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Review Article

Neuroblastoma

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Abstract

Neuroblastoma is one of the most common solid tumors in children and has a diverse clinical behavior that largely depends on the tumor biology. Neuroblastoma exhibits unique features, such as early age of onset, high frequency of metastatic disease at diagnosis in patients over 1 year of age and the tendency for spontaneous regression of tumors in infants. The high-risk tumors frequently have amplification of the *MYCN* oncogene as well as segmental chromosome alterations with poor survival. Recent advanced genomic sequencing technology has revealed that mutation of *ALK*, which is present in ~10% of primary tumors, often causes familial neuroblastoma with germline mutation. However, the frequency of gene mutations is relatively small and other aberrations, such as epigenetic abnormalities, have also been proposed. The risk-stratified therapy was introduced by the Japan Neuroblastoma Study Group (JNBSG), which is now moving to the Neuroblastoma Committee of Japan Children's Cancer Group (JCCG). Several clinical studies have facilitated the reduction of therapy for children with low-risk neuroblastoma disease and the significant improvement of cure rates for patients with intermediate-risk as well as high-risk disease. Therapy for patients with high-risk disease includes intensive induction chemotherapy and myeloablative chemotherapy, followed by the treatment of minimal residual disease using differentiation therapy and immunotherapy. The JCCG aims for better cures and long-term quality of life for children with cancer by facilitating new approaches targeting novel driver proteins, genetic pathways and the tumor microenvironment.

Key words: neuroblastoma, spontaneous regression, risk classification, predisposition, multidisciplinary therapy

Introduction

Neuroblastoma (NB) is one of the most common solid tumors of early childhood, occupying ~8% of pediatric malignancies, and occurs in ~150–200 children each year in Japan (1). NB is more common in boys than in girls; however, the genetic and epigenetic basis for this preponderance remains unclear (2). The incidence of NB in Japan appears similar to that in the United States, but both the incidence and phenotype of the disease are highly associated with race (2). For example, in North America, the incidence of disease is less common in black individuals compared with white individuals, and individuals with African ancestry are more likely to have a more malignant phenotype than individuals of European descent. However, the incidence figures among various ethnicities are not precisely known.

The likelihood of developing NB varies by age, with the highest number of cases detected in the perinatal period and then steadily decreasing over the first 10 years (2,3). NB rarely occurs in adolescents and young adults, but in this patient group, NB tends to be more indolent but lethal disease.

Of interest, NB in patients <18 months of age often regresses spontaneously (2–6). In Japan, the United States and some European

countries, the attempts to detect NB early using screening for catecholamine metabolites in urine revealed that approximately half of all NB cases that arise in the first year of life are mostly not detected owing to complete spontaneous regression (7–9).

The occurrence of spontaneous regression as well as the presence of autoimmune paraneoplastic manifestations in some patients such as opsoclonus myoclonus syndrome (OMS; a rare syndrome associated with lymphoid infiltrates in the tumor, anti-neuronal antibodies in the serum and a high survival rate) may suggest the possible involvement of an aberrant developmental and/or immune system that regulates such phenomena (2).

Recent advances have revealed the presence of several genomic alterations in NB. These include amplification of *MYCN* (10–12), mutations of *ALK* (13–16), and copy number aberrations of chromosomes (17,18). Aggressive NB often harbors *MYCN* amplification (in ~20% of tumors), deletions of a distal part of the short arm of chromosome 1 (1p loss), a gain of the long arm of chromosome 17 (17q gain) and a loss of a part of the long arm of chromosome 11 (11q loss), although these cases usually show a diploid or near-tetraploid karyotype (2,3,17,18). Thus, NB appears to be a copy number-driven cancer.

NB is diagnosed, like other cancers, using a combination of laboratory tests, radiographic imaging and pathology (2). The disease staging is the first-choice assessment. The patient is then stratified as very-low-risk, low-risk, intermediate, high-risk or ultra-high-risk based on clinical and molecular risk factors to aid clinicians in deciding the best course of treatment (19).

In 2006, the Japan Neuroblastoma Study Group (JNBSG) that covers almost all hospitals treating the childhood cancers in Japan was officially organized (1). Since then, several clinical study protocols for NB are now used among the registered Japanese hospitals. In 2016, the Japan Children's Cancer Group (JCCG) was founded (1). The JCCG included and unified all groups of childhood cancer studies in Japan. The JNBSG then changed its name to the NB Committee of JCCG. Herein, the current status of biological research, diagnoses and therapies for NB in Japan and other global areas are reviewed.

Development and pathogenesis of NB

NB originates from the precursor cells of the sympathoadrenal lineage that are derived from the neural crest (2). During embryonic development, the neural crest cells develop and migrate to differentiate into several different lineages, including melanocytic, sensory, enteric and sympathetic neurons (Fig. 1). All of these developing neurons undergo massive programmed cell death (PCD), mostly at the last stages of terminal differentiation, and about a half of the neurons die mainly owing to apoptosis (5,6). The surviving neurons differentiate terminally and become mature neuronal cells like ganglion cells with proper function. Of interest, NB occurs only from the precursor cells or stem cells of the sympathoadrenal lineage, but never from the other lineages derived from the neural crest cells. This suggests that the oncogenic events that cause NB may occur after the time point when the migrating cells receive the decision to differentiate into sympathetic neurons. Accumulating evidence obtained from developmental neurobiology research suggests that many transcription factors and/or molecules included in the transcriptional complex are involved in the regulation for deciding the fate of sympathetic lineage (5,6,20,21). These transcriptional regulators may include *Mycn* (10), *Mash1* (22,23), *Id2* (24), *dHAND* (25), *HIF* (26,27) and *Phox2* (28). The Mash1 and Phox2a/b regulatory network appears to be directly or indirectly regulated by Notch and BMP signaling pathways (29). Furthermore, one of the downstream targets of these pathways is the catecholamine metabolism pathway (29). Thus, the molecular mechanism of the transcriptional regulatory cascade at the sympathetic lineage fate determination may be very important to understanding why and how NB occurs.

Recently, Ohnishi et al. reported that premature termination of reprogramming in mice, in which reprogramming factor expression *in vivo* can be controlled temporally with doxycycline, causes tumors in the kidney that share a number of characteristics with Wilms tumor (30). This suggested that epigenetic regulation associated with developmental reprogramming error may drive the genesis of pediatric cancer.

Genomics and genetics of NB

Structural chromosomal aberration

NB patients who are younger than 1 year of age with localized disease and a very good prognosis generally have a hyperdiploid or near-triploid karyotype caused by mitotic dysfunction (3). These tumors generally have whole chromosome gains without structural rearrangements. However, patients over 1 year of age with advanced stage and rapidly progressive disease generally have a near-diploid or near-tetraploid DNA karyotype and show recurrent segmental chromosomal copy number variations (CNVs), including allelic losses of 1p, 3p, 4p, 6q, 11q and 14q and gains of 1q, 2p and 17q (2,3). Of these, 17q gain is the most common CNV and has been detected in over half of NB cases (31), while losses of 1p and 11q have been shown in around 20% and 30% of NBs, respectively (32). Both gain of 17q and loss of 1p correlate with *MYCN* amplification and poor prognosis, while loss of 11q is inversely correlated with *MYCN* amplification and is associated with high-risk disease (31–33). In addition, losses of 6q, 11q and 17p are frequently detected in relapse tumors (34).

Recent whole-genome sequencing analysis has identified chromothripsis (a local shredding of chromosomes) in nearly 20% of high-stage NB cases. These structural defects are associated with a poor outcome and recurrently affect *ODZ3*, *PTPRD* and *CSMD1*, which are involved in neuronal growth cone stabilization (35). In addition, recurrent genomic rearrangements that affect the chromosomal region at 5p15.33 proximal of the telomerase reverse transcriptase gene (*TERT*) occur in ~25% of high-risk NBs (36,37, see below).

Genetic aberrations in NB drivers

N-Myc

Approximately 20% of patients with NB present amplification of the *MYCN* locus located on chromosome 2p24, and the degree of amplification strongly correlates with advanced disease stage, unfavorable biologic features and a poor outcome (11,38) (Fig. 2).

MYCN encodes the transcriptional factor N-Myc, a member of the Myc family of proto-oncogenes that includes other Myc family members such as c-Myc and L-Myc. Distinct from the ubiquitous expression pattern of c-Myc, N-Myc expression is restricted mainly to the nervous system and mesenchymal tissues during particular embryonal stages and is expressed at a very low level in adult tissues (39,40). N-Myc plays an essential role in normal brain development. Because of the high structural and sequence homologies within the Myc family, N-Myc shares partial functional redundancy with c-Myc in the transcriptional regulation of genes involved in diverse

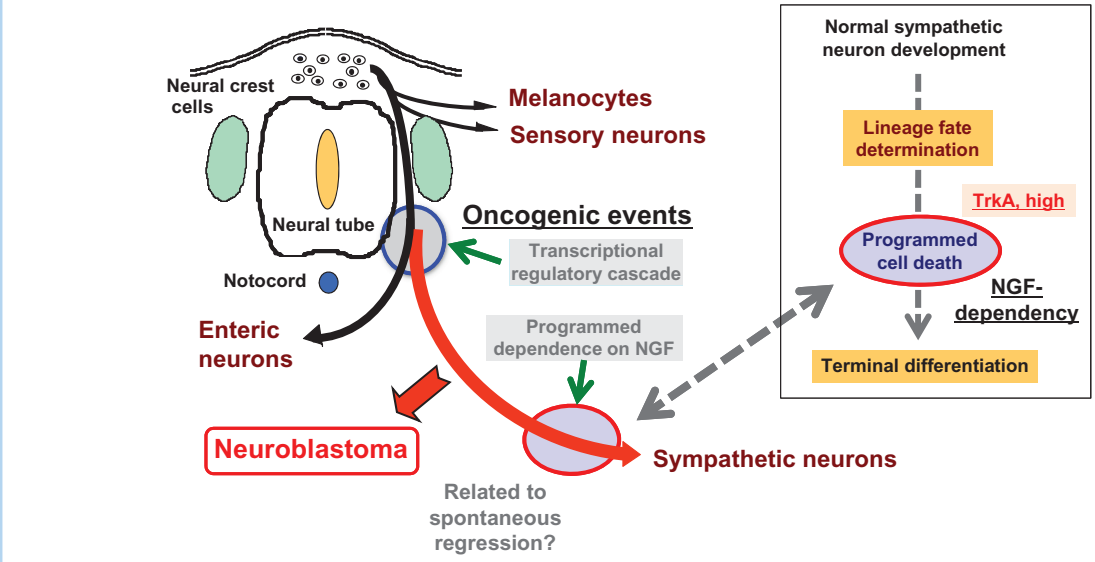

Figure 1. Neural crest development and genesis of NB.

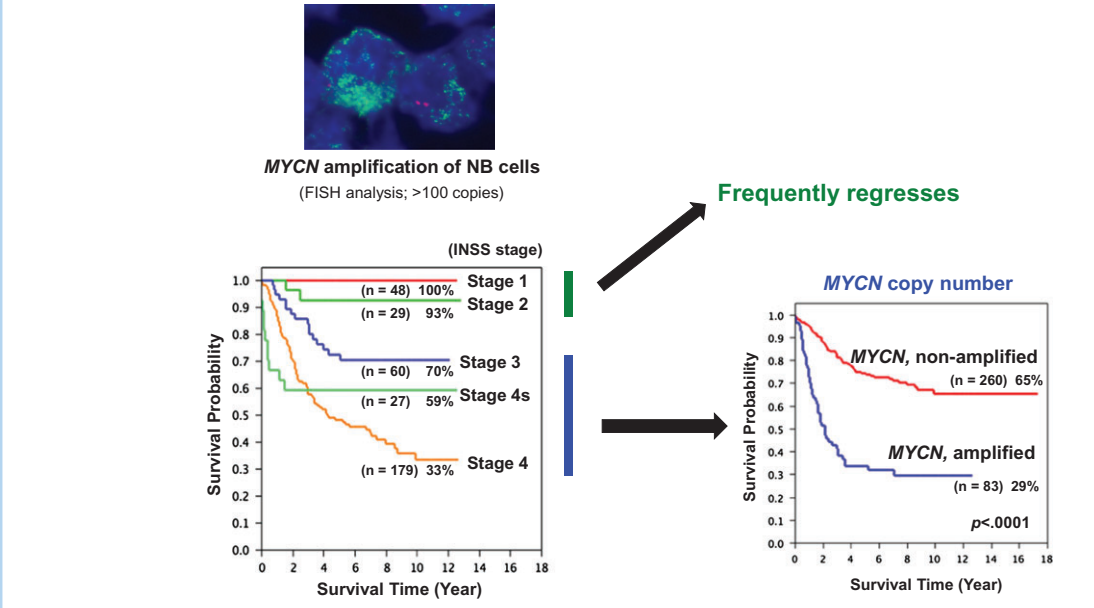

Figure 2. *MYCN* is a key oncogene in NB. The cumulative survival curves of 343 Japanese patients according to the INSS stages are shown. Among these patients, 256 patients with NB in Stage 3, 4 s and 4 were further analyzed according the status of *MYCN* copy number.

cellular functions such as cell growth and proliferation, metabolism, apoptosis and differentiation (41).

