accompanied by increased drug resistance and enhanced MRP1-mediated drug efflux (17). These studies provide evidence, suggesting that *MYCN* overexpression in NB is a possible biochemical pathway that contributes to the malignant behavior.

A recent discovery of RNA interference (RNAi), as a highly efficient method for gene knock-down, has been one of the major breakthroughs in molecular medicine (18,19). RNAi provides a new reliable method to investigate gene function that has many advantages over other nucleic-acid-based approaches such as antisense oligonucleotides, and which is therefore currently the most widely used gene-silencing technique in functional genomics. The previous extensive research on the development of therapeutic antisense nucleic acids should facilitate development of therapeutic siRNAs (20). Although several recent studies have demonstrated high efficiency and versatility of RNAi in cell cultures, the knock-down of *MYCN* in amplified NB cell line with RNAi has not been reported yet.

In this study, in order to elucidate the role of *MYCN* molecular and biological mechanisms, we transfected artificially synthesized siRNAs that were designed to target the *MYCN* gene by adopting lipofection method as a siRNA delivery system.

Materials and methods

Cell lines and culture condition. *MYCN*-amplified human NB cell line NB-1 was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). NB-1 cells were propagated and maintained in RPMI-1640 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (MP Biomedical, Inc., Eschwege, Germany) and anti-biotic-anti-mycotic solution (Nacalai tesque), and cultured in a 37°C humidified atmosphere containing 5% CO_2.

SiRNA oligonucleotides. A cocktail of three siRNA oligo-nucleotides targeting human *MYCN* with two thymidine residues (dTdT) at the 3'-end of the sequence was purchased from B-Bridge International Inc. (Sunnyvale, CA). These siRNA oligonucleotides corresponded to nucleotides 536-554, 1526-1544, and 1654-1672 of the human *MYCN* gene (GeneBank Access no. NM 005378). The sequences are as follows: si*MYCN*-1 (sense 5'-CGGAGATGCTGCTT GAGAA-3'), si*MYCN*-2 (sense 5'-CGGAGTTGGTAAAGA ATGA-3'), si*MYCN*-3 (sense 5'-CAGCAGTTGCTAAAGA AAA-3').

Each siRNA oligonucleotide included in the cocktail was separately available and used for preliminary experiments. To verify sequence specific effectiveness of the *MYCN*-siRNAs, we also used negative control siRNAs (NC-siRNA, B-Bridge International Inc.) that have no significant homology with any known sequences in the human genome.

Transfection. Transient transfection of siRNA was carried out using a commercially available transfection reagent (HiPerFect, Qiagen Inc., Valencia, CA), according to the instruction manual. Transfections were performed with a final concentration of 25 or 50 nM of siRNA in serum-free culture media. In this step, we adopted a reverse transfection method, in which cell seeding and transfection were performed simultaneously by adding the mixture of siRNA and the reagent onto the cells as soon as seeding the cells on the plates (21). Transfection efficiency of this transfection condition was estimated by green fluorescent protein (GFP) signals derived from a sham transfection of pEGFP-N1 Vector (Clontech Laboratories, Palo Alto, CA) to the NB-1 cell. Approximately 30% of the cells were fluorescent. To study the specific effect of *MYCN* silencing, we prepared the following four groups including several types of control: group 1 (*MYCN*-siRNA group), transfected siRNA against human MYCN; group 2 (NC-siRNA group), transfected negative control siRNA; group 3 (mock control group), the cells were treated with the reagent and PBS without any siRNAs to verify the influence of the transfection reagent; group 4 (no treatment group), the cells received no treatment.

RNA and cDNA preparation. The reverse transfection treatment was applied to 2.5×10^5 NB1 cells suspended in 2 ml medium in each well of a 6-well plate. At a later time indicated below, the cells were harvested for RNA extraction and cDNA synthesis. Total cellular RNA of each group was prepared by RNAqueous RNA isolation kit (Ambion, Austin, TX). First-strand cDNA was synthesized from 1 μg of total RNA using MMLV Reverse Transcriptase (Clontech Laboratories) and oligo(dT) primers.

Gene expression assays by real-time RT-PCR. To quantitate the level of mRNA of the *MYCN* and its relating genes Ha-*ras*, *TrkA*, *TrkB*, and *TrkC* that are associated with differentiation and prognosis of NB, real-time RT-PCR was performed on an ABI PRISM 7700 sequence detection system using Sequence Detector V1.7 software (PE Applied Biosystems Inc., San Jose, CA) (22). Human *GAPDH* was used as an internal control. Primers and TaqMan probes for *MYCN*, Ha-*ras*, *TrkA*, *TrkB*, *TrkC* and *GAPDH* were indicated in Table I. The relative expression levels of *MYCN*, Ha-*ras*, *TrkA*, *TrkB*, and *TrkC* mRNAs were standardized by that of *GAPDH*, and compared to that of no treatment control. *MYCN* mRNA expression was firstly evaluated at post-transfection 48 h, then, time-course of the expression for up to 6 days after transfection was independently investigated. Expressions of the Ha-*ras*, *TrkA*, *TrkB*, and *TrkC* mRNAs were evaluated at 48 h.

Western blotting. Ninety-six hours after siRNA transfection on 1.5×10^5 NB1 cells under the similar conditions as the gene expression assay, cells of each group were harvested to quantitate the MYCN protein level using Western blotting (23). Briefly, harvested cells were lysed in RIPA lysis buffer (Upstate, Lake Placid, NY) and protein amounts were measured by BCA protein assay set (Pierce Biotechnology, Inc., Rockford, IL). Then, 25 μg of protein was loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel (Criterion™ XT Precast Gel, Bio-Rad, Hercules, CA) for electrophoresis, and subsequently transferred onto a polyvinyl difluoride membrane. The membrane was soaked in a solvent (Can Get Signal, Toyobo, Osaka, Japan) including anti-MYCN monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 2.0 μg/ml, and incubated for 24 h at 4°C. The membrane was then incubated with a horseradish

Table I. Primers used in real-time quantitative PCR reactions.

Gene		Sequences (5'→3')
MYCN	Forward	GACCACAAGGCCCTCAGTACC
	Reverse	TGACCACGTCGATTTCTTCCT
	TaqMan probe	FAM-CCGGAGAGGACACCCTGAGCGA-TAMRA
GAPDH[a]	Forward	GAAGGTGAAGGTCGGAGTCA
	Reverse	GAAGATGGTGATGGGATTTC
	TaqMan probe	FAM-CAAGCTTCCCGTTCTCAGCC-TAMRA
TrkA	Forward	TTCACCTACGGCAAGCAGC
	Reverse	CCTGCGTGATGCAGTCGAT
	TaqMan probe	FAM-TGGTACCAGCTCTCCAACACGGAGG-TAMRA
TrkB	Forward	GTCTTTGAGTACATGAAGCATGGG
	Reverse	TCAGCACGGCATCAGGG
	TaqMan probe	FAM-ACCTCAACAAGTTCCTCAGGGCACACG-TAMRA
TrkC	Forward	CAAATATGGTCGACGGTCCAA
	Reverse	GAGTCCTCCTCACCACTGATGAC
	TaqMan probe	FAM-TTTGGAATGAAGGGTCCCGTGGC-TAMRA
Ha-*ras*	Forward	CCAGAACCATTTTGTGGACGA
	Reverse	CCCATCAATGACCACCTGC
	TaqMan probe	FAM-CGACCCCACTATAGAGGATTCCTACCGGA-TAMRA

[a]Human glutaraldehydes-3-phosphate dehydrogenase.

peroxidase conjugated anti-mouse antibody for 1 h and developed with an enhanced chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ).

Immunocytochemistry. For each group, $3.0x10^4$ NB-1 cells were treated and maintained in 0.5 ml medium per chamber on the four-chamber culture slides (BD Falcon™, BD Biosciences, San Jose, CA). At post-transfection 96 h, the cells were fixed in 4% paraformaldehyde, rinsed in PBS, and permeabilized with 1% Triton X-100 for 1 h. Then, they were incubated with anti-MYCN antibody (2.0 μg/ml) at 4°C overnight after a blockage step of 30 min performed in 10% normal rabbit serum. For visualization, FITC-conjugated rabbit anti-mouse immunoglobulins (6 mg/ml; Dako, Tokyo, Japan) were used as the second antibody, and nuclear staining was done in PBS. The slides were washed and mounted with fluorescence mounting medium (Dako) to be examined with photographs taken by Keyence VB6000 digital photography system (Keyence, Osaka, Japan) attached to Nikon Eclipse C1000 microscope (Nikon, Tokyo, Japan).

Cell viability assay and time-course evaluation. Cell viability was determined by WST-1 assay utilizing a colorimetric detection of mitochondrial dehydrogenase in viable cells (24). NB-1 cells were seeded at a density of $1x10^4$ cells in 100 μl of medium into each well of 96-well plates and maintained without medium change for up to 9 days after transfection. From post-transfection day 1, four wells were devoted for the assay everyday, and time-course of cell viability was monitored. In practice, 10 μl of WST-1 solution (Cell Count Reagent SF, Nacalai tesque) was added to each well, and samples were incubated at 37°C for 2 h. Then, the absorbency of the treated samples against a blank control was measured by an immunoreader apparatus (Immuno Mini NJ-2300, Nippon InterMed, Tokyo, Japan) under 414 nm as a detection wavelength and 630 nm as a reference wavelength, respectively.

