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Chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor-β1 mRNA ameliorates renal injury in hypertensive rats

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Objective **Transforming growth factor (TGF)-β is a critical factor in the progression of renal injury, regardless of the primary etiology. Such injury is characterized by glomerular sclerosis and tubulointerstitial fibrosis. To develop a ribozyme-based therapy for progressive renal diseases, we examined the effects of chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA on glomerulosclerosis in salt-loaded, stroke-prone spontaneously hypertensive rats (SHR-SP) and salt-sensitive Dahl (Dahl-S) rats.**

Methods **The chimeric DNA–RNA ribozyme to TGF-β1 was delivered by polyethylenimine to cultured mesangial cells from SHR-SP *in vitro* and to glomeruli in SHR-SP *in vivo*. The chimeric ribozyme reduced expression of TGF-β1 mRNA and protein, which was accompanied by inhibition of expression of extracellular matrix molecules such as fibronectin and collagen type I in mesangial cells from SHR-SP *in vitro*.**

Results **One intraperitoneal injection of 200 μg of chimeric DNA–RNA ribozyme to TGF-β1 *in vivo* markedly ameliorated thickening of capillary artery walls and glomerulosclerosis in salt-loaded SHR-SP and Dahl-S rats without a reduction in blood pressure. The chimeric ribozyme reduced expression of TGF-β1 and connective tissue growth factor (CTGF) mRNAs in renal cortex in salt-loaded Dahl-S rats. Chimeric ribozyme to TGF-β1 significantly reduced levels of protein in urine in the Dahl-S rats.**

Conclusion **These results suggest that chimeric DNA–RNA ribozyme to TGF-β1 may be useful as a gene therapy for progressive tissue injury in a wide variety of renal diseases, including hypertensive nephrosclerosis. *J Hypertens* 25:671–678 © 2007 Lippincott Williams & Wilkins.**

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Keywords: gene therapy, glomerulosclerosis, hypertension, ribozyme, transforming growth factor-β1

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Introduction

A number of studies have identified transforming growth factor (TGF)-β as a critical factor in kidney diseases such as glomerulosclerosis [1] and mesangioproliferative glomerulonephritis [2,3]. TGF-β mRNA and protein are expressed strongly in various animal models of progressive renal diseases such as hypertensive renal sclerosis, diabetic nephropathy and focal renal sclerosis [4–6].

Because there are still no effective treatments for progressive renal diseases, gene therapy is now being considered as a strategy. Ribozymes are RNA molecules that catalytically cleave a phosphodiester bond in the appropriate target RNAs in a sequence-specific manner, thereby inhibiting the expression of specific gene products. Ribozymes have progressed from being objects of scientific study to potential therapeutic agents for treatment of both acquired and inherited diseases [7]. We have demonstrated that ribozyme to TGF-β1 inhibits growth of vascular smooth muscle cells *in vitro* [8,9] and neointima formation of arteries after angioplasty *in vivo* [10]. However, ribozyme therapy for progressive renal diseases has not yet been reported.

Experimental evidence supports the notion that the pathogenesis of glomerulosclerosis with nephrosclerosis involves glomerular hypertension, mesangial dysfunction, such as mesangiolysis, and increased mesangial matrix [11]. Glomerular hypertension results from systemic hypertension and leads to increased glomerular capillary pressure with subsequent glomerular endothelial damage and sclerosis [12,13]. Stroke-prone spontaneously hypertensive rats (SHR-SP) show severe cardiovascular organ damage such as stroke, ventricular hypertrophy and glomerulosclerosis associated with TGF-β [5]. Salt-sensitive Dahl (Dahl-S) rats also show

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glomerulosclerosis, which is known to be associated with TGF-β [4].

In the current study, to develop ribozyme therapy for progressive renal diseases, we examined the in-vivo effects of chimeric DNA–RNA hammerhead ribozyme targeting TGF-β1 mRNA on glomerulosclerosis in SHR-SP and Dahl-S rats.

Methods

Our investigation conformed to standards of the *Guide for the Care and Use of Laboratory Animals* [14].

Synthetic chimeric DNA–RNA hammerhead ribozyme

The 38-base chimeric DNA–RNA hammerhead ribozyme, which contained deoxyribonucleotides instead of ribonucleotides at noncatalytic residues and two phosphorothioate linkages at the 3′ terminus, was designed to cleave the GUC sequence in the rat TGF-β1 mRNA [7]. The TGF-β1 specific ribozyme and a mismatch ribozyme with a single base change in the catalytic loop region were synthesized with the use of a DNA/RNA synthesizer (Model 394; Applied Biosystems Inc., Foster City, California, USA).

Isolation of glomeruli and culture of mesangial cells

Glomeruli were isolated from the kidneys of 4-week-old male SHR-SP/Izumo rats (SHR Corporation, Funabashi, Chiba, Japan). Glomeruli were isolated with a graded-sieve technique as described previously [15]. The renal cortex was excised and minced into small pieces under sterile conditions. The minced cortex was pressed through a 200-μm sieve and suspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, New York, USA). The suspension was passed through a 120-μm sieve, and glomeruli were collected on the surface of the sieve and resuspended in RPMI 1640. Mesangial cells were isolated from explants of whole glomeruli according to the differential growth capacities of glomerular epithelial and mesangial cells [15].

Delivery of chimeric DNA–RNA ribozyme to TGF-β1 *in vitro* and *in vivo*

For in-vitro delivery, mesangial cells were transfected with 1.0 μmol/l fluorescein isothiocyanate (FITC)-labeled chimeric DNA–RNA ribozyme to TGF-β1 with polyethylenimine for 120 min. After 24 h, cells were fixed with acetone at 4°C for 10 min and examined via fluorescence microscopy. Total intracellular FITC signal, as well as distribution of signal to the cytoplasmic or nuclear compartment, were noted.

For in-vivo delivery, 500 μg/kg of FITC-labeled chimeric DNA–RNA ribozyme to TGF-β1 in polyethylenimine was injected into SHR-SP subcutaneously, intraperitoneally or intravenously. After 24 h, kidneys were removed from SHR-SP and fixed in 10% neutral buffered formalin in a dark environment and embedded in paraffin for light microscopy with eriochrome black T stain.

Reverse transcription–polymerase chain reaction

mRNA was extracted from cultured mesangial cells of SHR-SP and renal cortex from Dahl-S rats loaded with 1% salt water. Aliquots of mRNA were reverse transcribed into single-stranded cDNA by incubation with avian myeloblastoma virus reverse transcriptase (Takara Biochemicals, Otsu, Shiga, Japan). Diluted cDNA products were then subjected to polymerase chain reaction (PCR). The primers used for amplification were as follows:

(1) TGF-β1: forward 5′-GCCCTGGATACCAACTAC TGCT-3′, reverse 5′-AGGCTCCAAATGTAGGG GCAGG-3′, product 161 base-pair (bp);
(2) fibronectin: forward 5′-TGCCACTGTTCTCCTA CGTG-3′, reverse 5′-ATGCTTTGACCCTTACA CGG-3′, product 312 bp;
(3) collagen type I: forward 5′-GGTGCTAGATCAG GAGCAGG-3′, reverse 5′-ATGCCCACTCCCTAA CAGTG-3′, product 182 bp; and
(4) connective tissue growth factor (CTGF): forward 5′-ATCCCTGCGACCCACACAAG-3′, reverse 5′-TT ACGTCTGGCGTCGAAGGC-3′, product 145 bp;
(5) human 18S ribosomal RNA was used as an internal control, the primers were as follows: forward 5′-TCAAGAACGAAAGTCGGAGG-3′, reverse 5′-GG ACATCTAAGGGCATCACA-3′, product 312 bp.

PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA), and products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualized by ultraviolet illumination.

Western blot analysis

Mesangial cells of SHR-SP (10^5 cells/cm^2) and renal cortex from Dahl-S rats loaded with 1% salt were disrupted with lysis buffer [50 mmol/lTris–HCl (pH8.0), 150 mmol/l NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100]. Total proteins were extracted and purified with 100 μl of chloroform and 400 μl of methanol. Protein samples were boiled for 3 min and subjected to electrophoresis on 8% polyacrylamide gels and then transblotted to nitrocellulose membranes (BioRad Laboratories, Hercules, California, USA). Blots were incubated with rabbit pan-specific polyclonal antibody for TGF-β (R & D Systems, Minneapolis, Minnesota, USA) or mouse monoclonal antibody specific for α-tubulin as a control (Sigma BioScience, St. Louis, Missouri, USA), diluted 1:500 in 5% nonfat milk in TBST solution (10 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, and 0.05% Tween 20) for 3 h at room temperature. The membrane was incubated with goat anti-mouse IgG for 1 h at room temperature, then washed with TBST once for 15 min,

and four times for 5 min. Immune complexes on the membrane were detected by the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Buckinghamshire, UK).

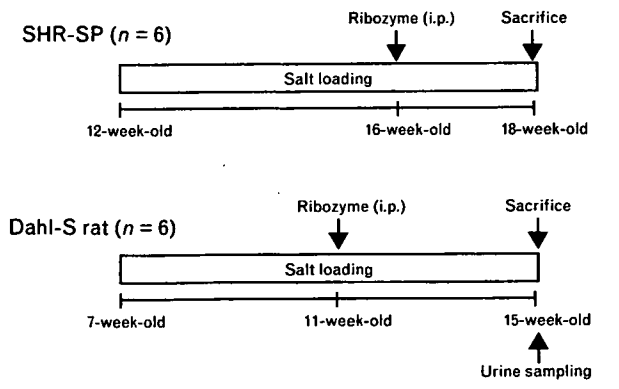
Experimental design

Twelve-week-old male SHR-SP or 7-week-old male Dahl-S rats of the Iwai substrain (Seac Yoshitomi Ltd, Fukuoka, Japan) were loaded with 1% salt water for 6 or 8 weeks, respectively. After 4 weeks of loading with salt water, the control group received an intraperitoneal injection of 1.5 ml of saline. The mismatch or ribozyme group received an intraperitoneal injection of mismatch ribozyme or chimeric DNA–RNA ribozyme to TGF-β1 (200 μg/body weight) with polyethylenimine (Fig. 1). Systolic blood pressure (SBP) was measured by the tail-cuff method.

Histological examination

Kidneys removed from SHR-SP or Dahl-S rats were fixed in 10% neutral buffered formalin and embedded in paraffin for light microscopic study. Sections (2 μm thick) were stained with Masson trichrome (MT) stain or hematoxylin–eosin (HE). Histological examination was done by a pathologist without any prior knowledge of the experimental groups. To semiquantify the glomerular matrix, 50 glomeruli were selected randomly. The percentage of each glomerulus occupied by mesangial matrix was estimated and given a score of 0, normal; 1, involvement of up to 25% of the glomerulus; 2, involvement of 25–50% of the glomerulus; 3, involvement of 50–75% of the glomerulus; or 4, involvement of 75–100% of the glomerulus. The glomerular injury score (GIS) was obtained by the following formula: $[(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/50$. To semiquantify the tubulointerstitial area, 20 areas of renal cortex were selected randomly. The percentage of each area that showed sclerofibrotic change was estimated and assigned a score of 0, normal; 1, involvement of less than 10% of the area; 2, involvement of 10–30% of the area; 3, involvement of 30–50% of the area; or 4, involvement of more than 50% of the area. The tubulointerstitial injury score (TIS) was calculated as $[(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/20$.

