Cytogenetic analyses classified neuroblastomas into four ploidy patterns: near-diploid, near-triploid, near-tetraploid, and near-pentaploid tumors [42]. The near-diploid and near-tetraploid tumors were usually detected in children older than 1 year and frequently had genetic abnormalities involving 1p deletion 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 [43].

Analyzing 1p deletion and ploidy using two-color FISH in tumors obtained during mass-screened program in Japan, event-free survival (EFS) was lowest in the disomy 1 with 1p deletion group, intermediate in the disomy 1 with normal 1p group and highest in trisomy 1 group regardless 1p status [44]. This indicates that cytogenetic 1p deletion is a poor prognostic factor for diploid tumors, but not for triploid tumors. The majority of trisomy 1 tumors with 1p36 deletion were detected by mass screening. Modern risk stratification used DNA ploidy in infant neuroblastoma but not in children who are diagnosed over 1 year of age [45]. Recently, DNA ploidy was proposed to be a clinically useful factor for prognostic and treatment stratification in children with *MYCN*-amplified, favorable-stage tumors [46]. Thus, DNA ploidy is a very intriguing biological character in neuroblastoma.

3. Chromosome loss and gain

Chromosome loss and gain in neuroblastoma have been reported in various chromosome regions, most frequently in chromosome 1p (Fig. 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 (Fig. 4) [47-50].

3.1. 1p loss

Several reports using microsatellite markers (Fig. 3) identified that chromosome 1p deletion occurs in approximately 35% of all neuroblastomas [51, 52] and a smallest region of overlapping deletion (SRO) was mapped to 1p34-p36 [53], suggesting that tumor suppressor genes for the development of neuroblastoma might be located in this lesion. Recently, the SRO was refined to a size of approximately 1 Mb within 1p36.3. Patients whose tumors had large 1p deletion showed poorer outcome than patients with short or interstitial deletions [54, 55], suggesting the existence of more than one tumor suppressor genes at 1p locus in neuroblastoma. The tumors with large

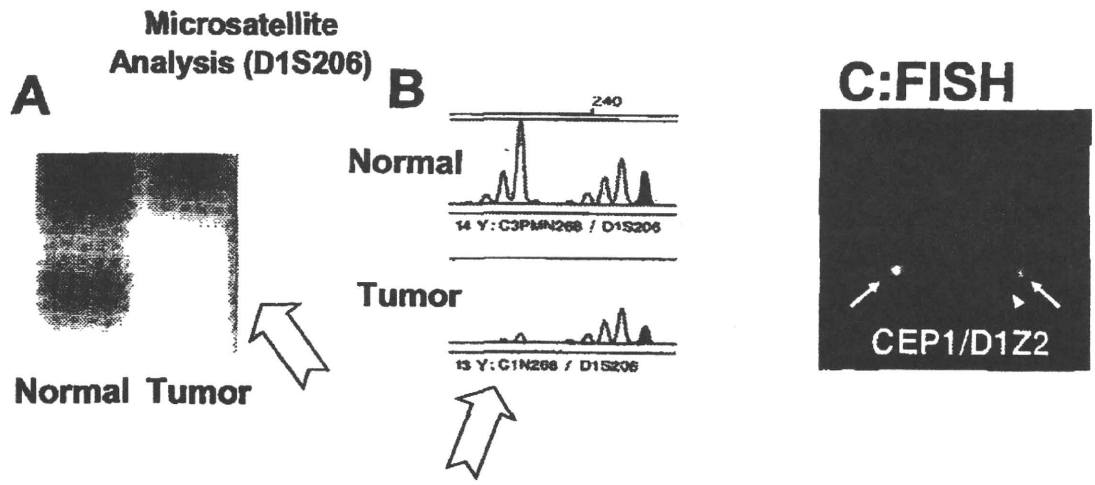

Figure 3. LOH analysis using microsatellite marker (A and B) and fluorescence *in situ* hybridization (FISH, C). The smaller-size allele was lost in the tumor sample in autoradiographic analysis (A) and fluorescent labeled fragment analysis (B) for microsatellite maker D1S206 (closed peak in B is a size marker). Two-color FISH analysis using a centromere probe of chromosome 1 (CEP1: green) and a telomere probe of chromosome 1p (D1Z2: red) revealed two green signals (arrow) and one red signal (arrow head), indicating a chromosome 1p loss in this tumor (C).

