

# Molecular Markers in Neuroblastoma

Eiso Hiyama<sup>1,2\*</sup>, Naomi Kamei<sup>2</sup>, Arata Kamimatsuse<sup>2</sup>

<sup>1</sup> Natural Science Center for Basic Research and Development and <sup>2</sup> Department of Pediatric Surgery, Hiroshima University Hospital, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Hiroshima, Japan

*\*Corresponding author: Eiso Hiyama, MD, Natural Science Center for Basic Research and Development, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Hiroshima, Japan  
(Phone: +81 822 55 59 51, Fax: +81 822 57 52 19, E-mail: eiso@hiroshima-u.ac.jp)*

## 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 makers 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.

## 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.

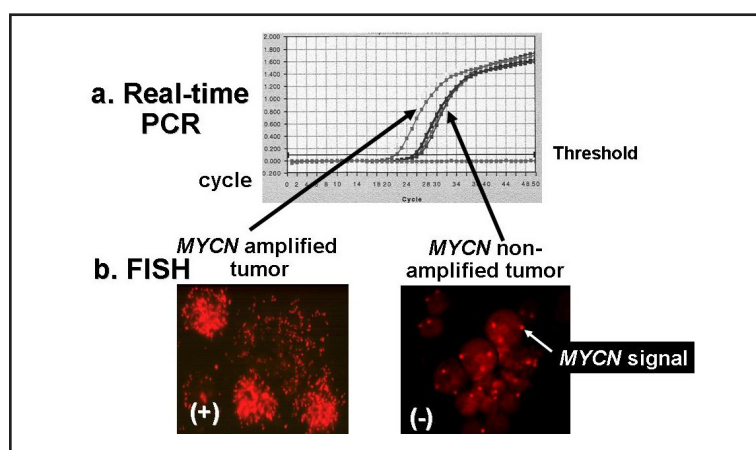
Clinically, to distinguish progressive tumors from favorable tumors is needed because multimodal

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

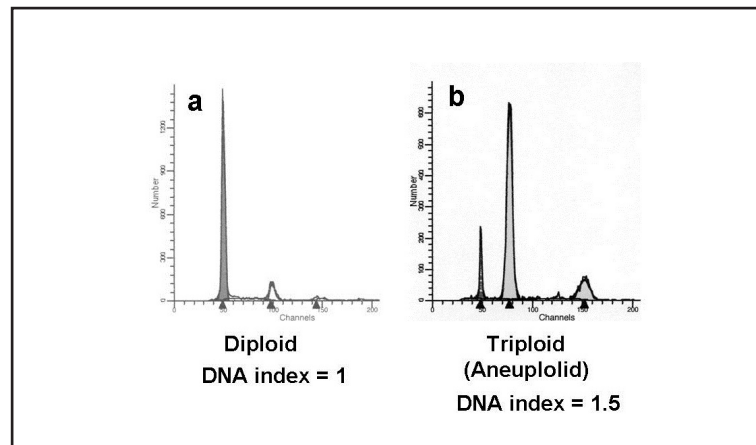
### *MYCN* amplification

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 advance 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>