Fig. 1



Experimental protocols for treatment of salt-loaded stroke-prone spontaneously hypertensive rats (SHR-SP) and salt-sensitive Dahl (Dahl-S) rats with chimeric DNA–RNA ribozyme targeting transforming growth factor (TGF)-β1 mRNA.

Statistical analysis

Results are given as the mean ± SEM. The significance of differences between mean values was evaluated by Student's *t*-test for unpaired data and by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

Results

Delivery of chimeric DNA–RNA ribozyme to TGF-β1

FITC-labeled chimeric ribozyme was observed in the cytosol of cultured mesangial cells *in vitro*, and the transfection efficiency was approximately 20% (Fig. 2b).

FITC-labeled chimeric ribozyme was clearly visible in glomeruli from SHR-SP 24 h after subcutaneous, intraperitoneal or intravenous administration (Fig. 2c, e, f), but not visible in glomeruli 72 h after subcutaneous administration (Fig. 2d). FITC-labeled chimeric ribozyme was also visible in capillary arteries 24 h after subcutaneous administration (Fig. 2c). Intravenous and intraperitoneal administration were more effective than subcutaneous administration for delivery of the ribozyme.

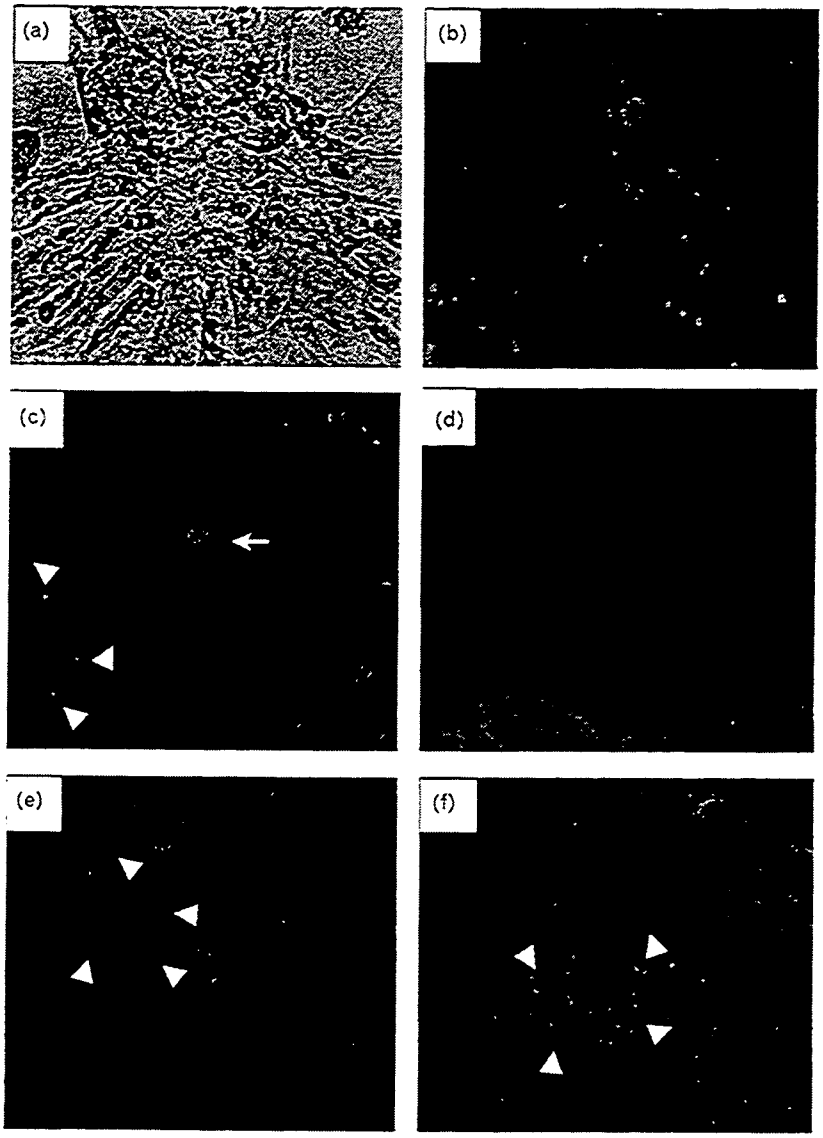
Effects of chimeric DNA–RNA ribozyme to TGF-β1 on expression of TGF-β1, fibronectin, and collagen type I mRNAs and TGF-β1 protein in mesangial cells from SHR-SP

Levels of TGF-β1 mRNA were lower with chimeric DNA–RNA ribozyme to TGF-β1 (0.1 and 1.0 μmol/l) than with mismatch ribozyme in mesangial cells from SHR-SP. This reduction was dose dependent (Fig. 3a). Chimeric DNA–RNA ribozyme to TGF-β1 (1.0 μmol/l) also reduced levels of TGF-β1 protein (Fig. 3b). Ribozyme to TGF-β1 decreased levels of fibronectin mRNA at a concentration of 1.0 μmol/l and collagen type I mRNA at concentrations of 0.1 and 1.0 μmol/l in comparison to expression levels with mismatch ribozyme (Fig. 3a).

Effects of chimeric DNA–RNA ribozyme to TGF-β1 on expression of TGF-β1 and CTGF mRNAs and TGF-β1 protein in renal cortex from Dahl-S rats

Intraperitoneal administration of 200 μg of chimeric DNA–RNA ribozyme to TGF-β1 markedly reduced expression of TGF-β1 and CTGF mRNAs and TGF-β1 protein in renal cortex from salt-loaded Dahl-S rats (Fig. 4).

Fig. 2



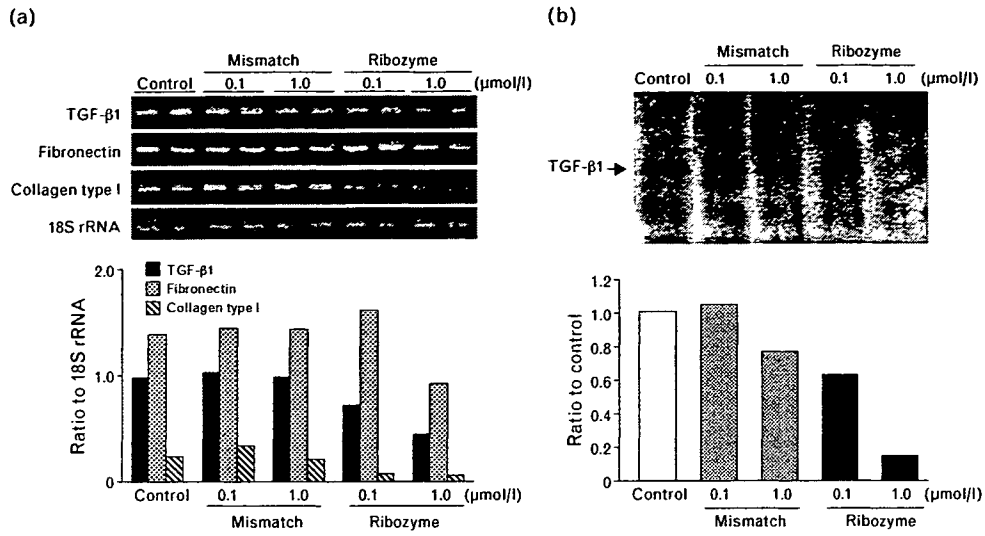
Delivery of chimeric DNA–RNA ribozyme to transforming growth factor (TGF)-β1 *in vitro* and *in vivo*. For in-vitro delivery, mesangial cells were transfected with 1.0 μmol/l fluorescein isothiocyanate (FITC)-labeled chimeric ribozyme with polyethylenimine for 120 min. Cells were fixed with acetone at 4°C for 10 min and examined via fluorescent microscopy without (a) or with (b) ultraviolet light. For in-vivo delivery, 200 μg of FITC-labeled chimeric DNA–RNA ribozyme to TGF-β1 polyethylenimine was injected into stroke-prone spontaneously hypertensive rats (SHR-SP) subcutaneously (c), intraperitoneally (d,e), or intravenously (f). After 24 h (c, e, f) or 72 h (d), kidneys were removed, fixed in 10% neutral-buffered formalin in the dark, and embedded in paraffin for light microscopy with eriochrome black T stain. Large arrows indicate the localization of labeled ribozyme in glomeruli and small arrows indicate capillary arteries.

Effects of chimeric DNA–RNA ribozyme to TGF-β1 on blood pressure and nephrosclerosis in SHR-SP and Dahl-S rats

The renal cortex of salt-loaded SHR-SP and of salt-loaded Dahl-S rats showed marked thickening of capillary artery walls and severe glomerulosclerosis with glomerular ischemia due to obstruction of the afferent artery. Intraperitoneal administration of 200 μg of chimeric DNA–RNA ribozyme to TGF-β1 or mismatch ribozyme had no effect on blood pressure in salt-loaded SHR-SP and Dahl-S rats with salt-loading (Table 1). Intraperitoneal administration of chimeric DNA–RNA ribozyme to TGF-β1 markedly ameliorated thickening of the capillary artery wall and glomerulosclerosis in kidneys of salt-loaded SHR-SP and Dahl-S rats. Administration of mismatch ribozyme had no effect on thickening or glomerulosclerosis (Fig. 5).