1p deletion were associated with other adverse prognostic factors, such as diploidy or tetraploidy and amplified *MYCN*, while small internal deletion of 1p were usually observed in favorable tumors in the triploid range. The regions of 1p deletions in *MYCN*-amplified tumors are very large including 1p32 to telomere [30]. In contrast, in *MYCN* non-amplified tumors, 1p deletions were described to be consistently smaller, and the commonly deleted region was mapped to 1p36.3. Thus, the second tumor suppressor gene, which is correlated with progressive neuroblastoma, was suggested to be located at 1p32 or 1p35-36.1, proximal to the SRO at 1p36.3, in *MYCN*-amplified cases [56, 57]. The SRO of the *MYCN* non-amplified tumors is included in the larger SRO of *MYCN*-amplified tumors, implying that more than two suppressor loci in 1p must be deleted in *MYCN*-amplified tumors.

Preferential 1p deletion of the maternal allele [58] would imply that the distal locus of tumor suppressor gene (TSG) at 1p36.3 may be subject to genomic imprinting. Another study indicated that the parental origin of the deleted allele is random in neuroblastoma with a large 1p deletion and amplified *MYCN* gene [59]. Other reports for preferential loss of the allele have given conflicting results [56, 58, 60, 61]. In this region, several candidate TSGs have been identified [62]. Among them, *CHD5* is the strongest candidate tumor suppressor gene that is deleted from 1p36.31 in neuroblastomas, and inactivation of the second allele may occur by an epigenetic

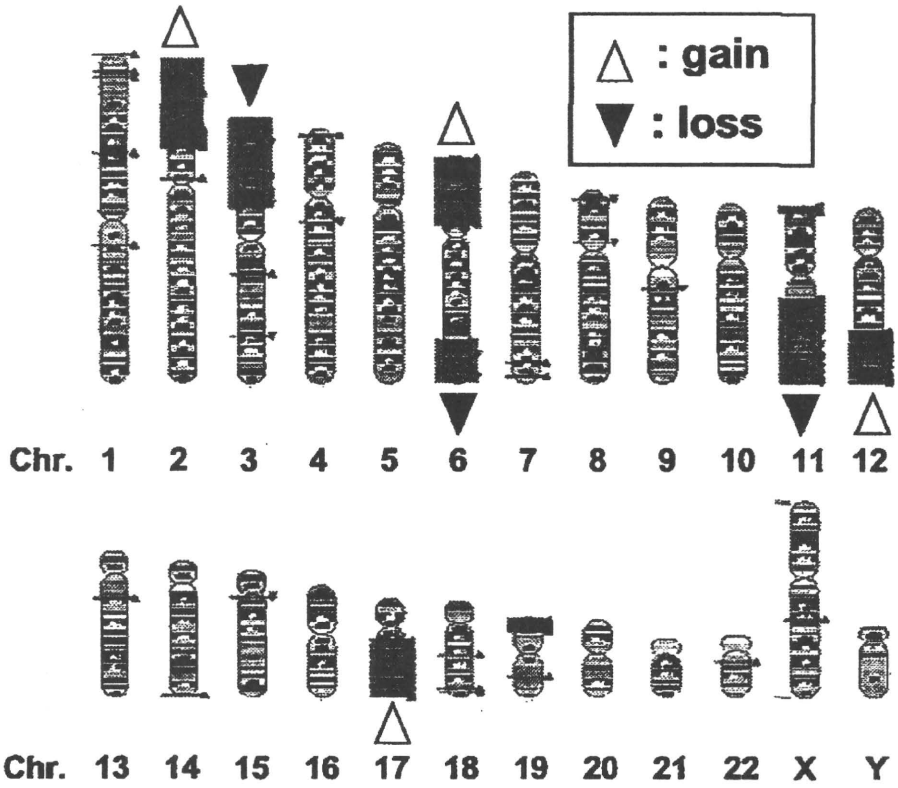

Figure 4. SNP (single nucleotide polymorphism) microarray analysis in a representative neuroblastoma sample. Approximately 1,000,000 SNPs were examined and the regions of gain (blue upward triangle) and the loss (red downward triangle) in each chromosome were described. This tumor showed the gain of chromosomes 2p, 6p, 12q and 17q and the loss of chromosomes 3p, 6q, and 11q regions.

mechanism [63]. The 1p alterations are frequently detected together with other genetic alterations, such as amplified *MYCN*, 17q gain, diploidy/triploidy, and each combination appears to have a divergent impact on tumor growth characteristics. In the recent study, LOH at 1p36 was a significant independent predictor of decreased event-free survival, but had no significant effect on overall survival in multivariate analysis [64]. In contrast, amplified *MYCN* was a more powerful prognostic factor for decreased overall survival. This implies that 1p36 allelic status may be useful for predicting disease progression in patients with neuroblastoma showing otherwise favorable clinical and biological features.

