Table 3 Intravenous nutritional requirements, types of enteral nutrition, and liver function

Patient no.	Age	PN (kcal/[kg d])	Type of enteral nutrition	AST (8-38 IU/L)	ALT (4-44 IU/L)	TBil (0.2-1.2 mg/dL)
1[a]	3 y	82.9	General diet + semistate digested nutrient	54	64	0.3
2	4 y	31.4	General diet + semistate digested nutrient	35	20	0.3
3	2 y	13.3	General diet + semistate digested nutrient	33	21	0.6
4	1 y	0.0	General diet + semistate digested nutrient	48	30	0.3

Values in parentheses are the standard values. PN indicates parenteral nutrition; ALT, alanine transaminase; AST, aspartate transaminase; TBil, total bilirubin.

[a] Initially treated in another hospital.

patients with intestinal failure [14-17]. Fortunately, none of our patients developed cholestatic liver disease during treatment (Table 3). Patients receiving at least some enteral nutrition have been shown to have partial protection from intestinal failure–associated liver disease compared with patients on total parenteral nutrition [18-22].

The preservation of the intestine is essential for improved outcomes in hypoganglionosis. We found a significant improvement in intestinal motility over time in one patient with hypoganglionosis (data not shown) with the intestinal manometry [23]. Intestinal preservation is also important for evaluating intestinal function in patients with hypoganglionosis. Previously, Okamoto et al [5] also recommended continued monitoring of intestinal function for 1 year without bowel resection. However, gut segments distal to the jejunostomy tend to atrophy and gradually deteriorate, as occurred with patient 1. Our experience with patients 2 to 4, in whom we administered infusions of GFO and lactobacillus as synbiotic preparations through the double-barrel jejunostomy, demonstrated that this might be effective for considerable functional development in distal intestinal segments. A minimal amount of nutrients may also be beneficial for neonates in attempting to achieve intestinal adaptation [24]. Moreover, refashioning a double-barrel jejunostomy into a Bishop-Koop type jejunostomy may help regulate intestinal flow and prevent an overload from occurring in the distal segment of the intestine. Aerobic bacteria of the colon metabolize unabsorbed fibers to short-chain fatty acids. These fatty acids are rapidly absorbed by the colonic mucosa and used as an energy source, and this may provide some additional anti-inflammatory effects [24]. The infusion of GFO and lactobacillus and refashioning to Bishop-Koop type jejunostomy may thus prevent future massive resection of the functional hypoganglionic distal intestine. However, further studies are required to determine the optimal timing for closure of Bishop-Koop–type jejunostomy.

5. Conclusions

Hypoganglionosis must be distinguished from extensive or total colonic aganglionosis during the initial surgery. Atrophy and resection of distal bowel segments may be preventable by maintaining gut viability through the use of synbiotics and a Bishop-Koop–type jejunostomy. As the nutritional management of hypoganglionosis is significantly influenced by the treatment method, our recent approach for hypoganglionosis (simultaneous jejunal and sigmoid colonic biopsies and the 2-stage surgical technique) may help to establish an early diagnosis and improve patient management and outcomes.

References

[1] Martucciello G, Pini AP, Puri P, et al. Controversies concerning diagnostic guidelines for anomalies of the enteric nervous system: a report from the Fourth International Symposium on Hirschsprung's Disease and Related Neurocristopathies. J Pediatr Surg 2005;40:1527-31.

[2] Feichter S, Meier-Ruge WA, Bruder E. The histopathology of gastrointestinal motility disorders in children. Seminars Ped Surg 2009;18:206-11.

[3] Taguchi T, Masumoto K, Ieiri S, et al. New classification of hypoganglionosis: congenital and acquired hypoganglionosis. J Pediatr Surg 2006;41:2046-51.

[4] Kubota A, Yamauchi K, Yonekura T, et al. Clinicopathologic relationship of hypoganglionosis. J Pediatr Surg 2001;36:898-900.

[5] Okamoto E, Toyosaka A, Tomimoto Y, et al. In: Okamoto E, Toyosaka A, editors. A report of the investigation of the collected cases of allied disorder of Hirschsprung's disease—a study for the pathogenesis, diagnosis, and treatment. Tokyo: Nagai Shoten Co, Ltd; 1996. p. 1-14. (Japanese).

[6] Martucciello G. Hirschsprung's disease, one of the most difficult diagnoses in pediatric surgery: a review of the problems from clinical practice to the bench. Eur J Pediatr Surg 2008;18:140-9.

[7] Toyosaka A, Miura K, Seki t, et al. In: Okamoto E, Toyosaka A, editors. Hypoganglionosis—pathogenesis and treatment in allied disorder of Hirschsprung's disease—a study for the pathogenesis and diagnosis of treatment. Tokyo: Nagai Shoten Co., Ltd; 1996. p. 1-14. (Japanese).

[8] Swaminathan M, Kapur RP. Counting myenteric ganglion cells in histologic sections: an empirical approach. Hum Pathol 2010;41: 1097-108.

[9] Watanabe Y, Ito F, Ando H, et al. Morphological investigation of enteric nervous system in Hirschsprung's disease and hypoganglionosis using whole-mount colon preparation. J Pediatr Surg 1999;34:445-9.

[10] Meier-Ruge W, Hunziker O, Tobler HJ, et al. The pathophysiology of aganglionosis of the entire colon (Zuelzer-Wilson syndrome). Morphometric investigations of the extent of sacral parasympathetic innervation of the circular muscles of the aganglionic colon. Beitr Pathol 1972;147:228-36.

[11] Rabah R. Total colonic aganglionosis: case report, practical diagnostic approach and pitfalls. Arch Pathol Lab Med 2010;134:1467-73.
[12] Sun CC, Caniano DA, Hill JL. Intestinal aganglionosis: a histologic and acetylcholinesterase histochemical study. Pediatr Pathol 1987;7: 421-35.
[13] Ikeda K, Goto S, Nagasaki A, et al. Hypogenesis of intestinal ganglion cells: a rare cause of intestinal obstruction simulating aganglionosis. Z Kinderchir 1988;43:52-3.
[14] Kubota A, Yonekura T, Hoki M, et al. Total parenteral nutrition-associated intrahepatic cholestasis in infants: 25 years' experience. J Pediatr Surg 2000;35:1049-51.
[15] Goulet O, Ruemmele F, Lacaille F, et al. Irreversible intestinal failure. J Pediatr Gastroenterol Nutr 2004;38:250-69.
[16] Goulet O, Ruemmele F. Causes and management of intestinal failure in children. Gastroenterology 2006;130(2 Suppl 1):S16-28.
[17] Kelly DA. Intestinal failure-associated liver disease: what do we know today? Gastroenterology 2006;130(2 Suppl 1):S70-7.
[18] Andorsky DJ, Lund DP, Lillehei CW, et al. Nutritional and other postoperative management of neonates with short bowel syndrome correlates with clinical outcomes. J Pediatr 2001;139:27-33.
[19] Kaufman SS. Prevention of parenteral nutrition-associated liver disease in children. Pediatr Transplant 2002;6:37-42.
[20] Weber TR, Keller MS. Adverse effects of liver dysfunction and portal hypertension on intestinal adaptation in short bowel syndrome in children. Am J Surg 2002;184:582-6.
[21] Wilmore DW. Factors correlating with a successful outcome following extensive intestinal resection in newborn infants. J Pediatr 1972;80:88-95.
[22] Vantini I, Benini L, Bonfante F, et al. Survival rate and prognostic factors in patients with intestinal failure. Digestive and Liver Disease 2004;36:46-55.
[23] Watanabe Y, Ito T, Ando H, et al. Manometric evaluation of gastrointestinal motility in children with chronic intestinal pseudo-obstruction syndrome. J Pediatr Surg 1996;31:233-8.
[24] Torres C, Sudan D, Vanderhoo J, et al. Role of an intestinal rehabilitation program in the treatment of advanced intestinal failure. J Pediatr Gastroenterol Nutr 2007;45:204-12.

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society
Pediatrics International (2011) **53**, 497–500 doi: 10.1111/j.1442-200X.2010.03268.x

Original Article

Urinary sulfated bile acid analysis for the early detection of biliary atresia in infants

Mitsuyoshi Suzuki,[1] Toshihiro Muraji,[2] Masayuki Obatake,[3] Masaki Nio,[4] Kiichi Ito,[5] Ken Suzuki,[6] Kunitaka Ota,[7] Shunichi Maisawa,[8] Yuichiro Yamashiro[1] and Toshiaki Shimizu[1]

[1]*Department of Pediatrics, Juntendo University School of Medicine, Tokyo,* [2]*Department of Pediatric Surgery, Ibaraki Children's Hospital, Ibaraki,* [3]*Division of Surgical Oncology, Department of Translational Medical Science, Nagasaki University Graduate School of Biomedical Science, Nagasaki,* [4]*Department of Pediatric Surgery, Tohoku University School of Medicine, Sendai,* [5]*Department of Health Science, Daito Bunka University, Saitama,* [6]*Tokyo Health Service Association, Tokyo,* [7]*Department of Pediatrics, Rokko Island Hospital, Kobe,* [8]*Department of Pediatrics, Morioka Children's Hospital, Morioka, Japan*

Abstract ***Background***: Measurement of urinary sulfated bile acid (USBA) is a non-invasive method to detect bile congestion. Our aim was to evaluate the feasibility of USBA analysis for the early detection of biliary atresia (BA).

Methods: We determined the USBA-to-creatinine ratio (USBA/cr) in 1148 infants at 10–40 days after birth. All infants were followed until the 3- to 4-month postnatal routine health check. The cutoff value for USBA/cr was 55.0 μmol/g creatinine.

Results: Among the infants tested, 47 (4.10%) had USBA/cr ratios that exceeded the cutoff value. Two of these 47 infants had liver disease; one was diagnosed with neonatal hepatitis syndrome, and the other was diagnosed with BA. The BA patient underwent USBA analysis for the first time on day 18 after birth and hepatoportoenterostomy on day 49. No other infants were diagnosed with hepatobiliary disease during the follow-up period.

Conclusion: This USBA analysis provided the correct assessment without fail and identified a case of BA. This approach could be used for the screening and early detection of BA when the false-positive rate is decreased by improving the methods for sample collection and urine storage.

Key words biliary atresia, hepatoportoenterostomy, jaundice, urinary sulfated bile acid.

Early diagnosis of biliary atresia (BA) is associated with improved outcomes following hepatoportoenterostomy (HPE; the Kasai procedure) and prolonged survival with the native liver. However, significant numbers of infants are referred for surgery after the first 60 days of life. Studies have identified a trend toward improved outcomes for infants who undergo HPE before 60 days of age compared to infants who undergo the procedure after 90 days.[1–3] The Japanese Biliary Atresia Registry suggested that improvement of the 10-year survival rate depends on liver transplantation; this conclusion was based on data showing no remarkable increase in the proportion of patients who underwent a corrective operation at the age of 60 days or younger.[2] Therefore, developing appropriate screening tests for BA is of considerable interest.

A direct enzyme assay for water-soluble urinary sulfated bile acids (USBAs) is a sensitive and rapid method for detecting cholestatic jaundice.[4–6] This assay has a possible use for selective screening of BA and neonatal hepatitis syndrome.[6] Furthermore, the USBA assay can differentiate breastfeeding jaundice from persistent jaundice caused by liver disease, including BA.[7] However, no previous study has examined the use of the USBA assay alone as a screening method for the early detection of BA.