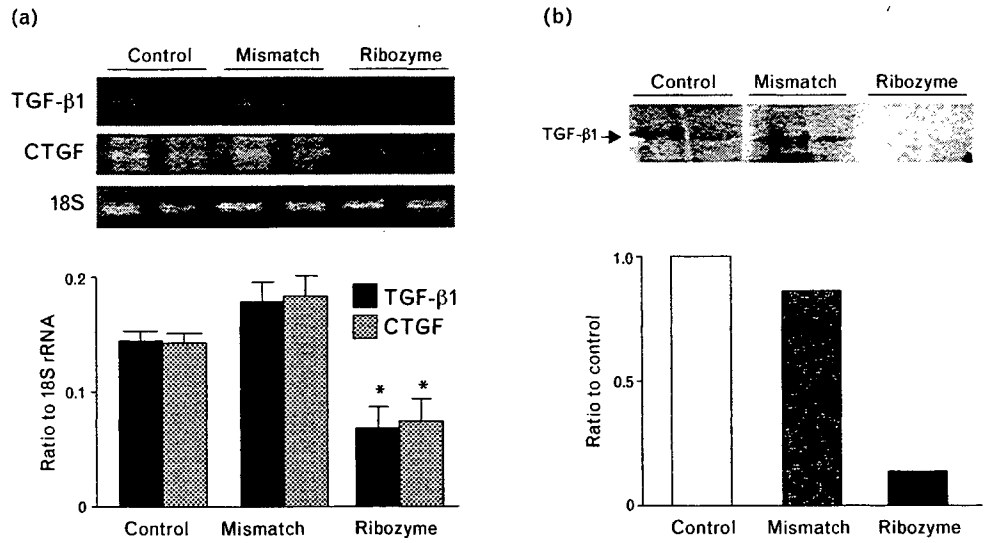
Intraperitoneal administration of chimeric DNA–RNA ribozyme to TGF-β1 significantly ($P < 0.05$) decreased the GIS and TIS in kidney from salt-loaded SHR-SP and Dahl-S rats. Mismatch ribozyme had no effect on GIS or

Fig. 3



Effects of chimeric DNA–RNA ribozyme to transforming growth factor (TGF)-β1 on expression of TGF-β1, fibronectin and collagen type I mRNAs (a) and TGF-β1 protein (b) in cultured mesangial cells from stroke-prone spontaneously hypertensive rats (SHR-SP). Levels of TGF-β1, fibronectin and collagen type I mRNAs were evaluated by reverse transcription-polymerase chain reaction (RT-PCR). The ratio of the abundance of TGF-β1, fibronectin and collagen type I mRNAs to that of 18S rRNA was evaluated by densitometric analysis. Data are the means of experiments carried out in duplicate (a). Levels of TGF-β1 protein in renal cortex were evaluated by Western blot analysis. The abundance of TGF-β1 protein was evaluated by densitometric analysis. Data are the means of experiments carried out in duplicate (b).

Fig. 4



Effects of chimeric DNA–RNA ribozyme to transforming growth factor (TGF)-β1 on expression of TGF-β1 and connective tissue growth factor (CTGF) mRNAs and TGF-β1 protein in renal cortex of salt-loaded salt-sensitive Dahl (Dahl-S) rats. Seven-week-old male Dahl-S rats were given 1% salt water for 6 or 8 weeks. After 4 weeks of salt loading, the mismatch or ribozyme group received an intraperitoneal injection of mismatch ribozyme or chimeric DNA–RNA ribozyme to TGF-β1 (200 μg/body weight). Levels of TGF-β1 and CTGF mRNAs were evaluated by reverse transcription-polymerase chain reaction (RT-PCR). The ratio of the abundance of TGF-β1 and CTGF mRNAs to that of 18S rRNA was evaluated by densitometric analysis. Data are the mean ± SEM ($n = 4$). *$P < 0.05$ versus mismatch ribozyme (a). Levels of TGF-β1 protein in renal cortex were evaluated by Western blot analysis. The abundance of TGF-β1 protein was evaluated by densitometric analysis. Data are the means of experiments carried out in duplicate (b).

Table 1 Systolic blood pressure in salt-loaded, stroke-prone spontaneously hypertensive rats (SHR-SP) and salt-sensitive Dahl (Dahl-S) rats treated without (control) or with mismatch ribozyme (mismatch) or chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor (TGF)-β1 mRNA (ribozyme)

	Systolic blood pressure (mmHg)	
	SHR-SP	Dahl-S
Control	272 ± 16	214 ± 28
Mismatch	275 ± 22	224 ± 36
Ribozyme	277 ± 10	205 ± 20

$n = 6$ in each group.

Table 2 Glomerular injury score (GIS) or tubulointerstitial injury score (TIS) in salt-loaded, stroke-prone spontaneously hypertensive rats (SHR-SP) and salt-sensitive Dahl (Dahl-S) rats treated without (control) or with mismatch ribozyme (mismatch) or chimeric DNA–RNA hammerhead ribozyme targeting transforming growth factor (TGF)-β1 mRNA (ribozyme)

Strain	Group	GIS	TIS
SHR-SP	Control	0.87 ± 0.01	0.67 ± 0.10
	Mismatch	0.84 ± 0.12	0.65 ± 0.18
	Ribozyme	0.68 ± 0.06*	0.50 ± 0.07*
Dahl-S	Control	1.01 ± 0.07	0.72 ± 0.18
	Mismatch	1.02 ± 0.14	0.78 ± 0.12
	Ribozyme	0.64 ± 0.09*	0.54 ± 0.26*

* $P < 0.05$ versus mismatch. $n = 4$ in each group.

TIS in kidney from salt-loaded SHR-SP and Dahl-S rats (Table 2).

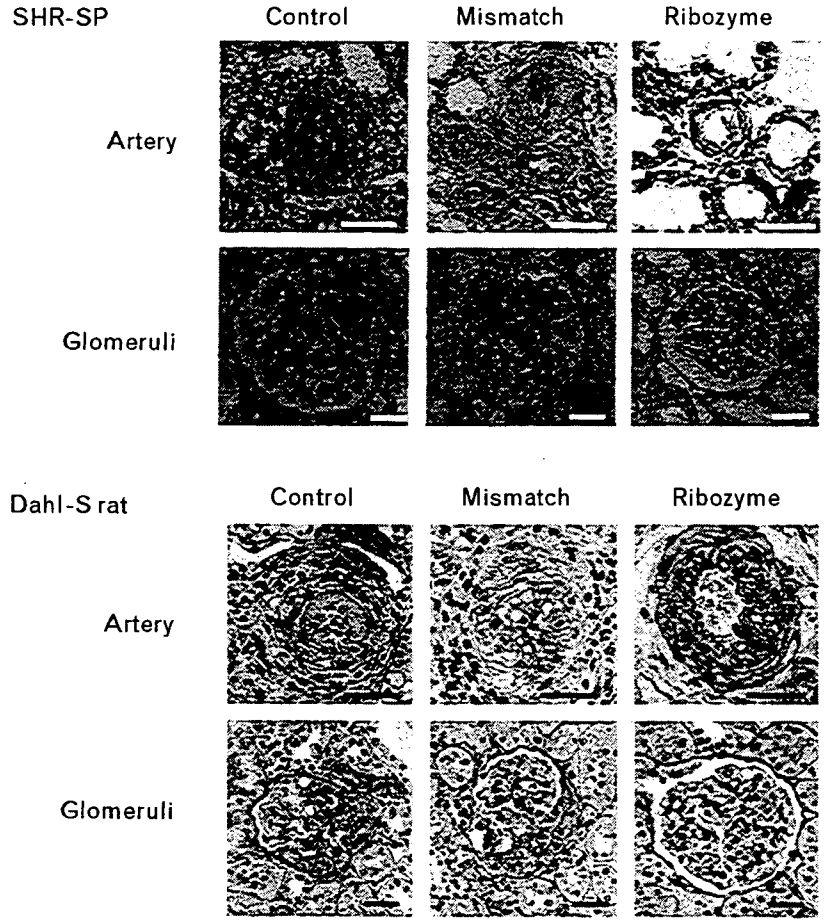
Effects of chimeric DNA–RNA ribozyme to TGF-β1 on proteinuria in Dahl-S rats

Intraperitoneal administration of chimeric DNA–RNA ribozyme to TGF-β1 to salt-loaded Dahl-S rats decreased excretion of protein by 50% ($P < 0.05$). Administration of mismatch ribozyme had no effect on proteinuria (Fig. 6).

Discussion

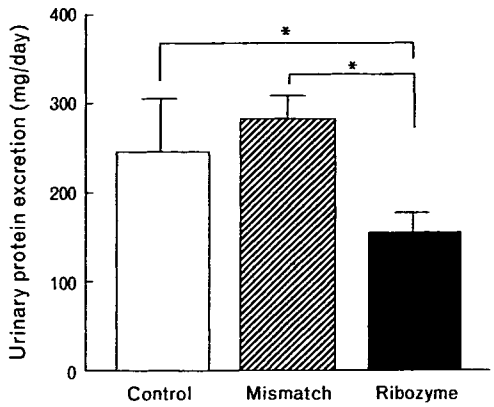
In the present study, salt-loaded SHR-SP and Dahl-S rats experience significant thickening of the capillary arteries,

Fig. 5



Histological findings in renal cortex of salt-loaded, stroke-prone spontaneously hypertensive rats (SHR-SP) and salt-sensitive Dahl (Dahl-S) rats treated with chimeric DNA–RNA ribozyme to transforming growth factor (TGF)-β1. Twelve-week-old male or 7-week-old male SHR-SP and Dahl-S rats were loaded with 1% salt water for 6 or 8 weeks, respectively. After 4 weeks of salt loading, the mismatch or ribozyme group received an intraperitoneal injection of mismatch ribozyme or chimeric DNA–RNA ribozyme to TGF-β1 (200 μg/body weight). Renal cortex was fixed and stained with Masson trichrome for SHR-SP or hematoxylin–eosin for Dahl-S rats. Bar = 20 μm.

Fig. 6



Effects of chimeric DNA–RNA ribozyme to transforming growth factor (TGF)-β1 on proteinuria in salt-loaded, salt-sensitive Dahl (Dahl-S) rats. Data are the mean ± SEM ($n = 4$). *$P < 0.05$ versus mismatch ribozyme and control.

glomerulosclerosis, atrophic tubules and interstitial fibrosis in kidney in response to the development of hypertension. We have demonstrated previously that expression of TGF-β1 mRNA is higher in glomeruli and capillaries of renal cortex from SHR-SP than in those of normotensive Wistar–Kyoto rats [5]. In addition, Dahl-S rats also developed severe hypertension, glomerulosclerosis, thickening of capillary artery walls and interstitial fibrosis in response to salt loading [4].

TGF-β1 stimulates extracellular matrix formation [16,17]. TGF-β-induced production of extracellular matrix proteins in glomeruli causes renal damage in rats with thymidylate synthase complementing protein (Thy-1)-induced nephritis [18], diabetic nephropathy [19] and interstitial nephritis induced by obstructive nephropathy [20]. Moreover, TGF-β has recently been reported to induce epithelial–mesenchymal transformation in renal tissue, which plays a critical role in the pathogenesis of nephritis [21]. Thus, TGF-β may associate the nephrosclerosis and interstitial nephropathy by its effects on extracellular matrix formation and epithelial–mesenchymal transformation in kidney.

Ribozymes hybridize to and cleave a target RNA. Once the target RNA is cleaved, the ribozyme can dissociate from the cleaved products and repeat this process with another RNA molecule [22]. Thus, ribozyme does not require any cellular components and has high specificity for inhibiting expression of a target gene. One problem with ribozymes is rapid degradation in tissue, which diminishes the availability of the ribozyme and reduces their efficiency as a gene therapy. There are a number of modifications that can improve stability, specificity and efficacy of ribozymes. Our chimeric DNA–RNA hammerhead ribozyme to rat TGF-β1 mRNA contained deoxyribonucleotides instead of ribonucleotides at noncatalytic residues to enhance catalytic turnover and improve stability, as reported previously [23]. In addition, two deoxyribonucleotides at the 3′-terminus of the chimeric DNA–RNA ribozyme were modified with phosphorothioate linkages to improve resistance to nucleases [24]. Moreover, to increase uptake of the ribozyme, we delivered our ribozyme with polyethylenimine because liposome-complexed molecules are preferentially transported to the cytoplasm [25].