Elevated N-Myc protein mainly due to *MYCN* amplification plays a critical oncogenic role in the pathogenesis of NB. Research performed by Kaelin's group revealed that the nerve growth factor (NGF)/TrkA pathway is targeted for suppression by mutations of c-RET, NF-1, VHL and SDH genes (42). This may cause familial pheochromocytoma originating from the sympathoadrenal lineage of neural crest cells. On the other hand, in NB with the same origin as pheochromocytoma, there are no mutations of these genes; instead, the NGF/TrkA pathway is strongly suppressed by amplification of *MYCN* (Fig. 3).

Studies in transgenic mice have shown that neural crest-specific expression of N-myc causes the development of NB (43). To date, a number of protein-coding genes, as well as non-protein-coding genes, including several microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have been identified as downstream targets of N-Myc, and the deregulation of these factors contributes to the

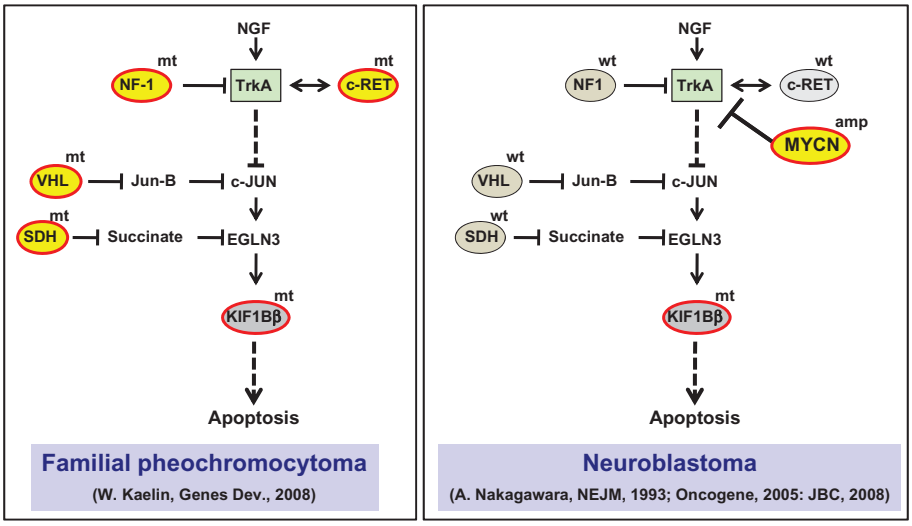

Figure 3. Both pheochromocytoma and NB share the same neurotrophin pathway. In familial pheochromocytoma, the TrkA pathway is suppressed by mutations of c-Ret, NF-1, VHL and SDH genes, while in NB, the same pathway is targeted by N-Myc to be suppressed, although there are no mutations in c-Ret, NF-1, VHL or SDH genes. KIF1Bβ, whose gene is mapped to chromosome 1p, is a common downstream target protein in both tumors.

development and progression of NB (41,44). Additionally, N-Myc functions as an epigenetic regulator to globally regulate gene expression through histone acetylation and DNA methylation in NB (41).

Stabilization of the N-Myc protein has been documented to be an important mechanism in facilitating its oncogenic activity in NBs. *NCYM* (also known as *MYCNOS*), a cis-antisense gene of *MYCN* that is co-amplified and co-expressed with *MYCN*, stabilizes the N-Myc protein by inhibiting the activity of GSK3β, a kinase that promotes N-Myc degradation (45). The mitotic Aurora kinase A (AURKA) can also stabilize N-Myc by inhibiting Fbxw7-mediated degradation of the N-Myc protein (46). AURKA itself is indirectly upregulated by N-Myc and is frequently overexpressed in *MYCN*-amplified NBs. The RNA binding protein LIN28B also elevates N-Myc expression through repression of the *let-7* miRNAs (see below).

N-CYM

NCYM is a *de novo* evolved cis-antisense gene of *MYCN* and is 100% co-amplified with the *MYCN* gene in human NBs. Its co-overexpression with *MYCN* mRNA in human NB is associated with an unfavorable prognosis (45). The N-CYM protein is a *de novo* evolved gene product only present in human and chimpanzee and is endogenously expressed in both normal human tissues and cancers (45). The N-CYM protein stabilizes the N-Myc protein by inhibiting the activity of GSK3β, whereas N-Myc induces *NCYM* transcription (45). Thus, the positive feedback regulation formed in *MYCN*/*NCYM*-amplified tumors promotes the aggressive behavior of human NB (Fig. 4). Furthermore, N-Myc also forms a positive feedback loop with N-CYM and OCT4, a core regulator of neural cell stemness, to contribute to the aggressiveness of *MYCN*-amplified NBs (47). N-CYM promotes cleavage of N-Myc to produce the anti-apoptotic protein Myc-nick (48). Both N-CYM and Myc-nick are induced at G2/M phase, and N-CYM knockdown induced apoptotic cell death accompanied by Myc-nick downregulation.

Notably, NBs that occur in *MYCN/NCYM* double transgenic mice are frequently accompanied by distant metastases, which resemble the tumors in advanced stages of human NB (45). Therefore, the *MYCN/NCYM* double transgenic mouse may be a suitable model for the screening of the new drugs against high-risk NBs.

ALK

Anaplastic lymphoma kinase (ALK) is an orphan receptor tyrosine kinase normally expressed only in the developing embryonic and neonatal brain. Formation of ALK fusion proteins due to chromosomal translocation that result in constitutive activation of ALK has been described in a variety of human malignancies. In NB, germline mutations in the tyrosine kinase domain of ALK have been discovered as the leading cause of most cases of familial NB (13,14), and these mutations are also somatically acquired in 7–12% of sporadic cases (15,16). Because of its proximal location (2p23) to *MYCN*, *ALK* can be co-amplified with *MYCN*. In addition, ALK has been identified as a direct transcriptional target of N-Myc (49). The mutated ALKs show increased kinase activity because of autophosphorylation. Constitutively activated ALK plays an oncogenic role by intensifying several downstream signaling pathways, such as the phosphoinositide 3-kinase (PI3K) pathway, the RAS/MAPK pathway and the RET pathway, and induces cell transformation *in vitro* and *in vivo* (50) (Fig. 4).

PHOX2B

Paired-like homeobox 2B (*PHOX2B*) is the first predisposition gene that was identified in NB. Germline mutations of *PHOX2B* occur in 6.4% of hereditary cases of NB (28,51,52). Mutations in *PHOX2B* have also been rarely found in germline and tumor cells of sporadic NBs (53,54). *PHOX2B* mutations usually occur in neural crest-derived disorders such as congenital central hypoventilation syndrome and/or Hirschsprung's disease (51,55), suggesting that *PHOX2B* may be the common genetic factor responsible for these diseases derived from the neural crest. Physiologically, PHOX2B functions as a transcription factor involved in the regulation of the differentiation program of the sympathetic nervous system. PHOX2B gene mutations that were identified in NB result in loss of function of PHOX2B (54).

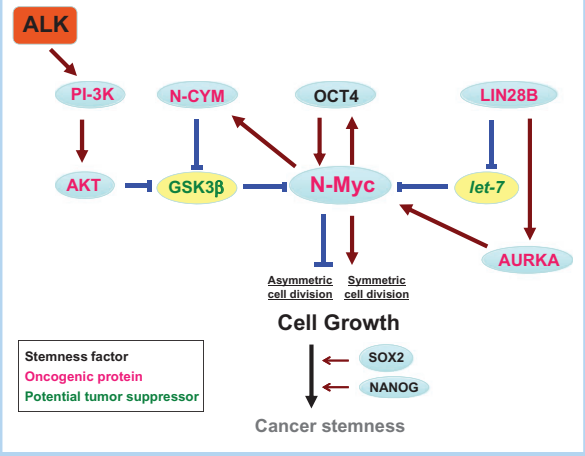

Figure 4. Molecular pathways of N-Myc regulation in growth of NB. N-Myc protein stability depends on the interaction partners. The receptor type-tyrosine kinase ALK activates downstream targets and eventually activates AKT. The activated AKT stabilizes N-Myc protein by inhibiting GSK-3β. N-CYM also stabilizes N-Myc by inhibiting the GSK3β-N-Myc interaction. N-Myc induces N-CYM expression. OCT4 and N-Myc form a positive feedback loop for their transcriptional expression. On the other hand, LIN28B inhibits miRNA *let-7* and contributes to the stability of N-Myc protein. AURKA (Aurora-A) also stabilizes N-Myc protein to inhibit FBXW7-dependent N-Myc ubiquitination. Thus, many oncogenic proteins are involved in N-Myc stability. N-Myc -dependent tumor cells display symmetric cell division and occasionally show a cancer stem cell-like property.

LIN28B

A genome-wide association study (GWAS) discovered several single nucleotide polymorphisms (SNPs) within the *lin-28 homolog B* (*LIN28B*) locus that are highly associated with the development of high-risk NB (56). Overexpression of LIN28B commonly occurs in high-risk NBs and is an independent risk factor for poor outcome (57). The *LIN28B* gene encodes a developmentally regulated RNA binding protein and is a key repressor of the *let-7* family of miRNAs, which act as potent tumor suppressors by post-transcriptionally repressing multiple oncogenic targets including *MYCN*. In NB, LIN28B elevates N-Myc expression through repression of the *let-7* miRNAs (57). On the other hand, it also promotes the activity of the oncogenic RAN GTPase and the stability of downstream AURKA (58), both resulting in NB tumorigenesis (Fig. 4). Moreover, *Lin28b*-transgenic mice develop NB with pathological characteristics similar to human NB (57). A recent report showed that *let-7* can be disrupted in NB by several mechanisms including genetic loss and sponging by abundant *MYCN* messenger RNAs as a competing endogenous RNA, bedsides LIN28B-mediated regression (59).

TERT

Recurrent *TERT* rearrangements at 5p15.33 have been discovered in ~25% of high-risk NBs and are strongly associated with unfavorable outcome (36,37). *TERT* rearrangements occur predominantly in tumors without *MYCN* amplification and *ATRX* mutations, resulting in strong transcriptional upregulation of *TERT*. Since *TERT* is a transcriptional target of N-Myc (60), TERT is elevated in *MYCN*-amplified NB tumors (36). Telomere lengthening caused by aberrantly activated TERT may represent a central mechanism for transformation of the aggressive subtype of NB.

ATRX

Somatic mutations in *ATRX*, which encodes the RNA-helicase ATRX, a member of the SWI/SNF family of chromatin remodeling proteins, were first identified in NB patients diagnosed at an older age, especially adolescent and young adult patients (~40% of these cases) (61). The *ATRX* mutations include missense, nonsense, frameshift, and in-frame deletion mutations and are mutually exclusive of *MYCN* amplification. *ATRX* deletions have also been detected in 11% of high-risk NBs without *MYCN* amplification and *TERT* rearrangement, and are associated with very poor prognosis (35–37). Structural variations and sequence mutations in *ATRX* result in loss of nuclear ATRX protein and alternative lengthening of telomeres (61).

LMO1

LIM domain only 1 (*LMO1*) at 11p15.4 has been identified as a NB susceptibility locus by GWAS (62,63). Aberrances in *LMO1*, including germline SNP risk alleles and somatic copy number gains, increase LMO1 expression and are robustly associated with more advanced disease and poor survival (62). In addition, a recently evolved polymorphism within a super-enhancer element in the first intron of *LMO1* can also influence NB susceptibility through differential GATA transcription factor binding and direct modulation of LMO1 expression *in cis* (63). These findings indicate that aberrant genotypes at the *LMO1* locus play a gain-of-function role in the tumorigenesis of NB.

Other driver genes

The GWAS identified a common CNV at 1q21.1 as highly associated with NB (64). A novel NB breakpoint family gene (*NBPF23*) was identified within this region, suggesting that the CNV at 1q21.1 probably plays a role in early tumorigenesis through disruption of *NBPF23*. Similar approaches have discovered several germline sequence variants at 2q35 within the *BARD1* locus as being significantly associated with aggressive NBs (65). Recent studies integrated with next-generation sequencing techniques have uncovered chromosomal deletions and sequence alterations in *ARID1A/ARID1B* (66,67) and recurrent missense mutations in *NRAS* (67,68) and *CHD9* (69) in primary NB tumors. *ARID1A/ARID1B* and *CHD9* encode ATP-dependent chromatin remodeling proteins. Genomic aberrations in *ARID1A/1B* are associated with early treatment failure and decreased survival (66). Gain-of-function mutations in *NRAS* and loss-of-function mutations in *CHD9* are both associated with aggressive NBs (67,69). Relapsed NB tumors harbor frequent mutations in the *RAS* family (*NRAS*, *HRAS* and *KRAS*) and other genes involved in the RAS-MAPK pathway (70,71).

Accumulated evidence has shown that genetic aberrances in several non-coding RNAs (ncRNAs) are involved in NB pathogenesis. Our group and others have identified *ncRAN* (non-coding RNA expressed in aggressive NB) and *lncUSMycN* as lncRNAs associated with aggressive NBs, and their expressions independently predict a poor outcome. *ncRAN*, which maps to chromosome 17q25.1, a region that is usually gained in NB, plays an oncogenic role in NB (72). *lncUSMycN*, located at the 2p 130-kb amplicon that is co-amplified with *MYCN*, upregulates N-Myc expression through the RNA binding protein NonO (73).