In the present study, we measured USBA levels in infants ranging in age from 10 to 40 days. This pilot study was performed to analyze the usefulness of the USBA assay for the early detection of BA, which increases the likelihood of a beneficial outcome after HPE.

Materials and methods

Subjects

The subject group consisted of 1855 healthy neonates born at Juntendo University, Nagasaki University, and their affiliated hospitals between August 2006 and May 2007. This survey was a multi-center trial. Neonates who were born before 36 weeks of gestation or at a body weight of <2000 g were excluded from the study. As a result, only 62% (1148/1855) of all births during the study period were included; these neonates both met our

Correspondence: Mitsuyoshi Suzuki, MD, PhD, Department of Pediatrics, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Email: f-mitsuo@zc5.so-net.ne.jp

Received 8 December 2009; revised 20 August 2010; accepted 21 September 2010.

© 2011 The Authors
Pediatrics International © 2011 Japan Pediatric Society

inclusion criteria and had parents who provided informed consent. Although parental education levels were not ascertained, all parents could read and comply with the study instructions (in Japanese) and provide informed consent, and no hindrances due to parental education level were encountered. As infant nutrition does not affect USBA levels, formula, breastfeeding, and mixture (formula and breastfeeding) were recorded for data analysis purposes only. All study participants received the 3- to 4-month postnatal routine health check, which is not mandatory but is undergone by 100% of infants. Infants who had no jaundice during the health check were judged to be free of BA. This study was approved by the Juntendo and Nagasaki University Institutional Review Board, and informed consent was obtained from the subjects' parents prior to study enrollment.

Urine sampling

Parents were instructed to collect their infant's urine in a small bottle at a randomly assigned time point (ranging from 10 to 40 days after birth). Parents collected urine by putting cotton balls in their baby's diaper and then squeezing the urine from the cotton balls into a small bottle, which is the same method that was used to collect urine during a neuroblastoma mass-screening campaign performed in Japan (ending in 2003).[8] Almost all parents successfully collected their baby's urine in one to three attempts. Parents then mailed the samples to our institution using regular postal services.

Assay

Urine specimens were frozen in an ordinary freezer at approximately –4°C until the USBA assay was performed. Urine specimens were stable for 2 weeks at –4°C, 7 days at 4°C, and 4–5 days at room temperature.[9] USBA levels were determined via a direct enzymatic assay using a commercially available kit (UBASTEC-AUTO; Japan Food & Liquor Alliance, Co., Ltd, Kyoto, Japan) with a TBA-120FR Automatic Analyzer (Toshiba Co., Ltd, Tokyo, Japan). Briefly, desulfation of the bile acid 3α-sulfates was catalyzed by bile acid sulfate sulfatase, followed by dehydrogenation of the desulfated product (3β-hydroxy bile acids) by β-hydroxysteroid dehydrogenase. The products were then subjected to conventional colorimetric assay to detect reduced nicotinamide adenine dinucleotide as formazan dye in the presence of nitrotetrazolium blue and diaphorase.[10,11] The only published values for the USBA-to-creatinine ratio (USBA/cr) at 1 month of age were from 91 infants with mean age ± SD of 30.9 ± 3.6 days.[6] The value at the 99th percentile of 55.0 μmol/g creatinine was taken as the cutoff.

Table 1 Subject characteristics

	Median (quartile range)
Gestation age (weeks)	39 (38–40)
Body weight at birth (g)	3080 (2858–3342)
Age at sample collection (days)	25.9 (19–33)
USBA/cr (μmol/g creatinine)	11.4 (7.3–17.5)

Results

The characteristics of the 1148 infants are summarized in Table 1. At the first analysis, 47 (4.10%) of the 1148 infants had a USBA/cr level greater than the cutoff level of 55.0 μmol/g creatinine. All infants below the cutoff value in first USBA analysis were confirmed to be free of BA by the 3- to 4-month postnatal routine health check. Thirteen of the 47 infants with values above the cutoff were without jaundice or abnormally pale-colored stools and did not undergo a second analysis; they were considered to be free of BA at their 3- to 4-month postnatal health check. The remaining 34 infants were analyzed a second time within 45 days after birth; at this second analysis, 27 infants had levels that were less than the cutoff value, while seven infants had levels that remained greater than the cutoff value (Table 2). Five of these seven infants did not have BA, as determined by blood analysis and routine clinical health checks until the age of 3 to 4 months. However, the remaining two infants had liver disease; one infant was diagnosed with neonatal hepatitis syndrome, and the other infant was diagnosed with BA.

In the patient with BA, a urine sample collected on day 18 showed a USBA/cr value of 134.4 μmol/g creatinine. A transabdominal ultrasound performed on day 22 clearly revealed dilation of the gallbladder and common bile duct. Thus, the presumptive diagnosis was choledochal cyst. Because the jaundice did not disappear, a cholangiogram was performed on day 43, and the patient was diagnosed with BA type I cyst (Fig. 1). HPE was performed on day 49, and the jaundice resolved.

The end-point of the study was the 3- to 4-month postnatal routine health check. The sensitivity, specificity, positive predictive value, and negative predictive value of the USBA screening

Table 2 Outcomes for infants with elevated USBA/cr values at the second test

Patients	USBA/cr values			Blood examination					Final diagnosis	Nutrition
	First test	Second test		AST	ALT	TB	DB	TBA		
1	133.9 (18)	170.6 (31)	Day 38	47	44	1.97	0.21	10.2	Normal	Breastfeeding
2	89.9 (17)	266.3 (43)	Day 51	54	50	2.79	1.12	96.3	Infantile hepatitis	Breastfeeding
3	98.7 (20)	240.0 (40)	Day 52	28	16	3.88	0.21	10.6	Normal	Breastfeeding
4	66.2 (23)	90.8 (41)	Day 55	32	21	0.72	0.15	27.0	Normal	Mix
5	125.6 (23)	130.4 (31)	Day 48	43	47	0.69	0.22	28.4	Normal	Formula
6	66.3 (16)	92.6 (26)	Day 33	44	39	1.55	0.23	19.6	Normal	Breastfeeding
7	133.4 (18)	179.0 (22)	Day 22	98	79	7.14	4.39	130.0	Biliary atresia	Mix

() indicate urine sampling day after birth. Mix, breastfeeding + formula. USBA/cr, USBA-to-creatinine ratio.
ALT, alanine aminotransferase; AST, aspirate aminotransferase; DB, direct bilirubin; TB, total bilirubin; TBA, total bile acid.

© 2011 The Authors
Pediatrics International © 2011 Japan Pediatric Society

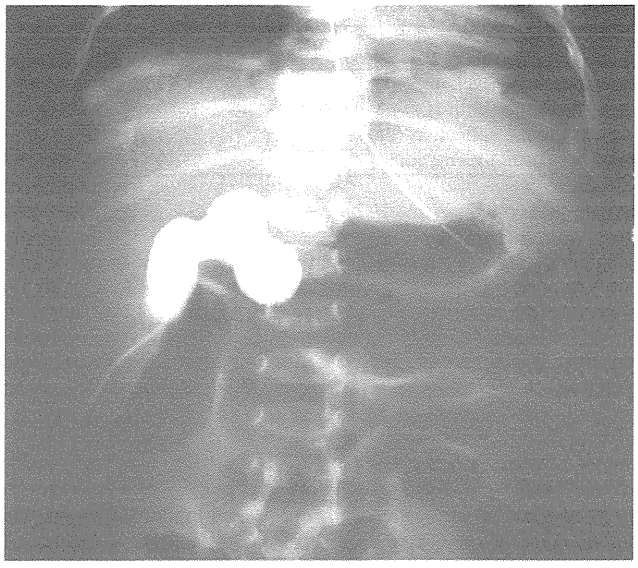


Fig. 1 Cholangiogram of the BA patient on day 43.

test in the detection of the cholestatic state in hepatobiliary disease were 100%, 96%, 4%, and 100%, respectively.

Discussion

The efficacy of two screening methods for the early detection of BA has been reported. The stool color card method devised by Matsui and Dodoriki[12] contains seven pictures of different stool colors, including three abnormal colors that represent BA. This card is included with the child health booklet. Before the routine 1-month postnatal health check, parents select the color on the card that most closely matches the color of their infant's stool and send it to a mass-screening center. Hsiao *et al.* reported that approximately 75% of all HPEs in Taiwan in 2005 were performed before the age of 60 days and were identified by use of the stool color card method.[13] The second screening method involves the measurement of direct (conjugated) bilirubin from the blood of 6- to 10-day-old infants, which is performed by public health nurses.[14,15] Direct bilirubin is a sensitive and specific marker of neonatal liver disease, including BA. However, neither screening method has achieved the goal of early HPE for all BA patients within the first 60 days of life.

The appearance of jaundice and the production of abnormally pale-colored stools are often the first indications of BA. However, infants with BA often pass cholic stool in an early phase, after which their stool becomes pale in color. According to the Japanese Biliary Atresia Registry, 72.9% (924/1267) of the parents of patients with BA initially recognized their baby's meconium as normal.[2] In addition, the parents of 30.4% (518/1702) of patients reported a normally colored stool (yellow to green) at the time of admission, and 3.5% (70/2019) of parents reported that their infant had a normal yellow stool after hospitalization. The obliteration of the bile duct in BA is probably not congenital, but instead develops postnatally as the result of several factors, such as occult viral infection, defects in morphogenesis, vascular insult, toxic agents, and maternal microchimerism.[16–18] Thus, the parents of some patients may fail to notice jaundice due to BA based on stool color alone.

USBA measurement as a screening method for BA has several advantages. First, the USBA test provides objective numerical results, while the use of stool color cards is more prone to error because parents may subjectively judge a light yellow stool to be yellow. Moreover, the numerical values provided by USBA analysis clearly identify abnormal results that exceed an accepted cutoff value. In our study, the infants whose USBA levels were less than the cutoff level did not have BA, as determined by clinical follow-up until the 3- to 4-month postnatal routine health check. A second advantage of USBA measurement is that it is a non-invasive method of detecting cholestatic syndrome; the urine samples are simply collected at home by parents. Third, increased USBA levels are not specific to BA; thus, this test can detect other cholestatic conditions including metabolic diseases such as galactosemia and tyrosinemia and hereditary diseases such as progressive familial intrahepatic cholestasis (*FIC1*, *BSEP*, and multiple drug resistance 3 gene deficiencies), alpha-1 antitrypsin deficiency, and cystic fibrosis.

In general, a re-examination rate of less than 1% is required for any mass-screening test. In Japan, mass screenings are most commonly performed to detect phenylketonuria (incidence 1:80 500), congenital adrenal hyperplasia (1:15 800), and hypothyroidism (1:3900); the re-examination rates for these tests are 0.2%, 0.3%, and 3.0%, respectively.[19–21] The high false-positive rate (greater than 3.0%) in our study is the most important obstacle to using this assay in a mass screening. Possible causes of the false-positive bias include temperature fluctuations and methods of collection, storage, transportation and measurement. The USBA assay has been reported to be applicable to chemically stable substances after freezing and long-term storage; however, the specimens lose stability after being held at temperatures above room temperature for longer than 7 days.[9,11] These results suggest that samples should be frozen immediately after collection or assayed as soon as possible to obtain reliable results. In the present investigation, the use of ordinary mail collection and delivery system with no temperature regulation and the 3- to 6-day interval between sampling and receipt at the lab may have increased the false-positive rate. Therefore, the establishment of an efficient collection and delivery system is essential to reduce the re-examination rate. In the mass screening for neuroblastoma in 6-month-old infants in Japan that ended in 2003, the cut-off level for urea creatinine was set at 5 mg/dL since the level of VMA (vanillylmandelic acid) is abnormally high in infants whose urine creatinine level is very low. Further studies are needed to establish an appropriate cut-off level for urea creatinine in order to decrease the false-positive rate.

