size of approximately 1 Mb within 1p36.3, which was defined by the region of LOH in a primary tumor

that extends distally from *D1S214*. Patients whose tumors had large 1p deletions showed poorer outcome than patients with short or interstitial deletions,<sup>37,38</sup> suggesting the existence of more than one deleted 1p locus in neuroblastoma. The tumors with large 1p deletions were associated with adverse prognostic factors, such as diploidy or tetraploidy and amplified *MYCN*, while the tumors with small interstitial deletions of 1p were in the triploid range in favorable tumors. The regions of 1p deletions of *MYCN*-amplified tumors are very large including a region from 1p32 to telomere.<sup>21</sup> In contrast, in *MYCN* single copy cases, 1p deletions were described to be consistently smaller, and a commonly deleted region maps to 1p36.3. Thus, a second tumor suppressor gene, which is correlated with progressive neuroblastoma, was suggested to be localized at proximal (1p32) or distal (1p35-36.1) to the deletion border of the smallest 1p deletion found in *MYCN*-amplified cases.<sup>39,40</sup>

Clinically, gain of 17q is more common at an advanced stage, in tumors from children aged over 1 year, and in tumors showing 1p loss, *MYCN* amplification and diploidy/ tetraploidy. On the other hand, triploidy with whole chromosome 17 gain is associated more often with neuroblastomas showing favorable clinical features.<sup>23</sup> The report from six European centers with more than 300 cases identified that 17q gain was the most powerful prognostic

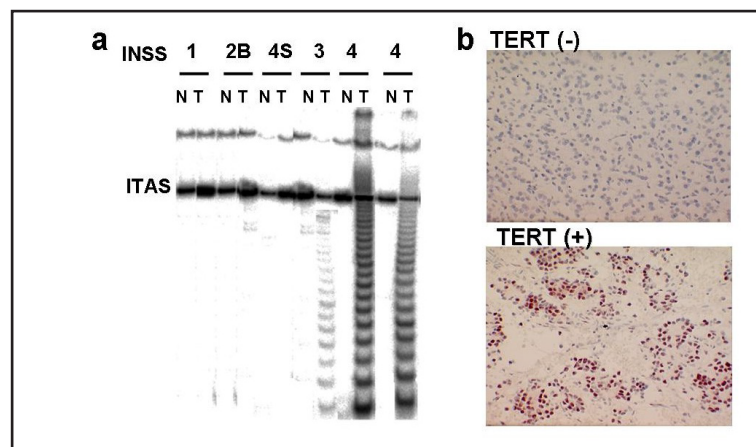


**Figure 3.** LOH analysis using microsatellite marker and fluorescence in situ hybridization (FISH) *a.* Autoradiographic analysis for microsatellite maker *D1S206*. The small band was lost in tumor DNA. *B.* Fluorescent labeled fragment analysis for microsatellite maker *D1S206*. The smallband was lost in tumor DNA. *c.* Two color FISH analysis: Centromere probe of chromosome 1 is *CDP1* (green) and telomere probe of choromosome 1p is *D1Z2* (red). The green signals were two but the red signal is one. These results indicated the LOH (loss of heterozygosity) of chromosome 1p in this tumor.

factor of survival in multivariate analysis with other clinical and tumor genetic parameters, including 1p deletion and *MYCN* amplification. In stepwise multivariate analysis, significant independent predictors of lethal outcome were 1p deletion ( $P = 0.02$ ), stage 4 disease ( $P = 0.004$ ), and 17q gain ( $P < 0.001$ ).<sup>23</sup> These studies suggested that unbalanced gain of distal 17q is the independent prognostic factor for predicting high risk for tumor progression and that the region of chromosome 17q gain includes a gene critical for tumor progression. Cytogenetic analyses have also reported 11q deletion in about 15% of neuroblastomas,<sup>41</sup> and LOH studies revealed 11q loss in 5-32% of the tumors.<sup>42-44</sup> Loss of the whole chromosome 11 was observed in 19%, while unbalanced 11q LOH was observed in 22% of primary neuroblastomas.<sup>45</sup> Loss of the whole chromosome 11 was mainly detected in low stage tumors, whereas unbalanced deletion of 11q was predominantly detected in high stage tumors without amplified *MYCN*.<sup>45,46</sup> Loss of 11q was significantly correlated with adverse clinical parameters, including age