Detection of apoptosis. To further assess influence of *MYCN*-siRNA on the cell survival, apoptotic features of NB-1 cells were evaluated. This was performed semi-quantitatively by using the TUNEL principle (ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit, Serologicals Corp., Norcross, GA). Cells were similarly seeded and transfected on the culture slides as the immunocytochemistry study. At post-transfection 96 h, the cells on the slides were fixed and subjected to the assay, according to the manufacturer's instructions. Apoptotic cells were observed and counted under a fluorescence microscope. Moreover, we evaluated the percentage of positive apoptosis cells in each group (25).

Morphologic change evaluation. To evaluate morphologic changes of the NB-1 cells induced by siRNA treatments, each group cells were stained with Phalloidin-Tetramethylrhodamine-B-isothiocyanate (Sigma-Aldrich Corp., St. Louis,

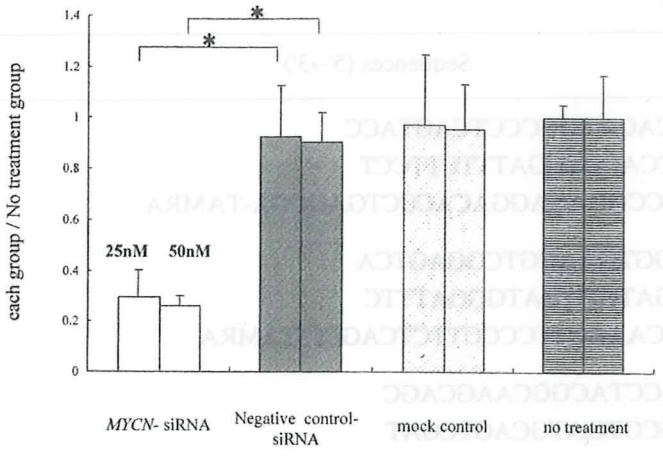


Figure 1. Assessment of relative mRNA expression of *MYCN* by real-time RT-PCR. The expression level of *MYCN* mRNA of the no treatment group defined as 1. The expression level of *MYCN*-siRNA group was significantly reduced to 30% of those of three control groups in NB-1 cell line. No significant change in the NC-siRNA group and mock control group was found compared to the no treatment group. Different *MYCN*-siRNA concentrations (25 and 50 nM) brought similar suppressive effect on the *MYCN* mRNA expression level (*p-value <0.05).

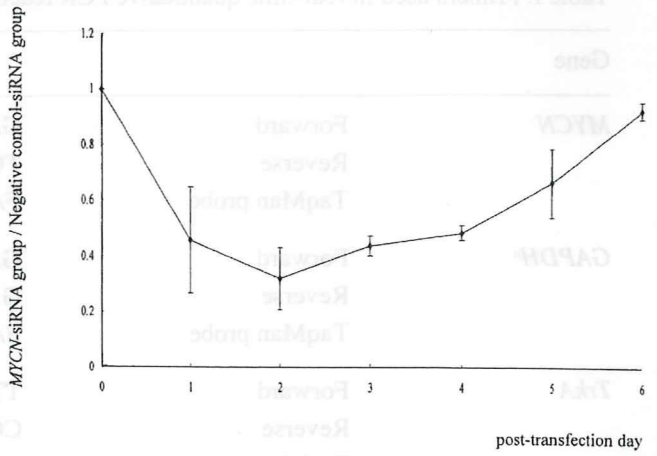


Figure 2. Time-course curve of *MYCN* mRNA expression level after *MYCN*-siRNA treatment. The expression level of the siRNA treated cells relative to that of Negative control-siRNA group on each day was calculated and plotted. Note that the maximum suppression occurred around post-transfection day 2.

MO) at 96 h. This staining highlighted cellular filamentous actin and differentiation appearance of the neuronal cells could be observed under a fluorescence microscope (26).

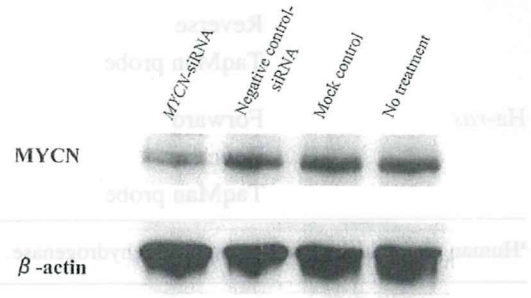
Statistical analysis. All experiments were performed at least three times and typical results were demonstrated. Data are presented as means together with standard deviation for each parameter. The statistical analysis was performed by an unpaired Student's t-test, and a p-value <0.05 was considered statistically significant.

Results

Effect of siRNA on MYCN mRNA expression. Forty-eight hours after *MYCN*-siRNA transfection, the expression level of *MYCN* mRNA significantly decreased to approximately 30% of group 4 ($p<0.05$) (Fig. 1), whereas the levels observed in groups 2 and 3 were similar to group 4. In these initial experiments, we separately treated the NB-1 cells with different *MYCN*-siRNA concentrations (25 and 50 nM), and found that both concentrations brought similar suppressive effect on the *MYCN* mRNA expression level (Fig. 1). When each of the three *MYCN*-siRNAs (si-*MYCN*-1-3) included in the cocktail was separately used for transfection at 25 nM concentration, we also found that *MYCN* mRNA expression was similarly reduced (data not shown). Then, in our subsequent experiments, we solely used the 25 nM cocktail.

Time-course curve of relative expression of *MYCN* mRNA is shown in Fig. 2. It appeared that *MYCN* knock-down lasted for 6 days after transfection. The nadir level of reduction occurred around post-transfection days 2, and consistently reached 30% of group 2. On post-transfection day 6, the expression of *MYCN* recovered to the same level observed in group 2.

Western blotting for MYCN. At post-transfection 96 h, Western blot assay revealed a reduction of MYCN protein level in group 1 compared to the other groups. MYCN protein expression did not differ among groups 2, 3 and 4 (Fig. 3A).



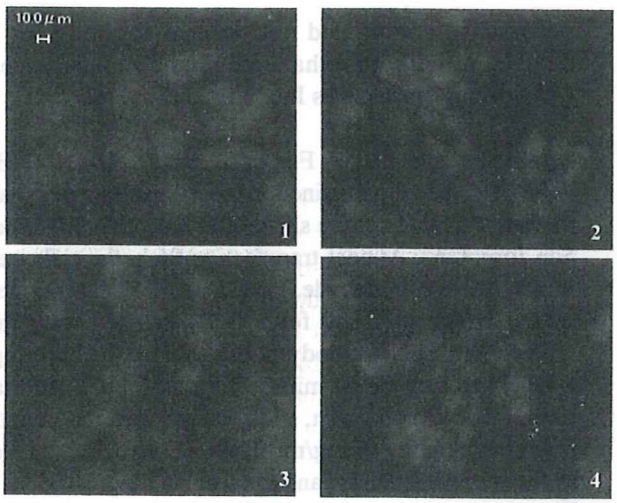


Figure 3. (A), Western blotting of MYCN and ß-actin. The study revealed reduction of cellular MYCN level in NB-1 cells treated by *MYCN*-siRNA. (B), Fluorescent immunocytochemical staining study demonstrating MYCN nuclear staining pattern in NB-1 cells. At 96 h of culture, nuclear staining of MYCN became very faint in the majority of the cells in the *MYCN*-siRNA group, whereas, those in other three control groups, almost all cells showed strong signals. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group.

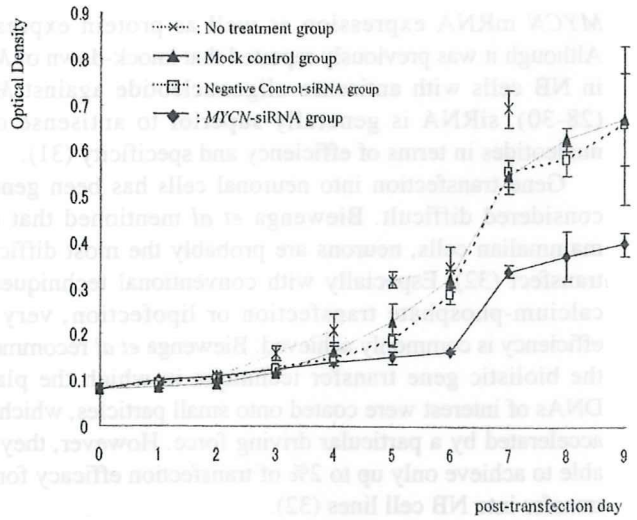


Figure 4. Time-course study assessed by WST-1 method demonstrated a significant reduction of NB-1 cell viability in the *MYCN*-siRNA group from day 5 (p-value <0.05). No significant change was found between the negative control-siRNA group and mock control group.

Immunocytochemistry for MYCN. Immunocytochemistry using anti-MYCN antibody showed intense immunoreactivity of nuclear staining groups 2, 3 and 4. In contrast, 96 h after siRNA treatment, nuclear staining of MYCN became very faint in the majority of the cells in group 1 (Fig. 3B).

RNAi suppressed NB-1 cell proliferation. In WST-1 assay, group 1 showed significantly reduced viable cell numbers, compared to groups 2 and 3. This significant suppression in cell proliferation became apparent on post-transfection day 5 and continued until day 9, when cells reached confluent growth in the NC-siRNA and mock groups and the time-course study was terminated ($p<0.05$) (Fig. 4). Cell proliferation modes of groups 2 and 3 were similar, but less propagated than that observed in group 4, probably reflecting some cytotoxic effect of the transfection reagent.

Apoptosis evaluation. Using *in situ* TUNEL assay, we identified significantly higher proportion of TUNEL positive cells in group 1 compared to the other groups ($p<0.0001$) (Fig. 5A). More than 50 cells per 100 were apoptotic in group 1, compared to 13 cells in group 2, and approximately 5 in groups 3 and 4 (Fig. 5B). These findings may indicate that siRNA treatment against MYCN activates an apoptotic process in NB-1 cells.