Japanese hospitals and obstetrics clinics in parts of Nagasaki and Okinawa prefectures have already implemented the USBA method for the early detection of neonatal liver disease, including BA, using an inexpensive commercial test kit ($5/sample). Prior to the 1-month postnatal heath check, parents collect urine from their infant (in the same manner as in our study) and then deliver it immediately to their local hospital or obstetric clinic. The

© 2011 The Authors
Pediatrics International © 2011 Japan Pediatric Society

specimen is stored in a regular freezer (−4°C) for no more than 4 days until the USBA level is measured. The cut-off level for urea creatinine is 2.5 mg/dL, which corresponds to the 5th percentile at that age. The false-negative rate achieved by this system is 1%–2%. We attribute the decreased false-positive rate to improvements in sample delivery and storage prior to testing.

Although the current form of this screening method is not perfect, it is a promising start. The USBA assay may be a useful tool for the screening and early detection of BA in infants, and it also seems to be an indicator of the cholestatic state in hepatobiliary disease. Further research should focus on reducing the re-examination rates and increasing the cost-effectiveness by referring to the experiences in Nagasaki and Okinawa prefectures.

Acknowledgments

We thank Ogata Haremi (LBS Co., Ltd, Yokohama, Japan) and Adachi Kenichi (Japan Food & Liquor Alliance Co., Ltd, Kyoto, Japan) for measuring USBA levels in urine specimens.

References

1 Nio M, Ohi R, Miyano T, Saeki M, Shiraki K, Tanaka K. Five- and 10-year survival rates after surgery for biliary atresia: A report from the Japanese Biliary Atresia Registry. *J. Pediatr. Surg.* 2003; **38**: 997–1000.
2 The Japanese Biliary Atresia Society and Department of Biliary Atresia Nationwide Registry. Summary of Japanese Biliary Atresia Registry from 1989 to 2006. *J. Jpn. Soc. Pediatr. Surg.* 2008; **44**: 167–76.
3 Mieli-Vergani G, Howard ER, Portman B, Mowat AP. Late referral for biliary atresia-missed opportunities for effective surgery. *Lancet* 1989; **1**: 421–23.
4 Simko V, Michael S. Urinary bile acids in population screening for inapparent liver disease. *Hepatogastroenterology* 1998; **45**: 1706–14.
5 Obatake M, Muraji T, Satoh S, Nishijima E, Tsugawa C. Urinary sulfated bile acids: A new simple urine test for cholestasis in infants and children. *J. Pediatr. Surg.* 2002; **37**: 1707–8.
6 Matsui A, Kasano Y, Yamauchi Y *et al.* Direct enzymatic assay of urinary sulfated bile acids to replace serum bilirubin testing for selective screening of neonatal cholestasis. *J. Pediatr.* 1996; **129**: 306–8.
7 Muraji T, Harada T, Miki K, Moriuchi T, Obatake M, Tsugawa C. Urinary sulfated bile acid concentrations in infants with biliary atresia and breast-feeding jaundice. *Pediatr. Int.* 2003; **45**: 281–3.
8 Hiyama E, Iehara T, Sugimoto T *et al.* Effectiveness of screening for neuroblastoma at 6 months of age: A retrospective population-based cohort study. *Lancet* 2008; **371**: 1173–80.
9 Obatake M. New strategy for the early diagnosis of biliary atresia using urine sulfated bile acid. *Jpn. J. Pediatr. Surg.* 2008; **40**: 56–60.
10 Tazuke Y, Matsuda K, Adachi K, Tsukada Y. Purification and properties of bileacid sulfate sulfatase from *Pseudomonas testosteroni*. *Biosci. Biotechnol. Biochem.* 1994; **58**: 889–94.
11 Tazuke Y, Kodama N, Adachi K *et al.* A new enzymatic assay method of sulfated bile acids in urine. *Jpn. J. Clin. Chem.* 1992; **21**: 249–58.
12 Matsui A, Dodoriki M. Screening for biliary atresia. *Lancet* 1995; **345**: 1181.
13 Hsiao CH, Chang MH, Chen HL *et al.* Universal screening for biliary atresia using an infant stool color card in Taiwan. *Hepatology* 2007; **47**: 1233–40.
14 Keffler S, Kelly DA, Powell JE, Green A. Population screening for neonatal liver disease: A feasibility study. *J. Pediatr. Gastroenterol. Nutr.* 1998; **27**: 306–11.
15 Powell JE, Keffler S, Kelly DA, Green A. Population screening for neonatal liver disease: Potential for a community-based programme. *J. Med. Screen.* 2003; **10**: 112–6.
16 Sokol RJ, Shepherd RW, Superina R, Bezerra JA, Robuck P, Hoofnagle JH. Screening and outcomes in biliary atresia: Summary of a National Institutes of Health workshop. *Hepatology* 2007; **46**: 566–81.
17 Sokol RJ, Mack C. Etiopathogenesis of biliary atresia. *Semin. Liver Dis.* 2001; **21**: 517–24.
18 Muraji T, Hosaka N, Irie N *et al.* Maternal microchimerism in underlying pathogenesis of biliary atresia: Quantification and phenotypes of maternal cells in the liver. *Pediatrics* 2008; **121**: 517–21.
19 Hisashige A. Health economic analysis of the neonatal screening program in Japan. *Int. J. Technol. Assess. Health Care* 1994; **10**: 382–91.
20 Pang SY, Wallace MA, Hofman L. Worldwide experience in newborn screening for classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Pediatrics* 1988; **81**: 866–74.
21 Toublanc JE. Comparison of epidemiological data on congenital hypothyroidism in Europe with those of other parts in the world. *Horm. Res.* 1992; **38**: 230–35.

© 2011 The Authors
Pediatrics International © 2011 Japan Pediatric Society

Pediatr Surg Int (2011) 27:713–720
DOI 10.1007/s00383-011-2856-x

ORIGINAL ARTICLE

Physical and psychological outcome in long-term survivors of childhood malignant solid tumor in Japan

Nami Honda · Shunichi Funakoshi · Hideo Ambo · Masaki Nio · Yutaka Hayashi · Hiroo Matsuoka

Accepted: 12 January 2011 / Published online: 4 February 2011
© Springer-Verlag 2011

Abstract
Purpose Few studies have assessed physical and psychological status in long-term survivors of childhood solid tumors in Japan. For children with such diseases diagnosed and treated in our hospital, our purpose was to clarify the physical and psychological status of long-term survivors and their parents.
Methods Subjects were 56 patients who were diagnosed at our institution as having a childhood malignant solid tumor between 1982 and 2005 and had been alive for at least 5 years after treatment. Surveys were sent and returned by mail.
Results Of the 56 patients surveyed, 32 responded. The current health condition and psychosocial status of survivors were evaluated as good by their parents. However, psychological tests revealed psychosocial problems in 28.1% of the children. Severe posttraumatic stress associated with the child's disease and its treatment was present in 15.6% of the parents.
Conclusion Physical status of long-term survivors of childhood malignant solid tumors was good in general. However, psychological tests revealed psychosocial problems in some of the children and posttraumatic stress in the parents. Considering the diversity of both the diseases and their clinical course, a qualitative study is warranted for further analysis.

Keywords Childhood malignant solid tumor · Long-term survivors · Late effect · Psychological problems

N. Honda (✉) · S. Funakoshi · H. Matsuoka
Department of Psychiatry,
Tohoku University Graduate School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
e-mail: nami47@med.tohoku.ac.jp

H. Ambo
Department of Human Development and Disabilities,
Tohoku University Graduate School of Education,
27-1 Kawauchi, Aoba-ku, Sendai 980-8576, Japan

M. Nio
Department of Pediatric Surgery,
Tohoku University Graduate School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Y. Hayashi
Miyagi Children's Hospital,
4-3-17 Ochiai, Aoba-ku, Sendai 989-3126, Japan

Introduction

Advances in the treatment of childhood cancers have led to a 70% cure rate. In Japan, 1 in every 500 to 1,000 adults aged 20 or older are said to have experienced cancer as a child, a nationwide total of nearly 100,000 people. However, these childhood cancer survivors can experience problems in adjusting over the long term [1]. Hence, there are calls for an all-encompassing approach to treatment that goes beyond medical care in the narrow sense and considers all the problems arising from childhood cancer, including psychological care of patients and families, financial advice and assistance, prevention and handling of late effects, and even issues relating to education, employment, and marriage [2].

Other countries have established follow-up systems for monitoring childhood cancer survivors, and considerable research has been conducted, not only on late effects but also from a psychiatric perspective [3, 4]. Many studies have investigated quality of life (QOL) [5, 6], psychosocial problems such as somatic complaints, attention deficit

disorder, and depressive states [7–9]; even research on post-traumatic stress disorder (PTSD) considers the unpleasant and arduous experience of struggling against disease to be a traumatic experience [10–14]. The long-term follow-up system for childhood cancer in Japan is not functioning particularly well, but it has yielded some research, including reports of at least 80% of childhood cancer patients exhibiting post-traumatic stress symptoms [15] and presence of PTSD as a late effect [16], as well as QOL surveys of long-term survivors [17].

Among pediatric cancer patients, those with childhood malignant solid tumors tend to suffer adverse effects over the longest period, since these cancers tend to be discovered in infancy and involve multimodality treatment combining surgery, chemotherapy, and radiotherapy. This has prompted other countries to study the specific late effects [18–22], psychological problems [19, 20, 23–25], and educational and social problems [21, 26] associated with childhood malignant solid tumors.

Although Japan has produced a few reports on late effects of solid tumors [27, 28], with relatively high levels of satisfaction in terms of QOL reported in a small number of cases [27], it has been noted that not enough data have been accumulated for solid tumor patients compared with those with hematologic malignancies. The situation regarding long-term follow-up is also unclear.

The psychosocial problems specific to childhood malignant solid tumor patients in Japan are not yet clear, but it is highly probable that treatment received in infancy can result in late effects such as growth impairments and hearing loss, as well as psychological, educational, and social problems. In this study, we surveyed long-term survivors of solid tumors to investigate their overall life situation and psychosocial state.

Materials and methods

Participants

Of the 204 cases of childhood malignant solid tumor diagnosed by the Department of Pediatrics at Tohoku University Hospital between 1982 and 2005, there were 48 deaths (mortality rate 23.5%), and of the remaining 156 expected to survive, there were 124 survivors currently aged 4–18 who were suitable for the psychological tests described below. Of these, 35 patient's case record were lost, 11 were treated for under 5 years, 5 were follow-up case with no medical treatment, 1 had a recurrence of cancer, 3 had moved to other community, and 9 did not receive regular outpatient treatment after diagnosis at all. After all, only 56 patients who had received inpatient treatment, survived for at least 5 years, and were confirmed as alive based on outpatient history for the previous 2 years were selected as the survey target group. There were 26 males and 30 females, with a mean age of 12.0 years. The diseases were neuroblastoma ($n = 38$), Wilms tumor ($n = 10$), hepatoblastoma ($n = 5$), primitive neuroectodermal tumor ($n = 2$), and adrenal adenocarcinoma ($n = 1$). This research was approved by the Ethics Committee of Tohoku University School of Medicine and the postal survey was administered in October 2006. Parents and children were informed in writing about the study objective and that participation was up to the free will of the parents and children. Returning the questionnaire was considered to indicate informed consent.