In the present study, the chimeric DNA–RNA ribozyme to TGF-β1 with polyethylenimine was transferred to cultured mesangial cells *in vitro* and to glomeruli in SHR-SP by subcutaneous, intraperitoneal or intravenous injection. The chimeric ribozyme reduced expression of TGF-β1 and inhibited expression of extracellular matrix proteins, such as fibronectin and collagen type I, in mesangial cells from SHR-SP *in vitro*, suggesting that ribozyme to TGF-β1 may inhibit extracellular matrix formation in glomeruli.

In the present experiments, a single injection of chimeric DNA–RNA ribozyme to TGF-β1 markedly ameliorated capillary artery hypertrophy and glomerulosclerosis in salt-loaded SHR-SP without any reduction in blood pressure. Because the effect of the ribozyme on glomerulosclerosis in SHR-SP was unexpectedly strong, to verify the efficiency we examined the effects of this ribozyme on glomerulosclerosis and proteinuria in salt-loaded Dahl-S rats. One injection of chimeric DNA–RNA ribozyme to TGF-β1 also markedly improved the capillary artery hypertrophy and glomerulosclerosis and significantly reduced proteinuria in salt-loaded Dahl-S rats without any reduction in blood pressure. These findings indicate that TGF-β contributes to both capillary artery hypertrophy and glomerulosclerosis in SHR-SP and Dahl-S rats, which were efficiently ameliorated by chimeric ribozyme independent of blood pressure. The chimeric ribozyme also reduced expression of TGF-β1 and CTGF mRNAs in renal cortex in salt-loaded Dahl-S rats. CTGF is a potent growth factor that stimulates growth of mesenchymal cells, including mesangial cells, and formation of extracellular matrix downstream of TGF-β1 [26]. These findings suggest that TGF-β1 causes glomerulosclerosis through induction of CTGF in Dahl-S rats. Ribozyme to TGF-β1 significantly decreased GIS and TIS of renal cortex in salt-loaded SHR-SP and Dahl-S rats, suggesting that TGF-β1 may also be involved in renal interstitial fibrosis due to extracellular matrix formation in these hypertensive rats. Dahly *et al.* [27] injected anti-TGF-β antibody every day for 2 weeks into salt-loaded Dahl-S rats and found that the antibody significantly reduced blood pressure, proteinuria and the degree of glomerulosclerosis and renal interstitial fibrosis. In the present study, the chimeric ribozyme to TGF-β1 ameliorated the renal injury

and proteinuria with only a single injection and without reduction of blood pressure in salt-loaded Dahl-S rats, suggesting that this ribozyme is an efficient strategy to ameliorate progressive renal injury.

Theoretically, nuclease-resistant ribozymes can be designed to cleave any target RNA. The recently completed sequencing of the human genome and of the genomes of several pathogenic species provide a large number of potential ribozyme targets that may benefit clinical medicine. These ribozymes can be chemically modified to prevent biological degradation, and thus, nuclease-resistant synthetic ribozymes are emerging as a new and broadly useful class of therapeutic agents. Gene therapy by nucleic acid-based therapeutics is currently in its infancy, but preliminary studies suggest that this type of therapy may be effective against diseases caused by genes encoding nontractable drug targets. We plan to develop chimeric DNA–RNA ribozyme targeting TGF-β1 mRNA as a clinical therapy for progressive renal diseases such as glomerulonephritis and hypertensive nephrosclerosis.

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Analyses of Novel Prognostic Factors in Neuroblastoma Patients

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Neuroblastoma (NB) is the most common malignant solid tumor in childhood. There are well-recognized prognostic factors in NB such as age at diagnosis, organ of origin, stages, *MYCN* gene amplification, and expression of H-*ras*, *trk*A and survivin. Moreover, we investigated the expression of vascular endothelial growth factor (VEGF), tyrosine hydroxylase (TH), p53, stem cell factor (SCF) and c-*kit* of its receptor with quantitative real-time polymerase chain reaction (PCR) in 22 NBs and 4 other tumors (one malignant lymphoma, one malignant teratoma, and 2 rhabdomyosarcomas) samples. The correlation between patients' prognoses and the expression of TH or c-*kit* was newly recognized, particularly the good prognosis in patients in whom c-*kit* highly expressed and the poor prognosis contrarily associated with low or no expression, although the SCF of its ligand had no relationship with patient prognosis. It is possible that tumors without c-*kit* expression can not react with SCF (*via* the autocrine or paracrine system) and remain immature. It may be that this is a new critical clinical event in NB patients.

Key words neuroblastoma; tyrosine hydroxylase; stem cell factor; c-*kit*

The neuroblastoma (NB) is the most common malignant pediatric solid tumor, which originates in the sympathetic nervous system and the adrenal gland. Many studies have demonstrated that both clinical and molecular biological factors are correlated with outcome.[1)] For example, patients under the age of 1 year at diagnosis usually have good prognoses, but those diagnosed over 1 year of age have poor prognoses.[2)] Increased/decreased expression of the molecular factors, *MYCN*, H-*ras*, and *trk*A is well known in NB.[3—11)]

A large body of basic research into genes and oncogenes has accumulated up till the present. Survivin has recently been described as a member of the inhibitor of apoptosis protein (IAP) family.[12)] It is expressed in many malignant tumors, including breast, lung, stomach, colon and pancreatic cancers, bladder tumors, malignant lymphomas, osteosarcomas and NBs.[13,14)] NBs are also characterized by elevated levels of catecholamine production. Tyrosine hydroxylase (TH) is very important as the first and rate-limiting step in the synthesis of catecholamines.[15—18)] Moreover, we analyzed the following three items: vascular endothelial growth factor (VEGF), which is concerned with tumor angiogenesis and tumor metastasis together with its receptors (VEGF-Rs),[19,20)] p53 (tumor suppressor gene)[21)]; and stem cell factor (SCF) as function of multipotent hematopoietic colony-stimulating factor (CSF) and its receptor (c-*kit*), based on tyrosine kinase activity.[22,23)] The present study into the expression of these genes demonstrates in particular that the degree of expression of TH and c-*kit* mRNA is a very useful prognostic indicator.

MATERIALS AND METHODS

Patients and Tumor Samples Between 1990 and 2004 in the Department of Pediatrics and Child Health, School of Medicine, Nihon University, 26 clinical tumor samples from 26 patients (22 neuroblastomas [NBs], one malignant lymphoma [ML], one malignant teratoma [MT], and 2 rhabdomyosarcomas [RMSs]) were examined. The clinical and pathological characteristics of these patients are shown in Table 1. The 22 NB patients consisted of 8 males and 14 females, with a mean age of 2.9 years (range 0.4 to 10.6 years), 2 cases at stage I, 6 cases at stage II, 3 cases at stage III, 10 cases at stage IV, and 1 case at stage IVS. One of the two RMS samples was from a recurrent tumor. These tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients were histopathologically confirmed. Written informed consent was obtained from all patients before they entered this study. The study underwent ethical review and approval according to the guidelines of institutional review board (IRB) at College of Pharmacy, Nihon University.

RNA Extraction Total RNA from the 22 NBs and the 4 other malignant tumor samples was extracted with TRI_{ZOL} reagent (Gibco BAL) by the acid-guanidium-phenol chloroform extraction method.[24)]

Quantitative Real-Time PCR and Gene Expression Total RNA from each tumor was reverse transcribed to cDNA using the Takara RNA PCR kit (AMV) Version 3.0 (Takara, Otsu, Japan) with oligo-dT as a primer (Table 2). Quantitative real-time PCR was carried out to detect h-GAPDH expression that was used to normalize the amount of cDNA of each sample. Equal amounts of cDNA from each sample were amplified using each primers to detect (the primers sequences are listed in Table 2). This experiment was carried out with a Smart Cycler II System with Sybr green fluorochrome. The cycling conditions were as follows: initial denaturation at 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 20 s. The degree of expression on each *trk*A, survivin (transcript variant 1), VEGF (VEGF-A), p53, TH, SCF, and c-*kit* was shown as the ratio of each gene/human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) (Figs. 1—7).

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Table 1. Clinical Characteristics and Behavior in Malignant Tumor Patients

Patient	Histology[a)]	Stage	Sex	Age	Origin	*MYCN* amp[b)]	Outcome
1	NB	I	F	7 m	Adrenal gland	ND[c)]	Alive
2	NB	I	F	1 y 11 m	Retroperitoneum	(−)	Alive
3	NB	II	F	9 m	Retroperitoneum	(−)	Alive
4	NB	II	F	6 m	Retroperitoneum	(−)	Alive
5	NB	II	F	10 m	Retroperitoneum	ND	Alive
6	NB	II	F	11 m	Mediastinum	ND	Alive
7	NB	II	M	7 m	Adrenal gland	ND	Alive
8	NB	II	F	9 m	Adrenal gland	(+)	Alive
9	NB	III	F	6 y	Adrenal gland	(−)	Alive
10	NB	III	F	8 m	Retroperitoneum	(−)	Alive
11	NB	III	M	4 m	Adrenal gland	ND	Alive
12	NB	IV	F	1 y	Mediastinum	ND	Alive
13	NB	IV	F	2 y	Adrenal gland	(−)	Dead
14	NB	IV	M	4 y 11 m	Adrenal gland	(−)	Dead
15	NB	IV	M	5 y 9 m	Adrenal gland	ND	Dead
16	NB	IV	F	10 y 7 m	Adrenal gland	(−)	Dead
17	NB	IV	M	3 y	Retroperitoneum	(−)	Alive
18	NB	IV	M	3 y	Adrenal gland	ND	Dead
19	NB	IV	F	4 y 4 m	Adrenal gland	(−)	Alive
20	NB	IV	M	3 y 3 m	Adrenal gland	(+)	Dead
21	NB	IV	M	9 y 6 m	Adrenal gland	(−)	Alive
22	NB	IVS	F	7 m	Adrenal gland	(−)	Alive
23	ML		F	7 y			
24	MT		F	17 y			
25	RMS		F	Unknown			
26	RMS		M	15 y			

a) NB: neuroblastoma, ML: malignant lymphoma, MT: malignant teratoma, RMS: rhabdomyosarcoma. *b*) *MYCN* amp: *MYCN* amplification. *c*) N.D.: not done.