Morphological evaluation. Original shape of NB-1 is round. In group 1, the cells treated by *MYCN*-siRNA exhibited multidirectional neurite extension. Additionally, size of these cells and nuclei became apparently larger than those observed in the other cell groups (Fig. 6). These morphologic changes were consistent with neural differentiation.

Relative expression of Ha-ras, TrkA, TrkB and TrkC. At forty-eight hours after the siRNA treatment, relative expressions of *TrkA* and *TrkC* mRNA were significantly up-regulated in group 1 compared with other groups ($p<0.05$). The expression

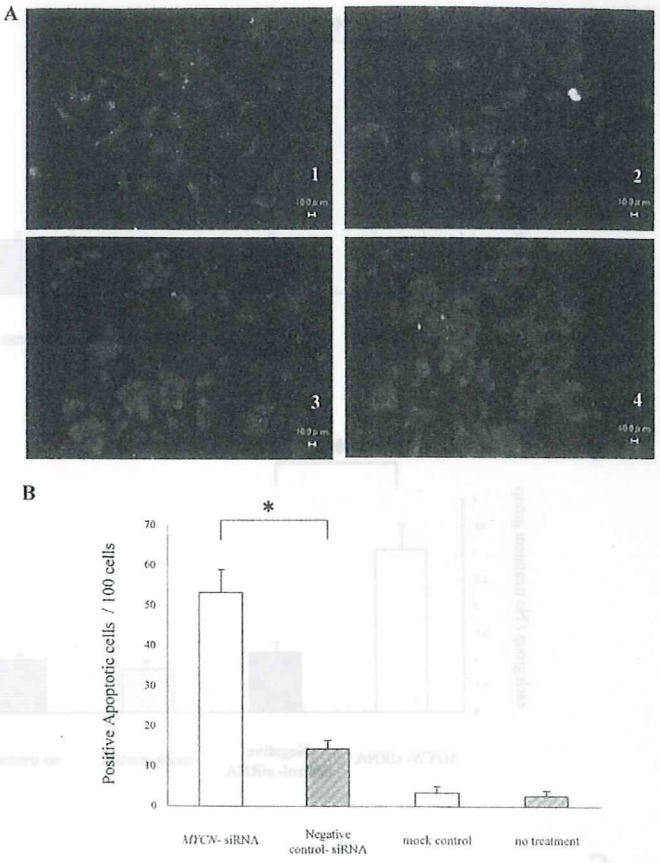


Figure 5. Detection of apoptosis. (A), Effect of *MYCN*-siRNA treatment on NB-1 culture at 96 h. The TUNEL positive cells were observed more frequently in the *MYCN*-siRNA group. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group. (B), Apoptotic cell counting per 100 cells at 96 h. Cells with apoptotic bodies were significantly increased in the *MYCN*-siRNA group (*p-value <0.0001).

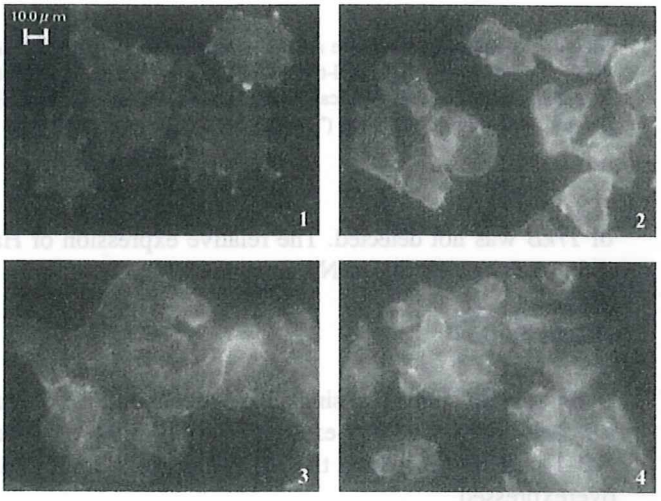


Figure 6. Effect of *MYCN*-siRNA treatment on NB-1 culture at 96 h. Phalloidin-TRTC staining of NB-1 exhibited multidirectional neurite extension in the *MYCN*-siRNA group. Additionally, sizes of the cells and the nucleus in this group became apparently larger than those observed in the other groups. These morphologic changes indicate differentiation effect of *MYCN*-siRNA treatment. Panel 1, *MYCN*-siRNA group. Panel 2, Negative control-siRNA group. Panel 3, Mock control group. Panel 4, No treatment group.

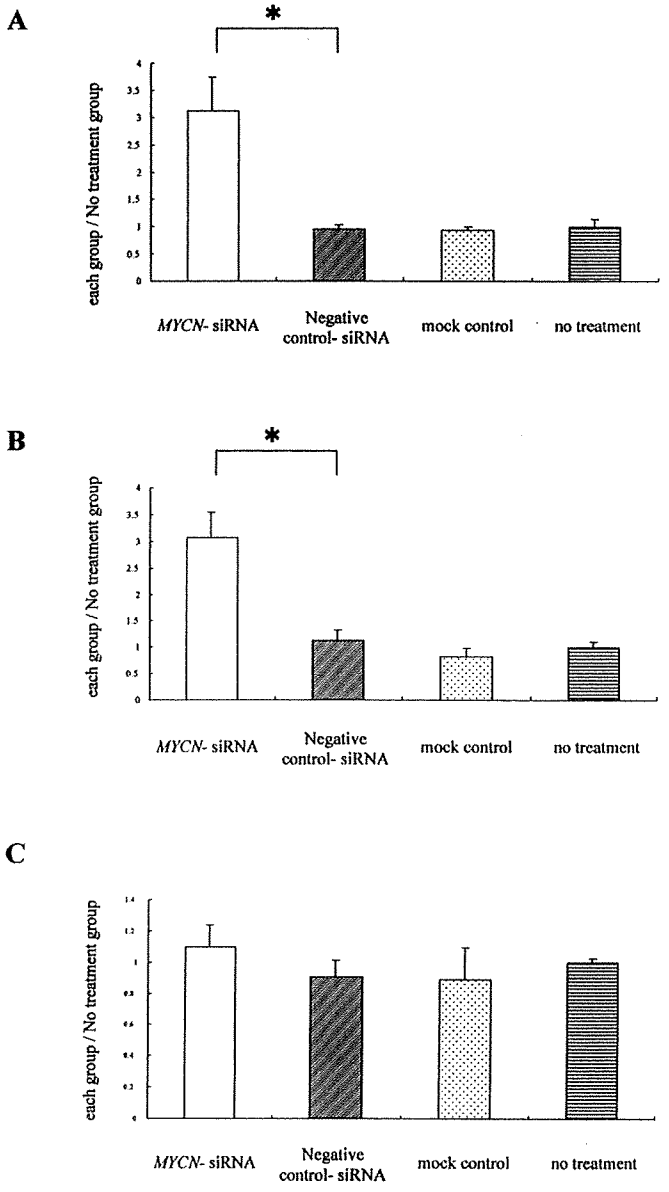


Figure 7. Assessment of relative mRNA expression levels each of *TrkA* (A), *TrkC* (B) and Ha-*ras* (C) by real-time RT-PCR. *TrkA* and *TrkC*, indicators of favorable prognosis, were significantly up-regulated. On the other hand, Ha-*ras* did not show significant change (*p-value <0.05).

of *TrkB* was not detected. The relative expression of Ha-*ras* did not change by the siRNA treatment (Fig. 7).

Discussion

In the present study, by using siRNA treatment, we conducted a knock-down of *MYCN* expression to examine its effect on NB-1 cell line in which the *MYCN* gene is amplified and overexpressed.

siRNA is a synthetic short double-stranded RNA that induces the destruction of homologous single-stranded RNA, when introduced into a cell. Therefore, siRNA has been evaluated as an effective tool for suppressing the target protein by specifically digesting its mRNA (18,27). In our experiment real-time RT-PCR and Western blotting definitely demonstrated that the treatment with siRNA targeted *MYCN* decreased *MYCN* mRNA expression as well as protein expression. Although it was previously reported that knock-down of *MYCN* in NB cells with antisense oligonucleotide against *MYCN* (28-30), siRNA is generally superior to antisense oligonucleotides in terms of efficiency and specificity (31).

Gene transfection into neuronal cells has been generally considered difficult. Biewenga *et al* mentioned that of all mammalian cells, neurons are probably the most difficult to transfect (32). Especially with conventional techniques like calcium-phosphate transfection or lipofection, very poor efficiency is commonly achieved. Biewenga *et al* recommended the biolistic gene transfer technique in which the plasmid DNAs of interest were coated onto small particles, which were accelerated by a particular driving force. However, they were able to achieve only up to 2% of transfection efficacy for gene transfer into NB cell lines (32).

To solve this problem, we adopted a reverse transfection method, in which cell seeding and transfection were performed simultaneously by adding the mixture of siRNA and the reagent onto the cells as soon as the cells had been seeded on the plates (21). Although we employed modified reagent for lipofection, reverse transfection method successfully achieved transfection efficacy around 30% and resulted in 70% reduction of *MYCN* expression. Our results show that reduction of *MYCN* mRNA expression induce growth inhibition of NB-1 cells.

After reduction of MYCN protein expression, the expression of target genes of *MYCN*, such as *ODC*, *MCM7* and *MRP1*, was probably suppressed. Those genes usually lead to cell progression through the G1 phase of the cell cycle (17,33,34). Thus, reduction of those gene expressions may lead to G1 cell cycle arrest and results in the suppression of cell proliferation.