Psychological tests

Parents were questioned about matters including the child's illness and treatment, current physical and psychological state, presence of late effects, and perception of tumor severity. We also conducted a psychological survey using the Child Behavior Checklist/4–18 (CBCL), Youth Self Report (YSR), and Japanese-Language Version of the Impact of Event Scale-Revised (IES-R-J) to ascertain the psychosocial condition of children and their parents and the presence of post-traumatic stress triggered by the illness.

The CBCL and YSR are standardized questionnaires developed by Achenbach and colleagues to provide a comprehensive assessment of emotions and behavior in children; the reliability and validity of these instruments have been confirmed [29]. Parents filled in the CBCL on their child's state, and the YSR consists of virtually identical questions for the child to answer. In Japan, the Association of Child and Adolescent Mental Health translated and standardized these tests and confirmed their reliability and validity [30]. Overall, there were 113 question items concerning the child's state at present or in the past 6 months. Each was evaluated as either "Not true (as far as you know)", "Somewhat or sometimes true", or "Very true or often true," and these were given scores of 0, 1, and 2 points, respectively. Scores were plotted to profiles by gender and by age, and were compared to the standardized T score for the scale: ≤ 59 points was the normal range, 60–63 points was the borderline range, and ≥ 64 was the clinical range. Nine symptom groups could be evaluated: "I Withdrawn", "II Somatic complaints", "III Anxious/depressed", "IV Social problems", "V Thought problems", "VI Attention problems", "VII Delinquent behavior", "VIII Aggressive behavior", and "IX Other problems". Also evaluable were 'internalizing', which combined scores for "I Withdrawn", "II Somatic complaints", and "III Anxious/depressed", and "Externalizing", which combined scores for "VII Delinquent behavior", and "VIII Aggressive behavior".

The IES-R was developed by Weiss and colleagues [31] as a self-questionnaire for measuring post-traumatic stress symptoms. It was translated into Japanese, and its reliability and validity were confirmed by Asukai and colleagues [32]. The 22 items of the IES-R measure the respondent's state in the past 7 days with regard to a highly stressful event. They include statements such as "Any reminder brought back feelings about it", "I avoided letting myself get upset when I thought about it or was reminded of it", and "I tried not to think about it"; the respondent rated each of these as "not at all", "a little", "moderately", "quite a bit", or "very much". These were scored from 0 to 4, respectively; a total score of 25 or more was considered to indicate the presence of post-traumatic stress caused by the event. The intensity of three subscales of intrusions, avoidance, and hyperarousal could also be determined: there were eight items for intrusions, including "Images of it popped into my mind" and "I had waves of strong feelings about it"; eight items for avoidance, including "I stayed away from reminders of it" and "I tried not to talk about it"; and six items for hyperarousal, including "I was jumpy and easily startled" and "Reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart". For this study we surveyed the children about symptoms in relation to "being in hospital" and the parents about symptoms in relation to their child "falling ill and being treated in hospital".

The relationship between the parent's and child's scores on the IES-R, CBCL, and YSR and age at the time of the survey, age at diagnosis, length of hospital admission, and years since discharge were analyzed using Pearson's correlation. A one-way analysis of variance was applied to the parent's IES-R, taking the parent's perception of the child's illness as the dependent variable. An unpaired t test was also carried out to compare these results with standardized data in the Japanese CBCL. SPSS 14.0J for Windows was used for statistical analysis.

Results

Responses were received from 32 of the 56 families sent the questionnaire (recovery rate: 57.1%). The children were 13 boys and 19 girls, with a mean age of 14.1 years. Diagnoses and other clinical data are presented in Table 1. Length of time in hospital was based on the parent's recollection and included periods in pediatric surgery and pediatrics departments, with the longest period being entered in the case of multiple stays. The mean stay was 3.7 months, and the median was 1.7 months, with more than half, namely 18 patients (56.3%), having been hospitalized for less than 2 months. In terms of treatment, two patients had received only chemotherapy, but biopsy by exploratory laparotomy under general anesthetic had been performed in all cases, and all children were left with abdominal scarring. The mean period from end of treatment until the survey was 11 years, and children attended outpatient follow-up appointments once every 9 months on average.

Table 1 Demographic, medical, and treatment characteristics

Variable	Survivors (n = 32)	
Age at survey (years)		
Mean ± SD	14.1 ± 3.5	
Sex (n)		
Male	13	
Female	19	
Educational status (n)		
Elementary school	9	
Junior high school	11	
High school	10	
College or University	2	
Age at diagnosis (months)		
Mean ± SD	21.4 ± 29.2	
Admission period (months)		
Mean ± SD	3.7 ± 4.8	
Period of remission (years)		
Mean ± SD	11.2 ± 3.3	
Diagnosis (n)		
Neuroblastoma	25	
Wilms' tumor	3	
Hepatoblastoma	2	
PNET	1	
Adenocortical adenocarcinoma	1	
Treatment	*N*	%
Surgery	7	21.9
Chemotherapy	2	6.2
Surgery + Chemotherapy	19	59.4
Surgery + Chemotherapy + Radiotherapy	2	6.2
Surgery + Chemotherapy + Radiotherapy + PBSCT	2	6.2

PNET primitive neuro-ectodermal tumor, *PBSCT* peripheral blood stem cell transplantation

Survey results

The child's illness was perceived as "mild" or "on the mild side" by six parents (18.7%) and as "on the serious side" or "serious" by 25 parents (78.2%). One parent did not answer.

Late effects had already appeared in five children (15.6%), with one case of growth impairment, one of spinal

deformity, and three of unilateral renal failure. Thirteen parents (40.6%) had not considered the possibility of relapse or late effects, while 14 parents (43.8%) had recognized the possibility to varying degrees.

The current physical condition of the child was rated as "good" or "on the good side" by 30 parents (93.7%) and "on the poor side" or "poor" by just 2 parents (6.2%). In general the children appeared to be in good health, apart from one child who suffered pain and difficulties in everyday life because of a spinal deformity following radiotherapy.

Regarding problems faced by the children in their current home and school life, 22 parents (68.8%) answered that there were none, while 5 (15.6%) answered that there were minor problems and 1 (3.1%) that there were major problems. However, in the comments section, parents reported a range of problems, mostly relating to surgical scars and after-effects of drug treatments and radiotherapy. Some comments also gave the impression that the child's contracting of disease had cast a shadow both psychologically and in practical terms.

When parents were asked "Who is there to support you at emotionally difficult times?" (they could give more than one answer) their partner (e.g., husband or wife) was cited in 20 cases, their mother in 10 cases, brothers and sisters in 6 cases, friends and acquaintances in 6 cases, father in 5 cases, relatives in 5 cases, mother-in-law in 1 case, work colleagues in one case, and others (church, daughter) in two cases (multiple responses included). Several responses from mothers stated that their supporting friends and acquaintances were the mothers of children in the same ward or with the same illness, and it could be assumed that mothers of children in the same ward supported each other during their children's stays in hospital.

When asked what they wanted from a support system, parents gave a wide range of answers, including material and personal support (e.g., "The ward was cramped, so space between the neighboring beds would have been good." "I would have liked for an assistant to be present to watch my child for short periods."), information provision (e.g., Information about my child's post-operative wound and why we should come into hospital." "On many occasions I wished I had been given a detailed explanation about relapse."), and counseling services for psychological support (e.g., "I think it would have helped if I would had the chance to speak to other people in the same situation". "Counseling for the patient of course, and also for the family." "I was psychologically unstable and needed advice.").

Behavioral and emotional problems of survivors of solid tumor

The CBCL reveals a child's behavioral and emotional problems from the parent's perspective (Table 2). Higher score means more behavior and emotional problems in survivors. Mean CBCL scores of survivors for internalizing, externalizing, and total problems were compared with standardized data in the Japanese CBCL. Compared with the standard group, girls' scores for externalizing (delinquent behavior, aggressive behavior) were found to be significantly lower ($t(19) = 3.422$, $p = 0.003$). Compared

Table 2 Raw score of Child Behavior Checklist/4–18

	Treatment	Sex	No. of cases	Internalizing (Mean ± SD)	Externalizing (Mean ± SD)	Total problem (Mean ± SD)
Survivors	Surgery	Male	4	7.50 ± 4.65	5.75 ± 7.23	27.00 ± 20.77
		Female	3	2.00 ± 2.00	0.67 ± 0.58	6.67 ± 1.53
	Chemotherapy	Male	2	3.00 ± 1.41	1.50 ± 0.71	9.00 ± 1.41
		Female	0			
	S + C	Male	6	4.83 ± 3.54	3.67 ± 2.25	14.33 ± 7.94
		Female	13	3.77 ± 4.87	2.92 ± 3.04	12.31 ± 10.96
	S + C + Radiotherapy	Male	0			
		Female	2	7.50 ± 0.71	0.00 ± 0.00	15.50 ± 0.71
	S + C + R + PBCST	Male	1	6.0	18.0	40.00
		Female	1	1.0	0.0	5.0
	Total	Male	13	5.46 ± 3.67	5.08 ± 5.69	19.38 ± 14.82
		Female	19	3.73 ± 4.33	2.11** ± 2.79	11.37 ± 9.43
Standard		Male	1,494	3.71 ± 4.17	5.31 ± 5.59	16.10 ± 14.47
		Female	1,579	3.77 ± 4.24	4.34 ± 4.86	14.35 ± 13.48

S surgery, *C* chemotherapy, *R* radiotherapy, *PBSCT* peripheral blood stem cell transplantation

** $P < 0.01$

with the standard group, boys' scores were high for internalizing ($p = 0.132$) and total problems ($p = 0.416$) and low for externalizing ($p = 0.883$), and girls' scores were low for internalizing ($p = 0.976$) and total problems ($p = 0.190$), but these differences were not significant.

Although it's so few cases in each treatment that mean scores may be inadequate to compare each other, the difference of mean score on each treatment may indicate psychological effect of treatment for child. Among boys, the survivors with surgery and S + C + R + PBSCT had extremely higher score of Internalizing and total problem, S + C + R + PBSCT was highest, and only surgery was higher of Externalizing than standard. Among girls, the survivors with S + C + R had very higher score of Internalizing problem, the other groups had lower score of Internalizing, and all groups had lower score of Externalizing than standard.

T score reveals clinical and borderline ranges that mean the children should receive medical treatment for their emotional and behavioral problems. In the present study, nine children (28.1%) showed scores ranging from borderline to clinical in Internalizing, Externalizing, or Total problems (Table 3). Specifically, scores were high for only Internalizing in two cases, only Externalizing in one case, Internalizing and Total problems in two cases, Externalizing and Total problems in one case, and all three measures in three cases.

The YSR reveals the child's emotional and behavioral problems as felt by the child him/herself. In the present study, four children (12.5%) had scores ranging from borderline to clinical, with high scores for only Internalizing in one case, Internalizing and Total problems in one case, Externalizing and Total problems in one case, and all three measures in one case (Table 4). Compared based on the treatment, the boys with surgery and S + C + R + PBCST showed scores ranging from borderline to clinical in Internalizing, Externalizing or Total problem and the girls with surgery, S + C and S + C + R showed that.