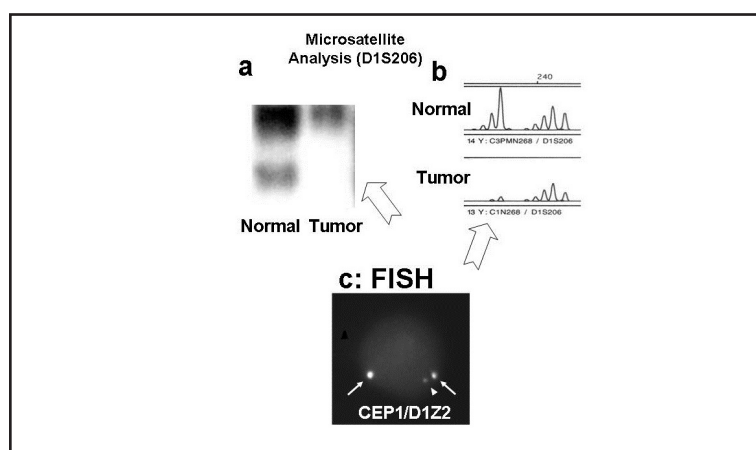
### Chromosome loss and gain

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>

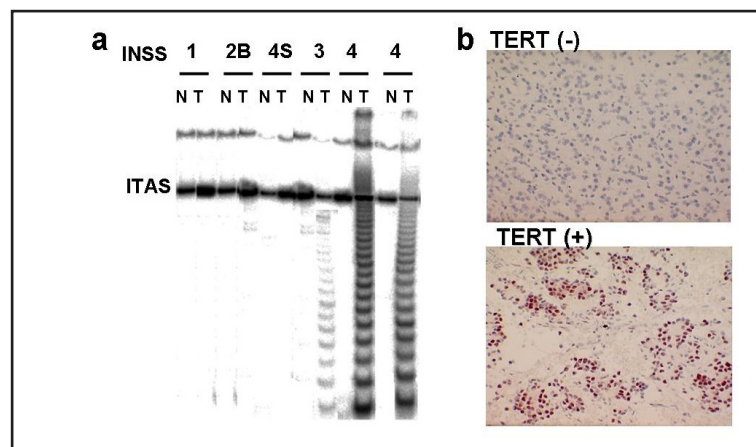
Clinically, gain of 17q is more common at an advanced stage, in tumors from children aged over 1 year, and in tumors showing 1p loss, *MYCN* amplification and diploidy/ tetraploidy. On the other hand, triploidy with whole chromosome 17 gain is associated more often with neuroblastomas showing favorable 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.

## References

1. Gurney JG, Ross JA, Wall DA, Bleyer WA, Severson RK, Robison LL. Infant cancer in the U.S.: histology-specific incidence and trends, 1973 to 1992. *J Pediatr Hematol Oncol.* 1997; 19: 428-32.
2. Evans AE, Gerson J, Schnauffer L. Spontaneous regression of neuroblastoma. *Natl Cancer Inst Monogr.* 1976; 44: 49-54.
3. Evans AE, Baum E, Chard R. Do infants with stage IV-S neuroblastoma need treatment? *Arch Dis Child.* 1981; 56: 271-4.
4. Haas D, Ablin AR, Miller C, Zoger S, Matthay KK. Complete pathologic maturation and regression of stage IVS neuroblastoma without treatment. *Cancer.* 1988; 62: 818-25.
5. Hiyama E, Iehara T, Sugimoto T, Fukuzawa M, Hayashi Y, Sasaki F, et al. Effectiveness of screening for neuroblastoma at 6 months of age: a retrospective population-based cohort study. *Lancet.* 2008; 371: 1173-80.
6. Bessho F, Hashizume K, Nakajo T, Kamoshita S. Mass screening in Japan increased the detection of infants with neuroblastoma without a decrease in cases in older children. *J Pediatr.* 1991; 119: 237-41.
7. Berthold F, Baillet A, Hero B, Schurr P, Nerenz A, Hunneman DH, et al. Which cases are found and missed by neuroblastoma screening at 1 year? Results from the 1992 to 1995 study in three Federal States of Germany. *J Clin Oncol.* 1999; 17: 1200.
8. Woods WG, Lemieux B, Tuchman M. Neuroblastoma represents distinct clinical-biologic entities: a review and perspective from the Quebec Neuroblastoma Screening Project. *Pediatrics.* 1992; 88: 114-8.
9. Brodeur GM, Maris JM, Yamashiro DJ, Hogarty MD, White PS. Biology and genetics of human neuroblastomas. *J Pediatr Hematol Oncol.* 1997; 19: 93-101.
10. 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.
11. Schwab M, Varmus HE, Bishop JM, Grzeschik KH, Naylor SL, Sakaguchi AY, et al. Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. *Nature.* 1984; 308: 288-91.
12. Schwab M. Human neuroblastoma: from basic science to clinical debut of cellular oncogenes. *Naturwissenschaften.* 1999; 86: 71-8.
13. Perez CA, Matthay KK, Atkinson JB, Seeger RC, Shimada H, Haase GM, et al. J Biologic variables in the outcome of stages I and II neuroblastoma treated with surgery as primary therapy: a children's cancer group study. *Clin Oncol.* 2000; 18: 18-26.
14. Berthold F, Hero B, Kremens B, Handgretinger R, Henze G, Schilling FH, et al. Long-term results and risk profiles of patients in five consecutive trials (1979-1997) with stage 4 neuroblastoma over 1 year of age. *Cancer Lett.* 2003; 197: 11-7.
15. Kaneko M, Nishihira H, Mugishima H, Ohnuma N, Nakada K, Kawa K, et al. Stratification of treatment of stage 4 neuroblastoma patients based on N-myc amplification status. Study Group of Japan for Treatment of Advanced Neuroblastoma, Tokyo, Japan. *Med Pediatr Oncol.* 1998; 31: 1-7.
16. Kaneko M, Tsuchida Y, Mugishima H, Ohnuma N, Yamamoto K, Kawa K, et al. Intensified chemotherapy increases the survival rates in patients with stage 4 neuroblastoma with MYCN amplification. *J Pediatr Hematol Oncol.* 2002; 24: 613-21.
17. Shapiro DN, Valentine MB, Rowe ST, Sinclair AE, Sublett JE, Roberts WM, et al. Detection of N-myc gene amplification by fluorescence in situ hybridization. Diagnostic utility for neuroblastoma. *Am J Pathol.* 1993; 142: 1339-46.
18. Squire JA, Thorner P, Marrano P, Parkinson D, Ng YK, Gerrie B, et al. Identification of MYCN Copy Number Heterogeneity by Direct FISH Analysis of Neuroblastoma Preparations. *Mol Diagn.* 1996; 1: 281-9.
19. Gotoh T, Hosoi H, Iehara T, Kuwahara Y, Osone S, Tsuchiya K, et al. Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction. *J Clin Oncol.* 2005; 23: 5205-10.
20. Brodeur GM, Hayes FA, Green AA, Casper JT, Wasson J, Wallach S, et al. Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. *Cancer Res.* 1987; 47: 4248-53.
21. Ohtsu K, Hiyama E, Ichikawa T, Matsuura Y, Yokoyama T. Clinical investigation of neuroblastoma