To investigate whether an apoptotic pathway was involved in suppression of cell proliferation after silencing of *MYCN*, we conducted TUNEL assay and confirmed that significantly higher proportion of TUNEL positive cells were observed in siRNA treated NB-1 cells. Galderisi *et al* showed that antisense oligonucleotide treatment of substrate adherent NB cells (S cells) resulted in a clear increase of the proapoptic Bax and Bak gene expression, along with a drastic decrease in the level of anti-apoptic Bcl-2 mRNA (35). Thus, these genes may have important roles in cell death after *MYCN* gene inhibition. Galderisi *et al* also found that the differentiation and apoptosis that followed antisense treatment persisted after the end of *MYCN* gene inhibition, indicating that a lasting *MYCN* downregulation is not required to induce these processes (35).

Moreover, after transfection with *MYCN*-siRNA we also observed that a pattern of the outgrowth was mostly multidirectional neurite extension of cell processes, and gradual long neurite elongation. Additionally, sizes of the cells and the nucleus in this group became larger than those observed in the other groups. These morphologic changes indicate differentiation effect of *MYCN*-siRNA treatment. Similarly previous studies had reported that *MYCN* suppression using antisense oligonucleotides resulted in cell differentiation in NB (28,30,35,36). These studies suggested that alterations in the regulation of *MYCN* expression can modulate the differentiation process of NB cells (28).

After silencing of *MYCN* expression by RNA interfering, relative expressions of *TrkA* and *TrkC* mRNA were significantly up-regulated. At the same time, morphological change corresponded to tendency of differentiation was observed. Evidence from several independent studies suggests that high expression of *TrkA* is an indicator of favorable outcome, and there is an inverse correlation between *TrkA* expression and *MYCN* amplification. *TrkC* is expressed in favorable NBs, essentially all of which also express *TrkA* (37-39). These findings suggest that suppression of *MYCN* up-regulated *TrkA* which activate specific signaling pathways linking to differentiation and survival.

Ha-*ras* genes are also closely associated with the growth, differentiation, and survival of neuronal tissues. Several observations suggest a role of Ha-*ras* p21 in promoting cellular differentiation and suppression of the proliferation activity of PC12, a tumor cell line originating from rat sympathetic nerve tissue (40). Thus, we examined Ha-*ras* expression after *MYCN* silencing. Consequently, expression of Ha-*ras* mRNA did not increase. Therefore, the differentiation after silencing *MYCN* may have a relation not to Ha-*ras* but rather to *TrkA* cascade.

In conclusion, the expression level of the *MYCN* mRNA was significantly reduced using the RNAi method. As a result, the knock-down of *MYCN* expression induced growth-inhibition, apoptotic activity and cell differentiation in *MYCN*-amplified NB-1 cell line. These data indicate that *MYCN* might be the key factor in the tumorigenesis and prognosis of NBs. Thus, silencing the *MYCN* gene by the RNAi method could be a potential tool for the treatment of NBs with *MYCN* amplification in the future.

Acknowledgements

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Intraoperative radiation therapy for advanced neuroblastoma: the problem of securing the IORT field

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Abstract To evaluate the efficacy of intraoperative radiation therapy (IORT) and the problem of securing the IORT field in advanced pediatric neuroblastoma. Between 1996 and 2005, 12 children received IORT for advanced pediatric neuroblastoma patients. Electron beam energies ranged from 10 to 12 MeV and median dose was 10 Gy (8–12 Gy). All of them had surgery with IORT against the primary tumor site and the abdominal aorta surroundings. A gross total resection (GTR) was achieved in 10 patients and subtotal resection (STR) was two patients. All of 12 patients were classified as high risk. Nine patients were alive 17–120 (mean 48 months) after diagnosis. Local tumor control was achieved in 100% of patients, of whom one experienced local recurrence outside the IORT field. At the operation, it was difficult to secure the IORT field because of the angle of the radiation cylinder in three patients. One of the three of these patients experienced local recurrence outside of the IORT field in the upper side of superior mesenteric artery and two of three patients had an external beam radiation after surgery, and there was no local recurrence. One patient had a postoperative ileus, and one patient had transient diarrhea and hydronephrosis. For advanced neuroblastoma patients, IORT produced excellent local control after surgery. However, there is a problem of securing the IORT field. For local control, it is necessary to add an external beam radiation after IORT when it is difficult to secure the IORT field.

Keywords Intraoperative radiation therapy · Neuroblastoma · External beam radiation therapy

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Introduction

In intraoperative radiation therapy (IORT) a high single radiation dose is delivered to the tumor site with protection of normal uninvolved organs and tissues [1–6]. Neuroblastoma is the most common solid tumor in childhood [7]. The clinical significance of intensive surgical therapy as a means to control the local lesion has been controversial in the treatment of advanced neuroblastoma [8, 9]. Renal vascular problems and other major complications were reported after extensive surgical resection of neuroblastoma [10, 11]. To prevent the major complications of the surgical treatment and excessive lymphadenectomy, we have been doing IORT. To evaluate the efficacy and problem of IORT in advanced pediatric neuroblastoma, our 10-year experience of IORT was reviewed in this study.

Materials and methods

Between 1996 and 2005, 12 patients received IORT for pediatric advanced neuroblastoma. The international neuroblastoma staging system (INSS) [12, 13] was determined for this study by retrospective interpretation of clinical,

Table 1 Induction chemotherapy regimens for advanced neuroblastoma following to JANB (≥1 year old) (mg/m^2)

MYCN status	1996–1997		1998–2005
	N-*myc*		All patients
	<10 copies	≥10 copies	
Regimen	91A1	91A3	98A3
Cyclophoshamide	1,200 × 1	1,200 × 2	1,200 × 2
Vincristine			1.5 × 1
Etoposide	100 × 5	100 × 5	
Pirarubicine	40 × 1	40 × 1	40 × 1
Cisplatin	90 × 1	25 × 5	25 × 5

radiological and surgical data. Shimada classification [14] was recorded by chart review and by blinded histopathological review of pathology specimens. N-*myc* copy number was recorded by chart review. At first we performed open biopsy for classification and diagnosis in all cases. After the diagnosis, all patients received induction chemotherapy with combination of cyclophoshamide, vincristine, etoposide, pirarubicine, and cisplatin, and according to the protocol proposed by the Japanese study group for advanced neuroblastoma (JANB) [15] (Table 1). After six courses of induction chemotherapy, a radical operation with IORT was performed. Response to induction chemotherapy was determined by magnetic resonance imaging (MRI) or computed tomographic (CT) scan, ^{131}I-metaiodobenzylguanidine (MIBG) scan, bone scan and bilateral bone-marrow biopsies. Ten of 12 patients had peripheral blood stem cell transplantation (PBSCT) and seven of 12 patients had total body irradiation (TBI) preoperatively.

The second operation was planed in the special IORT operating room. IORT was delivered in a linear accelerator chamber using high-energy electrons between 10 and 12 MeV. The radiation dose delivered was between 8 and 12 Gy. Depending on the depth of treatment desired, the energy levels used were modulated to attain a tissue penetration of 1–3 cm below the surface of the IORT field. At this point two surgeons and radiotherapist placed a radiation tube for localized irradiation under sterile conditions above the primary tumor site and the abdominal aorta surroundings leaving all other organs out of the IORT field, and the IORT team exited the room during the 2- to 3-min IORT delivery time while the patients and monitors were continuously observed via closed-circuit television. Special attention was given to the duodenum, small intestine, kidney, ureter and pancreas, which are susceptible to radiation. When we judged IORT to be insufficient for the primary tumor site during radical operation, we added external beam radiation therapy (EBRT) of 20 Gy within 1 month after IORT.

The clinical feathers and outcomes were analyzed retrospectively in relation to the control of the IORT field.

Results

Patient summary (Table 2)

Between 1996 and 2005, we treated 12 patients with neuroblastoma (nine with stage 4, three with stage 3 (INSS), who had tumor resection with IORT (8–12 Gy, 10–12 MeV of electron beam) against the primary tumor site and the abdominal aorta surroundings in our hospital. The age of patients at initial diagnosis ranged from 1 to 10 years (median 5.2 years), and included six boys and six girls. The primary tumors were located in the adrenal grand in nine cases and retroperitoneum in three cases. A complete gross surgical resection was achieved in 10 of 12 patients who received IORT during a second surgical procedure. While 10 patients had complete gross tumor resection at the time of the second operation, five of these patients did not have viable tumor on histopathologic examination (patients7, 8, 9, 10, and 11).

Pathological criteria relating to Shimada classification and N-*myc* copy number was available in all of 12 patients. All three patients with stage 3 had unfavorable criteria. One of these three patients with stage 3 showed N-*myc* oncogene amplification. Six of nine patients with stage 4 had unfavorable criteria. Two of nine patients with stage 4 showed N-*myc* oncogene amplification (also with unfavorable Shimada classification). Seven patients of all 12 patients had MIBG hot lesion before IORT (Patients 1, 2, 4, 5, 6, and 12). There were three patients who did not normalize the tumor marker (such as [Vanillylmandelic acid (VMA), homovanillic acid (HVA)]), and NSE (neuron-specific enolase) after induction chemotherapy (Patients 1, 6, and 12). All of these three patients who did not normalize the tumor marker died of relapse (patients 1, 6, and 12).