Post-traumatic stress of survivors and their parents

Only one child scored above 25 on the IES-R, and the mean score was very low at 4.0 (median 0.5, standard deviation 9.08). Mean scores on all subscales were also very low: 1.1 for intrusions, 2.1 for avoidance, and 1.0 for hyperarousal.

Five parents (15.6%) scored 25 or above on the IES-R, and were assumed to be continuing to experience post-traumatic stress as a result of their child's illness and hospitalization, even 10 or more years after the end of treatment. The mean score was 11.1 (median 6.5, standard deviation 11.85). The mean scores in subscales were 3.6 for intrusions (median 2.0, standard deviation 4.54), 5.4 for avoidance (median 3.0, standard deviation 5.87), and 2.1 for hyperarousal (median 0.5, standard deviation 2.99), the score for avoidance being slightly elevated. There were also seven parents (21.9) who showed no symptoms (total score = 0), while 24 (75%) were aware of a certain level of avoidance.

Factors affecting CBCL, YSR, and IES-R

There was no significant correlation between CBCL, YSR, or IES-R scores and the child's age at time of survey, length of hospital admission, or years since discharge. In addition, when looking at the relationship between the parent's IES-R score and their perception of the seriousness of the illness, awareness of the possibility of late effects, and actual presence of late effects, we found that the IES-R

Table 3 Range of Child Behavior Checklist/4–18

Treatment	Sex	No.	Internalizing			Externalizing			Total		
			Normal	Borderline	Clinical	Normal	Borderline	Clinical	Normal	Borderline	Clinical
Surgery	Male	4	2	0	2	2	1	1	2	0	2
	Female	3	3	0	0	3	0	0	3	0	0
Chemotherapy	Male	2	2	0	0	2	0	0	2	0	0
	Female	0									
S + C	Male	6	5	0	1	6	0	0	6	0	0
	Female	13	10	0	3	11	2	0	10	1	2
S + C + Radiotherapy	Male	0									
	Female	2	1	1	0	2	0	0	2	0	0
S + C + R + PBCST	Male	1	1	0	0	0	0	1	0	0	1
	Female	1	1	0	0	1	0	0	1	0	0
Total		32 (%)	25 (78.1)	1 (3.1)	6 (18.6)	27 (84.4)	3 (9.4)	2 (6.3)	26 (81.3)	1 (3.1)	5 (15.6)

S surgery, *C* chemotherapy, *R* radiotherapy, *PBCST* peripheral blood stem cell transplantation

Table 4 Range of Youth Self-Report

Treatment	Sex	No.	Internalizing			Externalizing			Total		
			Normal	Borderline	Clinical	Normal	Borderline	Clinical	Normal	Borderline	Clinical
Surgery	Male	4	4	0	0	4	0	9	4	0	0
	Female	3	2	0	1	3	0	0	2	0	1
Chemotherapy	Male	2	2	0	0	2	0	0	2	0	0
	Female	0									
S + C	Male	6	6	0	0	6	0	0	6	0	0
	Female	13	13	0	0	12	0	1	12	1	0
S + C + Radiotherapy	Male	0									
	Female	2	1	0	1	2	0	0	2	0	0
S + C + R + PBCST	Male	1	0	1	0	0	1	0	0	0	1
	Female	1	1	0	0	1	0	0	1	0	0
Total		32	29	1	2	30	1	1	29	1	2
		(%)	(90.6)	(3.1)	(6.3)	(93.8)	(3.1)	(3.1)	(90.6)	(3.1)	(6.2)

S surgery, *C* chemotherapy, *R* radiotherapy, *PBCST* peripheral blood stem cell transplantation

score was higher, but not significantly so, as perceived seriousness of the child's illness increased ($F_{(2,28)} = 0.115$, $p = 0.891$).

Discussion

Physical and psychological status of long-term survivors of malignant solid tumors and their parents.

Japan has not yet properly established a long-term follow-up system for survivors of childhood cancer across the board, and the Japanese Society of Pediatric Oncology is currently reviewing the state of the system. Follow-up of solid tumors is particularly inadequate; ours is the first Japanese study to look at the psychosocial problems associated with solid tumors, and the data from this study should prove valuable.

Three main points emerge from this study. First, the overall physical and educational circumstances of long-term survivors of childhood malignant solid tumors were generally good. This is similar to the findings by Toma and colleagues of a relatively high level of satisfaction in interviews of 20 cases [27], and to those of a study by Nathan and colleagues on neuroblastoma and Wilms tumor, which reported that survivors' physical condition was as good as that of normal individuals [20]. Studies also indicate that, for reasons unknown, neuroblastoma survivors tend to have poor educational and social status [21], and that diagnosis and treatment during very early infancy seems to impede normal social and educational development [26]. However, our survey revealed that apart from one case of spinal deformity, there appeared to be no problems in terms of education: the majority was leading a normal school life, and the CBCL and YSR comments sections gave a picture of children enjoying various activities and sports just like healthy children.

The late-effect incidence rate of 15.6% was lower than the 18% found in a previous Japanese survey [28]. In other countries, spinal deformity resulting from radiotherapy has been found to be a late effect characteristic of solid tumor [19], while 41.1% of long-term survivors of neuroblastoma have experienced musculoskeletal, neurologic, endocrine, sensory, and other late complications within 20 years after treatment, with reports that this percentage increases in patients who received two or more multimodality treatments [22]. Hearing loss is also common in neuroblastoma [22], affecting 31.4% of patients [21] and rising to 95% in advanced stage neuroblastoma [18].

The late effects found in our study were far fewer than in the previous studies. It is therefore possible that among solid tumor survivors, our survey respondents were a group whose severity of illness was low, who were treated relatively easily, and whose lives have been relatively free of any late effects.

Second, the psychological tests gave a clear picture of the children's psychological characteristics.

In CBCL, the externalizing behaviors of delinquency and aggression were significantly lower among girls when compared with the standard group. Boys' scores were slightly higher for internalizing and slightly lower for externalizing, although not significantly so.

Psychological problems identified in solid tumor survivors include somatization, anxiety, and depression [23, 24], poorer functioning for love/sex relationships, friendship, non-specific social contacts, and day-to-day coping in Wilms tumor survivors [25]; psychological problems resulting from scar formation [19]; academic and psychosocial difficulties in children with hearing loss [21];

somatoform disorder in children given radiotherapy [23, 24]; and an association between depression and alkylating agents [23, 24].

Our finding of slightly increased internalizing scores in boys indicates problems in the subscales of "withdrawal", "somatic complaints", and "anxiety/depression", with results thought to be similar to the studies cited above, which found somatoform disorders, anxiety, and depression in solid tumor survivors. Being female has been identified as one of the risk factors for psychological problems in solid tumor survivors [20, 23], but in this study the reverse was true: girls showed significantly fewer problems of delinquency and aggression than the normal group, though the reason for this is unclear.

On the CBCL, nine children (28.1%) had behavioral and emotional problems ranging from borderline to clinical. In Japan, problems in the borderline and clinical range on the CBLC are present in 25% of healthy children [30], 88% of those with psychiatric diseases [30], and 28% of those with chronic childhood diseases [33]. No disease-specific problems have been discovered in chronic childhood diseases [33], and our study was also unable to clarify the relationship between the nine cases of behavioral and emotional problems and the experience of developing and being treated for solid tumor.

Post-traumatic stress related to hospitalization and treatment was almost non-existent in these children, possibly because the experience was during infancy. However, many parents cited problems relating to scarring, and five parents stated that scarring was a cause of distress. One report found psychosocial problems resulting from scar formation in 12 of 79 solid tumor survivors [19], and the possibility was considered that the five children in our study whose parents mentioned distress were also burdened with psychosocial problems as a result of their scars.

Third, 15.6% of parents experienced post-traumatic stress in relation to their child's illness and treatment. In a survey by Pelcovits and colleagues [34], at an average of 3.3 years after the end of treatment for childhood cancer, 25% of mothers could be diagnosed with current PTSD, and 54% had met the criteria for PTSD in the past. A simple comparison with our study is not easy, since time from last treatment was over three times longer in our study and illness and treatment received were also different, but our finding is worthy of note considering that more than 10 years had passed since the last treatment. Even where PTSD was not apparent, 75% of parents noticed some kind of avoidance behavior in themselves, and many expressed anxiety about after-effects or the future, suggesting the possibility that some parents have continued to experience a state of anxiety over their child's illness.

Finally, although the long-term survivors of childhood malignant solid tumors in our study were generally in good physical condition, were receiving a normal education, and leading a normal social life, we showed that 28.1% of children exhibited psychosocial problems, some of which appeared to be the result of surgical scarring. Although the relationship between psychosocial problems and illness was unclear, the individual circumstances of the patient's family or the nature of the illness are likely to be factors, and there is a need for qualitative surveys to be performed in the future in order to more fully understand this relationship. In addition, 15.6% of parents experienced post-traumatic stress in relation to their child's illness and treatment. This means that, even if 10 years have passed since the child's treatment, some parents still show a level of stress requiring psychological therapy. Ideally, in the future, there should be consideration of long-term follow-up or approaches that take into account the psychological needs of the parent at the same time as those of the child.

Limitations

The number of subjects in our study was small, and compared with studies on solid tumors in other countries the illnesses were less serious and could treated relatively easily, and patients had fewer late effects. This suggests the high probability that the children and families currently experiencing a good quality of life may have been biased to respond to the questionnaire positively. Other possibilities considered include the fact that parents with a greater awareness of their child's problems responded to the questionnaire, and that children or parents with more intense PTSD were unable to complete a psychological survey. We therefore cannot rule out the possibility that these survey results reflect a biased cohort.

Acknowledgments This study was supported by a Grant-in-Aid from the Meiji Yasuda Mental Health Foundation.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Tanigawa K (2000) A research for education and guidance to children with cancer. The research report of Grant-in-Aid for Scientific research (Japanese)
2. Sato N, Kanai S, Matushita T (2005) Decide of patients with childhood cancer after graduation. Child Nurs 28:1227–1232 (Japanese)
3. Eshelman D, Landier W, Sweeney T et al (2004) Facilitating care for childhood cancer survivors: integration children's oncology group long-term follow-up guidelines and hearth links in clinical practice. J Pediatr Oncol Nurs 21:271–280
4. Landier W, Wallace WHB, Hudson MM (2006) Long-term follow-up of pediatric cancer survivors: education, surveillance and screening. Pediatr Blood Cancer 46:149–158