Genetic aberrations in NB tumor suppressors

TP53

Somatic mutations of the classical tumor suppressor TP53 or other members in the TP53 pathway are rare in primary NBs obtained at diagnosis. However, a germline missense variant in *TP53* has been identified as a candidate pathogenic mutation that is associated with high-risk NBs (67). Moreover, two rare germline variants (rs78378222 and rs35850753) that map to the 3′ untranslated region (UTR) and 5′ UTR of *TP53*, respectively, have been discovered to be strongly associated with NB susceptibility (74). In addition, p53 loss of function usually occurs in relapsed, treatment-resistant NBs, suggesting the role of p53 in the development of high-risk therapy-resistant NB.

KIF1Bβ

The kinesin motor protein KIF1Bβ is a candidate tumor suppressor mapped to chromosome 1p36.2, a region frequently deleted in NB. KIF1Bβ regulates apoptosis in the developing sympathetic nervous system through NGF-dependent signaling pathway (42,75). A recent study demonstrated that KIF1Bβ affects mitochondrial dynamics through calcineurin-mediated dephosphorylation of the mitochondrial fission protein DRP1 (76). Hemizygous deletion of *KIF1Bβ* is significantly correlated with advanced stages and *MYCN* amplification in NB (42). Germline loss-of-function *KIF1Bβ* missense mutations have been uncovered in NBs and pheochromocytomas, another neural crest-derived tumor (42), indicating that KIF1Bβ is a critical pathogenic target of these diseases and its loss of function contributes to tumorigenesis (Fig. 3).

CHD5

The chromatin remodeler *CHD5* resides in 1p36.31 and functions as a tumor suppressor that controls proliferation, apoptosis, and senescence via the p19(Arf)/p53 pathway (77). In high-risk NBs, CHD5 is expressed at extreme low levels or is absent owing to hemizygous 1p deletions and *CHD5* promoter hypermethylation (78,79). Conversely, high CHD5 expression is strongly correlated with favorable clinical/biological features and outcome (79), indicating that inactivation of CHD5 drives NB tumorigenesis and malignant features. Although somatically acquired *CHD5* mutations are rare in primary NBs, mutations in the *CHD5* coding region recurrently occur in the relapsed tumors (71), suggesting the clonal evolution of tumor cells harboring *CHD5* mutations in the primary tumors that may lead to therapy resistance and unfavorable outcome.

Other tumor suppressor genes

Decreased expression of CAMTA1, a haploinsufficient 1p36 tumor suppressor belonging to the calmodulin-binding transcription activator (CAMTA) protein family, is significantly associated with unfavorable features and a poor outcome in NB (80). In addition, miR-34a, a miRNA located at chromosome 1p36.23 that acts as a potential tumor suppressor by inducing apoptosis, is generally expressed at lower levels in unfavorable primary NB tumors and NB cell lines (81). Together with *KIF1Bβ* and *CHD5*, loss of these 1p36 genes plays a critical role in NB tumorigenesis and progression.

We have defined the smallest region of overlap (SRO) of deletion up to 10-Mb at 11q23 in primary NBs using an array-based comparative genomic hybridization (CGH) analysis (82). *TSLC1* (tumor suppressor in lung cancer 1), a putative tumor suppressor gene identified for lung and other cancers, resides in this SRO. We found that 35% of primary NBs harbor loss of heterozygosity on the *TSLC1* locus and that decreased expression levels of *TSLC1* are significantly associated with a poor prognosis (83).

A GWAS identified a high-risk NB-associated susceptible polymorphism in the intronic region of the lncRNA *NBAT1* (neuroblastoma associated transcript 1) on chromosome 6p22 (84). *NBAT1* normally functions as a tumor suppressor in the regulation of cell proliferation, invasion and neuronal differentiation. The risk-associated SNP genotype and promoter hypermethylation account for the decreased expression of *NBAT1* in high-risk NBs, and lead to impaired *NBAT1*-mediated epigenetic silencing of pro-tumor genes such as *SOX9*, *OSMR* and *VCAN* (85). The lncRNA *NDM29* (NB differentiation marker 29) maps to the 11p15.3 region, which is frequently deleted in NBs (86). Functional analyses have shown that *NDM29* possesses tumor suppressor properties, indicating that loss of *NDM29* may be involved in NB tumorigenesis.

Altered gene expressions

TrkB

The tropomyosin-related kinase (Trk) family consists of three members, TrkA, TrkB and TrkC, which function as the receptors of neurotrophins. TrkB and its ligand BDNF (brain-derived neurotrophic factor) are highly expressed in *MYCN*-amplified aggressive NBs with a poor prognosis (87). BDNF/TrkB signaling contributes to malignant biological behaviors and an unfavorable outcome in NB in an autocrine/paracrine manner through regulation of pathways involved in epithelial–mesenchymal transition-like transformation, metastasis, angiogenesis and chemotherapeutic resistance (88). In contrast to TrkB, elevated TrkA expression is correlated with favorable biological features including younger age at diagnosis, lower stage and absence of *MYCN* amplification, as well as a good outcome (89). TrkA and its ligand NGF transduce signals to promote survival and differentiation of NB cells (89) (Fig. 3). TrkC is also predominantly expressed in *MYCN*-unamplified lower-stage NB tumors (90).

NLRR1

Neuronal leucine-rich repeat protein-1 (NLRR1) is highly expressed in *MYCN*-amplified NBs with a poor clinical outcome in a mutually exclusive pattern with ALK expression (91,92). Our recent study indicated that NLRR1 negatively regulates ALK signaling through impairing ALK phosphorylation (91). NLRR1 is a direct transcriptional target of N-Myc, and in turn enhances EGF-mediated induction of N-Myc (93). The positive N-Myc/NLRR1 regulation feedback may play an important role in tumorigenesis and malignant progression of NBs that lack oncogenic ALK expression.

LMO3

LMO1, described above, belongs to the LIM-only (LMO) protein family of transcriptional regulators that are mainly expressed in the nervous system. Notably, LMO3, another member of the LMO family, also plays an oncogenic role in NB. LMO3 is overexpressed in unfavorable NBs and its expression levels significantly predict a poor prognosis (94). Studies using NB cell lines have demonstrated that LMO3 contributes to the tumorigenesis and malignant phenotype of NB in collaboration with the neural basic helix-loop-helix (bHLH) protein HEN2 through transactivation of downstream MASH1, a bHLH transcription factor involved in the neuronal commitment and differentiation (22).

Polycomb-group proteins

Several members of the polycomb-group protein family have been demonstrated to be involved in tumorigenesis and tumor progression of NB. A previous study showed that elevated polycomb

protein histone methyltransferase EZH2 is critical to maintain the undifferentiated and poor prognostic status of NB through epigenetic repression of multiple tumor suppressor genes including *CASZ1*, *CLU*, *RUNX3* and *NGFR* (95). The polycomb complex protein Bmi-1 has been identified as a direct target of N-Myc/c-Myc and E2F-1. Bmi-1 is highly expressed in up to 90% of primary NB tumors and plays an essential role in NB tumorigenesis through repression of tumor suppressors KIF1Bβ and TSLC1 (96–99).

ncRNAs

Deregulated expressions of miRNAs are involved in NB tumorigenesis. Oncogenic miRNAs such as miR-17–92 cluster, miR-18a, miR-128 and miR-380-5p are upregulated, while tumor suppressor miRNAs such as miR-9, miR-34a, miR-101/let-7 and miR-184 are downregulated in NB; both groups promote cell proliferation and inhibit neuronal differentiation (100). In addition, the lncRNA *CAI2* (CDKN2A/ARF Intron 2 lncRNA) maps to 9p21, which harbors the well-known tumor suppressor genes *CDKN2A* and *ARF* (101). Elevated expression of *CAI2* along with CDKN2A and ARF is significantly associated with a poor clinical outcome in NBs (101).

NB predisposition

Approximately 2% of patients with NB possess an underlying genetic predisposition with a penetrance in gene carriers estimated to be ~65%. Germline mutations in *ALK* and *PHOX2B* account for most familial NB cases (102,103). The other cancer predisposition syndromes, such as Li-Fraumeni syndrome, RASopathies and *SDHB* mutations, may also have an increased risk for NB. No protocols for NB surveillance have been established. These predisposed individuals have been expected to show an increased risk to develop NB into adulthood, but the risk is considered to be very low.

Tumor biology

NB arises from the cells that normally make up an embryonic structure called the neural crest (104). The neural crest cells consist of multipotent and migratory cell populations that give rise to diverse cell lineages including Schwann cells, melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons, and glia (104). Thus, neural crest cells serve as multipotent stem cells that differentiate into mature peripheral neural tissues. It is now assumed that the multipotent neural crest cells with genetic mutations and/or epigenetic changes may contribute to NB tumorigenesis and its stemness.

NB stem cells

Only a small number of highly malignant cells that produce heterogeneous cell populations are present in the cancer cell population. These cells are called 'cancer stem cells' and/or 'tumor initiating cells'. Emerging evidence now strongly suggests that these cells cause chemoresistance, metastasis and tumor recurrence. At the 2006 American Cancer Society Annual Meeting (AACR meeting), a cancer stem cell was defined as 'a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor' (105).

Several NB cell lines partially differentiate and can differentiate into multiple cell types (104). Intermediate type (I-type) NB cells have morphologic and biochemical features of both neuroblastic type (N-type) and substrate-adherent type (S-type) cells. Upon treatment of retinoic acids, I-type cells differentiate into N-type cells. On the other hand, upon adding BrdU to the culture medium, I-type cells differentiate into S-type cells. Moreover, I-type NB cells have higher tumorigenic potential than N-type and S-type cells. Thus, I-type NB cells show a cancer stem-like cell phenotype owing to their multiple cell differentiation potential and high malignancy. In fact, a number of reports have indicated that NB cell lines exhibit the expression of pluripotency-associated genes (47,57).

NCYM, a *de novo* evolved cis-antisense gene of *MYCN* that encodes a small 109 amino acid protein, induces expressions of not only N-Myc, LIN28B, NANOG and SOX2 but also the core-reprogramming factor OCT4 via N-Myc, which forms a positive feedback loop with N-Myc and OCT4 (47). Importantly, N-CYM promoted malignant transformation and metastasis of NB in *NCYM*/*MYCN*-double transgenic mice (45). These findings demonstrate that N-Myc cooperates with N-CYM to promote NB malignancy and their stemness.

Introduction of pluripotency inducing factors (OCT4, KLF4, SOX2 and MYC) into SH-IN cells, an I-type human NB cell line with properties similar to those of cancer stem cells (6), increases the expressions of LIN28 and hTERT, which are involved in NB malignancy (36,57), and cancer stem markers such as CD133 and ALDH1 (106), as well as drug resistance compared with the parental SH-IN cells. These results indicate that the pluripotency inducing factors initiate partial reprogramming in SH-IN cells and transform them into cancer stem cell-like cells.

On the other hand, Ikegaki and colleagues succeeded in inducing expression of stem cell-related genes by treating an NB cell line with epigenetic modifiers such as a histone deacetylase and creating malignant NB cells compared with the parental cells (107). These results also indicate the importance of epigenetic modifications in malignant NB.

Differentiation of NB cells

Several NB cell lines, especially SH-SY5Y, can be easily induced to differentiate into neuronal cells with drugs and growth factors *in vitro* (107). The most common drugs are all-*trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid (13-cis-RA). ATRA is used as a therapeutic agent to induce the differentiation of certain leukemia such as acute promyelocytic leukemia (APL). However, 13-cis-RA shows a higher effect than ATRA in the practical therapy of NB in the clinic. In an *in vitro* study, ATRA-treatment induced growth inhibition and neuronal differentiation as indicated by morphological and biochemical features such as neurite outgrowth, increased neuron specific enolase (NSE) activity and high expression of growth associated protein 43 (GAP43), a protein important for axonal growth (108). ATRA treatment also induced the upregulation of the neurotrophin receptor RET, resulting in glia-derived neurotrophic factor (GDNF) responsiveness. These results indicate that ATRA-treated NB cells can differentiate in response to GDNF.

Hypoxia and de-differentiation

For cancer cells, the hypoxic environment (hypoxia) favors the selection of more malignant cells. Under hypoxic conditions, the hypoxia-inducing factor (HIF) transcriptional factors are deeply involved with the survival of cancer cells. Among the HIFs, HIF-2α is correlated with advanced clinical stage and worse prognosis in NB (109). HIF-2α is also associated with the regulation of several stem cell-associated genes such as OCT4 and ABCG2 genes (110–112). Pietras et al. demonstrated that knockdown of HIF-2α decreases vascular endothelial growth factor (VEGF) expression and leads to

partial sympathetic neural differentiation of NB stem cells (110). Thus, these studies suggest that hypoxia and HIF pathways partly contribute to NB progression by maintaining immature, stem-like tumor cells. In fact, hypoxia caused de-differentiation of human NB cells toward an immature and neural crest-like phenotype. Furthermore, the hypoxic microenvironment may serve as a niche for highly tumorigenic cells.

Angiogenesis

Hypoxia also upregulates VEGF expression and in conjugation with Flt-1 plays a pivotal role in VEGF-mediated autocrine signaling of tumor growth and angiogenesis in NB (113). High angiogenesis activity correlates with unfavorable histology and aggressive behavior of NB. The treatment of NB with anti-VEGF agents results in a decrease in tumor vascularity. A recent report showed that the dual PI3K/mTOR inhibitor NVP-BEZ235 suppressed NB tumor proliferation and angiogenesis *in vivo* (114). The authors showed that NVP-BEZ235 inhibited PI3K/mTOR activity and caused GSK-3β reactivation, which in turn, facilitated proteasomal degradation of N-Myc protein. Since MYCN is known to upregulate VEGF expression, this suggests that NVP-BEZ235 indirectly inhibits MYCN-mediated VEGF paracrine signaling, which enhances tumor–vascular interactions in NB tissues.