There was no recurrence of disease in the IORT field in all of 12 patients with median follow-up of 44.2 months (range 17–120). At the time of IORT, seven of these 12 patients had viable tumor seen (patients 1, 2, 3, 4, 5, 6, and 12). There were three children who died of relapse in this study, and these three patients were included in the seven patients who had viable tumor at the time of IORT. There was one patient who had died of local recurrence (patient 12). She had local recurrence outside of the IORT field in the upper side of superior mesenteric artery after IORT and complicated the liver metastasis. The complication rate by IORT in this study was exceedingly low. One patient had postoperative ileus, which required treatment with ileus tube, and one patient had colitis and hydronephrosis.

Table 2 Patients summary

Patient	Sex	Age (year)	Primary site	Stage (INSS)	Extent of resection	Viable tumor in resected specimen Y or N	Shimada classification F or UF	N-*myc* oncogene copy no.	MIBG hot legion before IORT	NSE level before IORT	External Beam radiation	Complication	Survival status months A or D	FU months
1	Female	10	Adrenal	4	STR	Y	UF	1	Primary lesion	29	–	–	D	37
2	Male	9	Adrenal	4	GTR	Y	UF	1	Primary lesion	10	–	–	A	120
3	Female	9	Adrenal	3	GTR	Y	UF	1	Negative	3.7	–	–	A	65
4	Male	3	Retroperitoneum	4	GTR	Y	UF	1	Primary lesion, bone	10	–	Colitis, hydronephrosis	A	54
5	Female	7	Adrenal	4	GTR	Y	UF	1	Primary lesion	9	–	–	A	56
6	Female	3	Adrenal	4	GTR	Y	F	2–9	Primary lesion, bone	20	–	–	D	36
7	Male	5	Adrenal	4	GTR	N	F	1	Negative	10	–	Ileus	A	36
8	Female	1	Adrenal	4	GTR	N	UF	100	Negative	7.7	–	–	A	30
9	Male	2	Adrenal	4	GTR	N	UF	2–9	Negative	10	+	–	A	24
10	Male	3	Retroperitoneum	4	GTR	N	UF	150	Negative	10	+	–	A	17
11	Male	6	Retroperitoneum	3	STR	N	UF	2–9	Negative	7.6	–	–	A	33
12	Female	4	Adrenal	3	GTR	Y	UF	150	Primary lesion	15	–	–	D	22

INSS the international neuroblastoma staging system, *STR* subtotal resection, *GTR* gross total resection, *Y* yes, *N* no, *F* favorable, *UF* unfavorable, *NSE* level of neuron-specific enolase, *A* alive, *D* dead, *FU* follow-up

Although many patients received IORT to paraspinous field, no neuropathies have been seen.

The problem to secure the IORT field

At the time of the IORT, it was difficult to secure the radiation field because of the angle of the radiation cylinder in three patients (patients 9, 10, and 12). Patient 12 had a local recurrence outside of the IORT field in the upper side of superior mesenteric artery after IORT, and so we added the EBRT after the IORT to patient 9 and 10 because it was difficult to secure the IORT field. Neither of these two patients who had the EBRT after the IORT had any local recurrence.

Discussion

Intraoperative radiation therapy has been demonstrated to have an impact on local tumor control in a variety of adult cancers [16–18]. Recently IORT has been described in a cohort of pediatric patients with varying benign conditions and malignancy [5]. EBRT also has been described that it was effective in advanced neuroblastoma [19–21]. The importance of intensive local treatment for advanced neuroblastoma was emphasized in other studies [22–26]. In this study we have been able to evaluate the impact of IORT in 12 children diagnosed with advanced neuroblastoma over a 10-year period. Neuroblastoma already has several well-recognized prognostic factors such as age at diagnosis [26], amplification of the N-*myc* oncogene [27], and Shimada classification of diagnostic tissue [14, 28]. Open biopsy was used for diagnosis in all patients, and we could have data in all patients. The outcome for the patients reported in this study does relate to the histopathological characteristic at diagnosis with eight children with unfavorable Shimada classification surviving. One of the three patients who died had favorable Shimada classification. All of the three patients who had been admitted amplification of the N-*myc* oncogene had unfavorable Shimada classification, and one of them died of the local recurrence (patient 12). The prognosis of the patient who had been admitted unfavorable Shimada classification and amplification of the N-*myc* oncogene was in the bad run though it was not possible to say indiscriminately because the number of cases was little. All of the three patients who died had variable tumors at the point of IORT, though intensive chemotherapy was done. At the point of IORT, it seemed that the existence of variable tumor influenced the prognosis. Non-normalization of the tumor marker after induction chemotherapy and the existence of variable tumors at the point of IORT suggest that there is a possibility that the tumor cell exists locally and systemically and the prognosis is bad. Thinking about patient 12 who had local recurrence outside of IORT field in the upper side of superior mesenteric artery, seemed that we should add EBRT to para-aorta because it was difficult to secure the IORT field. When it was difficult to secure the IORT field and the variable tumor exists in addition to amplification of the N-*myc* oncogene, it seemed that it was necessary to add EBRT to para-aorta or supplemental chemotherapy to control the local part after IORT and it is important to improve the clinical outcome in advanced neuroblastoma. The combination IORT and EBRT appeared effective in advanced neuroblastoma for local control. Some investigators have attributed some complications of IORT and EBRT. These include episodes of intestinal bleeding, neuropathy, increased infection, and secondary neoplasms [29–33]. We did not show any of these complications in our series. We have virtually always been able to dissect the ureter off the tumor and exclude it from the IORT field. But one patient (patient 4) had hydronephrosis on both sides after the IORT.

Intraoperative radiation therapy seems to promote the local control in advanced neuroblastoma. The observation time, however, is still short and our series of patients rather small. Therefore, it is necessary to continue this study to evaluate the role of IORT in advanced neuroblastoma.

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Outcome of Early Surgery for Bilateral Congenital Cataracts in Eyes with Microcornea

SACHIKO NISHINA, EIICHIRO NODA, AND NORIYUKI AZUMA

• PURPOSE: To report the outcome of early surgery for bilateral congenital cataracts in eyes with microcornea.
• DESIGN: Interventional case series.
• METHODS: We retrospectively reviewed 22 eyes of 11 patients with microcorneas who underwent early surgery for bilateral congenital cataracts. All patients underwent lensectomy and anterior vitrectomy via the limbal approach by 12 weeks of age. The corneal diameters at the time of surgery ranged from 7.0 to 9.0 mm. The mean age at the time of surgery was 7.7 ± 3.3 weeks (range, two to 12 weeks); the follow-up period was 115 ± 58 months (range, 40 to 199 months). Aphakic eyes were corrected with spectacles or contact lenses. Visual acuities and the postoperative complications were evaluated periodically.
• RESULTS: The morphologic types of cataract were nuclear (12 eyes), complete (eight eyes), and membranous (two eyes). Other preoperative ocular abnormalities included iris hypoplasia in 10 eyes and persistent fetal vasculature in three eyes. Systemic abnormalities were found in four patients. Postoperative complications occurred in 11 eyes (50%), including glaucoma (nine eyes), exudative retinal detachment (two eyes), rhegmatogenous retinal detachment, and secondary membrane formation, in one eye each. The binocular visual acuity was 20/40 to 20/20 in six patients (55%), 20/200 to 20/100 in two patients (18%), and 2/100 or worse in three patients (27%) who developed postoperative glaucoma.
• CONCLUSION: Despite microcorneas, favorable visual outcomes were achieved after early surgery in this series. However, adequate management of postoperative complications, especially glaucoma, is required. (Am J Ophthalmol 2007;144:276–280. © 2007 by Elsevier Inc. All rights reserved.)

PROGRESS IN EARLY SURGERY HAS IMPROVED THE visual prognosis of congenital cataracts, and excellent visual acuities and stereopsis can be obtained in cases of bilateral cataracts. However, congenital cataract with a microcornea has a relatively poor prognosis attributable to associated ocular and systemic anomalies and the high rate of severe postoperative complications, including glaucoma, corneal opacity, and secondary membrane formation.[1–7] Early surgery for congenital cataracts in the presence of a microcornea is technically difficult because of the small hazy cornea, shallow anterior chamber, poor pupil dilation, and persistent tunica vasculosa lentis, and few reports have focused specifically on the surgical results in these eyes.[1] Several reports have suggested that early surgery for congenital cataract is a risk factor for postoperative complications including glaucoma.[8–10] The long-term prognosis and the most favorable time for performing cataract surgery remain unclear, especially for eyes with microcornea. We retrospectively evaluated the surgical outcomes of patients who underwent early surgery within 12 weeks of age for bilateral congenital cataracts associated with microcornea.

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METHODS

• PATIENTS: We retrospectively reviewed the records of 22 eyes of 11 patients (seven boys and four girls) who underwent surgery for bilateral congenital cataracts with microcornea within 12 weeks of age at the National Center for Child Health and Development, Tokyo, Japan, between 1990 and 2003. The corneal diameters at the time of surgery ranged from 7.0 to 9.0 mm.

• SURGICAL PROCEDURES: All patients underwent lensectomy and anterior vitrectomy via the limbal approach using a 20-gauge surgical system (MVS XII or Accurus Surgical System; Alcon, Tokyo, Japan). A 25-gauge surgical system was used with one patient (Patient 11) who had extreme microcorneas. The mean patient age at surgery was 7.7 ± 3.3 weeks (range, two to 12 weeks). Aphakic eyes were corrected with spectacles or contact lenses postoperatively.