5. Hudson MM, Mertens AC, Yasui Y et al (2003) Health status of adult long-term survivors of childhood cancer. A report from the childhood cancer survivor study. JAMA 290:1583–1592
6. Meeske KA, Petel SK, Palmer SN et al (2007) Factors associated with health-related quality of life in pediatric cancer survivors. Pediatr Blood Cancer 49:298–305
7. Jurberg N, Long A, Hudson M et al (2007) Self-report of somatic symptoms in survivors of childhood cancer: effects of adaptive style. Pediatr Blood Cancer 49:84–89
8. Bizer A, Sonneville LMJ, van den Heuvel-Eibrink MM et al (2005) Chemotherapy and attentional dysfunction in survivors of childhood acute lymphoblastic leukemia: effect of treatment intensity. Pediatr Blood Cancer 45:281–290
9. Patenaude AF, Kupst MJ (2005) Psychosocial functioning in pediatric cancer. J Pediatr Psychol 30:9–27
10. Butler RW, Rizzi LP, Handwerger A (1996) A brief report: the assessment of posttraumatic stress disorder in pediatric cancer patients and survivors. J Pediatr Psychol 21:499–504
11. Erickson SJ, Steiner H (2000) Trauma spectrum adaptation. Somatic symptoms in long-term pediatric cancer survivors. Psychosomatics 41:339–346
12. Hobbie WL, Stuber M, Meeske K et al (2000) Symptoms of posttraumatic stress in young adults survivors of childhood cancer. J Clin Oncol 18:4060–4066
13. Rourke MT, Hobbie WL, Schwartz L et al (2006) Posttraumatic stress disorder (PTSD) in young adult survivors of childhood cancer. Pediatr Blood Cancer 49:177–182
14. Kazak AE, Barakat LP, Alderfer M et al (2001) Posttraumatic stress in survivors of childhood cancer and mothers: development and validation of the impact of traumatic stressors interview schedule (ITSIS). J Clin Psychol Med Set 8:307–323
15. Ozawa M (2005) Psychological reaction of families with children with cancer. Jpn J Child Adolesc Psychiat 46:120–127 (Japanese)
16. Izumi M, Ozawa M, Hosoya R (2002) Focusing on posttraumatic stress of survivors of childhood cancer. J Jpn Pediatr Soc 106:464–471 (Japanese)
17. Kamibeppu K, Ozeki S, Ikeda M et al (2007) Late effect and quality of life in survivors with childhood cancer and their families. The research report of Grant-in-Aid for Scientific research, 65-47 (Japanese)
18. Laverdiere C, Cheung NV, Kushner BH et al (2005) Long-term complication in survivors of advanced stage neuroblastoma. Pediatr Blood Cancer 45:324–332
19. Pinter AB, Hock A, Kajtar P et al (2003) Long-term follow-up of cancer in neonates and infants: a national survey of 142 patients. Pediatr Surg Int 19:233–239
20. Nathan PC, Ness KK, Greenberg ML et al (2007) Health-related quality of life in adult survivors of childhood Wilms tumor or neuroblastoma: a report from the childhood cancer survivor study. Pediatr Blood Cancer 49:704–715
21. Gurney JG, Tersak JM, Ness KK et al (2007) Healing loss, quality of life, and academic problems in long-term neuroblastoma survivors: a report from the children's oncology group. Pediatrics 120:1229–1236
22. Laverdiere C, Liu Q, Yasui Y et al (2009) Long-term outcome in survivors of neuroblastoma: a report from the childhood cancer survivors study. J Natl Cancer Inst 101:1131–1140
23. Zebrack B, Zevon MA, Turk N et al (2007) Psychological distress in long-term survivors of solid tumors diagnosed in childhood: a report from the childhood cancer survivors study. Pediatr Blood Cancer 49:47–51
24. Zeltzer LK, Recklitis C, Buchbinder D et al (2009) Psychological states in childhood cancer survivor study. J Clin Oncol 27:2396–2404
25. Mackie E, Hill J, Kondryn H et al (2000) Adult psychosocial outcome in long-term survivors of acute lymphoblastic leukaemia and Wilms' tumour: a controlled study. Lancet 335:1310–1314
26. Barrera M, Shaw AK, Speechley KN et al (2005) Educational and social late effect of childhood cancer and related clinical, personal, and familial characteristics. Cancer 104:1751–1760
27. Toma M, Matuto B, Yokoyama J et al (1999) Late effect of childhood malignant solid tumors. Child Cancer 36:203–206 (Japanese)
28. Fukushige T, Takamatsu H, Ijichi O et al (2002) Late effects of treatment for childhood malignant solid tumors. Jpn J Pediatr Surg 34:893–897 (Japanese)
29. Achenbach TM (1991) Manual for the child behavior checklist/4–18 and 1991 profile. University of Vermont, Department of psychiatry, Burlington
30. Itani T, Negishi T, Nakata Y et al (2001) Standardization of the Japanese version of the Child Behavior Checklist/4–18. J Pediatr Psychiatr Neurol 41:243–252
31. Weiss DS, Marmar CR (1997) The impact of event scale-revised. In: Wilson JP, Kaene TM (eds) Assessing psychological trauma and PTSD. The Guilford Press, New York, pp 399–411
32. Asukai N, Kato H, Kawamura N et al (2002) Reliability and validigy of the Japanese-language version of the Impact of Event Scale-Revised(IES-R-J): four studies of different traumatic events. J Nerv Ment Dis 190:175–182
33. Okuyama M (2005) An overview of pediatric consultation/liaison psychiatry. Jpn J Child Adolesc Psychiatr 46:79–89 (Japanese)
34. Pelcovits D, Goldenberg B, Kaplan S et al (1996) Posttraumatic stress disorder in mothers of pediatric cancer survivors. Psychosomatics 37:116–126

Pediatr Surg Int (2011) 27:863–870
DOI 10.1007/s00383-011-2853-0

ORIGINAL ARTICLE

Therapeutic effects of vitamin A on experimental cholestatic rats with hepatic fibrosis

Ken-ichi Murakami · Tatsuru Kaji · Ryuichi Shimono · Yoshihiro Hayashida · Hiroshi Matsufuji · Shinichiro Tsuyama · Rie Maezono · Ken-ichiro Kosai · Hideo Takamatsu

Accepted: 12 January 2011 / Published online: 3 February 2011
© Springer-Verlag 2011

Abstract
Purpose The aim of this study is to investigate the role of hepatic stellate cells (HSCs) and the effect of vitamin A administration on liver damage induced by bile duct ligation (BDL) and administration of CCl_4.
Methods Two types of animal model were used; one was BDL as a model of biliary atresia, the other was CCl_4-induced hepatic fibrosis. Pathological changes of the liver with or without administration of vitamin A were compared by light and electron microscopy with focusing on HSCs in each experimental group. Immunohistochemical examination was performed with anti-keratinocyte growth factor (KGF), anti-alpha-smooth muscle actin (α-SMA), and anti-glial fibrillary acidic protein (GFAP) antibodies, as markers of fibrosis.
Results On light microscopic findings, periportal inflammation with bile ductular proliferation was obvious in BDL group and pericentral necrosis with fatty degeneration was observed in CCl_4 group, both of which were ameliorated by subcutaneous injection of vitamin A. Electron microscopy showed lipid droplets were almost depleted in the HSCs treated with BDL or CCl_4, which improved with vitamin A administration. Immunohistochemistry demonstrated that enhanced expression of all three fibrotic markers in the BDL group was diminished by vitamin A administration.
Conclusions Although most of our data are qualitative observation, vitamin A may ameliorate hepatic fibrosis in the BDL model by restoring vitamin A in the HSCs.

Keywords Biliary atresia · CCl_4 · Hepatic fibrosis · Vitamin A · Hepatic stellate cell · GFAP

K. Murakami (✉) · H. Matsufuji
Department of Pediatric Surgery, Kagoshima University Graduate School of Medical and Dental Sciences,
8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
e-mail: mkenwood@po.synapse.ne.jp

T. Kaji · R. Shimono · Y. Hayashida
Department of Pediatric Surgery,
Kagoshima University Hospital,
8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

S. Tsuyama · R. Maezono · K. Kosai
Department of Structural Cell Biology, Kagoshima University Graduate School of Medical and Dental Sciences,
8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

H. Takamatsu
Kagoshima University Hospital,
8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Introduction

Biliary atresia (BA) is one of a number of neonatal diseases that causes obstructive jaundice. Although pathogenesis still remains to be clarified, the standard therapy for BA is hepatoportojejunostomy: the 'Kasai procedure' [1]. The Kasai procedure relieves obstructive jaundice in some patients; however, two-thirds of BA patients require a liver transplantation due to progressive hepatic fibrosis leading to hepatic failure [2]. Therefore, one of the therapeutic targets for BA patients should be to suppress the progressive hepatic fibrosis.

In the last decade, the anti-fibrotic potential of vitamin A has been recognized. In the liver, hepatic stellate cells (HSCs) store a large amount of vitamin A as lipid droplets. HSCs have been shown to be intrinsically involved in hepatic fibrosis by changing morphologically and functionally after releasing intracellular vitamin A [3–7]. Once HSCs exhaust vitamin A, they transform into fibroblasts and promote fibrosis in the liver. Thus, vitamin A

supplementation was thought to prevent hepatic fibrosis [8]. In fact, recent investigations have shown the efficacy of vitamin A in animal models with liver dysfunction induced by carbon tetrachloride (CCl_4) [8, 9], and in patients with cirrhosis [10, 11]. However, there are few studies describing the therapeutic effects of vitamin A on BA [12] and there is no scientific information regarding the effect of vitamin A on the liver histologically in animal models with obstructive jaundice.

The purpose of this study is to investigate the effect of vitamin A administration and a role of HSCs on liver damage induced by bile duct ligation (BDL) as a rat model of biliary atresia and CCl_4 administration as a model of hepatitis.

Materials and methods

Animal models

Eighty male Wistar rats, 5-week-old weighing 150–250 g, were obtained from KYUDO Co., Ltd. (Kumamoto, Japan). The rats were housed under control in appropriate cages in a quiet room adjusted for temperature (23 ± 1°C) and humidity (50 ± 10%) with a 12–12 h light–dark cycle, and were given a standard diet and water ad libitum. The rats were randomly assigned to six groups. In the BDL group ($n = 40$), the biliary duct was ligated. In the CCl_4-treated group ($n = 20$), carbon tetrachloride was administered. In the control group for BDL group ($n = 20$), simple laparotomy was performed as a sham operation. Half of these groups were given vitamin A (BDL + vitamin A, CCl_4 + vitamin A, Sham + vitamin A).

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee of Animal Research at Kagoshima University, Kagoshima, Japan.

Surgical procedure

Each rat was weighed and anesthetized by intraperitoneal administration of ketamine (60 mg/kg b.w.) and medetomidine (0.6 mg/kg b.w.). The abdomen was shaved and disinfected with 10% povidone iodine. Following a midline incision, the common bile duct was exposed and double ligatured with 3/0 silk thread. After ligation, the bile duct was sectioned between the ligatures and the abdomen was closed. The sham operation was identical to the ligation procedure, including locating and manipulating the common hepatic duct, except that the bile duct was not ligated or sectioned. The rats were maintained on a standard diet after operation and sacrificed a week later.

CCl_4-induced hepatic fibrosis

Forty percent CCl_4 dissolved in liquid paraffin was administered with subcutaneous injection twice a week for 6 weeks (1.2 g/kg b.w./day). Food and water were supplied ad libitum. The rats were sacrificed 1 week after the last administration of CCl_4.

Vitamin A administration

Vitamin A (Eisai Co., Ltd., Tokyo, Japan) 100,000 I.U./kg/day was dissolved in 0.5 ml liquid paraffin and given subcutaneously on 3 days before the operation on a daily basis, and on the 1st and 3rd day after the BDL operation. The same dose of vitamin A was given administered twice a week for 6 weeks in the CCl_4 models.

Histological examination

In all groups, the whole liver was perfused with 4% paraformaldehyde–1% glutaraldehyde mixed solution via the superior mesenteric vein. After macroscopic observation, the liver was promptly removed and post-fixed additionally with 4% paraformaldehyde–1% glutaraldehyde mixture. The tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin.

For electron microscopy, the samples were prefixed with half-strength Karnovsky's fixative for 12 h at 4°C and were postfixed with 1% osmium tetroxide buffered with 0.1 M phosphate buffer (pH 7.2), and embedded in Epon 812 (TAAB Co., Ltd., Berkshire, UK).