Notably, VEGF signaling may directly affect cancer stem cells. Blanpain and colleagues found that neuropilin-1, a co-receptor of VEGF, was predominantly expressed and induced autocrine effects of VEGF in cancer stem cells (115). These results indicate angiogenesis as an attractive target for NB therapy.

Asymmetric cell division

Recent stem biology studies have indicated that N-Myc oncoprotein not only shows oncogenic activity but also plays a central role in the self-renewal of normal neural stem and precursor cells (116). NB cells are derived from multipotent neural crest cells and exhibit cancer stem cell-like properties owing to aberrant *MYCN* expression. In addition, human NB cultured cells show the ability to differentiate into neuronal cells with the treatment of retinoids as mentioned above, indicating that human NB cultured cells with the capacities of both proliferation and differentiation may possess cancer stem cell-like properties.

Asymmetric cell division (ACD) research was originally performed using model organisms such as *Caenorhabditis elegans* embryos and *Drosophila melanogaster* neuroblasts (117–119). These powerful genetic studies have revealed that the machinery of ACD is highly conserved (119). On the analogy of ACD studies using these organisms, it has been found that asymmetric cell division occurs in NB cells in an evolutionarily conserved manner (120) (Fig. 5). The magnitude of the *MYCN* gene expression affects the regulation of cell division fate: overexpression of *MYCN* induces symmetric cell division (self-renewal division), and reduced expression of *MYCN* tends to cause ACD (120). Moreover, the transcriptional activity of N-Myc is also important for inducing self-renewal division in human NB cells (120).

TRIM32 was identified as an ACD-inducer using human NB cells. Although previous studies established TRIM32 as an ubiquitin ligase that facilitates degradation of c-Myc protein during mammalian neurogenesis, the function of TRIM32 in human cancer was still unknown. In 2014, Izumi et al. found that TRIM32 facilitated the proteasomal degradation of N-Myc and induced ACD in human NB cells (121). In addition, overexpression of TRIM32 enhanced retinoid-induced neuronal differentiation of NB cells. These findings provide novel insights into the mechanisms of ACD to develop new cell differentiation therapy for NB (122). Current research is focusing on the lineage of cancer cell division to explore the mechanism of ACD involved in NB.

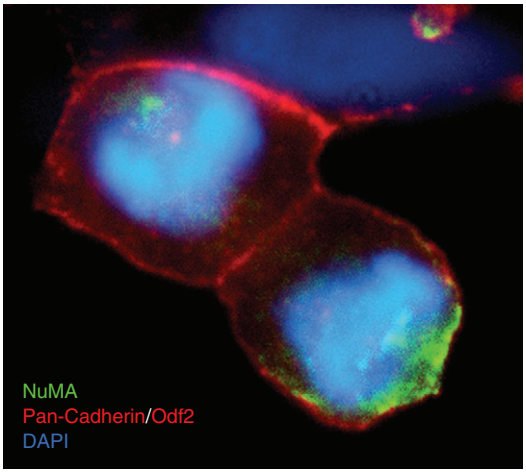

Figure 5. Asymmetric division of NB cells. Pictured is an asymmetric cell division of a human NB cell, NB69, with staining of the cell membrane (Pan-Cadherin, red), an older centrosome (Odf2, pink), NuMA crescent in green and chromosomes in blue. Izumi H and Kaneko Y found that human NB cell lines showed asymmetric cell division and that the expression of the *MYCN* gene is involved in the regulation of whether cells display asymmetric polarity cell division or symmetric self-renewal division. The authors also found that the centrosome with a younger mother centriole is inherited to the daughter cell, with NuMA preferentially localized to the cell cortex, while the older mother centrosome migrates to the other daughter cell, suggesting that these findings may be a fundamental role in cancer stem cells.

Tumor regression

Spontaneous regression of NB often occurs in young infants, especially in patients less than 1 year of age with Stage 4 s disease with distant metastases to the skin, liver or bone marrow (4). The mass screening program for NB performed in Japan, the United States and some European countries in patients at 6 months, 3 months or 18 months of age that measured urinary cathecholamine metabolites (vanillylmandelic acid (VMA), homovanillic acid (HVA) and/or dopamine levels standardized by creatinine levels) revealed many more NBs than the number of tumors that manifest later in life (7,123) (Fig. 6). The tumors found by mass screening usually showed non-amplified *MYCN*, hyperdiploid DNA karyotype and favorable prognosis of the patient (8,9), while the later-presenting NB in patients who received negative results from a screening test at 6 months of age often showed aggressive phenotype with *MYCN* amplification and diploid DNA karyotype (124). The large-scale mass screening program performed in Japan for 20 years showed that the incidence of NB became almost double after starting the program without a significant change in overall mortality, suggesting the presence of possibility of overdiagnosis (125,126).

The molecular mechanism underlying how NB in infants spontaneously regresses is still unknown. Unfortunately, research of NB with spontaneous regression is difficult because its frequency is very

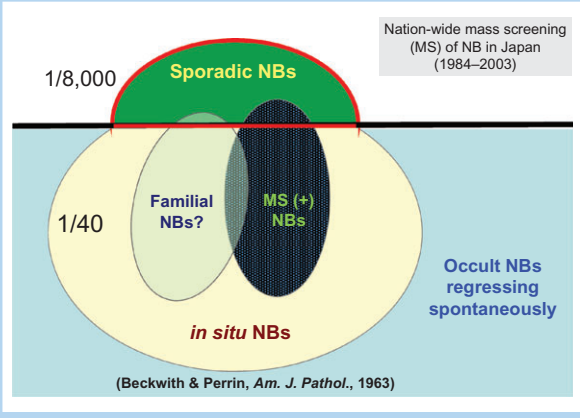

Figure 6. Occult NBs. In 1963, Beckwith and Perrin reported that during the human fetal development, NB-like cells (*in situ* neuroblastoma) appear at a frequency of ~1/40 but most disappear before birth, except some cases, in which such cells have a tendency to form a mass (Ref. 368) The mass screening program performed in Japan for nearly 20 years (1984–2003) suggested that the incidence of NB almost doubled after starting the program and that most of the NBs found by mass screening have a tendency to regress or differentiate spontaneously with favorable genetic markers. These suggest that there may be a large number of occult NBs that do not become sporadic NBs.

low and sampling tumor tissues is very difficult. Even so, the recent analyses of tumors found by mass screening as well as those in Stages 1 and 4s have shown interesting results. Expression of *TERT* is significantly low in regressing NB (123), suggesting that telomere crisis might have a role in tumor regression (2). The host immunity might also have a role in tumor regression, as suggested in the tumor of a patient with OMS (127,128), which is associated with anti-neuronal antibodies, differentiation of tumor and a favorable prognosis (129). One of the most likely key mechanisms underlying spontaneous regression of NB is the dependency of the tumor cells on NGF (5,89). Tumors with a tendency to regress express high levels of TrkA protein, a specific receptor for NGF (5,6,20,89). These tumor cells might be dependent on NGF for survival, similar to the developing normal sympathetic neurons that undergo PCD during the limited perinatal period time (5,89). However, since the amount of NGF is too low within the favorable tumor tissue for the tumor cells to survive, the tumor cells die (89,130). These tumor cells express high levels of tumor suppressor genes and proteins, such as UNC5D (131), DCC (132), KIF1Bβ (75), E2F1 (75,131), BMCC1/PRUNE2 (133) and NLRR3 (134), among others. On the other hand, aggressive tumors in advanced stages show high levels of oncogenes and their protein products like N-Myc (11), LMO3 (94), TrkB/BDNF (135), N-CYM (45), LIN28B (57) and others. Among these genes and proteins, the role of the dependence receptor UNC5D is very unique because it is induced after triggering the NGF depletion-induced neuronal cell death (131). As TrkA itself is a dependence receptor to trigger programmed neuronal cell death, the sequential activation cascade of the downstream molecules might be important. Thus, the balance between tumor suppressor genes and oncogenes in the NB tissue might decide the fate of the tumor cells.

Our study on the clinical genomic (CG) pattern of NB according to the array CGH analyses showed interesting results (136), revealing a difference between sporadic NBs and those found by mass screening program in Japan (Fig. 7). Notably, the CG2 group with 11q loss and 17q gain but without 1p loss or *MYCN* amplification showed poor survival in the patients with sporadic NB, but indicated a good survival in patients found by mass screening. These findings suggest that there might be some epigenetic differences between these NB groups.

Tumor immunology

In 1968, Hellström et al. reported that lymphocytes from NB patients show inhibited colony formation and exhibit cytotoxicity for their own and other allogeneic NB cells *in vitro* (137). As revealed by OMS syndrome and spontaneous regression cases, NB is thought to have a characteristic immune mechanism, and various studies on tumor-specific antigens have been conducted. Disialoganglioside (GD2) is one such NB cell-specific antigen and is highly expressed in most NB cells, suggesting that it is a useful target for therapy with monoclonal antibodies (mAbs).

M2 macrophages are thought to play an important role in these immune responses, and CD163+ M2 macrophages were expressed at higher levels in high risk NB than low risk NB. Asgharzadeh et al. reported that metastatic NB shows higher infiltration of tumor-associated macrophages (TAMs) than localized NB. A high expression of inflammation-related genes, such as FCGR3 (CD16), IL-6R and CD33, is associated with older age of patients (>18 months) and poor prognosis (138).

Prostaglandin E2 (PGE2) is one of the proinflammatory lipid mediators and contributes to the tumor growth and viability of NB cells through the maintenance of cancer-associated fibroblasts in the tumor microenvironment and elevation of cAMP. Previous studies suggested that the COX-2 inhibitor acts as a tumor suppressor by inhibiting the production of PGE2 (139,140).

IL-6 produced by tumor-associated CD68+ macrophages may also be involved in drug resistance of NB cells via activation of STAT3 (141).

Both T cells and natural killer (NK) cells mediate the immune response to NB cells and play a prominent role in immune-based therapies for NB. Normally, cytotoxic T cells (CTLs) exhibit cytotoxic activity upon presentation of MHC class 1, but expression of MHC class 1 is markedly downregulated in NB patients, especially in high-risk patients with *MYCN* amplification (142). This is one of the most common mechanisms by which high-risk NB escapes the host immune response, rendering any endogenous or therapeutic anti-tumor T cell responses ineffective. CTL exhibits cytotoxicity against MHC class 1 mismatched cells, whereas NK cells show cytotoxicity for all killer immunoglobulin-like receptor (KIR)/KIR-ligand (KIR-L) mismatched cells and lose cytotoxic activity for MHC class 1 matched cells. Therefore, NK cells have the excellent potential role for immunotherapy (143–147).

The next immunotherapy is antibody-dependent cell-mediated cytotoxicity (ADCC), such as administering anti-GD2 mAb in combination with ADCC-enhancing cytokines (IL2 and GM-CSF) (148–150).

Other clinical approaches to cellar immunotherapies for high-risk NB are the development of chimeric antigen receptor-modified T-cells (CAR-T) and Bi-specific T-cell Engager. Although these approaches mainly use GD2 as a tumor antigen, their clinical effectiveness in NB is not yet known (143,151).

Recently, high expression of programmed cell death-ligand-1 (PD-L1) was confirmed in patients with high-risk NB patients, and a correlation between the expression of N-Myc as well as *MYCN* amplification and poor prognosis was reported. The combination

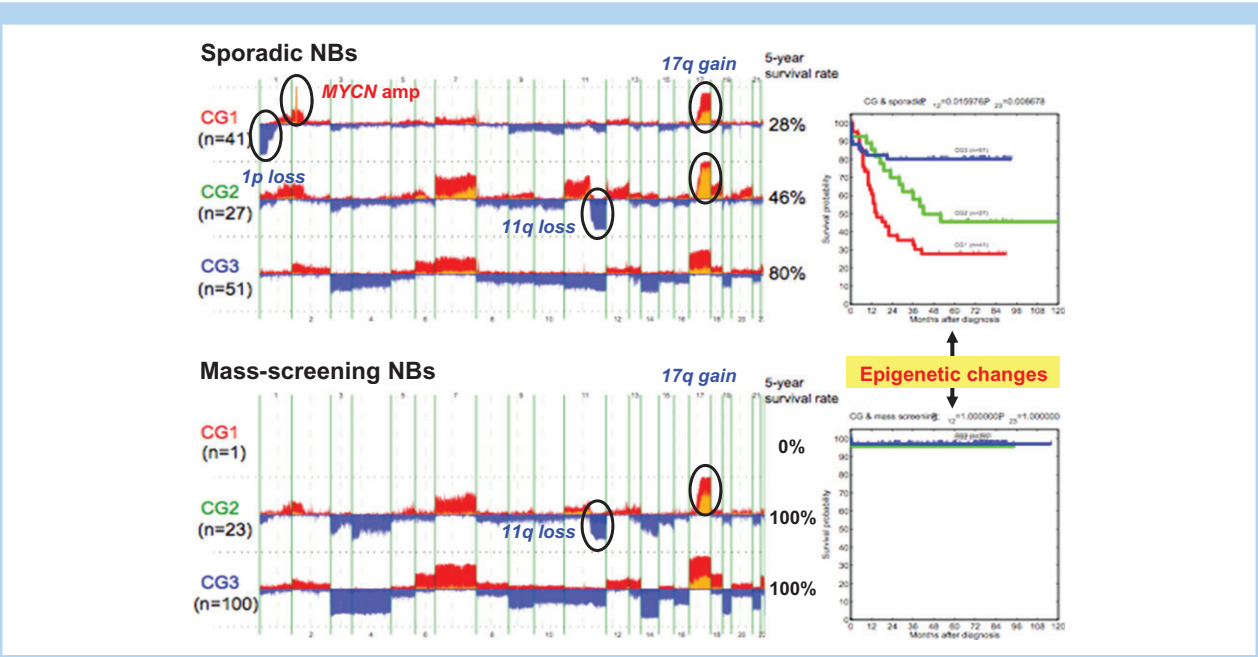

Figure 7. Differences of the pattern of genomic abnormalities and the survival rates between sporadic and mass-screening NBs. The sporadic NBs distribute similarly among the clinical genomic pattern (CG1, CG2 and CG3) with different prognosis: 28% in CG1, 46% in CG2 and 80% in CG3. On the other hand, the NBs found by mass screening show an incidence of ~20% in CG2 and 80% in CG3, but a very few in CG1, while the prognoses are 100% in both CG2 and CG3 groups. Thus, although a part of both sporadic and mass screening NBs share the common genomic aberration pattern (CG2), their prognoses are different, suggesting that there might be some differences in the epigenetic, but not genetic, changes between both NB groups.

with high expression of PD-L1 and low expression HLA class 1 allows NB cells to escape from tumor immunity. Therefore, blocking the PD-1/PD-L1 axis would be a new therapeutic target against high-risk NB (152–154).