3.2. 17q Gain

Cytogenetic studies also revealed additional copies of 17q in cell lines and primary tumors [65]. Several studies on neuroblastoma cells and tumors have indicated a high frequency of unbalanced translocations involving

chromosome 17q [39, 66, 67]. Clinically, gain of 17q is more common in tumors at an advanced stage, obtained from children aged over 1 year, and showing 1p loss, *MYCN* amplification and/or diploidy/tetraploidy. On the other hand, triploidy with whole chromosome 17 gain is associated more often with neuroblastomas showing favorable clinical features [32]. The report from six European centers with more than 300 cases demonstrated that 17q gain was the most powerful prognostic 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) [32]. 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. FISH analyses indicated that the breakpoints were clustered in the proximal half of 17q from *D17Z1* to *MPO* (17q23.1), with a shortest region of gain extending from *MPO* to 17qter [68, 69]. Several candidate genes have been proposed to be responsible for the 17q gain effect on tumor growth characteristics. Survivin, an anti-apoptosis protein, which was recently mapped to 17q25, is one candidate as its expression correlates strongly with adverse clinical data [70]. *NME1* (*NM23-H1*, non-metastatic cells 1, protein (NM23A) expressed in) located at 17q21-22 is another candidate because its overexpression has been demonstrated in aggressive neuroblastomas [71, 72].

3.3. 11q loss

Cytogenetic analyses have also detected 11q deletion in about 15% of neuroblastomas [73]. Constitutional aberrations of 11q, including a deletion of 11q23-qter, have been reported in some neuroblastoma patients [74-76]. These constitutional changes may predispose neuroblasts to the development of neuroblastoma. On the other hand, LOH studies revealed 11q loss in 5-32% of the tumors [77-79]. Loss of the whole chromosome 11 was observed in 19%, while unbalanced 11q LOH was observed in 22% of primary neuroblastomas [80]. 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* [80, 81]. Loss of 11q was significantly correlated with adverse clinical parameters, including age over 1 year, stage 4, and unfavorable histology [82]. The SRO in 11q23.3 locates between markers *D11S1340* and *D11S1299* in the tumors with 11q LOH [81]. Unbalanced 11q deletion is considered as a frequent event in the

MYCN non-amplified tumors, and COG study recently revealed that 11q deletion is an independent prognostic indicator for predicting high risk for tumor progression, especially in *MYCN* non-amplified tumors [47, 83]. Several candidate TSGs have been reported in this 11q region: *CDKN2A*, *CADM1*, *TSLC1* and so on [84-86].

3.4. 16p12 loss

Genome-wide linkage analysis revealed that a candidate locus for a part of familial neuroblastomas is 16p12 [87, 88], in addition to 1p [64, 89] and 2p (*ALK*) [34, 35]. Thus, tumor suppressor genes as well as an oncogene with dominant mutation would function to predispose to familial neuroblastomas.

3.5. Chromosome imbalance

Comparative genome hybridization (CGH) and microarray analyses including array-based CGH and single nucleotide polymorphism (SNP) array have been used for genome-wide screening of gains and losses in neuroblastoma [47-50, 84, 90]. With respect to ploidy changes in neuroblastoma, CGH analyses have confirmed the findings proposed by flow cytometry or classical cytogenetic analysis: triploid tumors with favorable outcomes are characterized by numerical chromosome imbalances, including the typical pattern of gains for chromosomes 1, 2, 6, 7, 8, 12, 13, 17, 18, and 22 and losses for chromosomes 3, 4, 9, 11, 14, and X, with only few structural abnormalities [49]. Partial loss of the chromosomes is usually detected in advanced-stage tumors. Similarly, gain of the whole chromosome 17 is predominantly observed in early-stage tumors, whereas partial gain of 17q is usually detected in advanced stage tumors [49]. A number of CGH studies have also confirmed the unbalanced 11q deletion in approximately 20% of primary neuroblastomas, and loss of whole chromosome 11 was a frequent finding in near-triploid early-stage neuroblastomas [47, 90, 91]. Unbalanced 11q deletion was detected in more than 50% of stage 4 neuroblastomas without *MYCN* amplification [47]. This genetic subgroup is also characterized by a positive correlation with deletion events, such as losses of 3p, 4p, and 14q, and an inverse correlation with 1p deletion, and furthermore 17q gain was consistently present [47, 49]. More recently, array-based CGH and SNP array, the powerful tools to survey whole chromosomal changes in tumor cells, revealed that major alternations in neuroblastoma cells were losses of 1p and 11q and gain of 17q [50, 92]. Thus, in addition to amplified *MYCN* gene, 1p deletion, 17q gain, 11q deletions, ploidy changes,

Table 1. Genetic and Molecular Abnormalities in Neuroblastoma.