• EYE EXAMINATIONS: Visual acuities and the development of postoperative complications were evaluated every two to three months. If amblyopia was detected during follow-up, part-time occlusion therapy was applied. The best-corrected visual acuity was measured with a standard Japanese visual acuity chart using Landolt rings or pictures at 5 m, and then converted to Snellen visual acuity. Intraocular pressure (IOP) was measured with the use of Goldmann applanation tonometry (Möller-Wedel GmbH, Wedel, Germany). During infancy and young age, the IOP

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TABLE 1. Preoperative Data and Outcome of Patients with Microcornea Undergoing Early Surgery for Bilateral Congenital Cataracts

Patient No.	Gender	Age at Surgery (weeks)	Corneal Diameter (mm) (OD, OS)	Type of Cataract (OU)	Associated Ocular/Systemic Anomalies
1	M	4	8.0, 7.5	Nuclear	Retinal degeneration (OS)/cystic kidney
2	F	12	9.0, 9.0	Nuclear	
3	F	2	8.0, 8.0	Membranous	PFV, pupillary occlusion (OS), retinal dystrophy (OU)/Smith-Lemli-Opitz syndrome
4	M	8	8.5, 8.5	Nuclear	
5	M	8	8.0, 8.0	Complete	
6	M	12	8.0, 8.0	Nuclear	
7	M	6	8.0, 8.0	Nuclear	Iris hypoplasia (OU)
8	F	5	8.0, 8.0	Complete	Iris hypoplasia, tunica vasculosa lentis (OU)
9	M	7	8.0, 8.0	Nuclear	Iris hypoplasia (OU)/developmental delay
10	F	11	9.0, 9.0	Complete	Iris hypoplasia (OU)
11	M	10	7.0, 8.0	Complete	Iris hypoplasia, retinal fold (OU)/Hallermann-Streiff syndrome

Postoperative Complications	Visual Acuity (OD, OS)	Visual Acuity (OU)	Follow-up (mos)
Glaucoma, retinal hole (OS)	20/40, 2/100	20/40	199
	20/40, 20/25	20/20	183
Glaucoma, corneal opacity (OU), rhegmatogenous RD (OS)	LP (+), LP (−)	LP (+)	174
Glaucoma (OU), secondary membrane (OS)	20/25, 20/200	20/25	168
	20/100, 20/200	20/100	120
	20/200, 20/40	20/30	103
	20/50, 20/30	20/20	87
Glaucoma, corneal opacity (OU)	LP (+), LP (+)	LP (+)	78
Glaucoma, corneal opacity (OU)	1/100, 2/100	2/100	69
	20/25, 20/30	20/25	44
Exudative RD (OU)	2/100, 20/200	20/200	40

F = female; M = male; OD = right eye; OS = left eye; OU = both eyes; PFV = persistent fetal vasculature; RD = retinal detachment; LP = light perception.

TABLE 2. Aphakic Glaucoma after Early Surgery in Eyes with Bilateral Congenital Cataracts with Microcornea

Patient No.	Gender	Eye	Age at Surgery (weeks)	Corneal Diameter (mm)	Type of Cataract	Associated Ocular Anomalies	Postoperative Time at Onset of Glaucoma (mos)	Treatment	Visual Acuity
1	M	OS	4	7.5	Nuclear	Retinal degeneration	53	Medication + trabeculotomy	2/100
3	F	OD	2	8.0	Membranous	Retinal dystrophy	1	Medication	LP (+)
		OS	2	8.0	Membranous	PFV, pupillary occlusion, retinal dystrophy	1	Medication	LP (−)
4	M	OD	8	8.5	Nuclear		100	Medication	20/25
		OS	8	8.5	Nuclear		68	Medication + trabeculotomy	20/200
8	F	OD	5	8.0	Complete	Iris hypoplasia, tunica vasculosa lentis	2	Medication	LP (+)
		OS	5	8.0	Complete	Iris hypoplasia, tunica vasculosa lentis	2	Medication + trabeculotomy	LP (+)
9	M	OD	7	8.0	Nuclear	Iris hypoplasia	14	Medication	1/100
		OS	7	8.0	Nuclear	Iris hypoplasia	5	Medication + trabeculotomy	2/100

M = male; F = female; OD = right eye; OS = left eye; PFV = persistent fetal vasculature; LP = light perception.

TABLE 3. Characteristics of Eyes With and Without Aphakic Glaucoma After Early Surgery for Bilateral Congenital Cataracts With Microcornea

Parameter	Eyes with Postoperative Glaucoma n = 9		Eyes without Postoperative Glaucoma n = 13	
Age at cataract surgery (weeks)	2–8 (mean, 5.3)*		4–12 (mean, 9.4)	
Corneal diameter (mm)	7.5–8.5 (mean, 8.1)		7.0–9.0 (mean, 8.2)	
Type of cataract	Nuclear 5, complete 2, membranous 2		Nuclear 7, complete 6	
Associated ocular anomalies	Iris hypoplasia	4	Iris hypoplasia	6
	PFV, tunica vasculosa lentis	3	PFV, tunica vasculosa lentis	0
	Retinal dystrophy, degeneration	3	Retinal fold	2
Visual acuity	20/50–20/25	1	20/50–20/25	9
	20/200–20/100	1	20/200–20/100	3
	LP–2/100	7	LP–2/100	1

PFV = persistent fetal vasculature; LP = light perception.
*P = .0014 (Welch t test).

was measured using a Perkins hand-held applanation tonometer with the patient under general anesthesia. Glaucoma was defined as an IOP exceeding 21 mm Hg and increased cupping of the optic nerve head. The mean follow-up period was 115 ± 58 months (range, 40 to 199 months).

RESULTS

THE PREOPERATIVE CHARACTERISTICS AND POSTOPERATIVE results for all patients are shown in Table 1. The corneal diameter at the time of surgery was 9.0 mm in four eyes, 8.5 mm in two eyes, 8.0 mm in 14 eyes, and 7.5 mm or smaller in two eyes. The minimum corneal diameter was 7.0 mm. The morphologic types of cataract were nuclear in 12 eyes, complete in eight eyes, and membranous cataracts in two eyes. No patient had a morphologic difference in the type of cataract between the eyes. Fundus features could not be visualized by indirect ophthalmoscopy in any eye owing to the dense lens opacity.

Other preoperative ocular abnormalities included iris hypoplasia in 10 eyes and persistent tunica vasculosa lentis in three eyes. Persistent fetal vasculature (PFV) in the anterior segment including tunica vasculosa lentis caused pupillary occlusion in one eye. Pupil dilation using mydriasis was incomplete in all eyes. Seven eyes required pupilloplasty to perform lensectomy in periphery. Preoperative ultrasonography failed to detect any posterior segment anomalies; however, one patient had bilateral retinal dystrophy postoperatively, another patient had bilateral small retinal folds between the disk and fovea, and one eye of another patient had peripheral retinal degeneration. Systemic abnormalities were found in four patients (36%), including the Smith-Lemli-Opitz syndrome, Hallermann-Streiff syndrome, developmental delay, and cystic kidney.

Intraoperative complications did not occur in any eyes, while postoperative complications occurred in 11 eyes (50%) of six patients. The complication that occurred most frequently was glaucoma, which developed in nine eyes (41%). Table 2 shows the data from patients with postoperative aphakic glaucoma. Table 3 shows the features of eyes with and without glaucoma. All nine eyes with glaucoma underwent cataract surgery within eight weeks after birth. Thus, the incidence of the postoperative glaucoma in the 14 eyes in which cataract surgery was performed within eight weeks was higher (64%) than the overall (41%). Table 3 shows a significant difference in age at cataract surgery between eyes that developed glaucoma and eyes that did not (Welch t test, P = .0014). The corneal diameters at the time of cataract surgery in the nine eyes in which glaucoma developed ranged from 7.5 to 8.5 mm. Glaucoma did not develop in eyes with a corneal diameter of 9.0 mm in this series. Seven (78%) of the nine eyes with aphakic glaucoma had associated ocular anomalies besides microcorneas, including iris hypoplasia, PFV, and retinal dystrophy. All eyes had open angles, except for partial peripheral synechia at the incision site of cataract surgery by gonioscopy. The period between cataract surgery and the onset of glaucoma varied from one month to eight years. Four (44%) eyes of two patients (Patients 3 and 8) developed glaucoma within two months after cataract surgery; severe corneal opacity and deep amblyopia developed that resulted in poor visual prognosis. Persistent fetal vasculature was present in three of these four eyes. Glaucoma was controlled with medication alone in five (56%) eyes, while four (44%) eyes required trabeculotomy. At the final examination, the IOP was well controlled in all eyes; however, corneal opacity remained in six eyes (67%) of three patients (Patients 3, 8, and 9).

Other postoperative complications included exudative retinal detachment in two eyes (9%) of one patient, and a secondary membrane formation, a rhegmatogenous retinal detachment, and a retinal hole in one eye each (4.5%). An exudative retinal detachment developed five months after

lensectomy in both eyes of a patient with the Hallermann-Streiff syndrome. The detachments, which may have been related to the uveal flow of microphthalmos, spontaneously regressed in the left eye; however, the right eye required subscleral sclerectomy. The secondary membrane that formed two months after lensectomy by proliferation of residual lens epithelial cells in an eye with poor pupil dilation was removed during a second operation. The rhegmatogenous retinal detachment that occurred at three years of age in the patient with the Smith-Lemli-Opitz syndrome, who also was mentally challenged with a propensity for self-mutilation, was successfully treated with cryopexy and scleral buckle encircling. A hole in the peripheral retinal degeneration was found when the patient was seven years of age and photocoagulation was applied.