over 1 year, stage 4 tumors and unfavorable histology.<sup>47</sup> The SRO in 11q23.3 is between markers *D11S1340* and *D11S1299* in the tumors with 11q LOH.<sup>46</sup> Unbalanced 11q deletions are considered as a frequent event in the *MYCN* non-amplified tumors and COG study recently revealed that 11q deletion is as independent prognostic indicator for predicting high risk for tumor progression, especially in *MYCN* non-amplified tumors.<sup>31,48</sup> Several candidate TSGs have been reported in this 11q region: *CDKN2A*, *CADM1*, *TSL1* and so on.<sup>49-51</sup>

Recently, array-based CGH and SNP array, a powerful tool for survey whole chromosomal changes in the tumor cells, revealed that major alternations in neuroblastoma cells are loss of 1p, 11q, and gain of 17q.<sup>34,52</sup> In addition to amplified *MYCN* gene, 1p deletion, 17q gain, 11q deletions, and ploidy changes, further genetic alterations exist in neuroblastomas (table 1). These methods will be clarified the genetic differences in heterogeneity of neuroblastoma.



**Figure 4.** Telomerase activity and TERT expression in neuroblastoma specimens

*a.* Telomerase activity in adjacent noncancerous adrenal gland (N) and neuroblastoma tissue (T). Telomerase activity was measured by TRAP assay in 6 representative neuroblastoma samples with each normal adrenal gland tissue. Noncancerous adrenal gland tissues did not show telomerase activity. In neuroblastoma tissues, cases with stage 1, 2B, 4S tumors showed no detectable activity, while the stage 3 cases showed low activity and 2 stage 4 cases showed high activity.

*b.* TERT expression was analyzed by immunohistochemistry. Positive TERT signals were detected in nuclei in the lower case.

## Expression of neurotrophin receptors

The neurotrophin receptors (*NTRK1*, *NTRK2*, and *NTRK3* encoding TrkA, TrkB, and TrkC) and their ligands (NGF, BDNF, and neurotrophin-3, respectively) are important regulators of survival, growth, and differentiation of neural cells.<sup>53</sup> Thus, Trk receptors encoding the high-affinity receptor tyrosine kinases for neurotrophins are important to regulate growth, differentiation and apoptosis of neuroblastoma. High TrkA expression is seen in favorable neuroblastomas with good outcome.<sup>54,55</sup> Explanted neuroblastoma cells with high TrkA expression differentiate when exposed to NGF or undergo apoptosis in the absence of NGF.<sup>55</sup> Thus, NGF/TrkA signalling could provoke differentiation or regression in favorable neuroblastomas depending on the particular microenvironment. Recently, a neurodevelopmentally regulated oncogenic splice variant of TrkA (TrkAIII) has been identified that antagonises the anti-oncogenic NGF/TrkA signaling and promotes neuroblastoma tumor growth.<sup>56</sup> On the other hand, the levels of *TrkA* expression is extremely low in aggressive tumors with MYCN amplification and/or 1p loss.<sup>57,58</sup> In contrast, *TrkB* is expressed in aggressive neuroblastomas and its preferred ligands, BDNF and NT-4/5, are also expressed together with an autocrine/paracrine manner.<sup>59,60</sup> *TrkC* is expressed rather in favorable neuroblastomas at variable levels,<sup>61</sup> but its preferred ligand, *NT-3*, is usually undetectable by RT-PCR in primary tumors.<sup>58</sup> Thus, expression levels of Trks show one of heterogeneous characteristics in neuroblastoma.

## Telomere and telomerase biology

Normal cells have a limited life span, only dividing 20 to 80 times before growth arrest (senescence) and eventually dying. Telomere, specialized DNA-protein structure at the ends of eukaryotic chromosomes, consists of a large number of tandem repeats of short guanine-rich sequence which is highly conserved throughout evolution.<sup>62,63</sup> The gradual erosion with each cell division of chromosomal telomeres plays an integral role in cell senescence and activation of a mechanism for maintaining telomeres is a key to cell immortality.<sup>64</sup> Telomerase is a unique reverse transcriptase capable of maintaining telomere

length that is expressed in germ-line cells and immortal cells, not in most somatic cells, due to the repression of telomerase during development. Expression of sufficient telomerase activity and stabilization of telomeres is frequently found in highly malignant neuroblastoma.<sup>65</sup>