Diagnosis, staging and risk classification

Clinical presentation

NB patients present extremely heterogeneous clinical courses, which are mainly contingent on the age at diagnosis, the site of the primary tumor and the stage of the disease. The median age at diagnosis of NB is 18 months, with 40% of patients diagnosed at infancy and 90% of patients at <10 years of age (2). The majority of NB cases are sporadic, with only ~1–2% of cases arising in the familial setting. Infant patients <18 months of age usually have a better overall survival than those >18 months of age, even with disseminated disease. Primary tumors can arise anywhere along the sympathetic nervous system, but are most frequent in the adrenal medulla and paraspinal ganglia. Tumors in the adrenal glands are correlated with poorer survival than primary tumors in other regions. More than 50% of all NB patients are diagnosed with metastatic diseases, frequently in the regional lymph nodes, bone marrow, bone, liver and skin. Some patients present specific clinical signs and symptoms such as Horner syndrome and spinal cord compression, which are caused by local aggressiveness or infiltration of primary lesions to neural tissue.

Laboratory testing

NB is designated as a neuroendocrine-related tumor with the characteristics of secreting various regulatory peptides. Increased levels of plasma catecholamines, dopamine (DA) and norepinephrine, as well as their metabolites in urine, such as HVA and VMA, have been used in clinical diagnosis and for early detection in screening programs of NB. Low levels of urinary VMA and VMA/HVA ratios or high levels of DA/VMA and DA/HVA ratios are associated with unfavorable features and poor prognosis in aggressive NB (155). Other biological tumor markers tested in clinical practice include chromogranin A (CgA), NSE, lactate dehydrogenase (LDH) and ferritin; their serum levels are increased in some patients with advanced-stage tumors but are not specific to NB (156).

The genetic markers identified as important prognostic tools for NB include *MYCN* amplification, DNA index (ploidy), chromosome 1p loss and chromosome 17q gain, which have been incorporated into the diagnosis and risk stratification of NB patients. *MYCN* amplification status is frequently determined by fluorescent *in situ* hybridization or quantitative PCR. DNA ploidy is assessed by flow cytometry. Array CGH or other methods are used to detect chromosomal alterations. In addition, genomic analysis for *ALK* mutations is recommended for the diagnostic evaluation of NB to guide appropriate therapy.

Radiographic imaging

Radiography is a useful diagnostic tool for primary tumor imaging and metastatic evaluation in NB. Computed tomography (CT) or magnetic resonance imaging (MRI) is frequently used for local assessment of primary tumors, providing information for surgical excision as well as staging of the tumor. The extent of metastatic lesions is mainly assessed using iodine-123 (^{123}I) radiolabelled metaiodobenzylguanidine (MIBG) scan, based on the fact that MIBG has a similar structure to the neurotransmitter noradrenaline. Because of the expression of the noradrenaline transporter in NB tumor cells, nearly 90% of NBs have a high uptake of MIBG, which enables MIBG scan to reach a sensitivity of 90% and a specificity of 99% (156). Semi-

quantitative MIBG-based scoring methods, including the Curie scoring system and the International Society for Pediatric Oncology Europe Neuroblastoma group (SIOPEN) scoring system, are currently being evaluated for their prognostic significance at diagnosis of NB and during follow-up (156). Imaging techniques that are independent of MIBG uptake, such as technetium-99 bone scintigraphy and 18 F-fluorodeoxyglucose PET-CT can be applied for the metastatic evaluation of the NB patients who are not MIBG-avid.

International Neuroblastoma Pathological Classification (INPC)

The INPC was established in 1999 based on the Shimada classification and was partly revised in 2003 (157–159). The INPC combines histopathological indicators including the amount of stromal Schwann cells in the tumor, the grade of tumor differentiation and mitosis-karyorrexis index with age at diagnosis to classify NBs into two categories: favorable histology and unfavorable histology. The patients with favorable histology tumors usually have a better prognosis than those with unfavorable histology tumors (158). As one of the important prognostic variables, the INPC is currently incorporated into risk stratification of NB patients.

International Neuroblastoma Staging System (INSS)

The INSS, which was first established in 1986 and revised in 1993, stratifies NBs into Stages 1, 2A/2B, 3, 4 and 4S according to the extent of surgical excision of tumor at diagnosis and metastases (160). Generally, patients with early stage tumors (Stages 1 and 2) have a better prognosis than those with advanced tumors (Stages 3 and 4). However, infant patients less than 1 year of age always have a good outcome, even if they present disseminated diseases limited to liver, skin, or bone marrow (Stage 4S).

The INSS defines a postsurgical staging of NB. To stage patients before any treatment, in 2009 the International Neuroblastoma Risk Group (INRG) developed the INRG staging system (INRGSS), a new clinical staging system for pretreatment risk classification, based on clinical criteria and image-defined risk factors (IDRFs) (161). In the INRGSS, localized tumors are designated as Stage L1 or L2 based on the absence or presence of one or more of 20 IDRFs, respectively. Distantly metastatic tumors are defined as Stage M, except for Stage MS, in which metastases are confined to the skin, liver and/or bone marrow in children younger than 18 months of age (161). As a preoperative staging system, the INRGSS can facilitate consensus assessment and comparison of clinical trials across different cooperative groups and thereby provide insight into optimal treatment strategies for NB patients.

Genomic risk classification

To date, several research groups have established risk classification systems of NB using genomic signatures to predict prognosis. Our group has developed a genomic subgrouping system based on array CGH analysis for the risk stratification of NB (Table 1). We first used a DNA chip carrying 2464 bacterial artificial chromosome clones at a resolution of ~1 Mb to examine genomic aberrations in 236 Japanese primary NBs (82,136). To validate this risk classification, we further examined an independent cohort of 107 primary NBs using another genome platform (Agilent oligo-microarray) (68). Our array CGH analyses of a total of 343 NBs have categorized NBs into three risk groups with distinct genomic profiles and different prognoses: a genomic group (GG) of silent pattern, almost without any chromosomal aberrations except *MYCN* amplification (GG-S); a group with partial gains and/or losses pattern (GG-P); and a group with whole gains and/or losses pattern (GG-W), especially the gain of chromosome 17, and that rarely exhibit *MYCN* amplification. Each group was further classified into several subgroups on the basis of clinical outcome and known genomic signatures, including *MYCN* amplification, 1p loss, 11q loss and 17q gain. In general, the S group without *MYCN* amplification (the Ss subgroup) as well as the GG-W group have a good prognosis with an 8-year cumulative survival rate (SR) of 82% for the Ss subgroup ($n = 67$) and 80–100% for the GG-

Table 1. Risk classification of NB according to the pattern of the genomic copy number aberrations

Genetic group (GG)	Chromosomal aberration				Frequency ($n = 343$)	5-year survival
	MYCN	1p	11q	17		
Silent chromosomal aberration					(%)	(%)
Ss	–	–	–	–	19	80
Sa	Amp	–	–	–	2	0
Partial chromosomal gain/loss						
P1s	–	Loss	–	17q Gain	<1	67
P2s	–	Loss	Loss	17q Gain	4	26
P3s	–	–	Loss	17q Gain	14	41
P4s	–	–	–	17q Gain	6	52
P5s	–	–	–	–	1	–
P1a	Amp	Loss	–	17q Gain	17	38
P2a	Amp	Loss	Loss	17q Gain	2	17
P3a	Amp	–	Loss	17q Gain	1	–
P4a	Amp	–	–	17q Gain	<1	–
P5a	Amp	–	–	–	<1	–
Whole chromosomal gain/loss						
W1s	–	Loss	–	17pq Gain	1	100
W2s	–	Loss	Loss	17pq Gain	<1	–
W3s	–	–	Loss	17pq Gain	3	86
W4s	–	–	–	17pq Gain	25	94
W5s	–	–	–	–	2	80
W4a	Amp	–	–	17pq Gain	2	–

W group ($n = 106$), while the GG-P group has a poor outcome with an overall 8-year SR of 0–53% ($n = 130$) (68,82).

Janoueix-Lerosey and colleagues conducted a whole-genome DNA copy number analysis for the overall assessment of genomic aberration in 493 French NB patients using array CGH (162). Similar to our findings, the authors demonstrated that the tumors presenting whole chromosome CNVs are associated with excellent survival, whereas those with any type of segmental chromosome alterations have a high risk of relapse. Their results indicate that segmental chromosome alterations are the strongest predictor of relapse regardless of *MYCN* status.

Several recent lines of evidence have indicated that the aberrant DNA methylation phenotype is associated with clinical features and prognosis, suggesting that epigenetic aberrations contribute to the NB biological phenotype. Alaminos et al. found hypermethylation profiles of 10 genes that are associated with clinical risk groups in 140 NB tumors (163). Abe et al. revealed the presence of the CpG island methylator phenotype characterized by the methylation of a set of five CpG islands in the NB tumor genome, which is strongly associated with poor survival with extremely high hazard ratios in both Japanese and German NB cohorts (164). Olsson et al. recently identified two clusters with different methylation levels in NB and found that patients in the hypermethylated cluster are associated with the INRG Stage M and have a poor survival (165). The authors identified *TERT*, *PCDHGA4*, *DLX5* and *DLX6-AS1* as the genes with the highest number of hypermethylated CpG sites in INRG Stage M tumors.

The INRG classification system

Pretreatment risk classification provides insights into optimal treatment strategies for NB patients. For this purpose, the INRG proposed a risk classification system through comprehensive regression tree analyses of 8800 NB patients from several regions in the world, based on the following prognostic variables: stage (INRGSS), age at diagnosis (an age cutoff: 18 months), histologic category (INPC), grade of tumor differentiation, the status of the *MYCN* oncogene, chromosome 11q status, and DNA ploidy (19) (Table 2). According to the INRG, NB patients are stratified into very-low-risk, low-risk, intermediate-risk and high-risk groups, with a 5-year event-free survival (EFS) of >85%, >75–≤85%, ≥50–≤75%, or <50% for each group, respectively. Currently, the INRG system is widely adopted around the world to guide clinical therapy and management of patients with NB.

With the evolution of genomic techniques and integration of next-generation sequencing, more genomic and genetic aberrations will be likely included to refine the risk groups for a more accurate risk assessment and personalized therapy.

Surgery

Since NB is heterogeneous disease in terms of clinical behavior as well as tumor biology, the pediatric oncology surgeon should pay attention to minimize any complications that could be caused during the surgical management depending on the disease stage and age. The tumor originates from the sympathetic nervous system and invades into the important surrounding organs by encasing major vessels. Low stage tumors with favorable biology are usually treated by surgical resection alone. However, tumors with poor biological profiles are subsequently managed with aggressive chemotherapy after surgery. In particular, as the high-risk tumors are often resistant to complete resection, they undergo biopsy followed by aggressive high-dose chemotherapy. The responders receive surgical resection of the residual primary tumor followed by additional chemotherapy and radiation.

Neonatal NB

Perinatal NBs are often found as suprarenal masses by ultrasound examination during pregnancy or postnatally. The SIOPEN recommends observation if the mass is cystic or solid ≤5 cm in diameter (166).