The final best-corrected visual acuity results are shown in Table 1. The patient ages at the final examination ranged from three to 16 years (mean, 10 years). All but one patient (Patient 3) showed latent or manifest-latent nystagmus. The best-corrected visual acuity measured in each eye was 20/50 to 20/25 in nine eyes (41%), 20/200 to 20/100 in five eyes (23%), and 2/100 or worse in eight eyes (36%). The binocular best-corrected visual acuity, which was better than the monocular best-corrected visual acuity in patients with latent or manifest-latent nystagmus, was 20/40 to 20/20 in six patients (55%), 20/200 to 20/100 in two patients (18%), and 2/100 or worse in three patients (27%). In the eight eyes with poor visual outcomes, four eyes of two patients (Patients 3 and 8) developed postoperative glaucoma within two months after cataract surgery, persistent corneal opacity and deep amblyopia, and light perception vision. Another three eyes also developed glaucoma and amblyopia, and one eye had retinal fold and developed exudative retinal detachment. Thus, seven (78%) of the nine eyes with postoperative aphakic glaucoma had a poor visual outcome of 2/100 or worse, although the IOP was controlled in all eyes at the final examination.

DISCUSSION

IN THE PRESENT SERIES, THERE WERE NO INTRAOPERATIVE complications in any eyes; however, several postoperative complications occurred in 50% of eyes. Glaucoma is the most frequent postoperative complication. The overall prevalence of glaucoma in our series was 41% during the average follow-up period of nine years, seven months.

Glaucoma develops even in eyes without a microcornea after congenital cataract surgery. The prevalence of aphakic glaucoma has been reported to vary from 0% to 32%,[3,5,8,11–13] which increased with longer follow-up. A recent large series of aphakic glaucoma revealed that modern lensectomy/vitrectomy techniques could not prevent the development of later-onset open-angle glaucoma.[12] Several risk factors have been implicated, such as microcornea, early surgery, cataract type, retained lens material, aphakia, and associated ocular anomalies.[5,7–10,12]

The extremely early cataract surgery in eyes with microcornea and associated anomalies may be a risk factor for the development of postoperative glaucoma.[10] In the current study, the prevalence was much higher; 64% of 14 eyes in which cataract surgery was performed within eight weeks. Seven (78%) of the nine glaucomatous eyes had preoperative associated ocular anomalies including iris hypoplasia and PFV. In these complicated eyes, the period between cataract surgery and the onset of glaucoma was shorter than in eyes without ocular anomalies. In three eyes with PFV, glaucoma developed within two months after cataract surgery and resulted in the worst visual outcome. These results suggest that the eyes with microcornea also have some degree of anterior segment dysgenesis, including malformation of the trabecular meshwork, the Schlemm canal, or both, even though all glaucomatous eyes appeared to have open angles by gonioscopy. Persistent fetal vasculature and iris hypoplasia indicate the apparent immaturity or dysgenesis of the anterior segment. Early-onset glaucoma may be triggered by extremely early surgical intervention and postoperative inflammation, especially in eyes with microcornea and PFV. In our series, all eyes had incomplete or poor pupil dilation, and pupilloplasty was needed in 32%. In these eyes, retained lens material may be a risk factor for the development of postoperative glaucoma.

The incidences of other serious postoperative complications such as development of retinal detachment and secondary membrane[13] decreased as the result of modern lensectomy/vitrectomy technique. To avoid these complications, we performed lensectomy/vitrectomy via the limbal approach; however, one eye with poor pupil dilation required reoperation for secondary membrane.

The final visual acuity results were favorable in eyes without postoperative early-onset glaucoma. Overall, 73% patients obtained binocular visual acuity exceeding 20/200, and 55% of patients obtained binocular visual acuity exceeding 20/40. These favorable results depend on early surgical intervention to prevent deep deprivation amblyopia, postoperative optical correction, and successful prevention of intraoperative and postoperative complications. However, extremely early surgery for the eyes with microcornea and associated anomalies, especially PFV, may result in the worst prognosis because of the early development of glaucoma and corneal opacity. In these eyes with microcornea and PFV, it may be better to perform cataract surgery after eight weeks of age in order to avoid the marked inflammation caused by surgical intervention.

Our study indicated that good visual prognosis can be obtained in bilateral congenital cataracts with microcornea after early surgery without postoperative early-onset glaucoma. To prevent corneal opacity and deep amblyopia, adequate management of postoperative glaucoma including periodic examinations with the patient under general anesthesia may be required in eyes with microcornea.

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第35回日本小児神経外科学会 教育講演

小児脳腫瘍に伴う内分泌障害の診断と治療

横谷 進[1]

小児脳腫瘍では，頭蓋咽頭腫，胚細胞腫をはじめとして，視床下部下垂体近傍に好発する腫瘍が比較的多く，また，髄芽腫など離れた部位の腫瘍であっても放射線治療が行われた場合にも，下垂体機能異常が起こり得る．しかも，こうした内分泌障害は，正常な成長・性発達の経過を巻き込んで発症するため，診断上も治療上も成人の内分泌障害とは異なった知識と対応が必要である [1].

ここでは，小児脳神経外科医が脳腫瘍を診療する上で知っておくべき事項について述べる．

術前・術後の内分泌学的評価

視床下部下垂体近傍の腫瘍では，診断前に既に成長ホルモン（GH）分泌不全による成長速度の低下や中枢性尿崩症を合併していることも多い．手術療法などの腫瘍学的（oncologic）治療を安全に行うためにも，また，治療前後の比較のためにも，術前からの内分泌学的評価が必須である．

ホルモン基礎値としては，遊離サイロキシン（FT_4），インスリン様成長因子 I（IGF-I），コルチゾール（朝の採血，および可能なら24時間蓄尿）が有用である．下垂体前葉負荷試験では，LHRH と TRH の2者を同時負荷して，ゴナドトロピン（黄体化ホルモン LH と卵胞刺激ホルモン FSH）と甲状腺刺激ホルモン（TSH）とプロラクチン（PRL）とを測定する．GH と副腎皮質刺激ホルモン ACTH を評価するために，上記の2者にインスリンを加えた3者負荷もしばしば行われる．しかし，インスリン負荷は十分な監視が必要で，患児にも負担がかかることから，インスリンの代わりに GHRH と CRH を追加する4者負荷も行われる．ただし，GHRH 負荷では，視床下部障害による GH 分泌低下の場合に低下反応がみられないことがある．GHRH の代わりに GHRP-2 を使用すると，視床下部障害による GH 分泌低下も検出できると考えられるが，GHRP-2 負荷試験での小児におけるカットオフは定められていない（経験的には，GHRP-2 への GH の反応は他の負荷試験よりも4～5倍高い）．下垂体後葉ホルモン（抗利尿ホルモン ADH）については，多飲多尿がなく副腎機能不全がなければ，早朝尿で十分な尿濃縮（通常は，尿比重 1.022 以上，尿浸透圧 800 mOsm/kg 以上）を確認することで足りる．尿崩症の疑いがあれば，水制限試験を過剰にならない負荷時間で行い診断する．

術中術後の急性期管理 [2]

術前の評価で ACTH や TSH の分泌不全が認められれば，後述の維持量でヒドロコルチゾン（コートリル®）やレボチロキシンナトリウム（チラーヂン S®）を開始しておく．チラーヂン S® は，術後2～3日間の休薬は支障がない．GH を急性期に補充すべき必要性は指摘されていない．

手術当日のステロイドカバーには，ACTH 分泌不全のある場合は，当日朝に維持量（後述するが，ここでは 12 mg/m²）の3倍ほどのコハク酸ヒドロコルチゾンナトリウム（ソルコーテフ®）などを静注する．手術当日のうちに，術中から術後に維持量の10倍ほどのソルコーテフ®などを2回投与する．ACTH 分泌不全が術後に初めて合併する危険があれば，その場合にも術中または直後より同様の補充を行う．患児の術後の全身状態にもよるが，翌日・翌々日は維持量の10倍，3～7日目は5倍，8日目より2倍，数週後より維持量の補充を基本として投与する．経口が可能になった時点で，同量のコートリル®内服に置き換えてよい．

尿崩症を伴う場合の水電解質管理はしばしば困難を伴う．術前から尿崩症を合併している場合には，手術当日の朝に，夕方まで有効なはずの量の DDAVP（デスモプレシン®）を点鼻する．通常の輸液をしながら，術中・術後に多尿（尿崩症の診断基準に用いられる尿量 3,000 ml/m²/day に相当する 125 ml/m²/hr を超える尿量が目安になる）を呈した時点から，筆者らはアルギニン・バゾプレシン（ピトレシン®）0.3 mU/kg/hr で持続点滴静注を開始している．この量で大部分の症例で尿量が適正化するが，効果が不十分な場合には，ピトレシン® 投与速度を 1 hr

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ごとに増加する．この方法では，血中アルギニン・バゾプレシンは生理的血中濃度までの上昇で働いている．通常量の皮下注・筋注よりも投与量が極めて少ないので，ピトレシン® は 10,000 倍オーダでの希釈が必要である．生理食塩水を用いて 100 ～ 200 倍ずつの 2 段階で正確に希釈し，投与速度が調節できるように側管からシリンジポンプで投与する．尿量の目安は，通常に設定した輸液量から不感蒸泄を減じ代謝水を加えた量がゼロバランスのための尿量であるので，その 0.7 ～ 2 倍の範囲に入るようにピトレシン® の投与速度を調節する．もし多尿による負バランスが生じた場合には，5% ブドウ糖の追加輸液により，毎 2 hr 遅れで補充する．この方法により，水分バランスについては安定的に理論通りにコントロールすることが可能である．ただし，この方法による治療中に低ナトリウム血症を来すことが多く，筆者らの例では，中枢性塩喪失症候群であることが確認されている．喪失したナトリウムを，4 hr ごとに補充して待つことにより，1 ～ 4 日で自然に回復する．状態が安定してデスモプレシンの点鼻が可能になった時点で，デスモプレシン点鼻による通常の補充療法（後述）に移行する．

