ATIII activity ≤60% received postoperative prophylactic treatment with ATIII concentrates (ATIII[+] group), and the remaining 16 patients with preoperative ATIII activity >60% did not receive ATIII prophylaxis (ATIII[−] group). In addition to the difference in ATIII activity, Child-Pugh score was worse and splenomegaly, as shown by spleen weight and SVD, was more severe in the ATIII(+) than in the ATIII(−) group. A higher percentage of patients in the ATIII(+) group experienced surgical difficulties. Postoperative PVT was significantly less frequent in the ATIII(+) than in the ATIII(−) group (8.1% vs 43.8%; p = 0.005). Using the initial criteria based on ATIII activity alone, the overall prevalence of PVT was 19% (10 of 53 patients).

High frequency of portal vein thrombosis after splenectomy in the antithrombin III(−) group

Although we expected that patients in the ATIII(−) group would be at lower risk for PVT, the prevalence of PVT in this group was 43.8%. We had previously reported that large SVD was associated with PVT after splenectomy in patients with liver cirrhosis and portal hypertension.[19] We therefore evaluated the occurrence of PVT based on ATIII activity and SVD (Fig. 2). Portal vein thrombosis rates in patients with ATIII activity >60% but <70% and ≥70% (normal range) were 100% (3 of 3) and 30.8% (4 of 13), respectively. In the latter group, 66.7% (4 of 6) of patients with SVD ≥10 mm had PVT, compared with 0% (0 of 7) of patients with SVD <10 mm. Using ATIII activity <70% or SVD ≥10 mm as a threshold to predict the incidence of PVT had a sensitivity of 100% and a specificity of 77.8%; using ATIII activity <70% or SVD ≥9 mm, the sensitivity was 100% and the specificity was 55.6%.

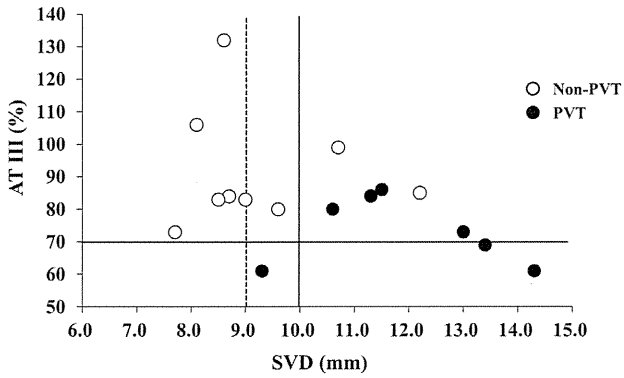

Figure 2. Relationship of portal vein thrombosis (PVT) with antithrombin III (AT III) activity and splenic vein diameter in 16 cirrhotic patients with ATIII activity >60% who received no prophylactic treatment with ATIII concentrates. Splenic vein diameter thresholds of 9 mm and 10 mm are indicated by dotted and solid lines, respectively. A threshold of 70% for ATIII activity is indicated by a solid line.

Prevention of portal vein thrombosis after splenectomy with antithrombin III concentrates and its therapeutic limitation

Although 37 patients with ATIII activity ≤60% and who received prophylactic ATIII concentrates were thought to be at higher risk for PVT after splenectomy, PVT developed in only 3 (8.1%) (Fig. 3). Of the first 21 patients, treated from April 2008 to December 2009, PVT developed in 3, despite prophylactic treatment with ATIII concentrates. Portal vein thrombosis was detected in these 3 patients by CT on POD 7, followed by immediate intravenous administration of danaparoid sodium (2,500 U/day) for 14 days followed by warfarin for 3 months, until there were no indications of PVT. These findings indicated that prophylactic ATIII cannot always prevent PVT after splenectomy. At a threshold of SVD ≥15 mm, the sensitivity and specificity for predicting PVT were 66.7% and 94.4%, respectively; at a threshold of SVD ≥13 mm, the sensitivity and specificity were 100% and 77.8%, respectively. These findings indicated that ATIII monotherapy was not suitable as a primary prophylaxis for patients with SVD ≥15 mm. We therefore modified treatment of these patients, starting with ATIII concentrates (1,500 U/day for 3 days), followed by danaparoid sodium (2,500 U/day for 14 days) and subsequent warfarin for 3 months or until PVT was completely eliminated. Of the 16 patients