with partial deletion in the short arm of chromosome 1. *Clinical Cancer Research*. 1997; 3: 1221-8.

22. Hiyama E, Hiyama K, Yokoyama T. Neuroblastoma with DNA amplification and rearrangement in the N-myc gene region. *Cancer Res*. 1991; 51: 1946-51.

23. Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, et al. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med*. 1999; 340: 1954-61.

24. Amler LC, Schwab M. Amplified N-myc in human neuroblastoma cells is often arranged as clustered tandem repeats of differently recombined DNA. *Mol Cell Biol*. 1989; 9: 4903-13.

25. McDermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. *Cancer Res*. 2008; 68: 3389-95.

26. Mosse YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, et al. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature*. 2008; 455: 930-5.

27. Gansler T, Chatten J, Varello M, Bunin GR, Atkinson B. Flow cytometric DNA analysis of neuroblastoma. Correlation with histology and clinical outcome. *Cancer*. 1986; 58: 2453-8.

28. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, et al. Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol*. 1991; 9: 581-91.

29. Kaneko Y, Kanda N, Maseki N, Sakurai M, Tsuchida Y, Takeda T, et al. Different karyotypic patterns in early and advanced stage neuroblastomas. *Cancer Res*. 1987; 47: 311-8.

30. Ladenstein R, Ambros IM, Potschger U, Amann G, Urban C, Fink FM, et al. Prognostic significance of DNA di-tetraploidy in neuroblastoma. *Med Pediatr Oncol*. 2001; 36: 83-92.

31. Plantaz D, Vandesompele J, Van Roy N, Lastowska M, Bown N, Combaret V, et al. Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification. *Int J Cancer*. 2001; 91: 680-6.

32. Lastowska M, Nacheva E, McGuckin A, Curtis A, Grace C, Pearson A, et al. Comparative genomic hybridization study of primary neuroblastoma

tumors. *United Kingdom Children's Cancer Study Group. Genes Chromosomes Cancer*. 1997; 18: 162-9.

33. Vandesompele J, Van Roy N, Van Gele M, Laureys G, Ambros P, Heimann P, et al. Genetic heterogeneity of neuroblastoma studied by comparative genomic hybridization. *Genes Chromosomes Cancer*. 1998; 23: 141-52.

34. Hiyama E, Yamaoka H, Kamimatsuse A, Onitake Y, Hiyama K, Nishiyama M, et al. Single nucleotide polymorphism array analysis to predict clinical outcome in neuroblastoma patients. *J Pediatr Surg*. 2006; 41: 2032-6.

35. Fong CT, Dracopoli NC, White PS, Merrill PT, Griffith RC, Housman DE, et al. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci USA*. 1989; 86: 3753-7.

36. Kohno T, Morishita K, Takano H, Shapiro DN, Yokota J. Homozygous deletion at chromosome 2q33 in human small-cell lung carcinoma identified by arbitrarily primed PCR genomic fingerprinting. *Oncogene*. 1994; 9: 103-8.