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

* 1p36 deletion is not correlated with poor prognosis

and further genetic alterations exist in neuroblastomas (Table 1). These methods will clarify the role of genetic aberrations in biological heterogeneity of neuroblastoma.

4. Epigenetic modification: DNA methylation

As an epigenetic modification, the methylation of cytosines within the dinucleotides CpGs in gene promoter region is an essential regulatory mechanism for normal cell development. The aberrant methylation of CpG-rich regions (CpG islands, CGi) within gene promoter regions, in cooperation with histones acetylation and deacetylation that can modify nuclear chromatin conformation interfering with the transcriptional machinery, epigenetically alters the relative gene expression [93, 94]. In this respect, it isgenerally agreed that genetic factors (e.g., mutations, deletions, and chromosome rearrangements) cooperate with epigenetic mechanisms (e.g.,

methylation and acetylation) in inactivation of the pathways biologically important to induce cell transformation. On the other hand, demethylation of specific sequences may reactivate the expression of potential oncogenes. Thus, aberrant methylation status is considered to be a crucial step leading to cancer development. Recently, several reports described the role of methylation in neuroblastoma and in particular its clinical significance.

Indeed, when the pattern of methylation of the caspase 8 (*CASP8*) gene was first analyzed [95-98], it appeared that this gene was epigenetically silenced in *MYCN* amplified tumors. However, a detailed analysis of the structure of this gene revealed that the region previously considered as 'regulatory' was intragenic and lacked evident promoter activity [99]. Furthermore the identification and characterization of a *CASP8* promoter region showed that the effect of DNA methylation on its expression was indirect [99, 100]. Nevertheless, the level of methylation of that intragenic region near the third exon of *CASP8* is higher in *MYCN* amplified tumors as compared to that in single copy ones. Now, we are developing an oligoarray system for detecting *CASP8* methylation.

Epigenetic inactivation of *RASSF1A* (Ras-association domain family 1, isoform A) is one of the most common molecular changes in cancer. Hypermethylation of the *RASSF1A* promoter CpG island silences expression of the gene in many cancers including lung, breast, prostate, glioma, neuroblastoma and kidney cancers. Several recent studies have illustrated the prognostic potential of *RASSF1A* methylation in neuroblastoma. Genome-wide methylation might be correlated with tumor cell biology in neuroblastoma. Now, DNA methylation array is available so provides the evidence, for interrogation of genome-wide tumor-specific changes in DNA methylation and to identify and validate genes with aberrant DNA methylation in neuroblastomas, as shown in other childhood cancers [101].

5. Expression of neurotrophin receptors

Neuroblastoma originates from sympathetic neurons, which are derived from the neural crest. The migration of neural crest cells during embryonic development is controlled by several molecules including the bone morphogenetic proteins, *ASCL1* (*MASH1*, achaete-scute complex homolog 1), *RET*, *MYCN*, and Trk family tyrosine kinase 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 [102]. 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 [103, 104]. Explanted neuroblastoma cells with high TrkA expression differentiate when exposed to NGF or undergo apoptosis in the absence of NGF [104]. Thus, NGF/TrkA signaling could provoke differentiation or regression in favorable neuroblastomas depending on the particular microenvironment. A trophic theory is that normally developing sympathetic neurons survive and differentiate by the target-derived supplement of neurotrophins. The stromal cells such as schwannian cells and fibroblasts, as shown in normal sympathetic neurons, may supply a limited amount of NGF which partly regulates differentiation and programmed cell death of the neuroblast [105]. More recently, a neurodevelopmentally regulated oncogenic splice variant of TrkA (TrkAIII) that antagonises the anti-oncogenic NGF/TrkA signaling and promotes neuroblastoma tumor growth has been identified [106]. On the other hand, the levels of *NTRK1* (TrkA) expression is extremely low in aggressive tumors with *MYCN* amplification and/or 1p loss [107, 108]. In contrast, *NTRK2* (TrkB) is expressed in aggressive neuroblastomas and its preferred ligands, *BDNF* and *NTF4* (NT-4/5, neurotrophin 4), are also expressed simultaneously in an autocrine/paracrine manner [109, 110]. Thus, aggressive neuroblastomas shut off TrkA signals by down-regulating its expression or disturbing the downstream signaling cascades, whereas BDNF or NT-4/TrkB autocrine system may stimulate their growth. *NTRK3* (TrkC) is expressed rather in favorable neuroblastomas at various levels [111], but its preferred ligand, *NT-3*, is usually undetectable by RT-PCR in primary tumors [108]. Thus, expression levels of Trks show one of heterogeneous characteristics in neuroblastoma. Co-expression might compose an autocrine or paracrine survival pathway that additionally promotes chemotherapy-resistance and metastases.