長期フォローアップにおける内分泌障害への対応

内分泌障害を予測する

腫瘍そのものや腫瘍に対する手術療法が下垂体機能異常を起こすかどうかは，腫瘍の部位や術式によってある程度予想できる．また，頭部放射線照射も，主に線量によって，下垂体機能異常の合併の有無や時期を予想できる．

下垂体前葉ホルモンのうちで，原因によらずにいちばん障害されやすいのは GH である．放射線照射では，小児には 7 ～ 15 Gy でも GH 分泌不全が起こることがあり，30 ～ 50 Gy では，5 年後までに 50 ～ 100%，10 年後までに 100%GH 分泌不全が起こるといわれている．線量が少ないと発症が年の単位で遅くなり，また，部分的な低下から始まって完全な分泌不全に進行したり，部分的な分泌低下に留まったりすることもある．したがって，長期のフォローアップと，再評価の反復が必要である．

ゴナドトロピンの分泌は，視床下部からのゴナドトロピン放出ホルモン GnRH により促進される．思春期前は GnRH の脈動的分泌の振幅は小さくコントロールされているが，思春期にはそれが増幅して，それによりゴナドトロピン分泌が増加する．視床下部への傷害の結果（腫瘍による圧迫にしても，放射線療法にしても），GnRH の分泌が，思春期前にあるべき抑制的コントロールから逸脱して大きくなることにより，ゴナドトロピン分泌が増加し，思春期早発症が引き起こされることがある．放射線照射では，30 ～ 50 Gy で（女子では 18 Gy 以上でも）思春期早発症が起こることが珍しくない．それ以上の線量では，むしろ性腺機能不全が起こりやすいが，思春期早発症の後にゴナドトロピン分泌が低下して，最終的に性腺機能低下症になることもある．

TSH と ACTH は，30 ～ 50 Gy でときどき分泌低下が起こるが，10 年後までには半数程度の症例で分泌が低下すると考えられる．

プロラクチンは，視床下部からの抑制が解除されると高プロラクチン血症を来すが，臨床的に問題になる（治療が必要になる）ことはほとんどない．

ADH は，頭蓋咽頭腫や胚細胞腫でしばしば分泌が低下して術前から尿崩症を来すが，視床下部・下垂体茎に侵襲が及ぶ手術でも術後に尿崩症の合併は多い．術後の尿崩症は一過性のことがあるが，6 カ月以上持続したものは回復しがたい．放射線療法やトルコ鞍内に留まる経蝶形骨洞手術では，尿崩症は起こりにくい．

内分泌障害の早期発見

内分泌障害の合併は，前述のようにだいたい予測可能である．その予測に基づいて，起こり得る内分泌障害を，長期フォローアップの中で積極的に早期発見する努力としくみが必要である．

GH 分泌不全は，それが始まったときから成長速度が低下するので，成長曲線を標準成長曲線に記入していけば，成長曲線が横に寝てくることにより早期発見できる．例外として，頭蓋咽頭腫の術後では，約 20% の症例に GH 分泌不全にもかかわらず成長速度が低下しないことがある（growth without GH）．GH 分泌不全では，IGF-I が低値であり GH 分泌刺激試験により確定診断できる．TSH 分泌不全による甲状腺機能低下症でも成長速度が低下するが，年 1 ～ 2 回の定期的な FT_4 の測定を行っていれば，その低下により，先に気づかれる．

思春期早発症は，二次性徴が通常より早く出現するので，フォローアップにおいては二次性徴に注目していることが大切である．女子では，7 歳 6 カ月以前の乳房発達（乳輪の隆起の始まり），10 歳 6 カ月以前の初経，男子では，9 歳 0 カ月以前の精巣の腫大（4 ml 以上），10 歳未満の陰毛発生などが診断の基準に挙げられている．性ホルモン（特にエストロゲン）は骨の成長をもたらすので，思春期早発症では成長スパートを伴うことが多く，成長曲線が早い年齢で立ち上がることも重要な手がかりである．確定診断には，LHRH 試験での LH の高い反応（頂値が 10 mIU/ml を超える）と性ホルモン（エストラジオールおよびテストステロン）の高値が，必要である．

性腺機能不全は，女子で 13 歳以上，男子で 14 歳以上で，二次性徴が発現しないか進行が停止することにより疑われる．思春期年齢でも成長曲線に成長スパートが認められない．LHRH 試験では低反応ないし思春期前の反応を

示す．

ACTH分泌不全による慢性副腎皮質機能不全では，活力がないなどの症状から疑うことができるが，必ずしも明らかでないことがある．慢性副腎機能不全を合併するリスクのある患児では，症状がなくても年1回程度の朝のコルチゾール測定が勧められる．確定診断は，24 hr尿中コルチゾール，CRH試験でのACTH・コルチゾールの低反応などによるが，判定が困難なこともある．ボーダーラインの低下が認められるときは，間を詰めて再検査を行い，また，身体的ストレス時（上気道炎，下痢など）には急性副腎不全を起こす可能性があるので，後述のように対応の準備が必要である．

内分泌障害の治療 [2, 3]

GH分泌不全では，成人身長が低くならないようにGH治療を開始するのが原則である．脳腫瘍後のGH治療に関する大規模な疫学調査では，GH治療は脳腫瘍の再発を促進しないと報告されている．通常は，脳腫瘍に対する治療後に2年（少なくとも1年）の経過で再発がなければGH治療を検討する．理論的には，それより短い期間でGH治療を開始してもよいが，もともと腫瘍の再発の多い時期をはずした方が，GH治療が中断されず継続しやすい．

思春期早発症では，二次性徴の進行が本人の人格成熟の上で，または社会生活上に早すぎるか，あるいは，無治療の場合に成人身長が著しく低くなると予測される場合に，LHRHアナログ製剤である酢酸リュープロレリン（リューブリン®）の4週間ごとの注射により治療する．

性腺機能不全では，二次性徴の発現・進行・完成を目標に治療する．女子では，乳房の発育が10歳で開始するのが標準なので，あまり遅れずに12歳の頃には治療が開始できるようにする．通常は，経口薬で結合型エストロゲン（プレマリン®0.625 mg/錠）を1/10錠，1/5錠，1/2錠，1錠と，6～12カ月ごとに増量する方法が勧められる．これにより，正常児と同様の約2年間で二次性徴が完成し，また，この間に骨成熟が早く進みすぎる心配がない（骨成熟が早く進むと，低い成人身長をもたらしやすい）．エストロゲン製剤は経口薬の場合に，肝の一次通過の際に非生理的なエストロゲン作用が肝に働いて，凝固因子の増加を招くなどの好ましくないことも起こすので，その点では経皮吸収剤の方が優れている．エストラジオール経皮吸収剤（エストラダームM® 0.72 mg/枚）では，1/8枚，1/4枚，1/2枚，1枚（いずれも隔日貼り替え）というように，6～12カ月ごとに増量する方法が良いと考えられる．いずれの方法でも月経がみられるか，または1錠ないし1枚で数カ月が経過したら，黄体ホルモン製剤（プロベラ錠®，ヒスロン錠®など）を併用して周期的に服用する，いわゆるHRTに移行する．これにより月経が周期的に起こるが，排卵を伴わないので，生殖を目的とする治療にはゴナドトロピン療法など別の方法が必要である．

男子の性腺機能低下症では，二次性徴の開始の標準が11歳なので，あまり遅れずに13歳の頃には治療が開始できるようにする．日本では，注射製剤以外に適切なテストステロン製剤が承認されていないので，エナント酸テストステロン（エナルモン・デポー®）を50～75 mg筋注4週ごと（6～12カ月間），引き続いて125 mg 3～4週ごと（6～24カ月間），更に，成人と同じ維持療法125 mg 2～3週ごと，または250 mg 3～4週ごとに移行する．これにより，外陰部は成熟し，勃起・射精の能力も完成する．しかし，ゴナドトロピン分泌能が残っていない限り，精巣は大きくならず精子形成も望めないので，生殖能力の獲得のためにはゴナドトロピン療法などの導入が必要である．リコンビナントFSH製剤（ゴナールエフ®）とhCG製剤（プロファシー®）の組合せによるゴナドトロピン療法が，2006年に初めて男子の性腺機能低下症に承認されて在宅自己注射（皮下注）が可能になったので，二次性徴の発現から生殖能力の獲得までを目標に，初めからそれらを用いる方法も選択できるようになった．

甲状腺機能低下症では，維持療法におけるチラーヂンS®の補充量の目安を表1に示した．部分的な低下ではこれより少量で十分なことが多い．適切な用量の決定は，血中FT_4が正常範囲で安定していることを目標に行う．TSHは分泌不全のためにコントロールの指標にならない．

慢性副腎皮質機能不全では，表2のようにコートリル®の維持量は12 mg/m²/dayを標準として，分2で朝：昼または朝：夕を2：1に分割して服用する．用量が多すぎる

表1　甲状腺ホルモンの補充（維持療法）

チラーヂンS	μg/kg/day　分1
乳児	6～10
幼児	5～7
6～12歳	3～5
13～18歳	2～4

FT_4を指標に用量調節

表2　副腎皮質ホルモン補充

維持療法
　コートリル　12 mg/m²/day
　　分2（朝2/3，昼or夕1/3）
　　活力を主な指標に，過量を避ける

身体的ストレス時
　発熱・下痢・抜歯などでは，3倍量の服用を指導しておく