For immunohistochemical staining at electron microscopic level, the samples were prefixed with 4% paraformaldehyde—0.5% glutaraldehyde mixture with 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C, and embedded in Lowicryl K4 M (EMS, Fort Washington, PA, USA). For morphological observation, ultrathin sections were cut and mounted on nickel grids, stained with 2% aqueous uranyl and Reynolds' lead citrate and examined with an H-7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.

Immunohistochemical determination

Immunohistochemical staining for light microscopy

Sections were stained with an immunoperoxidase technique using a monoclonal antibody of Keratinocyte Growth Factor (KGF, Santa Cruz, CA, USA), α-smooth muscle actin (α-SMA, DAKO, Glostrup, Denmark), and glial fibrillary acidic protein (GFAP, PROGEN, Maaß strasse, Heidelberg, Germany) for immunohistochemistry, as the primary antibody.

The sections were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity at room temperature for 20 min. After PBS wash for 3 min, nonspecific binding was blocked by 1% bovine serum albumin (BSA) in PBS for 10 min. The sections were incubated with rabbit anti-KGF antibody (diluted 1:20 with 1% BSA in PBS), mouse anti-α-SMA antibody (diluted 1:100) or mouse anti-GFAP antibody (diluted 1:10) overnight at 4°C. Then, they were immersed in the respective immunoglobulin antibodies (diluted 1:200) at 37°C for 60 min. After washing with PBS, they were dipped in streptavidin–horseradish peroxidase conjugate (DAKO, Glostrup, Denmark) (diluted 1:100) at 37°C for 60 min, followed by DAB reaction. After several washings, the sections were counterstained with hematoxylin. Staining-controls were treated with PBS alone instead of primary antibodies.

Immunohistochemical staining for electron microscopy

The BDL group and BDL ± vitamin A group were immunostained for anti-GFAP antibody for electron microscopy. Briefly, ultrathin sections mounted on nickel grids were floated on PBS with 1% BSA in PBS for 15 min, and incubated with mouse anti-GFAP antibody (diluted 1:8 with 1% BSA in PBS) overnight at 4°C. Then, they were incubated with biotinylated rabbit anti-mouse immunoglobulin antibody (1:100) for 60 min at room temperature. After washing with PBS, the sections were labeled with streptavidin-colloidal gold (15 nm) complex, diluted with 1% BSA in PBS for 1 h at room temperature, counterstained with uranyl acetate and lead acetate, and examined as described above.

Morphometric analysis with anti-GFAP antibody labeling

The labeling density of anti-GFAP antibody staining in HSC was analyzed with IMAGE-J software (Ver. 1.33 u, National Institutes of Health, USA). The number of gold particles in the nucleus of hepatocytes was counted on electronmicrographs taken at ×6,000 magnification in 20 areas each in the BDL group or in the BDL + vitamin A group and compared between them. The labeling density was expressed as the number of gold particles per μm^2 of the nuclear area.

Statistics

For the assessment of the GFAP labeling-ratio of nucleus, the results were statistically analyzed by t test using Microsoft Excel software. The differences between groups were evaluated by t test. $P < 0.05$ was considered significant. The results are expressed as the arithmetic mean ± SE.

Results

In the BDL group, cystic dilatation of the common bile duct was observed at autopsy after 7 days of operation. Light microscopy showed obstructive cholestasis in the number of dilated biliary ductules. In the periportal region of the lobule, there was a proliferation of bile ductules with inflammation in the BDL group (Fig. 1a). In the CCl_4 group, necrotic changes around the central veins and fatty degeneration of the hepatocytes were seen (Fig. 1c). Administration of vitamin A appeared to ameliorate the observed hepatic histological changes in both experimental groups (Fig. 1b, d).

In the electron microscopic observations, lipid droplets in the HSCs of the BDL- and CCl_4-treated groups were smaller in size than those in control group (Fig. 2a, b). The amount of lipid droplets apparently increased almost to the normal level in both groups by administration of vitamin A (Fig. 2d, e).

KGF was expressed in the periportal area of the BDL group (Fig. 3a), but the expression of KGF was reduced in the BDL + vitamin A group (Fig. 3b). The expression of α-SMA in the BDL + vitamin A group was also less than that in the BDL group (Fig. 4a, b). In the CCl_4 + vitamin A administered group, the expressions of KGF and α-SMA were slightly weaker than those in the CCl_4 group (data not shown).

GFAP was expressed in the periportal area and around the proliferating ductules in the BDL group (Fig. 5a, b), whilst the expression of GFAP was diminished in the BDL + vitamin A group (Fig. 5c, d). Distinct localization of GFAP was observed in the nuclei of HSCs in BDL group with immunohistochemical electron microscopy (Fig. 6a–f).

Statistical analysis using IMAGE-J revealed that GFAP expression in the nuclei of HSCs in the BDL + vitamin A group was significantly lower than that in BDL group (225 ± 81 vs. 141 ± 64, $P < 0.0005$). These findings suggest that administering vitamin A suppresses the expression of GFAP in the HSCs in the BDL group (Fig. 7).

Discussion

In this study, we used two models known to induce hepatic fibrosis; one was the CCl_4-induced model, the other was a cholestatic BDL model. Generally, the BDL model exhibits a proliferation of bile ductules, and periportal cell infiltration [13], while the CCl_4 model shows necrosis of hepatocytes around the central vein and connective tissue septa linking portal canals and central veins [8, 13]. As the pathology of BA showed cholestatic change co-proliferating with reactive bile ductules [14], the BDL model was

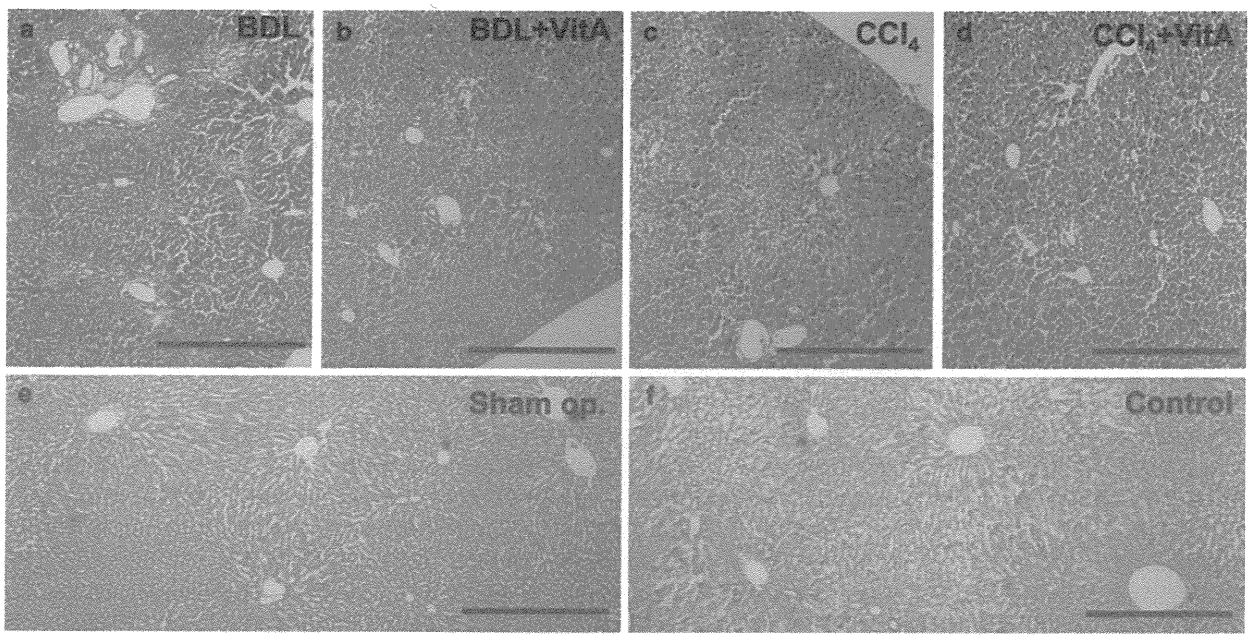
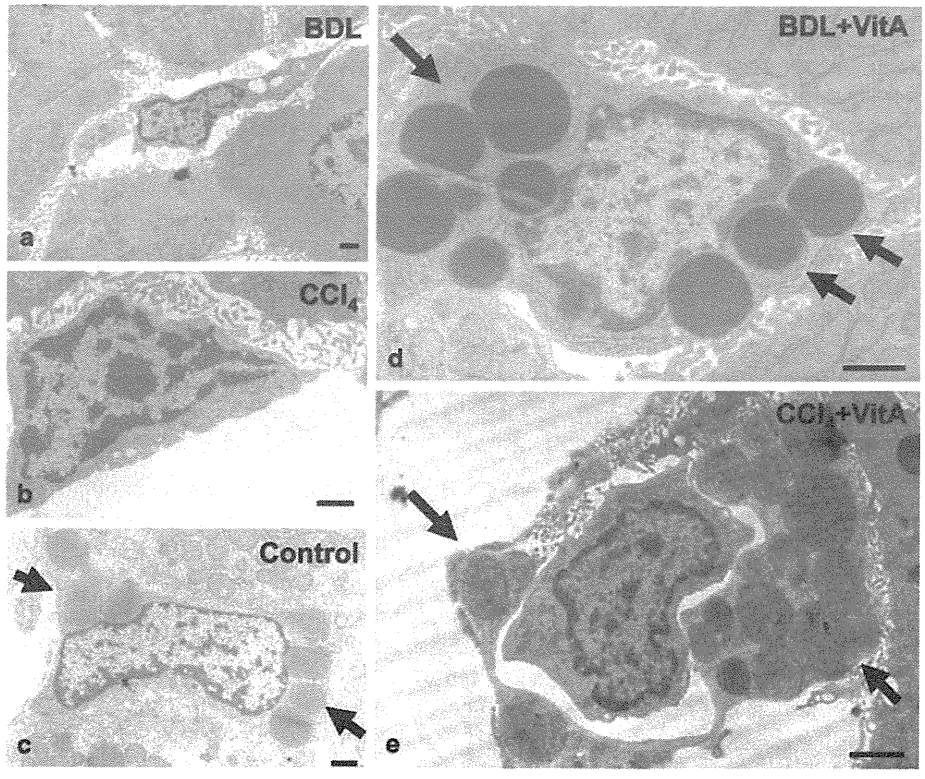


Fig. 1 Light microscopic photographs of rat liver tissues treated with BDL or CCl_4 and stained with hematoxylin/eosin. BDL or CCl_4 treatment induced a portal inflammation. Especially in the BDL-treated group (**a**), a proliferation of bile ductules was observed in comparison with sham operation group (**e**). Pericentral necrotic change was also seen in the CCl_4-treated group (**c**). Vitamin A administration improved these histological changes in both groups (**b** and **d**) to some extent in comparison with intact control (**e** and **f**). ×70 (*bar* 500 μm)

Fig. 2 Electron microscopic photographs of hepatic stellate cells. Lipid droplets (*arrows*) in HSCs in the BDL (**a**) and the CCl_4 (**b**) groups were smaller in size and number than those in the control group (**c**). Each experimental group with administration vitamin A (**d** and **e**) had more lipid droplets than that of the group without administration. **a** ×3,500, **b** ×7,000, **c** ×5,000, **d** ×8,000, **e** ×7,000 (*bar* 1 μm)