Table 2. Primers Used for PCR

Genes	Primer sequence	Size (bases)
*trk*A	F: 5′-TTGGCATGAGCAGGGATATCTACA-3′	117
	R: 5′-TCTCGGTGGTGAACTTACGGTACA-3′	
Survivin	F: 5′-AAGGCTGGGAGCCAGATGAC-3′	63
	R: 5′-AAGCGCAACCGGACGAAT-3′	
VEGF	F: 5′-GAGCCTTGCCTTGCTGCTCTAC-3′	148
	R: 5′-CACCAGGGTCTCGATTGGATG-3′	
p53	F: 5′-TGCCCAACAAACCAGCTC-3′	123
	R: 5′-CCAAGGCCTCATTCAGCTCTC-3′	
TH	F: 5′-GGAGTTCGGGCTGTGTAAGCA-3′	165
	R: 5′-GACTGGTACGTCTGGTCTTGGTAGG-3′	
SCF	F: 5′-CCCTTAGGAATGACAGCAGTAGCA-3′	166
	R: 5′-GCCCTTGTAAGACTTGGCTGTCTC-3′	
c-*kit*	F: 5′-AGGATTCCCAGAGCCCACAATAG-3′	113
	R: 5′-ACGGTGGCCCAGATGAGTTTAG-3′	

Statistical Analysis All statistical analyses were performed using GraphPad Prism 4 (Graphpad, San Diego, CA, U.S.A.). Data were expressed as means±standard deviation. Mean values of continuous variables were compared using analysis of variance (ANOVA) and did Tukey's test after transforming the data to logarithmic values. The significance level was set at p-values equal to or less than 0.05.

RESULTS AND DISCUSSION

Advanced NBs are the most malignant pediatric solid tumors, patients suffering from which have poor prognoses, so it is important to identify patients with unfavorable outcomes for whom we can then select appropriately strong chemotherapeutic regimes. Moreover, we can also improve the prognoses of these patients.

We examined 26 malignant tumor samples including the 22 NBs and four other tumors (one ML, one MT, and 2 RMSs (one is a recurrent case)). The clinical characteristics and behavior of the 22 NB patients in particular are shown in Table 1. As for staging, stages I, II, III, and IV accounted for 2, 6, 3, and 11, respectively, and 1 was a special case at stage IVS. It has been generally recognized that stage I, II, and IVS (special stage IV with favorable outcome) patients have good prognoses and stage III, and IV (so called "advanced NB") have poor prognoses.[1—3,25)] Regarding gender and age at diagnosis, 8 cases were males and 14 cases females, with a mean age of 2.9 years (range 0.4 to 10.6 years). It has been reported that patients younger than 1 year old have a much more favorable outcome than older patients.[1—3,25)] The organs of origin were the retroperitoneum in 6, the adrenal gland in 14 and the mediastinum in 2 cases (in general, NBs of adrenal gland origin have poor prognoses).[1—3,25)] *MYCN* gene amplification is very much related to patient prognosis and was recognized only each of the stage II and stage IV patients. As for patient outcome, all patients at stages I, II, III, and VIS are alive, on the contrary, 5 of the 11 stage IV patients are alive and 6 have died.

We measured some genes expressions (*trk*A, survivin, VEGF, p53, TH, SCF, and c-*kit*) in the NB clinical samples with quantitative real-time PCR. Moreover, we quantified individual gene expression among the 22 NBs and 4 other tumor samples; among the stage I, II, and IVS (early stages) samples and stage III and IV (advanced stages) samples; and between patients who survived and died.

*Trk*A is a nerve growth factor (NGF) receptor, which plays an important role in the growth and differentiating of neural cells. It has been reported that having little or none of this gene expression is a critical event and is related to the prog-

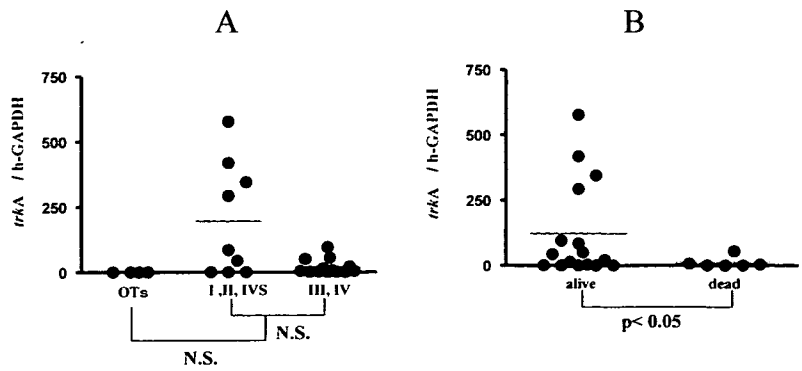

Fig. 1. The Expression of *trk*A mRNA in 26 Malignant Tumor Samples

(A) We classified 26 samples into 2 different groups: 22 NBs and 4 other malignant tumors (OTs: one malignant lymphoma [ML], one malignant teratoma [MT], and 2 rhabdomyosarcomas [RMSs]); stages I, II, and IVS (early stages), and satges III and IV (advanced stages). We performed statistical analyses among the 22 NBs and 4 OTs and among the early and advanced stages (as above) in 22 NBs for the degree of *trk*A expression (relative to h-GAPDH as the internal marker). (B) In addition to the 2 groups detailed above, performed a statistical analysis between surviving and dead NB patients by the same method. The lines in the figure represent the median. N.S.: not significant, *p*: *p*-value (<0.05).

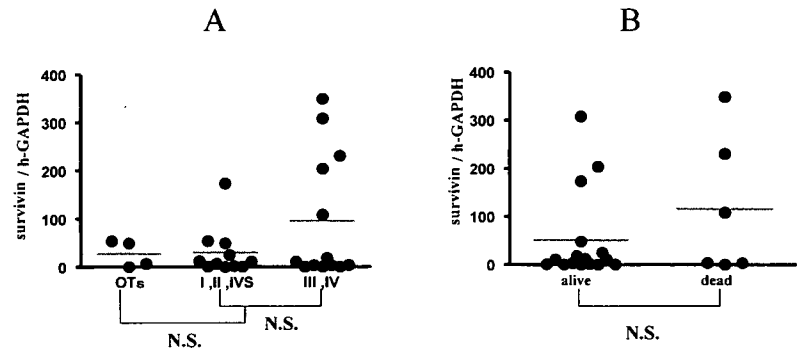

Fig. 2. The Expression of Survivin mRNA in 26 Malignant Tumor Samples

(A) Statistical analyis among the 22 NBs and 4 OTs and among early and advanced stages in 22 NBs for the degree of survivin expression (relative to h-GAPDH as the internal marker). (B) Statistical analysis using the same method between surviving and dead NB patients. The lines in the figure represent the median. N.S.: not significant.

nosis for NB patients.[7—9] Although the expression of *trk*A was not statistically significant among the 22 NBs and 4 other malignant tumors (OTs), and among the early stages (stage I, II, IVS) and advanced stages (stage III, IV) samples, it was, however, statistically significant between the surviving patients and those who died ($p<0.05$) (Fig. 1). This means that the existence of high or low *trk*A expression is also a good prognostic factor in NB patients as many researchers have reported.[7—9]

Survivin is abundantly expressed during fetal development but not in adult human tissues.[26] We have previously suggested that it is an important prognostic factor in NB (14 cases) and osteosarcoma patients (22 cases).[27,28] However, in the larger number of 22 NB cases in the present study we could not find any difference among the 22 NBs and 4 OTs, the early stages (stage I, II, IVS) and advanced stages (stage III, IV), and between surviving and dead patients (Fig. 2). Although survivin mRNA has been detected in various human cancers,[29,30] the lack of statistical significance in our 22 NB cases is possibly due to the low number of patients who died (6) compared with those who survived (12).

Vascular endothelial growth factor (VEGF), together with its receptors (VEGF-Rs), is concerned with tumor angiogenesis and tumor metastasis.[19,20] However, the correlation between prognosis and VEGF mRNA expression levels has not been evaluated based on surgically resected samples. Recently it has been reported in a phase III trial that the combination of bevacizumab (a human VEGF antibody) with irinotecan, fluorouracil, and leucovorin was very effective for advanced metastatic colorectal cancer patients.[31] We therefore investigated the expression of VEGF (VEGF-A) mRNA in our 26 tumor samples, but were unable to see any statistically significant differences in the three groups we compared (22 NBs and 4 OTs, early and advanced stages, and surviving and dead NB patients) (Fig. 3). This means that the level of VEGF-A mRNA have no prognostic correlation for NBs, but it may be an important target protein to treat advanced NB patients.

p53 is termed the "tumor suppressor gene," and gene alteration of more than 50% (that is loss or point mutation on specific DNA binding region) has been found in various tumors.[32,33] This alteration of p53 is concerned with resistance to radiation and anti-tumor drugs.[34,35] We therefore analyzed the expression of the p53 gene, but once again no statistically significances could be seen in the three groups we compared (Fig. 4). It is possible that p53 has no prognostic correlation in NB patients because no p53 gene mutation has been found in NB surgical samples.[36]

TH is very important as the first and rate-limiting step in the synthesis of catecholamines, and NB is characterized by elevated levels of catecholamine production.[15—18] We investigated the 26 clinical tumor samples with the same method. We found that TH mRNA was much higher expressed in the NB samples than the OTs. Moreover, in the surviving NB pa-

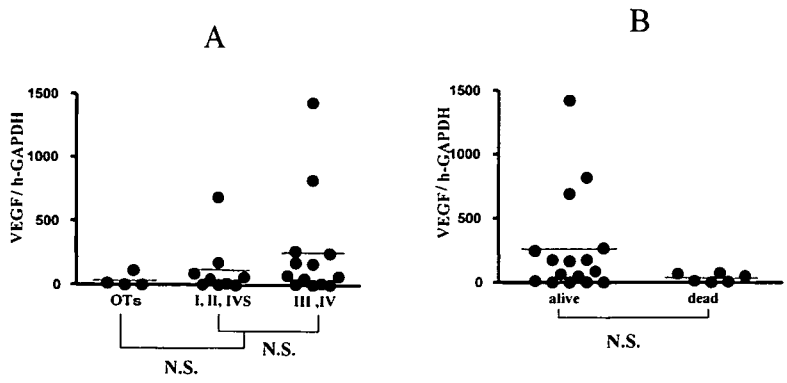

Fig. 3. The Expression of Vascular Endothelial Growth Factor (VEGF-A) mRNA in 26 Malignant Tumor Samples

(A) Statistical analysis among the 22 NBs and 4 OTs and among the early and advanced stages in 22 NBs for the degree of VEGF-A expression (relative to h-GAPDH as the internal marker). (B) Statistically analysis using the same method between surviving and dead NB patients. The lines in the figure represent the median. N.S.: not significant.

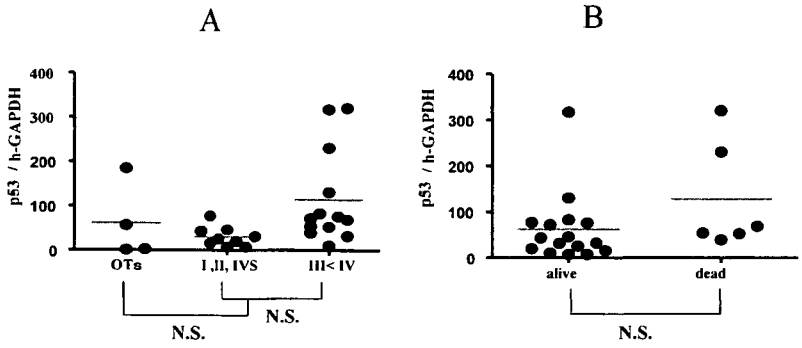

Fig. 4. The Expression of p53 mRNA in 26 Malignant Tumor Samples

(A) Statistical analysis among the 22 NBs and 4 OTs and among the early and advanced stages in 22 NBs for the degree of p53 expression (relative to h-GAPDH as the internal marker). (B) Statistical analysis using the same method between surviving and dead NB patients. The lines in the figure represent the median. N.S.: not significant.