6. Tumor specific mRNA in neuroblastoma

Using microarray gene expression profiles, neuroblastoma specific mRNA markers have been searched, but no definitely specific marker is identified. Most microarray data identified the correlation between biology and well-know genes such as *MYCN*, Trk, and cyclin D1 pathways [112-114]. Since accurate risk assessment of this disease is essential for the treatment, molecular markers for metastatic disease have been desired. Real-time quantitative RT-PCR (QRT-PCR) for bone marrow samples is a sensitive method for the detection of minimal disease (MD) and may improve monitoring of disease status and stratification of patients for therapy. To exploit the molecular mRNA markers to detect MD in children with

neuroblastoma, several microarray analyses revealed candidate markers for MD including *TH*, *PHOX2B*, and *DCX* mRNA [115, 116]. To validate the usefulness of these markers, clinical testings for a large corpus of patients are needed. Moreover, for the detection of MD in peripheral blood and bone marrow samples in children at diagnosis, on therapy, on follow-up, and at relapse, a panel of targets should be used to increase sensitivity and specificity by covering the heterogeneity of neuroblastoma and low-level expression in normal hematopoietic compartments [117]. In future, these mRNA markers might become useful indicators for the assessment of metastasis and effectiveness of therapy.

7. MicroRNAs

MicroRNAs (miRNAs) are located either in introns or other non-coding regions of chromosomes and are evolutionarily conserved, endogenous, small, noncoding RNA molecules of about 22 nucleotides in length that function as posttranscriptional gene regulators. Recent evidences have shown that miRNAs have diverse functions, including the regulation of cellular development, differentiation, proliferation, and apoptosis. They are deemed to play a crucial role in the initiation and progression of human cancer, and those with a role in cancer are designated as oncogenic miRNAs (oncomiRs) [118]. Since neuroblastoma is an embryonic tumor and microRNAs are major regulators of differentiation and development, microRNA analysis bears the potential to deliver new insights into the pathogenesis of this malignancy. Seven microRNAs revealed to be up-regulated by MYCN *in vivo* and *in vitro* in neuroblastoma: miR-92, miR-106, let-7b, miR-17, miR-93, miR-99, and miR-221 [119, 120]. In a very elegant attempt to integrate array CGH and microRNA data, Stallings' group analyzed microRNAs located in the commonly deleted 1p36 region. miRNA-34a was not only down-regulated in advanced neuroblastomas with 1p36 loss, but also its re-expression resulted in reduced proliferation of neuroblastoma cells and down-regulation of E2F3 [120]. MiRNA-125a/b were identified to be induced upon treatment with retinoic acid, and to modulate expression of the TrkC neurotrophin receptor, which itself is involved in the regulation of neuronal differentiation [121].

Further analysis of microRNA expression and function in neuroblastoma would contribute to the solution of some of the long-standing enigmatic questions about neuroblastoma tumor biology [122]. This will require microRNA expression profiling in a much larger cohort of well defined neuroblastomas. A single microRNA could serve as a prognostic biomarker or better identify distinct patient subgroups, providing new factors to improve individualized treatment of the patient. MicroRNA inhibitors, so-called

'Antagomirs' based on chemically modified nucleic acids, can be administered *in vivo* [123]. Nucleic acid mimicking microRNAs could be delivered in the same way, thereby functionally replacing tumor suppressor microRNAs down-regulated during oncogenesis. The functional implications of microRNA deregulation in neuroblastoma, as well as the evaluation of their potential application as novel anticancer targets using 'Antagomirs' deserve attention in future studies for neuroblastoma treatment [122].