Table 2. International Neuroblastoma Risk Group Staging System (INGSS)

Risk group for treatment	INRG stage	IDRFs in primary tumor	Distant metastases	Age (month)	Histological category	Grade of differentiation	MYCN status	Genomic profile	Ploidy
Very-low	L1	Absent	Absent	Any	GNB nodular, NB	Any	–	Any	Any
	L1 or L2	Any	Absent	Any	GN, GNB intermixed	Any	–	Any	Any
	MS	Any	Present	<12	Any	Any	–	Favorable	Any
Low	L2	Present	Absent	<18	GNB nodular, NB	Any	–	Favorable	Any
	L2	Present	Absent	≧18	GNB nodular, NB	Differentiating	–	Favorable	Any
	M	Any	Present	<18	Any	Any	–	Any	Hyperdiploid
Intermediate	L2	Present	Absent	<18	GNB nodular, NB	Any	–	Unfavorable	Any
	L2	Present	Absent	≧18	GNB nodular, NB	Differentiating	–	Unfavorable	Any
	L2	Present	Absent	≧18	GNB nodular, NB	Poorly differentiated, undifferentiated	–	Any[a]	Any
	M	Any	Present	<12	Any	Any	–	Unfavorable and/or diploid	
	MS	Any	Present	12–18	Any	Any	–	Favorable	Any
	MS	Any	Present	<12	Any	Any	–	Unfavorable	Any
High	L1	Absent	Absent	Any	GNB nodular, NB	Any	+	Any	Any
	L2	Present	Absent	≧18	GNB nodular, NB	Poorly differentiated, undifferentiated	+	Any	Any
	M	Any	Absent	12–18	Any	Any	–	Unfavorable and/or diploid	
	M	Any	Present	<18	Any	Any	+	Any	Any
	M	Any	Present	≧18	Any	Any	Any	Any	Any
	MS	Any	Present	12–18	Any	Any	–	Unfavorable	Any
	MS	Any	Present	<18	Any	Any	+	Any	Any

[a]Some clinical trial group consider unfavorable pathology with Stage L2, over 18 months of age. GN, ganglioneuroma; GNB, ganglioneuroblastoma.

The mass should subsequently be evaluated as follows (166):

- Repeat ultrasound examination and abdominal MRI scan
- Blood and urinary examinations
- MIBG scan

Surgery is then considered if the mass increases in size by 50% or with other evidence of progressive disease. The majority of tumors in this age often regress spontaneously without surgery and chemotherapy.

The role of surgery in management of NB is schematically shown in Fig. 8.

Surgical complications

Major complications at the resection of localized NB are as follows (167):

(i) Vascular injury: injury of major vessels including aorta, vena cava and renal and mesenteric vessels, which usually result in a serious operative mortality.
(ii) Renal loss: tumor invasion, arterial damage and renal vein thrombosis result in total nephrectomy. However, this is a disadvantage to subsequent chemotherapy.
(iii) Chylous acites or chylothrax: lymphatic leakage during surgery frequently results in postoperative chylous ascites or chylothrax. This revolves usually within several weeks or months but usually results in delayed subsequent adjuvant chemotherapy. Management is usually at first conservative. To decease lymphatic flow, fasting with total parental nutrition is required. In failure, surgical repair is adopted, including direct ligation, fibrin glue application or peritoneo-venous shunt. It is important to consider the timing of operative intervention before deterioration of the general condition.
(iv) Postoperative diarrhea: diarrhea is considered to result from the damage to inhibit sympathetic nervous supply when dissected celiac and supramesenteric artery.
(v) Horner's syndrome of the apical NB.
(vi) Adhesive bowel obstruction.
(vii) Neural injury: when the tumor extends into the neural foramina and the spinal cord (Dumbbell type), surgical complication may involve intra-spinal hematoma, its cord compression or direct cord injury, resulting in pelvic nerve palsy, fecal incontinence, neurogenic bladder, and/or erectile dysfunction. In case of left posterior mediastinal paraspinal tumors, the cord ischemia might occur owing to the injury of the Adamkiewicz artery, which typically derives from the aorta and left dorsal branch of the posterior intercostal artery at 9–12th level.

Image-defined risk factors

To minimize these surgical complications and to develop an effective strategy for individual patients, the clinical staging system has considered the most important guide. From this viewpoint, the INRG Project proposed a new staging system (INRGSS) in 2009. This composed of two factors: presence or absence of IDRFs (168,169).

Role of surgery in advanced disease

In advanced disease, surgery is now restricted in treatment so as not to interfere with chemotherapy and/or radiation. A recent study revealed that aggressive surgery, such as gross total resection, is effective in the Stage 3 tumor for survival, but in Stage 4 it is still under challenge (170,171).

Treatment according to risk classification

According to the INRG risk classification criteria, NB patients are currently stratified into very-low-risk, low-risk, intermediate-risk and high-risk groups, as described above (Table 2). Clinical therapeutic strategies are established based on the risk classification of NB.

Very-low-risk and low-risk NB

General statement and current results

In the COG risk classification, INSS Stages 1 and 2A/2B without *MYCN* amplification and INSS Stage 4s under 1 year old with non-amplified *MYCN*, favorable histology and DNA index >1 are

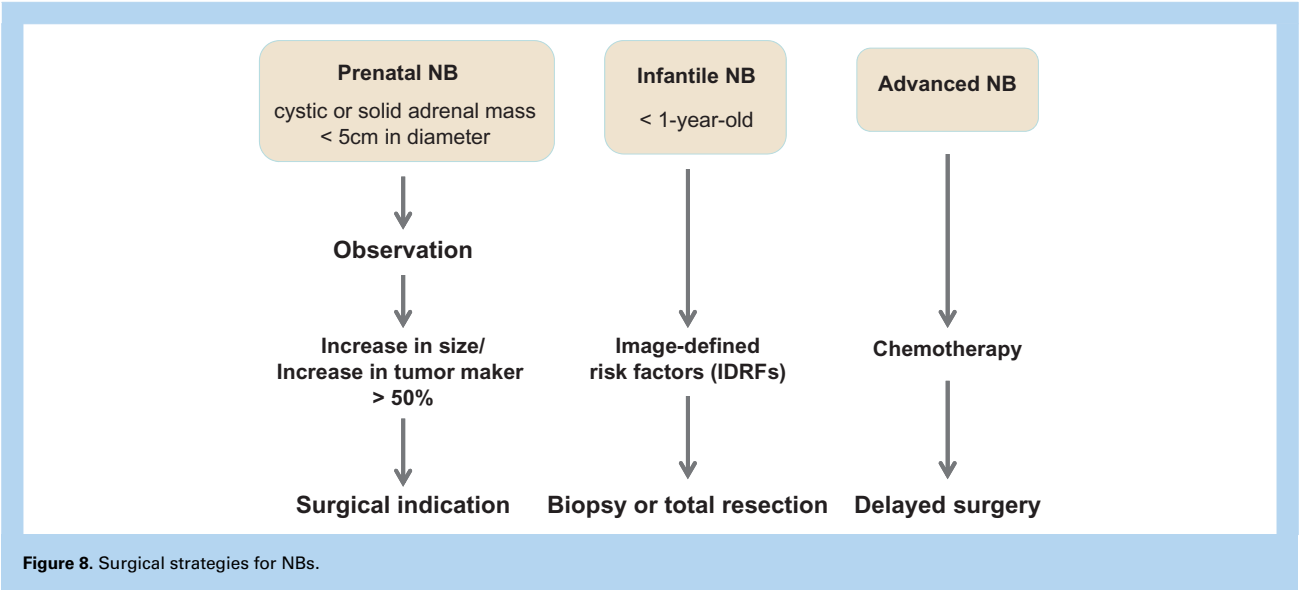

Figure 8. Surgical strategies for NBs.

classified as low-risk. Approximately 50% of newly diagnosed NB cases belong to this group, and excellent prognosis has been reported for these cases. The 5-year EFS rate is over 75% and the 5-year overall survival (OS) rate is 90–100% (2,19,169,172–180).

In particular, cases with a 5-year EFS of >85% (INSS Stage L1/L2 with ganglioneuroma maturing or ganglioneuroblastoma intermixed histology, INSS Stage L1 with non-amplified *MYCN* and INSS Stage MS in children younger than 18 months of age without 11q loss) are classified as very-low-risk group. The 5-year OS is 95–100%. This group accounts for 28% of all patients (19,169,172,175).

The standard treatment policy of this group is only surgical resection, but chemotherapy is performed when residual tumor is found or when surgical removal is difficult. Other treatment options include chemotherapy for the progressive disease after surgery and/or relapse, an emergency irradiation for the cases with neurological symptoms, or follow-up observations (2,173–175,177).

COG has reported that expectant observation as primary therapy for patients younger than 6 months with small adrenal masses leads to an excellent 3-year EFS (97.7 ± 2.2%) and 3-year OS (100%) by conducting periodic examinations including tumor imaging (sonogram, MRI or CT) and urinalysis (urinary VMA and HVA) (178).

Japanese protocols

In Japan, a nationwide mass-screening program measuring urinary VMA and HVA was conducted from 1985 to 2004 for infants at 6 months old with the purpose of improving the prognosis of NB by early detection (123,181,182). For the cases found in the mass-screening, a unified nationwide clinical trial (#9405) started in 1994, and in 1998 the protocol was revised (#9805) based on the risk classification of INSS. As a result, although the 5-year OS rate was as high as 98% in both protocols, excessive chemotherapy as well as surgical and late complications in some cases became a problem. In addition, infant patients found by mass-screening often included those showing spontaneous regression, suggesting that they might have received unnecessary surgery and chemotherapy (179,180). Since 2010, the JNBSG (now the JCCG) started observational research on low-risk NBs (JN-L-10) that determines the timing of surgery based on the preoperative risk classification according to IDRF. This protocol is currently tracking the cases. Determining the timing of surgery based on IDRF is logical and secure; however, there is not much that neoadjuvant chemotherapy will negate IDRF and make surgical resection safely possible. According to a report from Japan that examined whether IDRF would be changed by neoadjuvant chemotherapy, among the 15 patients with INRGSS Stage L2, only four patients (27%) had negative IDRF, for whom surgical excision became possible, however there was no change in IDRF in six patients (40%) (183). Furthermore, as most infant NBs show a good prognosis without aggressive treatment, non-treatment follow-up observation had been carried out in some facilities from long ago (184–188). Further, the NB screening program in Japan on infants 6 months of age demonstrated that while the screening may be effective to decrease the mortality of NB, it also may increase the risks of unnecessary chemotherapy and surgery (123). At present, for the low-risk NBs in patients less than 18 months of age, the JNBSG protocol of non-treatment follow-up observation (no biopsy, no surgical excision and no chemotherapy) (JN-L-16) is ongoing by introducing measurement of *MYCN* amplification in serum.

Follow-up

Attention should be paid to platinum-induced hearing loss and impaired renal function as late complications, especially in the case of single kidney of composite resection and scoliosis or sort stature in paravertebral tumor. Some patients also may need to be treated for blood transfusion-related hepatitis C virus infection (189–195). This means that unnecessary chemotherapy and surgery must be avoided. In particular, for the infants less than 2 months old with NB in Stage 4s with hepatomegaly, early initiation of chemotherapy should be considered because of the risk for rapid disease progression (196,197).

Intermediate-risk NB

General statement and current results

In general, non-low-risk NB patients who do not have poor prognostic factors are classified into the intermediate-risk group of NB. In the COG risk classification, this group includes patients under 1 year of age in INSS Stage 3/4 without *MYCN* amplification, patients aged 12 months to 18 months in INSS Stage 4 with favorable histology without *MYCN* amplification, patients under 1 year old in INSS Stage 4 s (*MYCN* non-amplified, unfavorable histology and DNA index > 1), and patients under 1 year old in INSS Stage 4 s (*MYCN* non-amplified, favorable histology and DNA index = 1) (Table 2). Overall, this group has a poor prognosis compared with the low-risk group and has a 5-year EFS rate of 50–75% and a 5-year OS rate of 60–85% (19,172–174,177). However, it is a very heterogeneous subgroup, for example, including patients from a good prognosis group with INSS Stage 3 under 1 year of age to a poor prognosis group with bone metastasis (169,176,198,199). *MYCN* gene status is the most potent prognostic factor in this group (19,173,174,182,198,200–204).

Japanese protocol

In Japan, clinical trials specialized for this group have not been conducted. Therefore, there is no comprehensive report on the treatment outcome of patients in INSS Stage 3 (over 1 year old) and INSS Stage 4 (12–18 months old) without *MYCN* amplification in Japan. However, for infant NBs, Japanese nationwide clinical study #9405 and #9805 data have been collected. In these protocol studies, patients in this group received nine courses of LI-B regimen chemotherapy (vincristine (VCR)/cyclophosphamide (CPA)/THP-adriamycin (THP-ADR)) or six courses of LI-D regimen chemotherapy (VCR/CPA/THP-ADR/cisplatin (CDDP)). According to these clinical trials, the 5-year EFS rate is considered to be ~70% for infant Stage 4 cases without *MYCN* amplification in Japan at the present time (205–207). Currently, JNBSG is undergoing Phase II clinical trial for the cases classified as intermediate-risk NB using the COG risk classification (JN-I-10) (206–208). In this clinical trial, the timing of surgery and additional chemotherapy is determined depending on the presence or absence of IDRF.

Follow-up

For the patients who receive irradiation therapy owing to metastases (bone, liver) (for example, INSS Stage 4 infants) as well as epidural compression, attention should be paid to the late skeletal complications and dysfunction of irradiated organs, secondary cancer, growth disorder and scoliosis. Of course, we should also note other complications that need attention in the low-risk group (209–212).

Among the patients with symptoms of spinal cord compression at diagnosis, ~70% of patients may have residual impairments (210,213).

High-risk NB

General statement and current results

Many patients of this group are over 18 months of age. The mass screening at 6 months of age in Japan did not yield data to help improve the incidence of high-risk NBs (185,214–219).