Using a highly sensitive, polymerase chain reaction-based assay for measuring telomerase activity,<sup>66,67</sup> which is known as the TRAP (telomeric repeat amplification protocol) assay, several studies have reported telomerase activity in neuroblastoma tissue.<sup>68-72</sup> Telomerase activity was not detectable in adrenal gland or in ganglioneuromas, but was detectable in almost all untreated neuroblastoma specimens except for stage 4S tumors.<sup>68,69</sup> Moreover, high expression of telomerase activity has been shown to correlate with advanced stages of disease and with tumor biological features that predict an adverse prognosis.<sup>68-70,73</sup> As shown in figure 4, favorable neuroblastoma could retain low telomerase activity of normal fetal neuroblast from a failure to repress telomerase activity during development.

Neuroblastomas with high telomerase activity have various telomere lengths, but these are presumably stabilized and maintained at a constant length, and in some cases are elongated far beyond that detected in normal cells (figure 4). These tumors were associated with advanced stages and more than one half of patients with high telomerase activity tumors died of disease.<sup>69</sup> On the other hand, none of those tumors with low or undetectable telomerase activity have elongated telomeres and those with shortened telomere lengths may be the result of repeated replication without sufficient telomerase activity. Indeed, most stage 4S tumors examined showed shortened telomeres relative to normal tissue, suggesting that telomere shortening with low or absent telomerase activity may be a factor in promoting the spontaneous regression of the tumors seen in some patients.

Human telomerase activity is associated with the expression of two major components: human telomerase RNA (*hTR*),<sup>63</sup> and human telomerase reverse transcriptase (TERT).<sup>74</sup> Recent studies have targeted the expression of these two components as surrogates of telomerase activity and discussed the feasibility of their quantitative evaluation. Since *hTR* is expressed at low level even in cells without

telomerase activity,<sup>75</sup> detection of *TERT* mRNA expression is believed to be a more reliable marker for existing cancer cells in neuroblastoma.<sup>76-78</sup> In situ hybridization (ISH) of telomerase components (hTR and *TERT* mRNA) and TERT immunohistochemistry (IHC) are applicable to fixed cells (figure 4).<sup>79-81</sup>

## Perspectives

Neuroblastoma, despite many advances in the understanding of its biological heterogeneity and developmental molecular pathways, has remained a serious disease in young children. Basic research and clinical efforts will lead to an understanding of the molecular pathways governing in occurrence, progression and spontaneous regression of neuroblastoma. Neuroblastoma mass-screening project

revealed that more than half of infant neuroblastomas regress or mature, while some favorable tumors might transit to unfavorable phenotype. These events should provide the platform to identify new diagnostic and prognostic markers including regression and progression indicators and might develop the new diagnostic and prognostic strategies for neuroblastoma under the well-understanding of neuroblastoma biology. Using recent advances of genome-wide genetic aberrations and gene expression profiles, more precise definition of the molecular markers in neuroblastomas may allow for more specific diagnostic and therapies with subsequent improvements in overall rates and quality of cure

**Table 1.** Genetic and molecular abnormalities in neuroblastoma.

Abnormalities	Associated genetic/ molecular abnormalities	Prognosis
Triploid	Unknown	Good
Diploid/ tetraploid	MYCN amplification	Poor
LOH 1p	MYCN amplification	Poor*
2q gain	Unknown	Poor
LOH 3p	LOH 11q, 14q, MYCN normal	Intermediate
LOH 4p	Unknown	Unknown
LOH 9p	Unknown	Unknown
LOH 11q	LOH 3p, 14q, MYCN normal	Intermediate
LOH 14q	LOH 3p,11q, MYCN normal	Intermediate
17q gain	t (1;17) or t (11; 17)	Poor
	NM23-H1 overexpression	
	Survivin overexpression	
	MYCN amplification	
MYCN amplification	LOH 1p, 17q gain	Poor
	High telomerase activity	
CCND1 amplification	CCND1 overexpression	Unknown

**LOH: Loss of heterozygosity.**  
\* 1p 36 deletion is not correlated with poor prognosis.

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