37. Takeda O, Homma C, Maseki N, Sakurai M, Kanda N, Schwab M, et al. There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chromosomes Cancer*. 1994; 10: 30-9.

38. Hiyama E, Hiyama K, Ohtsu K, Yamaoka H, Fukuba I, Matsuura Y, et al. Biological characteristics of neuroblastoma with partial deletion in the short arm of chromosome 1. *Med Pediatr Oncol*. 2001; 36: 67-74.

39. Caron H, Peter M, van Sluis P, Speleman F, de Kraker J, Laureys G, et al. Evidence for two tumour suppressor loci on chromosomal bands 1p35-36 involved in neuroblastoma: one probably imprinted, another associated with N-myc amplification. *Hum Mol Genet*. 1995; 4: 535-9.

40. Spieker N, Beitsma M, Van Sluis P, Chan A, Caron H, Versteeg R. Three chromosomal rearrangements in neuroblastoma cluster within a 300-kb region on 1p36.1. *Genes Chromosomes Cancer*. 2001; 31: 172-81.

41. Mertens F, Johansson B, Hoglund M, Mitelman F. Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. *Cancer Res*. 1997; 57: 2765-80.

42. Takita J, Hayashi Y, Kohno T, Shiseki M,

Yamaguchi N, Hanada R, et al. Allelotype of neuroblastoma. *Oncogene*. 1995; 11: 1829-34.

43. Fong CT, White PS, Peterson K, Sapienza C, Cavenee WK, Kern SE, et al. Loss of heterozygosity for chromosomes 1 or 14 defines subsets of advanced neuroblastomas. *Cancer Res*. 1992; 52: 1780-5.

44. Takeda O, Handa M, Uehara T, Maseki N, Sakashita A, Sakurai M, et al. An increased NM23H1 copy number may be a poor prognostic factor independent of LOH on 1p in neuroblastomas. *Br J Cancer*. 1996; 74: 1620-6.

45. Guo C, White PS, Hogarty MD, Brodeur GM, Gerbing R, Stram DO, et al. Deletion of 11q23 is a frequent event in the evolution of MYCN single-copy high-risk neuroblastomas. *Med Pediatr Oncol*. 2000; 35: 544-6

46. Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, et al. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. *Oncogene*. 1999; 18: 4948-57.

47. Maris JM, Guo C, White PS, Hogarty MD, Thompson PM, Stram DO, et al. Allelic deletion at chromosome bands 11q14-23 is common in neuroblastoma. *Med Pediatr Oncol*. 2001; 36: 24-7.

48. McArdle L, McDermott M, Purcell R, Grehan D, O'Meara A, Breatnach F, et al. Oligonucleotide microarray analysis of gene expression in neuroblastoma displaying loss of chromosome 11q. *Carcinogenesis*. 2004; 25: 1599-609.

49. Caren H, Erichsen J, Olsson L, Enerback C, Sjoberg RM, Abrahamsson J, et al. High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene. *BMC Genomics*. 2008; 9: 353.

50. Michels E, Hoebeek J, De Preter K, Schramm A, Brichard B, De Paepe A, et al. CADM1 is a strong neuroblastoma candidate gene that maps within a 3.72 Mb critical region of loss on 11q23. *BMC Cancer*. 2008; 8: 173.

51. Ando K, Ohira M, Ozaki T, Nakagawa A, Akazawa K, Suenaga Y, et al. Expression of TSLC1, a candidate tumor suppressor gene mapped to chromosome 11q23, is downregulated in unfavorable neuroblastoma without promoter hypermethylation. *Int J Cancer*. 2008; 123: 2087-94.

52. Hackett CS, Hodgson JG, Law ME, Fridlyand J,

Osoegawa K, de Jong PJ, et al. Genome-wide array CGH analysis of murine neuroblastoma reveals distinct genomic aberrations which parallel those in human tumors. *Cancer Res*. 2003; 63: 5266-73.

53. Nakagawara A. Trk receptor tyrosine kinases: a bridge between cancer and neural development. *Cancer Lett*. 2001; 169: 107-14.

54. Tanaka T, Hiyama E, Sugimoto T, Sawada T, Tanabe M, Ida N. trk A gene expression in neuroblastoma. The clinical significance of an immunohistochemical study. *Cancer*. 1995; 76: 1086-95.

55. 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.

56. Tacconelli A, Farina AR, Cappabianca L, Desantis G, Tessitore A, Vetusch A, et al. TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. *Cancer Cell*. 2004; 6: 347-60.

57. 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.