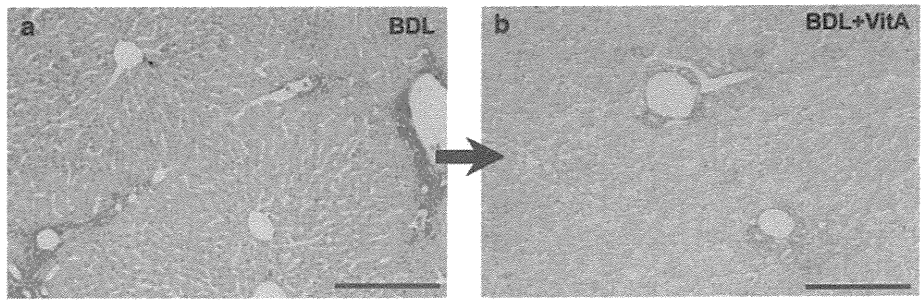


Fig. 3 Immunohistochemical staining with anti-KGF antibody and with hematoxylin counterstaining. KGF was expressed in the periportal area of the BDLtreated group (**a**), while the expression was reduced in the BDL + vitamin A group (**b**). ×175 (*bar* 200 μm)

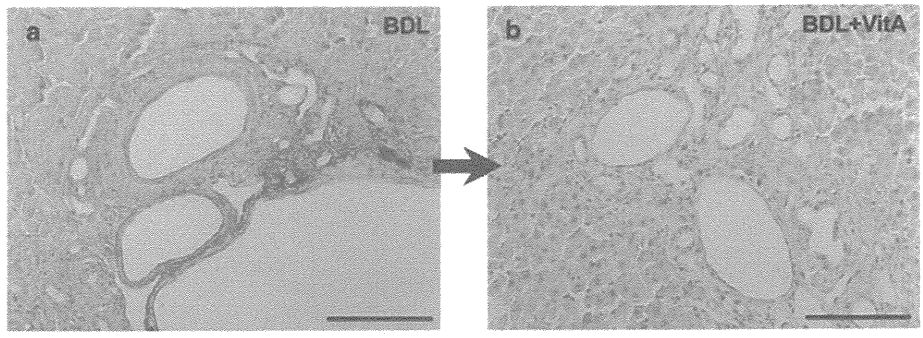


Fig. 4 Immunohistochemical staining with α-SMA antibody. α-SMA was expressed around the component cells of hepatic triad of the BDL group (**a**). The expression of α-SMA decreased in that area in BDL + vitamin A group (**b**). **a** ×350 (*bar* 100 μm), **b** ×700 (*bar* 50 μm)

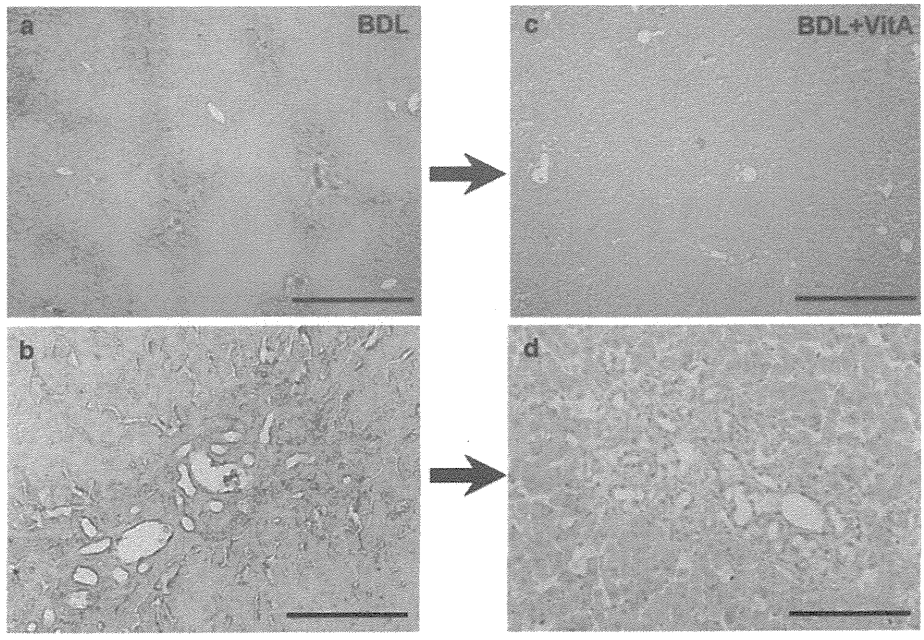


Fig. 5 Immunohistochemical staining with GFAP antibody. GFAP was intensely expressed in the periportal area and also around proliferating ductules in the BDL-treated group (**a**, **b**). The expression was diminished in BDL + vitamin A group (**c**, **d**). **a**, **c** ×70 (*bar* 500 μm), **b**, **d** ×175 (*bar* 200 μm)

considered to be representative of human BA pathology rather than the CCl_4-induced fibrotic model.

We administered vitamin A in each model, and evaluated its efficacy in repairing hepatic histological damage induced by different causes. Seifert et al. [7]. reported that vitamin A deficiency potentiates CCl_4-induced liver fibrosis. Senoo and Wake [8] reported an anti-fibrotic effect of vitamin A in the CCl_4-induced animal model. Noyan et al. [9] also investigated the suppressive effect of vitamin A on the transformation of Ito cell (HSC) into fibroblast in CCl_4-induced hepatic injury using histochemical, immunohistochemical and ultrastructural methods. However, little has been reported concerning the effectiveness of vitamin A on fibrotic change in the BDL model. Our results also confirmed that vitamin A administration is effective on the repair of liver injury induced by BDL. In order to investigate the effects of vitamin A on liver histology, we performed electron microscopy with focusing on HSCs.

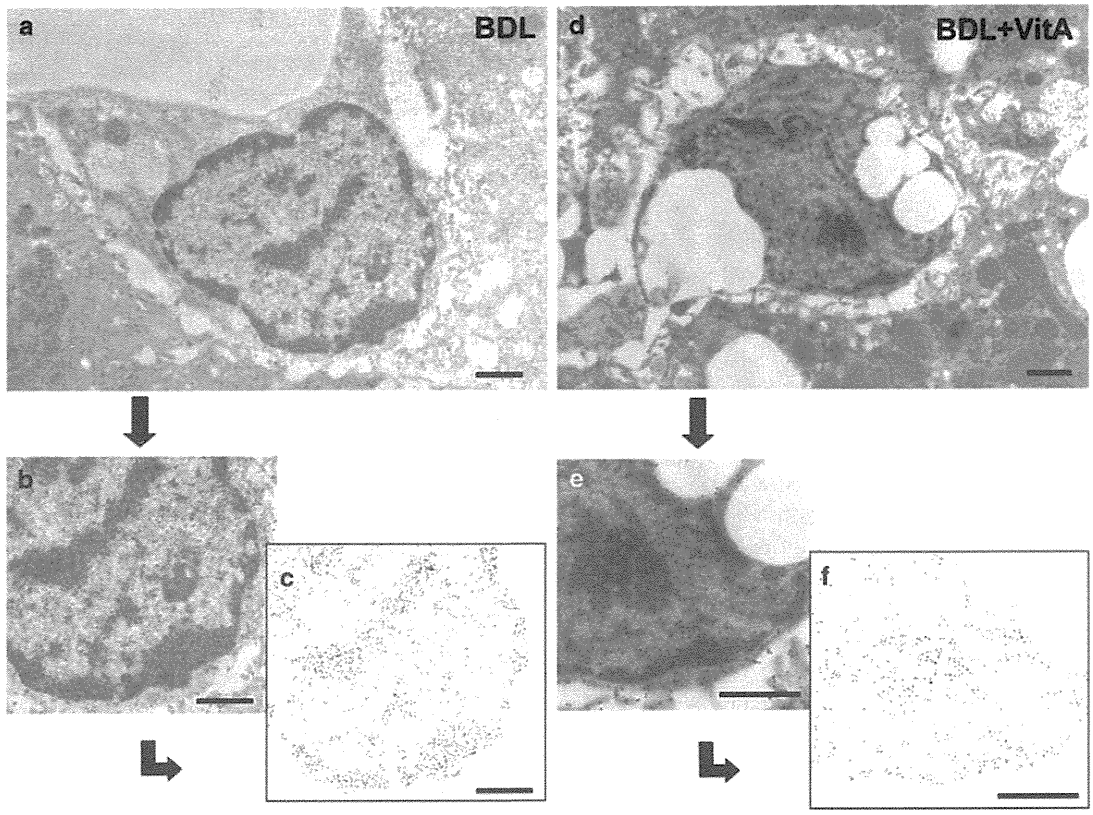


Fig. 6 Immunogold staining with anti-GFAP antibody in rat hepatocytes. The HSCs in BDL-treated group were stained with anti-GFAP antibody at electron microscopic level. The reduced expression of GFAP was observed with the vitamin A administration. Labeling gold particles in photographs **c**, **f** were extracted from **b**, **e**, and counted by "Image J software". ×6,000, **a**, **d** *bar* 1 μm; **b**, **c**, **e**, **f** *bar* 0.5 μm

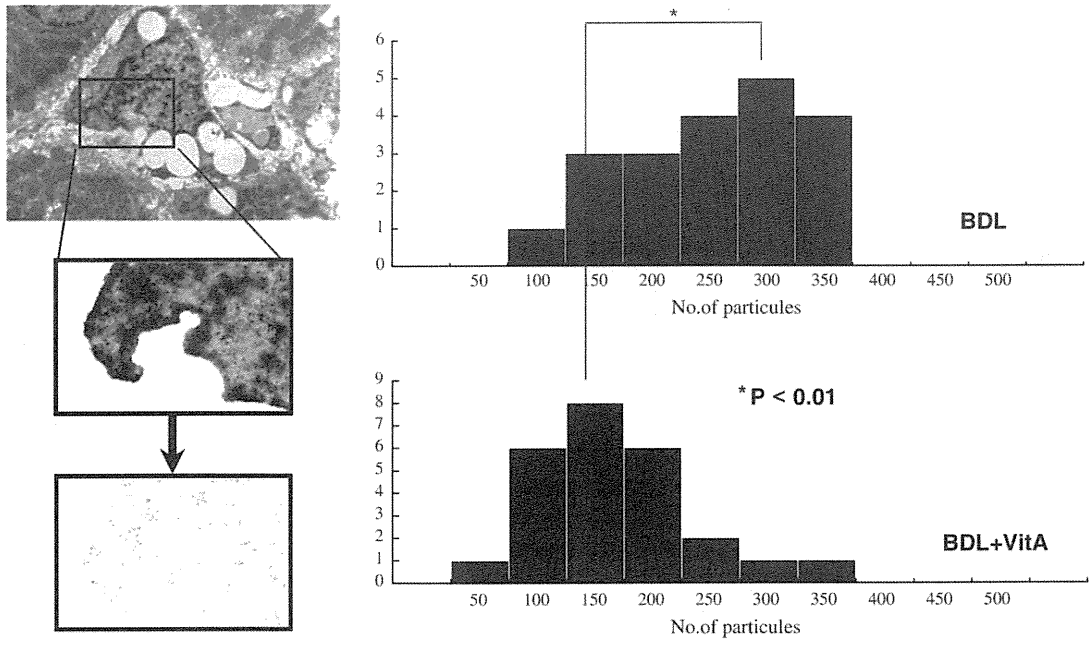


Fig. 7 Statistical analysis of GFAP plotting. GFAP expression in the nuclei of HSCs in the BDL + vitamin A group was significantly lower than in the BDL group ($P < 0.0005$)