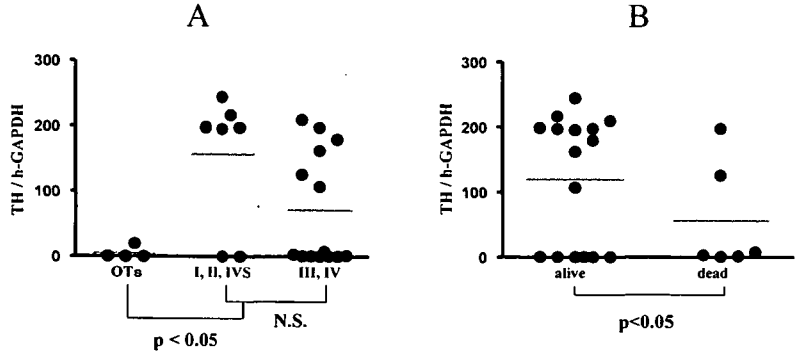

Fig. 5. The Expression of Tyrosine Hydroxylase (TH) mRNA in 26 Malignant Tumor Samples

(A) Statistically analysis among the 22 NBs and 4 OTs and among the early and advanced stages in 22 NBs for the degree of VEGF expression (relative to h-GAPDH as the internal marker). (B) In addition to the 2 groups detailed above, we performed a statistical analysis between surviving and dead NB patients by the same method. The lines in the figure represent the median. N.S.: not significant, *p*: *p*-value (<0.05).

tients it was also much higher than in those who had died. This is very useful for diagnosing NB and detecting minimal residual disease in clinical bone marrow (BM) samples and autologous BM and peripheral blood stem cell samples. Moreover, we could also use TH expression in assessing the prognosis of NB patients (Fig. 5).

Stem cell factor (SCF) is a function of the multipotent hematopoietic colony-stimulating factor (CSF). SCF exerts its biological effects by binding to the tyrosine kinase receptor c-Kit, which is located on the cell surface.[22,23,37] Signal transduction by this receptor plays a critical role in hematopoiesis, in mast cell development and function, and in development of melanocytes and germ cells. It has also been demonstrated that this ligand-receptor pair has an anti-apoptosis effect on primordial germ cells.[38] Neuroblastic tumors (including NB, ganglioneuroblastoma, and ganglioma) are also derived from the primordial neural crest cells, that ultimately populate the sympathetic ganglia and adrenal medulla. The variations in tumor locations and degrees of histopathologic differentiation result in an array of diverse clinical and biological characteristics and behavior.[25]

We investigated the expression of SCF and its receptor (c-*kit*) in clinical NB tumor samples. No statistically significance was observed regarding SCF expression among the 22

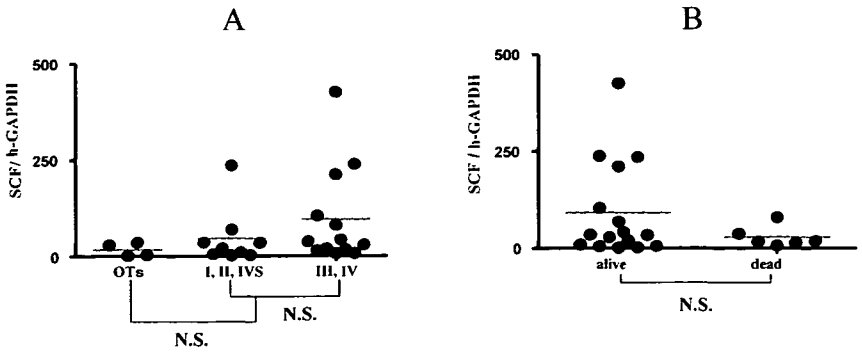

Fig. 6. The Expression of Stem Cell Factor (SCF) mRNA in 26 Malignant Tumor Samples

(A) Statistical analysis among the 22 NBs and 4 OTs and among the early and advanced stages in 22 NBs for the degree of SCF expression (relative to h-GAPDH as the internal marker). (B) Statistical analysis using the same method between surviving and dead NB patients. The lines in the figure represent median. N.S.: not significant.

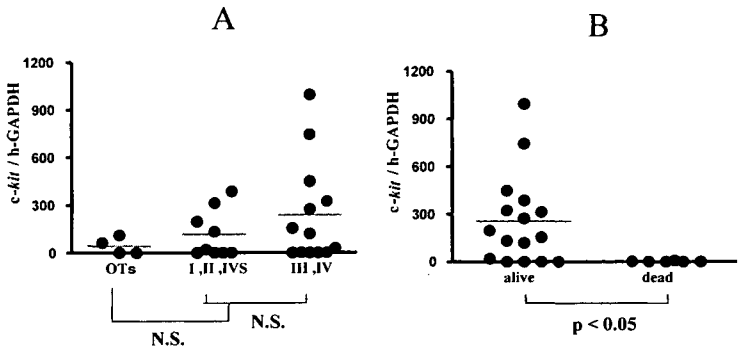

Fig. 7. The Expression of c-*kit* mRNA in 26 Malignant Tumor Samples

(A) Statistical analysis among the 22 NBs and 4 OTs and among the early and advanced stages in 22 NBs for the degree of c-*kit* expression (relative to h-GAPDH as the internal marker). (B) In addition to the 2 groups detailed above, we performed a statistical analysis between surviving and dead NB patients by the same method. The lines in the figure represent median. N.S.: not significant, *p*: *p*-value (<0.05).

NBs and 4 OTs, The early or advanced stage specimens or between the surviving and dead NB patients (Fig. 6). In c-*kit* expression, on the other hand, statistical significance was found between the surviving and dead NB patients ($p<0.05$) (Fig. 7). Based on these findings, the level of c-*kit* expression is not useful for diagnosing NB or detecting minimal residual disease as is the case with TH, but it could be a useful prognostic factor in NB patients.

It has been reported that autocrine growth in various tumors (gynecological tumors, small cell lung cancer, and pancreatic cancer) is mediated by the coexpression of SCF and c-*kit*.[39—41] Moreover, one report has said that NB tumors which have been immunohistochemically confirmed as being c-Kit or SCF protein positive have an unfavorable histology and the c-*kit* receptor becomes an important clinical therapeutic target.[42] On the contrary, another report stated that c-Kit positive NBs have favorable prognoses based on the same immunohistochemical method.[43] Based on our findings, although the SCF mRNA expression had no prognostic value for our NB patients, surviving NB patients had high expression of c-*kit* mRNA compared with little or no c-*kit* expression in the NB patients who subsequently died. It is possible that NB tumor cells with c-*kit* expression-positive NB tumor cells can react with SCF (by autocrine or paracrine system), but tumor cells with no such expression can not react, and these tumors continue to be immature. This might well be a new critical clinical event in NB patients.

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Insights into Infant Neuroblastomas Based on an Analysis of Neuroblastomas Detected by Mass Screening at 6 Months of Age in Japan

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- mass screening
- biology

Mots-clés
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Palabras clave
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- detección poblacional
- biologíca

Schlüsselwörter
- Neuroblastom
- Massenscreening
- biologische Eigenschaften

Abstract

Background/Purpose: Mass screening (MS) for neuroblastoma (NB) at 6 months of age in Japan was discontinued in 2004. We have previously reported that the majority of NB detected by MS showed a good prognosis, with only a few cases demonstrating an unfavorable outcome (J Pediatr Surg 2002, Cancer 2001). This study aims to provide insights into infant NB by assessing the details of the clinical courses in patients treated with a standard regimen and the biological features of such cases using highly sensitive methods at one institution in Japan.

Methods: In 76 NB detected through MS treated at Kyushu University Hospital, the clinical features and *MYCN* amplification, 1p deletion, 17q gain, the expression level of *TrkA* using FISH and the quantitative PCR were analyzed.

Results: Of these 76 persons with NB treated at one institution, 97% are still alive, while 2 cases died from other diseases. Three patients experienced a recurrence after complete remission (CR), and 2 patients demonstrated refractory disease since the initial diagnosis. Two of the 3 NB patients with recurrence have demonstrated a 2nd CR, while one case still has multiple active diseases. Regarding the findings of highly sensitive biological analyses, 5/74 (7%) showed *MYCN* amplification, 2/24 (8%) cases had a 1p deletion, 3/33 (9%) cases had a 17q gain, 5/50 (10%) cases had diploidy, 1/25 (4%) cases had a low expression of *TrkA*, and 2/76 (3%) cases had an unfavorable histology. Of the 76 NB, 13 tumors (17%) had one or more unfavorable factors (UF). Of the 5 refractory NB, 1 case had 3 UF, 1 case had 2 UF, 1 case had 1 UF, and 2 cases had no UF. As a result, 60% of the refractory NB had one or more UF.

Conclusions: Of the NB detected by MS at one institution in Japan, 17% had one or more unfavorable factors (UF) and might have a higher risk of recurrence than the patients with no UF, although the unfavorable biology of several refractory cases is still unclear even after highly sensitive analyses. At least one-fifth of the NB cases detected by MS are anticipated cases. In infantile neuroblastomas, it may therefore be most important to analyze biologically prognostic factors using highly sensitive methods followed by immediate surgical intervention. Since the MS program has been discontinued in Japan, it will be necessary in future to assess the mortality and characteristics of NB detected clinically.

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Introduction

Since 1985, a nationwide mass screening program (MS) for neuroblastoma was conducted in 6-month-old infants throughout Japan [1,5]. To date, more than 2000 Japanese children have been diagnosed as having neuroblastoma on the basis of the MS at 6 months of age. The outcome of these patients has been excellent, and more than 97% of all such patients are still alive [3,7]. Since MS was first started, the number of patients with neuroblastoma has increased. Likewise, the number of patients under 1 year of age as well as the number of cases with early stage neuroblastoma has also increased. However, the number of advanced-stage neuroblastoma patients over 1 year of age has not changed substantially [8,17]. These findings imply that a number of tumors in this age group (6 months of age) have the capacity to either spontaneously regress or mature, and thus they may not be detected clinically.