8. Cancer stem cells

Spontaneous tumor regression is frequently observed in patients in the first year of life with a special pattern of disseminated disease (stage 4S) [3]. These tumors show no consistent genetic aberrations except for the aberrant number of chromosomes (triploid), indicating that these tumors might occur from normal stem cells (SCs) but not from cancer stem cells (CSCs). Importantly, 20–25% of neuroblastoma cases are usually detected in elderly patients who show poor prognosis. The differential clinical course may relate to patient age at diagnosis and its origin in the embryonic neural crest, i.e., whether the malignancy has arised from CSCs in the neural crest, as described more than 20 years ago [124]. Indeed, tumors with a favorable prognosis, generally diagnosed in children younger than one year, have no or very few cells that are capable of indefinite replication. By contrast, tumors with a high mortality rate, generally found in older children, are less differentiated and have a greater number of indefinitely self-renewing CSCs [125, 126]. Thus, continuously replicating (immortal) cell lines have been established almost exclusively from patients who ultimately succumbed to their disease. Childhood cancers arise from dysregulation of developing normal SCs, and some of which likely turn out the aggressive tumors which contain CSCs after additional events. This assumption is supported by a Japanese mass-screening project for neuroblastoma which led to increased detection of favorable infant neuroblastomas which are considered not to contain CSCs and consequently a significant decrease of unfavorable tumors in elderly patients which probably contain CSCs [6]. This CSC theory indicates that relapse or metastasis of cancer may be caused by a rare CSC that is relatively resistant to conventional chemotherapy. CSCs are considered to be basically immortal and CSCs or their descendant cells have a very high proliferative potential even if most CSCs are dormant. This speculation is based on the observation that starting from a few CSCs, a large number of cell divisions is required to generate visible metastatic or recurrent tumors containing heterogeneous cells.

Recently, β-catenin signaling was reported to be involved in the maintenance and expansion of neural crest stem cells [127, 128] and neural progenitors [129]. An engineered gain-of-function β-catenin allele targeted to neural tissues caused marked neural progenitor expansion by promoting cell cycle entry at the expense of differentiation [128]. This pathway might contribute to maintenance of neuroblastoma stem cells as well, raising the question of whether emerging inhibitors of this pathway might have therapeutic usefulness [130]. Further elucidation of CSC biology and markers in neuroblastoma may provide the important molecular markers for clinical course as in other malignancies.

9. 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 tightly conserved throughout evolution [131, 132]. 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 [133]. Telomerase is a unique reverse transcriptase capable of maintaining telomere length that is expressed in germ-line cells and immortal cells, but not in most somatic cells, due to the repression of telomerase during development. Expression of sufficient telomerase activity and stabilization of telomeres are frequently found in highly malignant neuroblastomas that would contain so-called CSCs or their progenitor cells [134].

Using a highly sensitive, polymerase chain reaction-based assay for measuring telomerase activity, which is known as the TRAP (telomeric repeat amplification protocol) assay [135, 136], several studies have reported telomerase activity in neuroblastoma tissues [91, 137-140]. 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 [137, 138]. 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 [137-139, 141].

TRF (terminal restriction fragment) length, an indicator of telomere length, of normal adrenal glands in neuroblastoma patients ranged between 8 and 15 kb [141, 142], which are similar in size to other normal somatic cells. 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 (Fig. 5). These tumors were associated with advanced stages and more than one half of patients with high telomerase activity tumors died of disease [138]. 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. Alternative