The cases classified into this group (Table 2) still have a poor prognosis even after treatment with intensive chemotherapy, surgical resection, radiotherapy, or myeloablative hematopoietic stem cell transplantation. The prognosis of this group still shows a 5-year EFS rate of 30–50% (19). Nevertheless, these outcomes have been slightly improved by new drugs such as targeted biological agents and combined immunotherapy (2).

Some of the new drugs include 13-cis-RA, ALK inhibitor, anti-GD2 antibody and CAR-T therapy. According to the recent reports, these new approaches applied after intensive chemotherapy to eliminate minimal residual disease (MRD) have gradually improved the outcome of high-risk NBs. The therapeutic strategies for high-risk NBs are summarized in Fig. 9.

Japanese protocols

The first nationwide clinical trial for advanced NB in Japan was the JANB 85 trial that started in 1985, and 157 cases were registered in this trial by 1990. In this study, the patients received six course of intensive induction therapy (regimen A1) consisting of CPA (1200 mg/m^2), VCR (1.5 mg/m^2), THP-Adriamycin (40 mg/m^2) and CDDP (90 mg/m^2) followed by three different consolidation therapies (nitrosourea, dacarbazine and bone marrow transplantation). According to the results of this trial, the 5-year OS rate was 76.9% for Stage 3 over 1 year of age and 34.4% for Stage 4; however, the patients who did not achieve complete remission after six courses of induction chemotherapy all died (220–223).

After 1991 when the presence or absence of *MYCN* amplification was established as a prognostic factor, the induction therapy for Stage 4 cases with *MYCN* amplification was strengthened to regimen A3 consisting of CPA (2400 mg/m^2), THP-ADR (40 mg/m^2), etoposide (VP-16) (500 mg/m^2) and CDDP (125 mg/m^2). This clinical trial (JANB91) continued until 1998, and patients with *MYCN* amplification receive stem cell transplantation after induction therapy. The 5-year OS rate of the *MYCN*-amplified patients was 34%, and the relapse-free-survival (RFS) rate was 36%. Although higher doses of chemotherapy may ameliorate the outcome of *MYCN*-amplified patients, there was no significant difference of the 5-year OS rate between JANB85 and JANB91 studies (224,225).

Based on these results and introduction of the INSS classification, the JANB98 study was started in 1998. In the JANB98 protocol, all patients received six courses of induction therapy (regimen 98A3) consisting of CPA (2400 mg/m^2), VCR (1.5 mg/m^2), THP-ADR (40 mg/m^2) and CDDP (125 mg/m^2) in addition to the delayed surgical resection with or without myeloablative stem cell transplantation. By September 2003, 147 eligible cases were enrolled in this trial, but the detailed results have not been reported (206).

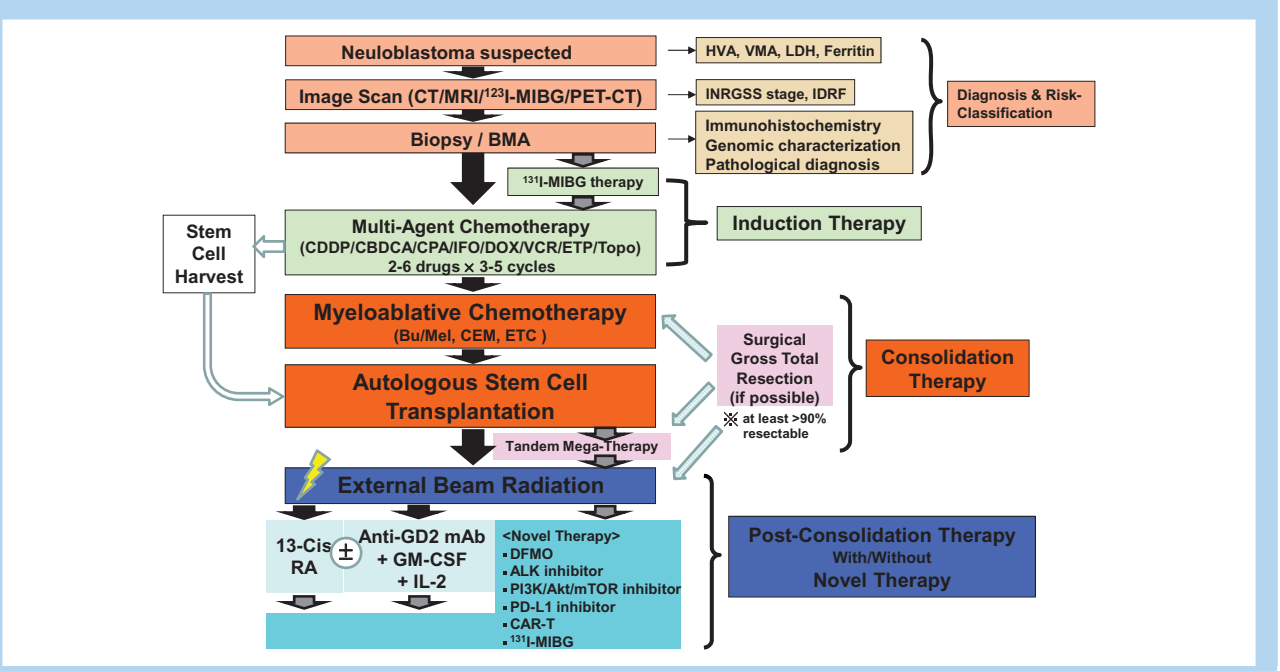

Figure 9. Therapeutic strategies for high-risk NBs. For high-risk NB, analysis of genetic background together with clinical symptoms and imaging diagnosis is very important. If it is judged to be a high-risk NB in the INRGSS classification, induction therapy is generally performed by multi-agent chemotherapy. Following induction therapy, myeloablative chemotherapy with autologous stem cell transplantation as consolidation therapy is performed. At this time, if residual tumor is found and if surgical gross total resection can be done, the tumor is removed at an appropriate time for local control. After chemotherapy, external beam radiation is performed for the purpose of preventing local recurrence as necessary. In recent years, performing immunotherapy and differentiation therapy is becoming standard therapy internationally, but in Japan it has not yet been confirmed with regulatory approval. Also, various novel therapies have been tried on the ultra-high-risk group, which is thought to be hard to cure in existing treatments.

Since the founding of JNBSG in 2006, a clinical research system was established and the Japan nationwide clinical study for high-risk NB has started. The first clinical trial of JNBSG aimed for safety assessment of delayed local treatment. However, this clinical trial was discontinued owing to the increasing cases with progressive disease or treatment-related deaths.

The second clinical trial of JNBSG (JN-H-07) was started in March 2007. This clinical trial adopted the COG classification and was a protocol for three courses of induction therapy followed by delayed local therapy, autologous stem cell transplantation and irradiation. For induction therapy, regimen 98A3, which reduced cyclophosphamide and cisplatin, was adopted. In addition, melphalan, etoposide and cyclophosphamide were used for pre-transplantation treatment. Fifty cases were enrolled in this trial, and the results were similar to those of the SIOPEN study without maintenance phase; the 5-year OS rate was 48.4% and the EFS rate was 32.2% (206).

The third clinical trial of JNBSG (JN-H-11) was started in March 2011. In this trial, the main objective was to evaluate the safety and efficacy of delayed surgical therapy and irradiation as local control after high-dose myeloablative chemotherapy. Induction therapy consisted of five courses of four drugs (CPA/VCR/THP-ADR/CDDP), and L-PAM/VP-16/CBDCA were adopted for pre-transplant treatment. At present, the observation period is over and the clinical data is being evaluated (206).

The JN-H-15 study is currently being conducted, in which ICE therapy (ifosfamide, carboplatin and etoposide) is incorporated into induction therapy and strengthened stem cell transplantation pre-treatment with Bu/L-PAM is combined (206).

Follow-up

To cure patients with high-risk NB, a multidisciplinary treatment strategy combining myeloablative chemotherapy, surgical resection, radiation therapy, immunotherapy and other treatments is performed. Because the treatment combination and intensity differ from case to case, it is necessary to individualize the follow-ups for each patient.

Since the survivors in the high-risk group had received multimodality treatment, we should pay attention to late mortality due to second malignancy (especially secondary acute myeloid leukemia in patients treated with VP-16), cardiovascular disease (especially cardiomyopathy in patients treated with anthracycline) and other late complications (226–229).

As described above, high-risk NB patients have various kinds of treatment contents for each patient, and contents to be followed up are different for each patient. For details, it is better to refer to the COG guidelines. COG long-term follow-up guidelines 'Long-Term Follow-Up Guidelines for Survivors of Childhood, Adolescent, and Young Adult Cancers' are available on the website (http://www.survivorshipguidelines.org/).

Ultra-high-risk NB

A European HR-NBL1/SIOPEN study stated that high levels of tyrosine hydroxylase (TH) and *PHOX2B* mRNA in peripheral blood at diagnosis objectively identify ultra-high-risk NB patients. Patients with high TH and high PHOX2B levels were seen in 19% of NB patients and the 5-year EFS and OS rates were both 0% (230). Novel treatment approaches should be considered and pursued for these patients (231). In another approach, Saarinen-Pihkala et al. reported that patients with combined *MYCN* amplification and bone metastases are suitable candidates to detect the ultra-high-risk NB subgroup (5-year EFS rate: 12.5 ± 8.3%) within the high-risk NBs, who should be given novel alternative upfront therapies (232).

Treatment option for high-risk NB

Induction therapy

The most common induction therapy for high-risk NB is intensive chemotherapy with four or five agents including alkylators (CPA, ifosfamide (IFO)), anthracycline (doxorubicin (DXR), THP-ADR), platinum compounds (CDDP, carboplatine (CBDCA)), topoisomerase-II inhibitors (VP-16), and vinca alkaloid (VCR) (220,233–235). Most recently, the topoisomerase-I inhibitor topotecan was used (236–239). Although the response to induction therapy correlates with prognosis, even the more intensive and condensed treatments do not appear to improve prognosis. Moreover, some reports suggested that the ICE regimen is effective for high-risk NB, and has been adopted in the latest clinical trials in Japan (220,233).

As another option, iodine-131 metaiodobenzylguanidine (^{131}I-MIBG) therapy was used for upfront treatment (238,240–245). The first report of ^{131}I-MIBG therapy for NB was published in 1986 (246) and its anti-tumor effect against NB was demonstrated in many trials (244,247–251). Several trials indicated increased myelosuppression and increased venoocuulusive liver disease, hypothyroidism, secondary thyroid cancer and radiation complications (241–243,252,253). In particular, when ^{131}I-MIBG therapy is performed in combination with chemotherapy or when administered over 12 mCi/kg MIBG, it is necessary to pay attention to prolonged myelosupression, especially thrombocytopenia, and some patients need to be supported by stem cell transplantation (241–243,249,251,254–256). In recent years, however, good results have been reported by introducing ^{131}I-MIBG into the initial treatment of high-risk NB. In addition, in some pilot studies for upfront treatment of high-risk NB using ^{131}I-MIBG, the studies use 200 mCi ^{131}I-MIBG for first 2 weeks and 150 mCi ^{131}I-MIBG for the next 2 weeks. Results have shown that ^{131}I-MIBG therapy is feasible and toxicity is permissible, and hematopoietic stem cell harvest was also possible (241).

Local control

In response to induction therapy, local control of the primary tumor becomes the next task. Current local treatment involves surgery and irradiation. However, for high-risk NB, systemic chemotherapy should be a high priority over topical therapy, and local treatment is generally performed after myeloablative chemotherapy with stem cell transplantation.

Role of surgery in high-risk NB

The effect of aggressive gross total resection (GTR) of the primary tumor on the prognosis of high-risk NBs with metastasis is still controversial (257–264). When surgery ended in partial resection (50–90%) or less, it does not impact prognosis. Improvement of prognosis can only be expected when the near-GTR (>90% but with palpable or visible tumor remaining) is completed (265–267). In addition, considering the long-term outcome, even if GTR of the tumor is possible, it is necessary to avoid resection of the kidney, or laminectomy for cases without neuropathic symptoms.

Role of radiation therapy in high-risk NB

Previous studies demonstrated that local recurrence decreases upon external irradiation of 21–36 Gy on the preoperative tumor bed after systemic chemotherapy and surgery (268–271). External

irradiation is superior to other therapies as local control for bone metastases (272). However, when the field of radiation includes important organs such as lungs, kidneys, ovary and orbital cavity, careful setting of the radiation field is required. Furthermore, attention must be paid to second malignancies and growth problems at any site included in the irradiation field, especially thyroid, bones, and large blood vessels (273–275).

To date, one study has demonstrated that local control can be performed in 50.4% of cases by re-resection and intraoperative irradiation (IORT) for local recurrent cases (276). However, only few reports have shown the clear effectiveness of IORT as local control for high-risk NB.

Although there is only a limited number of reports on proton radiotherapy for high-risk NB, there is a possibility that it can be effective and applied safely to cases in which external irradiation is difficult (277–280). In addition, it is possible to reduce the dose to normal tissue; therefore, proton radiotherapy as well as carbon particle beam therapy should be considered as one of the safer and more effective treatments against high-risk NBs in the future (281).