Based on these assessments, MS for neuroblastoma at 6 months of age in Japan was discontinued in 2004. We have previously reported that the majority of NB detected by MS show a good prognosis [9], while only a few cases demonstrated an

Table 1 Clinical characteristics of 76 neuroblastomas detected by MS in one institution

Clinical characteristics		No. of patients
Stage	stage 1, 2, 4S	65 (86%)
	stage 3, 4	11
Primary site	not adrenal	34 (45%)
	adrenal	42
Operation	complete resection	53
	incomplete resection	23
Chemotherapy	not done	19
	done	57
Outcome	alive	74
	dead	2

Table 2 Biological prognostic factors of 76 MS cases

Biological prognostic factors		No. of patients
MYCN amplification	not amp.	69
	amp.	5 (7%)
1p deletion	no deletion	22
	deletion	2 (8%)
17q gain	no gain	30
	gain	3 (9%)
TrkA expression	high expression	24
	low expression	1 (4%)
DNA ploidy	aneuploid	45
	diploid	5 (10%)
Shimada histology	favorable	74
	unfavorable	2 (3%)

unfavorable outcome [16]. This study aims to offer insights into infantile neuroblastoma by assessing the details of the clinical courses of the patients treated by the standard regimen and the biological features using highly sensitive methods at one institution in Japan.

Materials and Methods

The details of the MS protocol in Japan have been published elsewhere [10]. In the Kyushu area, the presence of urinary vanillylmandelic acids (VMA) and homovanillic acid (HVA) is measured by high performance liquid chromatography.

Seventy-six neuroblastoma patients detected through MS between 1985 and 2003 were treated at the Department of Pediatric Surgery, Kyushu University. The following clinical features were recorded: the stage at the time of diagnosis, the site of the primary tumor, the type of operation used to treat the primary tumor, the initial chemotherapy, and the outcome of the patients. The follow-up time of the patients ranged from 2 years to 18 years. In addition, in relapsed cases, the time from initial therapy to relapse, the pattern of relapse, the treatment method for relapse, and the prognosis after relapse were all investigated. The following biological features were analyzed: *MYCN* amplification, 1p deletion, 17q gain, the expression level of *TrkA*, DNA ploidy, and the Shimada histology [6]. The *MYCN* amplification status was examined using the FISH method, quantitative PCR and the Southern Blot method [12]. The 1p deletion was determined by the 2-color FISH method using both the centromere of chromosome 1 and 1p36 region (D1Z2) probes [14]. The 17q gain was evaluated by the gene dosage of Survivin using the quantitative PCR [15]. The expression level of *TrkA* was determined using both the quantitative PCR and the Northern Blot method. DNA ploidy was assessed using flow cytometry and the chromosome 1p status by the FISH method was done using the centromere probe of chromosome 1. The details of the FISH method and the quantitative PCR method have been described in detail in our previous reports [12,14,15].

Results

Clinical features of 76 neuroblastomas detected by MS in one institution

The clinical characteristics of the 76 MS cases treated at Kyushu University Hospital between 1985 and 2003 are presented in ○ **Table 1**. There were 65 stage 1 or 2, 4S (86%) cases, and 11 stage 3 or 4 cases. The primary tumor sites were the adrenal gland region in 42 cases and the non-adrenal gland region in 34 cases (45%). Complete resection of the tumor was performed in 53 of the 76 cases. In addition, 23 patients underwent an incomplete resection, 57 of the 76 cases received mild chemotherapy (low doses of cyclophosphamide and vincristine), 4 received multidrug chemotherapy (a combination of cyclophosphamide, vincristine, cisplatin, etoposide, and tetrahydropyranyl adriamycin), while 19 had no chemotherapy. Two patients out of the 76 cases detected by MS died, but none of them died of the disease. One stage 2 patient died of viral nephritis after completion of the therapy and demonstrating a complete remission (CR) (4 postoperative years) and another stage 2 patient died of ALL (Ph1 positive) after 2 years of mild chemotherapy in CR (15 postoperative years).

Biological features of 76 neuroblastomas detected by MS in one institution

The biological features of the 76 MS cases based on highly sensitive analyses are presented in ○ **Table 2**. Regarding the status of *MYCN* amplification, 5 (7%) of the 74 examined cases demonstrated an amplification of *MYCN* by highly sensitive analyses using a combination of FISH and quantitative PCR, in addition to the Southern Blot method. Regarding the 1p deletion, 2 (8%) of the 24 examined cases showed a deletion using the 2-color FISH method. Regarding the 17q gain, 3 (9%) of the 33 examined cases revealed such a gain based on the gene dosage of Survivin using quantitative PCR. Regarding the expression level of *TrkA*, 1 (4%) of the 25 examined cases demonstrated a low expression level using the quantitative PCR and Northern Blot method. Regarding DNA ploidy 5 (10%) of the 50 examined cases had a diploid pattern based on flow cytometry and an analysis of the chromosome 1p status by FISH. Regarding Shimada's histological classification, 2 (3%) of the 76 examined tumors were considered to have an unfavorable histology.

In summary, a total of 13 (17%) of all 76 cases presented with one or more biologically unfavorable factors. The clinical features of 13 cases with unfavorable factors are presented in ○ **Table 3**. All 13 cases are still alive; however, 2 of the 13 cases had recurrent disease, while 1 of the 13 cases presented with a residual tumor of liver metastasis at 6 years after the initial diagnosis. Three (cases 3, 8, 9) of these 13 cases had multiple unfavorable factors, of which 2 had either recurrent or refractory disease.

Table 3 Clinical features of 13 cases with unfavorable factors

Case no.	Number of positive unfavorable factors	Stage (INSS)	Initial operation of primary tumor	Initial chemotherapy	Recurrence	Outcome
1	1	3	incomplete resection	multi-drug	–	CR
2	1	1	complete resection	mild	–	CR
3	2	3	incomplete resection	mild	–	CR
4	1	1	complete resection	mild	–	CR
5	1	3	incomplete resection	mild	–	CR
6	1	1	complete resection	–	–	CR
7	1	1	complete resection	mild	–	CR
8	3	1	complete resection	–	+	2nd CR
9	3	4S	complete resection	mild, multi-drug	–	*RT
10	1	1	complete resection	–	–	CR
11	1	2A	incomplete resection	mild	–	CR
12	1	1	complete resection	–	–	CR
13	1	1	complete resection	–	+	CR → PD

CR: complete response; PD: progressive disease; *RT: residual tumor of liver metastasis

Table 4 Recurring neuroblastomas in 76 MS cases

Case no.	Stage	Initial operation chemotherapy	Time from initial therapy to recurrence	Pattern of recurrence treatment for recurrence	Outcome
8	1 (C1N0B0V0bm0H0D0)	complete resection (–)	21 months	bone and bone marrow New A1 → CPA, THP-ADR	2nd CR
14	1 (C1N0B0V0bm0H0D0)	complete resection (–)	7 months	local recurrence, lung, liver partial resection → A1	PD
15	2A (C3N0B0V0bm0H0D0)	partial resection mild (1 year)	48 months	local recurrence partial resection → A1	2nd CR

Mild: low dose of CPA and VCR; New A1: CPA, VP-16, THP-ADR, CDDP; A1: CPA, VCR, THP-ADR, CDDP; CR: complete response; PD: progressive disease

Table 5 Refractory neuroblastomas in 76 MS cases

Case no.	Stage	Initial operation of primary tumor	Chemotherapy	Time from initial therapy	Outcome
9	4S (C1N0B0V0bm0H1D0)	complete resection	mild → new A1 → C and A2 (30 months)	7 years	residual multiple liver metastasis
16	3 (C3N0B0V0bm0H0D0)	biopsy	mild (1 year)	6 years	residual local tumor (size: no change)

New A1: CPA, VP-16, THP-ADR, CDDP; C and A2: CPA, DTIC and CPA, VCR, THP-ADR, CDDP; Mild: low dose of CPA and VCR

The characteristics of recurring or refractory neuroblastomas in 76 neuroblastomas detected by MS in one institution

Three of the 76 patients experienced relapses with either local or metastatic recurrences. Their clinical courses are shown in **Table 4**. Regarding the staging of the 3 relapsed patients, 2 cases were stage 1 while the third case was stage 2A. Two cases underwent complete resection of the primary tumor, and 1 case had an incomplete resection of the tumor. Regarding the initial chemotherapy, 1 case received mild chemotherapy (a low dose of CPA and VCR), while 2 cases received no chemotherapy. The time from initial therapy to relapse ranged from 7 months to 48 months. The patterns of relapse included one local relapse, 1 metastatic recurrence, and 1 local and metastatic recurrence. Regarding the treatment for relapse, 2 patients (cases 14 and 15) underwent partial resection for local recurrences, and all 3 cases received high dose multi-drug chemotherapy such as the A1 protocol without peripheral blood stem cell transplantation. Regarding the outcome after relapse, 2 cases demonstrated a 2nd CR, 1 case has multiple progressive tumors.

Two patients of the 76 patients have shown refractory disease. Their clinical courses are shown in **Table 5**. Case 9 was diagnosed at 6 months of age to be right adrenal neuroblastoma with multiple liver metastatic lesions. Mild chemotherapy (a low dose of CPA and VCR) was administered after a complete resection of the primary tumor. However, the size and number of the multiple liver metastases did not change. As a result, intensive multiple drug chemotherapy (CPA, VCR, THP-ADR, CDDP) was started. After 3 courses of intensive chemotherapy, a liver biopsy was performed due to the fact that no change in the size of the liver metastases was observed. A number of viable ganglioneuroblast cells were present in the liver biopsy sample, which was similar to the findings in the liver metastasis at the primary operation. As a result, more intensive chemotherapy (CPA, VP-16, THP-ADR, CDDP) was administered after performing a liver biopsy. Thus, intensive chemotherapy was continued for 15

Table 6 Prognostic factors of recurrent or refractory neuroblastomas

Case	MYCN amp.	1p deletion	DNA ploidy	TrkA expression	Shimada	17q gain
8	–	–	diploid	low	favorable	+
14	–	–	diploid	high	favorable	–
15	–	–	aneuploid	high	favorable	–
9	6 copies	+	diploid	high	favorable	–
16	–	–	aneuploid	high	favorable	–

months. The patient has remained alive for 6 years since the initial diagnosis without any change in the size and number of liver metastases. Case 16 was diagnosed at 6 months of age as having a stage 3 retroperitoneal neuroblastoma involving the celiac artery. The patient was observed for 6 months without any surgical intervention according to the parents' wish because complete resection of the retroperitoneal mass was deemed to be impossible. However, the level of urinary VMA and HVA increased gradually, and therefore an open biopsy of the primary tumor was performed at 6 months after the initial diagnosis. Mild chemotherapy (a low dose of CPA and VCR) was administered for 1 year after the open biopsy of the primary tumor. However, the size of the tumor and the level of urinary VMA and HVA did not change. The patient has remained alive for 6 years since the initial diagnosis with no change in the size of the tumors and a slight decrease in the level of urinary VMA and HVA.

Table 6 shows the prognostic factors for the primary tumor in recurrent or refractory neuroblastomas. Three cases had one or more unfavorable factors, while 2 cases had no unfavorable factors. Only one case (case 9) showed *MYCN* amplification (6 copies). In cases 14 and 15, the biological findings of recurrent tumors corresponded to the findings of the primary tumor.