58. Nakagawara A. Molecular basis of spontaneous regression of neuroblastoma: role of neurotrophic signals and genetic abnormalities. *Hum Cell*. 1998; 11: 115-24.

59. 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.

60. Matsumoto K, Wada RK, Yamashiro JM, Kaplan DR, Thiele CJ. Expression of brain-derived neurotrophic factor and p145TrkB affects survival, differentiation, and invasiveness of human neuroblastoma cells. *Cancer Res*. 1995; 55: 1798-806.

61. Yamashiro DJ, Nakagawara A, Ikegaki N, Liu XG, Brodeur GM. Expression of TrkC in favorable human neuroblastomas. *Oncogene*. 1996; 12: 37-41.

62. Zakian VA. Structure and function of telomeres. *Annu Rev Genet*. 1989; 23: 579-604.

63. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, et al. The RNA component of human telomerase. *Science*. 1995; 269: 1236-41.

64. Harley CB, Kim NW, Prowse KR, Weinrich SL, Hirsch KS, West MD, et al. Telomerase, cell immor

- tality, and cancer. Cold Spring Harb Symp Quant Biol. 1994; 59: 307-15.
65. Hiyama E, Hiyama K. Telomere and telomerase in stem cells. Br J Cancer. 2007; 96: 1020-4.
  66. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994; 266: 2011-5.
  67. Piatyszek MA, Kim NW, Weinrich SL, Hiyama K, Hiyama E, Wright WE, et al. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci. 1995; 17: 1-15.
  68. 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.
  69. Hiyama E, Hiyama K, Ohtsu K, Yamaoka H, Ichikawa T, Shay JW, et al. Telomerase activity in neuroblastoma: is it a prognostic indicator of clinical behaviour? Eur J Cancer. 1997; 33: 1932-6.
  70. Reynolds CP, Zuo JJ, Kim NW, Wang H, Lukens JN, Matthay KK, et al. Telomerase expression in primary neuroblastomas. Eur J Cancer. 1997; 33: 1929-31.
  71. Brinkschmidt C, Poremba C, Christiansen H, Simon R, Schafer KL, Terpe H, et al. Comparative genomic hybridization and telomerase activity analysis identify two biologically different groups of 4s neuroblastomas. Br J Cancer. 1998; 77: 2223-9.
  72. Poremba C, Willenbring H, Hero B, Christiansen H, Schafer KL, Brinkschmidt C, et al. Telomerase activity distinguishes between neuroblastomas with good and poor prognosis. Ann Oncol. 1999; 10: 715-21.
  73. Hiyama E, Hiyama K, Yokoyama T, Ichikawa T, Matsuura Y. Length of telomeric repeats in neuroblastoma: correlation with prognosis and other biological characteristics. Jpn J Cancer Res. 1992; 83: 159-64.
  74. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, et al. Telomerase catalytic subunit homologs from fission yeast and human. Science. 1997; 277: 955-9.
  75. Koyanagi Y, Kobayashi D, Yajima T, Asanuma K, Kimura T, Sato T, et al. Telomerase activity is down regulated via decreases in hTERT mRNA but not TEP1 mRNA or hTERC during the differentiation of leukemic cells. Anticancer Res. 2000; 20: 773-8.
  76. Krams M, Hero B, Berthold F, Parwaresch R, Harms D, Rudolph P. Full-length telomerase reverse transcriptase messenger RNA is an independent prognostic factor in neuroblastoma. Am J Pathol. 2003; 162: 1019-26.
  77. Poremba C, Hero B, Heine B, Scheel C, Schaefer KL, Christiansen H, et al. Telomerase is a strong indicator for assessing the proneness to progression in neuroblastomas. Med Pediatr Oncol. 2000; 35: 651-5.
  78. Hiyama E, Hiyama K. Clinical utility of telomerase in cancer. Oncogene. 2002; 21: 643-9.
  79. Hiyama E, Hiyama K, Shay JW, Yokoyama T. Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. Neoplasia. 2001; 3: 17-26.
  80. Kumaki F, Kawai T, Hiroi S, Shinomiya N, Ozeki Y, Ferrans VJ, et al. Telomerase activity and expression of human telomerase RNA component and human telomerase reverse transcriptase in lung carcinomas. Hum Pathol. 2001; 32: 188-95.
  81. Kumaki F, Takeda K, Yu ZX, Moss J, Ferrans VJ. Expression of human telomerase reverse transcriptase in lymphangioleiomyomatosis. Am J Respir Crit Care Med. 2002; 166: 187-91.