Myeloablative chemotherapy with stem cell transplantation

Myeloablative chemotherapy with autologous hematopoietic stem cell transplantation (AHSCT) is conducted as consolidation therapy for high-risk NB. Several randomized trials have shown improved prognosis with AHSCT compared with conventional chemotherapy (234,235,282–285). However, recent reports have indicated that myeloablative chemotherapy with AHCST improves the EFS rate but does not affect the OS rate, especially for the patients who receive some immunotherapy (282,286). At the same time, myeloablative chemotherapy has many adverse effects, and thus how to reduce the risk of complications and improve quality of life should be clarified in future studies (2,282,287).

In recent years, total body irradiation (TBI) is no longer used as a pre-transplant conditioning regimen owing to the concerns of radiation late effects (such as growth failure, secondary malignancy, and infertility) (282,287). Instead, melphalan/etoposide/carboplatin (MEC) or busulfan/melphalan (BU/L-PAM) is more frequently used as a conditioning regimen (288–290). In comparing the toxicity of the MEC regimen with the BU/L-PAM regimen for serious infections, veno-occlusive disease, hepatic toxicity, and treatment-related mortality, there is no clear answer as to which regimen is more toxic. However, the MEC regimen requires a longer duration of hospitalization and intensive care and shows a higher association with renal toxicity. On the other hand, pulmonary toxicities including interstitial pneumonia and artificial respiratory management have been associated with the BU/L-PAM regimen (282,288–290). The BU/L-PAM regimen has better prognosis than other regimens containing L-PAM. The BU/L-PAM regimen is currently adopted in the ongoing JNBSG Phase II trial.

As a conditioning regimen, the use of drugs that have not been administered by previous chemotherapy is an option to address concerns of drug resistance. Furthermore, in some trials, attempts to improve the prognosis of high-risk NB include increasing the treatment intensity by using scheduled tandem high-dose chemotherapy (291–296).

There are few studies showing that allogenic-stem-cell transplantation (allo-SCT) is much superior to autologus-stem-cell transplantation (auto-SCT) (285,297–299). The graft-versus-tumor effect is very limited, and there is no relevance between prognosis and acute or chronic GVHD (299–304). Therefore, it is inappropriate to determine chemoresistance as the absolute indication for allo-SCT. However, if the patient is in complete remission (for example, MIBG-negative) before transplantation and the performance status is good, performing a devised transplant (i.e., KIR-ligand mismatched donor) may lead to an improvement in prognosis (299,305–307).

Maintenance therapy for MRD

In recent years, with the aim of reducing the risk of local recurrence and improvement of long-term survival rate, differentiation-inducing therapy with isotretinoin (13-cis-RA) or immunotherapy with anti-GD2 antibody after myeloablative chemotherapy with AHSCT has been performed in many countries to eliminate MRD (2,234,235). However, these new drugs have not been approved in Japan and thus have not been clearly investigated.

Differentiation-inducing therapy with 13-cis-RA

Previous studies showed that 13-cis-RA can induce differentiation in NB cell lines established from refractory tumors. Although no significant effect was observed as maintenance therapy after myeloablative chemotherapy, 13-cis-RA resulted in some improvement in OS (2,231,308–311). Currently, 13-cis-RA is used together with anti-GD2 antibody, GM-CSF and interleukin-2 in immunodifferentiation therapy (234,235,312–314).

Anti-NB drugs

Targeting ALK

ALK is a critical mediator of oncogenesis and a therapeutic target of small molecule inhibitors and immunotherapeutic approaches in NB (13,15,16,315–317). In preliminary early-phase clinical trials, the ALK inhibitor crizotinib showed a limited activity against the various ALK mutants identified from NB patients (318–320). Recently, many studies have been carried out to explore the effectiveness of inhibiting ALK activity. These approaches include new ALK inhibitors and concominant drugs as well as immunotherapy targeting ALK.

Targeting *MYCN*

Aurora kinase inhibitor

MYCN is an oncogenic transcription factor and its amplification is closely associated with aggressive NB and poor prognosis. *MYCN* encodes N-Myc protein, which is a therapeutic target for high-risk NB (321–324). However, to date, there are no molecules that directly inhibit N-Myc. Alisertib (MLN8237) is a selective AURKA inhibitor, and tozasertib (VX680, MK-0457) is a pan-Aurora kinase inhibitor. These Aurora kinase inhibitors reduce the stability of N-Myc, induce G2/M cell cycle arrest, decrease phosphorylation of the Aurora kinase substrate histone H3, and induce apoptosis (Fig. 4) (325–328). As a new approach to high-risk NB therapy, some clinical trials are ongoing to evaluate the effect and toxicity of Aurora kinase inhibitors in combination with chemotherapy such as irinotecan and temozolomide (329).

Difluoromethylornithine (DFMO)

In 2006, Saulnier-Sholler et al. reported that a 5-year-old girl who had chemotherapy-resistant aggressive NB responded to difluoromethylornithine (DFMO; nifurtimox) therapy combined with topotecan and cyclophosphamide, resulting in clinical remission (330). Nifurtimox had first been used to treat Chagas disease (a parasitic disease caused by *Trypanocoma cruzi*). In using nifurtimox to treat Chagas disease, nitro anion free radicals and oxyradicals appeared to kill the parasite.

DFMO is an irreversible inhibitor of ornithine decarboxylase, which targets MYC, leading to a reduction of *MYCN* expression at

Table 3. Early phase clinical trials for high-risk NB

NCT trial number	Novel agent	Mechanism of action	Chemotherapy backbone	Phase
NCT00601003	Nifurtimox (DFMO)	Ornithine decarboxylase (ODC) inhibitor	Cyclophosphamide, Topotecan	II
NCT00911560	Adjuvant OPT-821	Anti-GD2L/GD3L vaccine	none	I/II
NCT00931931	HSV1716	Oncolytic virus	none	I
NCT01114555	Bevacizumab	VEGF Inhibitor	Temozolomide, Irinotecan	II
NCT01586260	DFMO	ODC Inhibitor	none	II
NCT01601535	MLN8237	Aurora Kinase A Inhibitor	Irinotecan, Temozolomide	I/II
NCT01606878	Crizotinib	ALK Inhibitor	Cyclophosphamide, Topotecan, Vincristine, Doxorubicin	I
NCT01742286	LDK378	ALK Inhibitor	none	I
NCT01822652	Pembrolizumab	PD-L1 antibody	none	I
NCT01956669	Pazopanib (GW786034)	VEGF Inhibitor	none	I
NCT02013336	MM-398	Topoisomerase I Inhibitors	Cyclophosphamide	I
NCT02030964	DFMO	ODC Inhibitor	Celecoxib, Cyclophosphamide, Topotecan	I
NCT02139397	DFMO, Bortezomib	ODC Inhibitor, Proteasome Inhibitor	DFMO, Bortezomib	I/II
NCT02163356	Fenretinide/LXS	Synthetic Retinoid Derivative	Ketoconazole, Vincristine	I
NCT02298348	Sorafenib	Multi kinase Inhibitor	Cyclophosphamide, Topotecan	I
NCT02304458	Nivolumab ± Iplimumab	Immune Checkpoint Inhibitor	none	I/II
NCT02308527	Bevacizumab	VEGF Inhibitor	Temozolomide ± Irinotecan	II
NCT02311621	Anti-CD171 CAR-T	CAR-T	none	I
NCT02332668	Pembrolizumab(MK-3475)	PD-L1 antibody	none	I/II
NCT02337309	SF1126	PI3K Inhibitor	none	I
NCT02541604	Atezolizumab(MPDL3280A)	PD-L1 antibody	none	I/II
NCT02630043	Tolcapone	OCMT Inhibitor	Oxaliplatin	I
NCT02679144	DFMO	ODC Inhibitor	none	II
NCT02761915	Anti-GD2 CAR-T	CAR-T	Cyclophosphamide, Fludarabine	I
NCT02765243	Anti-GD2 CAR-T	CAR-T	none	II
NCT02919046	Anti-GD2 CAR-T	CAR-T	none	I/II
NCT02982941	Enoblizumab (MGA271)	Humanized IgG1 mAb	none	I
NCT02998983	Racotumomab	N-glycolyl GM3 Inhibitor	none	II

mRNA and protein levels (331,332). DFMO also suppresses basal and TrkB-mediated Akt phosphorylation and may contribute to the cytotoxicity of NB cells (333). Since DFMO has been long used for treatment of Chagas disease, the adverse drug effects of nifurtimox on children are likely very limited. Currently, Phase I/II clinical trials of nifurtimox in combination with topotecan and cyclophosphamide in patients with high-risk NB are ongoing (334) (Table 3).

PI3K/AKT/mTOR pathway inhibitors

In recent years, aberrant activation of phosphatidylinositol 3-kinase (PI3K), Akt and mammalian target of rapamysin (mTOR) has been identified in high-risk NB with *MYCN* amplification. Activation of the PI3K/Akt/mTOR pathway is thought to induce tumor cell growth and survival as well as treatment resistance and cause poor outcome (335–337). Therefore, PI3K, Akt and mTOR each could be a potential target for therapy for NB, and inhibitors for each of these components have been studied from both the basic and clinical aspects (338–343). To date, many inhibitors have been developed, including PI3K inhibitors (such as SF1126, GDC-0941, NVP-BEZ235 and PI103), Akt inhibitors (such as MK2206 and TAE) and mTOR inhibitors (sirolimus, Tron2 and ridaforolims (MK-8669, AP23573)), each of which is being evaluated for its synergistic anti-tumor effect in combination with chemotherapy (335,344–347) (Table 3).

Immunotherapy

Recently, research in the development of effective cancer immunotherapy has advanced rapidly. This field began with the first-generation cancer vaccine, and mAbs are now evolving to cell therapy using genome editing technology. Various immune-based therapies have also been applied for high-risk NB.

Anti-GD2 chimeric mAb

Immunotherapy using an anti-GD2 chimeric mAb is currently being evaluated as a treatment for MRD after high-dose chemotherapy in high-risk NB (2,310,314,348). The COG has reported that patients treated with the anti-GD2 mAb (ch14.18) plus GM-CSF, interleukin-2 and isotretinoin show significantly better outcome than treatment with isotretinoin alone (66 ± 5% vs. 46 ± 5% at 2-year EFS, respectively, $P = 0.01$, and 86 ± 4% vs. 75 ± 5% at 2-year OS, respectively, $P = 0.02$) (314).

GD2 is a glycolipid molecule abundantly expressed in NB cells with limited to no expression in most normal tissues except nerve fibers that signal pain. Thus, the anti-GD2 mAb (ch14.18) may induce severe neuropathic pain that only opioids can relieve. Neuropathic pain is the most problematic adverse event in anti-GD2 mAb therapy. Other toxicities include hypotension, respiratory toxicity and fluid retention (348–350). Recent trials have considered increasing the length of the mAb infusion to reduce adverse events and to investigate the necessity of IL-2, which may cause other adverse events, such as infusion reaction and fever (305,351,352).

ADCC induced by NK cells has also been reported to be important for the effect of immunotherapy with anti-GD2 mAb. Therefore, the anti-tumor effect may be affected by the combination of HLA class 1 molecule and KIRs. Delgado et al. reported that response or improvement of relapsed/refractory NB patients after immunotherapy is associated with autologous KIR/KIR-ligand mismatch (305). Forlenza et al. also reported that KIR3LD1 and HLA-B allele

combinations can have a prognostic impact on patient survival after treatment with the anti-GD2 mAb, which relies on NK-ADCC (353). The survival advantage seen in noninteracting combinations supports the therapeutic disinhibition of individuals with strongly interacting KIR and ligand pairs.

While the anti-GD2 mAb has not been approved for clinical application in Japan by the Pharmaceuticals and Medical Devices Act, a doctor-led Phase I clinical trial has been completed and preparations for Phase II trials are ongoing.

Immune checkpoint inhibitors

As a target of new immunotherapy, immune checkpoint inhibitors are being rapidly applied to the treatment of various refractory cancers. Immune checkpoint inhibitors act to maintain the activation of CTLs by inhibiting the programmed cell death-1 (PD-1) pathway including CTL associated protein 4 (CTLA4), PD-1 and PD-L1 as targets. Several preliminary studies have confirmed that PD-L1 is expressed in high-risk NB cells (354–356). Therefore, early clinical trials of immunity checkpoint inhibitors targeting PD-L1 (such as atezolizumab (MPDL3280A) and pembrolizumab (MK-3475)) are already being planned (Table 3).

CAR-T therapy

CAR-T therapy was first reported by Gross and colleagues from Israel (357). Since then, practical use of CAR as a novel cellular immunotherapy has been studied in various cancers. In particularly, the CD-19-specific CAR-T has shown impressive activity against B-cell malignancies (358–360).

In NB, a method to prepare CAR-T more efficiently with higher therapeutic effects, mainly using GD2 as a target antigen, has been examined. The conventional method to prepare CAR-T had the disadvantages of high cost, complexity and low efficiency (361–364). In recent years, easier, cheaper and more efficient methods to prepare CAR-T using CRISPR/Cas 9 technique has attracted attention (365–367).

In summary, the overall survival rate in patients with NB is gradually improving according to the introduction of multidisciplinary treatments. Nevertheless, the outcome of high-risk as well as ultra-high-risk NB is still poor and further development of innovative therapeutic tools that include new drugs, novel immunotherapies, particle beam radiation therapies and others is required. In addition, the long-term follow-up and care of NB survivors are necessary because most of the survivors receive very aggressive therapies that may often cause late effects, including not only physical but also psychological problems, and even second malignancies.

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Conflict of interest statement

None declared.

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