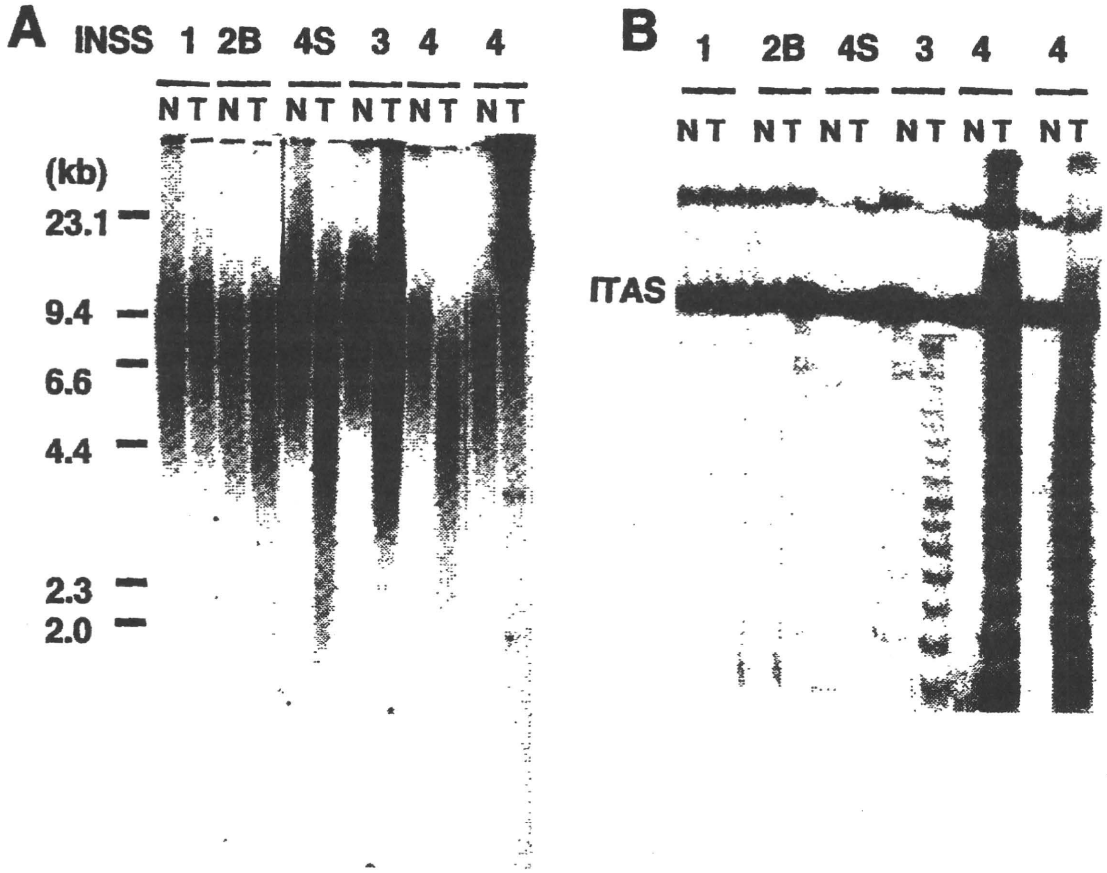

Figure 5. Telomere length measured by Southern blot analysis (A) and telomerase activity measured by TRAP assay (B) in normal adrenal gland (N) and neuroblastoma tissue (T) of 6 representative neuroblastoma cases. Stages were classified according to International Neuroblastoma Staging System (INSS). In cases of stages 1, 2B, and 3, telomere length in neuroblastoma tissue was indistinguishable from that of each corresponding normal adrenal gland (A), and telomerase activity in neuroblastoma tissues at stages 1, 2B, and 4S was undetectable as was in all noncancerous adrenal gland tissues (B). In the stage 4S case, telomere length was shorter than that of normal adrenal gland (A), suggesting that the telomere shortening without telomerase activity may cause regression of this tumor. Whereas 2 tumor samples in stage 4 showed shorter and longer telomeres, respectively (A), telomerase was highly activated (strong 6-base ladder signals in B) in both tumors, indicating that telomere lengths were maintained at these lengths.

telomere lengthening (ALT), which is alternative mechanism to elongate telomeres in some immortal culture cells, has not been reported in primary neuroblastoma. Most tumors with low or undetectable activity showed favorable outcomes. Indeed, most stage 4S tumors examined showed shortened telomeres relative to normal tissues, 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.

In human germ cells, telomerase is expressed to maintain telomere length, while in embryonic somatic tissues telomerase is likely repressed gradually before birth as cells differentiate [143]. Fetal adrenal gland tissues containing neuroblasts at 16 and 18 weeks of gestational age exhibited low telomerase activity. As shown in Fig. 6, favorable neuroblastoma could retain telomerase activity of normal fetal neuroblast from a failure to repress telomerase activity during development. Alternatively, reactivation of telomerase could occur in the tumor cells during progression of these cells, and consequently unfavorable aggressive neuroblastoma with high telomerase activity is likely to come out. The fact that most of these aggressive tumors are found in children older than one year is also compatible with the hypothesis that such tumor cells likely have emerged after accumulating several genetic changes during the course of multiple cell divisions. Such aggressive tumors are considered to include CSCs because of high recurrent and metastatic rates. Favorable neuroblastomas appear to share many features of neuroblasts. Most of these favorable tumors occur in infants (< one year old) and appear to have few genetic aberrations as described before in this chapter. Since these tumors are likely derived from dysregulation of normal neuroblasts, they senesce or regress consequently (mortal).