Discussion

We have previously reported that the majority of neuroblastoma detected by MS have a good prognosis, while a few cases have an unfavorable outcome. The purpose of MS at 6 months of age was to decrease the number of advanced-stage neuroblastoma patients over 1 year of age and reduce the mortality due to neuroblastoma. Regrettably, this purpose was not achieved [8,17]. However, we have obtained different insights into infantile neuroblastoma based on the clinical and biological features of neuroblastoma detected through MS at 6 months of age in Japan.

In the present study, the clinical characteristics of the 76 MS cases at our institution were almost the same as these reported by others [2,13]. However, detailed reports on the clinical features of MS cases with biologically unfavorable factors and the biological features of recurrent or refractory MS cases are rare. A total of 13 of the 76 MS cases presented with one or more biologically unfavorable factors. Our result demonstrates that at least 17% of the neuroblastomas detected by MS have biologically unfavorable factors. The frequency (23%) of recurrent or refractory cases (3 patients) in the 13 cases with biologically unfavorable factors was significantly higher than that (3%) of recurrent or refractory cases (2 patients) in the 63 cases without any biologically unfavorable factors ($p < 0.05$, Fisher's exact test). Furthermore, 2 out of 3 cases with multiple unfavorable factors presented with recurrent or refractory disease. Of the 5 recurrent or refractory cases, 3 cases had one or more biologically unfavorable factors, while 2 cases had no biologically unfavorable factors. This finding suggests that biologically unfavorable factors are associated with a high risk of recurrence in infantile neuroblastomas. Regarding the *MYCN* amplification status in the 5 recurrent or refractory cases, only one case revealed *MYCN* amplification using the Southern Blot method, FISH method and the quantitative PCR method. Two of the recurrent or refractory cases had other unfavorable factors than *MYCN* amplification. In many previous reports on infantile neuroblastomas, the real outcome of neuroblastoma with other prognostic factors than *MYCN* amplification was unclear. Our study suggests that some of these tumors may progress and eventually some patients may die, if they are not detected during mass screening at 6 months of age and, as a result, do not undergo any immediate surgical intervention with or without additional treatment. The immediate surgical intervention with or without additional treatment is therefore essential and should be beneficial for such patient groups. It may therefore be important to analyze other prognostic factors than just the *MYCN* amplification status in infantile neuroblastoma cases. On the other hand, 2 recurrent or refractory cases had no unfavorable factors, and the reason for this remains unclear. A prospective evaluation of these factors in larger patient cohorts is required to establish their prognostic significance.

Recent reports describe the clinical course of MS cases managed with non-treatment and observation (non-surgical intervention and no chemotherapy) [4,18]. The Committee for the Japanese Association of Pediatric Oncology analyzed 82 MS cases at 17 institutions managed by non-treatment and observation in 1998 [11]. Of these 82 cases, 22 subsequently underwent surgical intervention because of an increased tumor size, either with or without an increase in the urinary VMA and HVA levels ($n = 15$) or at the parents' request ($n = 7$). Of the 60 cases initially observed without surgical intervention, tumors disappeared in only 17 cases while they persisted in 43. All 82 patients in the cohort are still alive. However, the clinical course of the non-treatment cases may not be representative of the natural course of infantile neuroblastomas because the non-treatment cases included few patients with stage 1 or 2 disease associated with relatively low levels of urinary VMA and HVA, and had no invasion into the surrounding organs and vessels. Therefore, these findings do not support management by non-treatment and observation for infantile neuroblastomas. Instead, biological factors appear best able to predict whether an infantile neuroblastoma will regress spontaneously or grow aggressively.

In conclusion, regarding the neuroblastoma detected by MS at one institution in Japan, 17% had one or more biologically unfavorable factors and might thus have a higher risk of recurrence than the patients without such biologically unfavorable factors, although the unfavorable biology of several refractory cases remains unclear, even after performing highly sensitive analysis. At least one-fifth of the neuroblastomas detected by MS are anticipated cases. In infantile neuroblastomas, the most important

action may be to biologically analyze the prognostic factors using highly sensitive methods after performing immediate surgical intervention. Since the MS program has been discontinued in Japan, it will be necessary in future to assess the mortality and the characteristics of neuroblastoma detected clinically.

Résumé

Intérêt pour la neuroblastome de l'enfant du dépistage systématique à 6 mois d'âge au Japon

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But: Le dépistage systématique (MS) des neuroblastomes (NB) à 6 mois d'âge a été cessé au Japon en 2004. Nous avions publié que la majorité des NB détecté par MS avait un bon pronostic avec peu de cas évoluant défavorablement (J Pediatr Surg 2002, Cancer 2001). Cette étude a pour but de prouver l'intérêt chez l'enfant avec un NB en précisant l'évolution avec un protocole standard et le profil biologique de tels cas.
Méthode: Chez 76 NB détectés avec MS traités à l'hôpital universitaire de Kyustru, l'aspect clinique, l'amplication MYCV, la délétion 1p, le gain 17q, l'expression du taux de TrKA en utilisant Fish et la PCR quantitative étaient étudiés.
Résultats: Parmi ces 76 patients traités pour NB à notre institution, 97% sont en vie alors que 2 décédaient pour une autre raison. 3 patients présentaient une récidive après rémission complète (CR) et 2 patients présentaient une maladie réfractaire depuis le diagnostic initial. Deux des patients avec une récidive ont présenté une seconde CR alors que un cas avait encore une lésion active. Concernant les découvertes des analyses biologiques, 5/74 (7%) montraient une amplication MYCV, 2/25 (8%) avaient une délétion 1p, 3/33 (9%) avaient un gain 17q, 5/50 (10%) avaient une diploïdie, 1/25 (4%) avait une expression basse de TrKA et 2/76 (3%) avaient une histologie défavorable. Parmi ces 76 NB, 13 tumeurs (17%) avaient un ou plusieurs facteurs défavorables (UF). Parmi les 5 NB réfractaires, 1 avait 3 UF, 1 avait 2 UF, 1 avait 1 UF et deux cas n'avaient aucun UR. Ainsi, 60% des NB réfractaires avaient un ou plusieurs UF.
Conclusions: Parmi les NB détectés par MS dans notre institution au Japon, 17% avaient un ou plusieurs facteurs défavorables (UF) et avaient un risque de récidive supérieur que les patients sans facteur défavorable, quoique la biologie défavorable de plusieurs cas réfractaires ne soit pas claire même après des analyses biologiques très précises. Au moins 1/5 des NB diagnostiqués ont été détectés par MS. Dans le neuroblastome infantile, il peut-être très important de faire une analyse biologique suivie d'une intervention chirurgicale. Depuis que le MS a été arrêté au Japon, il paraît nécessaire dans le futur d'analyser la mortalité et les caractéristiques des NB détectés.

Resumen

Características del neuroblastoma infantil basadas en el screening masivo a los 6 meses de edad en Japon

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Objetivo: La detección poblacional (MS) del neuroblastoma (NB) a los 6 meses de edad se abandonó en Japón en 2004. Previamente hemos demostrado que la mayoría de los NB detectados por MS tienen buen pronóstico con solamente algunos casos de evolución desfavorable (J Pediatr Surg 2002, Cancer 2001). Este estudio pretende evaluar los resultados en NB infantil tratados con un regimen standard y sus carácterísticas biológicas estudiadas con métodos muy sensibles en unas institución japonesa.
Métodos: En 76 NB detectados por MS en Kyushu University Hospital, se analizaron los datos clínicos, la amplificación del NMYC, la deleción de 1p, la ganancia de 17q, la expresión de TrkA mediante FISH y PCR cuantitativa.
Resultados: De los 76 NB, 97% siguen vivos y 2 murieron de otras enfermedades. Hubo 3 recidivas tras remisión completa (CR) y 2 niños sufren enfermedad refractaria desde el inicio. Dos de los 3 NB recidivados tuvieron una segunda CR mientras que el restante tiene aún enfermedad extensa. En lo que se refiere a la biología, 5/74 casos (7%) tenían amplificación del NMYC, 2/24 (8%) delección de 1p, 3/33 (9%) ganancia de 17q, 5/50 (10%) diploidia, 1/25 (4%) baja expresión de TrkA y 2/76 (3%) histología desfavorable. De los 76 NB, 13 (17%) tenían más de un factor desfavorable (UF). De los 5 NB refractarios, 1 tenía 3 UF, 1 tenía 2 UF, otro 1 UF y 2 ninguno. En total, 60% de los casos refractarios tenían uno o más UF y pueden tener más riesgo de recidiva que los que no tienen UF aunque la biología desfavorable de varios casos refractarios es aún dudosa incluso tras análisis en profundidad. Por lo menos un quinto de los NB detectados por MS son casos anticipados. En el NB infantil puede ser más importante analizar biológicamente los factores pronósticos con métodos muy sensibles antes de la extirpación del tumor. Como se ha abandonado el programa de MS en Japón, será necesario evaluar en el futuro la mortalidad y las características de los NB detectados clínicamente.

Zusammenfassung

Einblick in Neuroblastome bei Kindern basierend auf einer Analyse von Neuroblastomen, die durch Massenscreening im Alter von 6 Monaten in Japan ermittelt wurden

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Zielsetzung: Das Massenscreening auf Neuroblastome bei Kindern im Alter von 6 Monaten wurde in Japan 2004 ausgesetzt. Über die Ergebnisse wurde andernorts berichtet (J Pediatr Surg 2002; Cancer 2001). Die Mehrzahl der entdeckten Neuroblastome hatte eine gute Prognose. Allerdings zeigten einige wenige auch einen ungünstigen Verlauf. Die vorliegende Studie möchte Einblicke in Details des klinischen Verlaufs bei Patienten, die mit einer standardisierten Therapie behandelt wurden, vorstellen. Die biologisch-genetischen Eigenschaften dieser Tumoren werden im Hinblick auf den Krankheitsverlauf untersucht.
Methode: Bei 76 Neuroblastomen, die im Rahmen des Massenscreenings im Kyushu Universitätskrankenhaus entdeckt wurden, wurden der klinische Verlauf, die MYCN-Amplifikation, der 1p-Verlust, das Auftreten von 17q, die Höhe des TrkA-Spiegels unter Verwendung von FISH und die quantitative PCR analysiert.
Ergebnisse: Von diesen 76 Neuroblastompatienten der Klinik leben noch 97%. Zwei Kinder verstarben an anderer Ursache. Drei Patienten erlitten ein Rezidiv nach kompletter Remission und zwei Kinder zeigten einen therapieresistenten Verlauf. Bei zwei der drei NB mit einem Rezidiv kam es zu einer zweiten kompletten Remission, bei einem Kind bestehen jedoch noch multiple aktive Herde. Im Hinblick auf die sehr sensitiven biologischen Parameter zeigten 4 von 74 Patienten (7%) MYCN-Amplifikationen, 2/24 (8%) einen 1p-Verlust, 3 von 33 (9%) ein Auftreten von 17q, 5 von 50 eine Diploidie. Ein Patient von 25 (4%) wies einen niedrigen TrkA-Spiegel und 2 von 76 (3%) wiesen eine ungüns-