Human telomerase activity is associated with the expression of two major components: human telomerase RNA (hTR) [132] and human telomerase reverse transcriptase (TERT) [144]. 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 [145], detection of *TERT* mRNA expression is believed to be a more reliable marker for existing telomerase-positive cancer cells in neuroblastoma [146-148]. However, the existence of splicing variants of *TERT* mRNA that do not produce telomerase activity [149] is also problematic in detection of *TERT* mRNA as a surrogate of telomerase activity. *In situ* hybridization (ISH) of telomerase components (hTR and *TERT* mRNA) and TERT immunohistochemistry (IHC) are applicable to fixed cells [150-152]. However, hTR is detectable at low level in cells without telomerase activity, and it does not

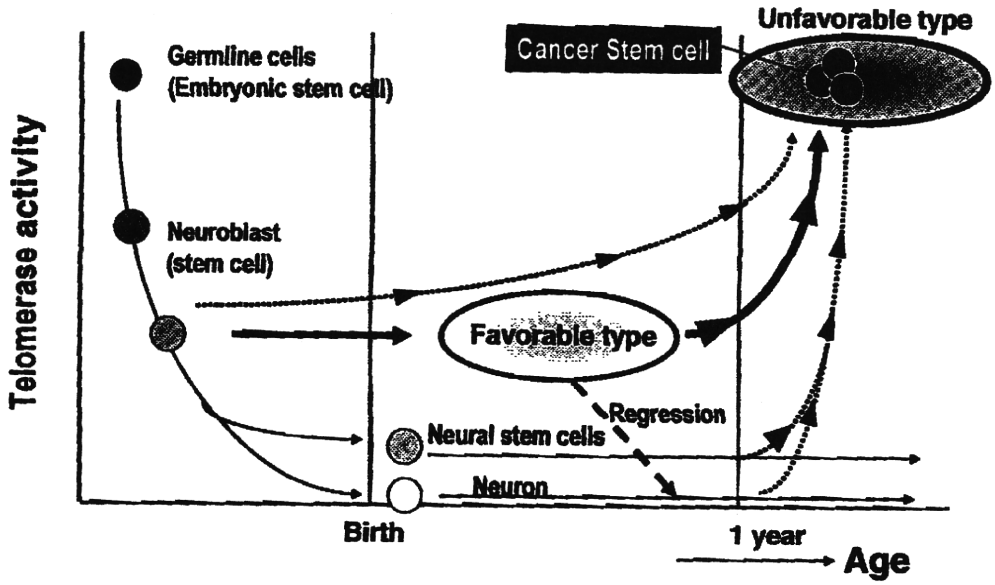

Figure 6. Hypothesis of Neuroblastoma Development. Normal neuroblasts, which develop from germline cells with high telomerase activity, gradually reduce the telomerase activity level according to fetal development. Thus, telomerase activity is repressed in normal neuroblasts (normal stem cells) and other somatic cells except for neural stem cells before birth. We hypothesized that favorable neuroblastoma has developed from the neuroblasts retaining the fetal expression level of telomerase activity, and this tumor is considered to be derived from normal stem cells by developmental dysregulation. Most of these tumors occur in infants and some of them regress concomitant with a loss of telomerase activity. Telomere lengths of these tumors are indistinguishable or shortened from those of normal tissues. On the other hand, unfavorable tumors are considered to have occurred with telomerase reactivation. In these tumors, other genetic aberrations such as *MYCN* amplification promote cell division and shortening of telomeres. Critical shortening of telomeres causes chromosomal instability which contributes to the accumulation of additional genetic alterations and telomerase reactivation. This reactivation maintains telomeres at various lengths and allows cells capable of unlimited proliferation. These tumors occur in older children and frequently show poor prognosis. These tumors might develop in the progression of favorable tumors, and cancer stem cells with telomerase activity appear in the tumor. The high rates of recurrence and metastasis are likely derived from cancer stem cells. Thus, telomerase expression may be required as a critical step in the multigenetic process of tumorigenesis, and cancer stem cells may play an important role in the development of unfavorable neuroblastoma.

always represent telomerase activation. On the other hand, ISH of *TERT* mRNA and TERT IHC are preferable to evaluate telomerase activation [150, 151, 153]. In neuroblastoma, the level of TERT expression in each cell differed between the unfavorable tumors with high telomerase activity and the favorable ones with low telomerase activity. The levels of TERT

expression may reflect the differentiating process of this tumor cells as described before [150]. The detail analysis of TERT, hTR, and telomerase associated proteins should contribute to clarify the biology of neuroblastoma in future and understanding of the biology of telomeres and telomerase in both normal and neuroblastoma cells might contribute to the development of antitelomerase agents as a novel therapeutic strategy for neuroblastoma [154].

Perspectives

Neuroblastoma, despite many advances in the understanding of its biological heterogeneity and developmental molecular pathways, remains a serious disease in young children. Basic research and clinical efforts will lead to an understanding of the molecular pathways governing 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 transform to unfavorable phenotype [6]. 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 technologies for genome-wide genetic aberrations and gene expression profiles, more precise definition of the molecular markers in neuroblastomas may allow more specific diagnostics and therapies with subsequent improvements in overall rates and quality of cure.

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