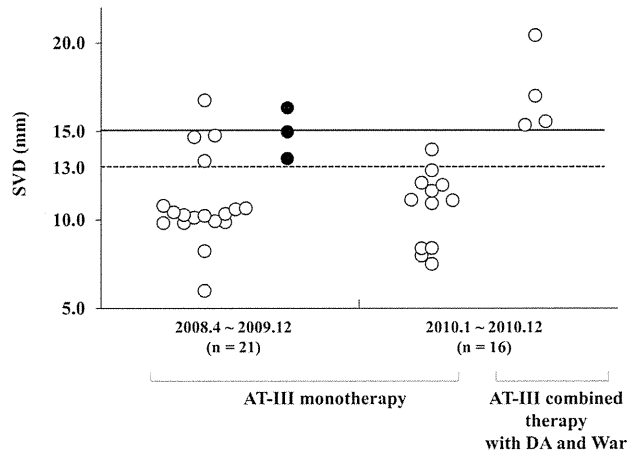

Figure 3. Relationship of portal vein thrombosis (PVT) to splenic vein diameter (SVD) in 37 cirrhotic patients with antithrombin III (ATIII) activity ≤60% who received prophylactic treatment with ATIII concentrates alone (ATIII monotherapy) or ATIII concentrates followed by danaparoid sodium (DA) and warfarin (War) (ATIII combined therapy with DA and War). Splenic vein diameter thresholds of 13.0 mm and 15.0 mm are indicated by dotted and solid lines, respectively. White dot, non-PVT; black dot, PVT.

treated from January to December 2010, four patients with SVD ≥15 mm received this schedule of prophylactic treatment, with none having PVT after the operation.

Risk stratification of portal vein thrombosis after splenectomy in patients with liver cirrhosis and portal hypertension

Based on the results of the testing cohort, we stratified the risk level of PVT after splenectomy in patients with liver cirrhosis and portal hypertension. Low risk was defined as ATIII activity ≥70% and SVD <10 mm; high risk as ATIII activity <70% and/or SVD ≥10 mm; and highest risk as SVD ≥15 mm (Table 2). Although patients at low risk received no prophylactic treatment for PVT, those at high risk received ATIII monotherapy, and those at highest risk received ATIII combined therapy, followed by danaparoid sodium and warfarin.

Validation cohort

Validation of risk stratification of portal vein thrombosis after splenectomy in patients with liver cirrhosis and portal hypertension

Table 3 shows the characteristics of the 57 patients in the validation group categorized by risk level of PVT after splenectomy. Only 2 (3.5%) of these patients had PVT under the new classification, a prevalence significantly lower than under the initial criteria ($p = 0.013$). None of the 14 patients classified as low risk experienced PVT, despite the lack of prophylaxis (Fig. 4). Of the 32 patients at high risk for PVT who received ATIII monotherapy, only 2 (6.3%) experienced PVT. Among the 11 patients at highest risk for PVT who received ATIII combined therapy followed by danaparoid sodium and warfarin, 8 showed partial and temporal PVT, extending from the splenic vein to the splenoportal confluence, on CT by POD 7, with PVT disappearing in all 8 by 3 months after splenectomy. None of the other 3 patients showed evidence of PVT after splenectomy, although they were maintained on warfarin for 3 months after the operation.

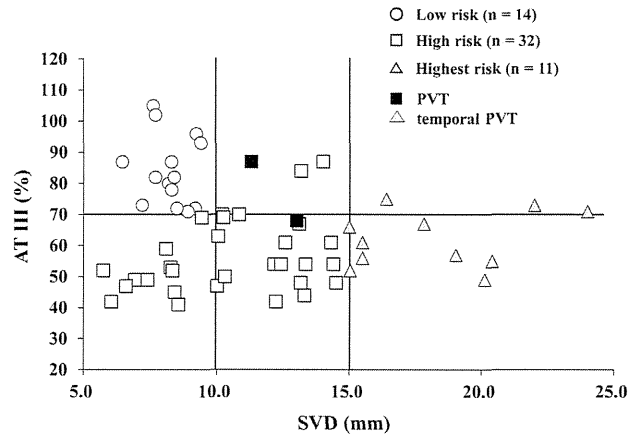

Figure 4. Relationship of portal vein thrombosis (PVT) with antithrombin III (AT III) activity and splenic vein diameter (SVD) in 57 cirrhotic patients who received prophylactic treatment of PVT according to the risk level of PVT after splenectomy. Fourteen patients at low risk received no prophylactic treatment, 32 patients at high risk received ATIII concentrates, and 11 patients at highest risk received ATIII concentrates followed by danaparoid sodium and warfarin. Thresholds of 70% for ATIII activity and 10.0 mm and 15.0 mm for SVD are indicated by solid lines.

DISCUSSION

Our previous study demonstrated that ATIII activity plays a crucial role in the development of PVT after laparoscopic splenectomy in cirrhotic patients.[18] Preoperative ATIII activity was found to be an independent predictor of PVT after laparoscopic splenectomy. The level of ATIII activity was significantly lower on PODs 1 and 4 than before surgery, but recovered to the preoperative level by POD 7, consistent with the observation that most PVTs developed within 7 days after splenectomy. Administration of ATIII concentrates (1,500 U/day) for 3 days corrected ATIII activity to near-normal range, as well as dramatically reduced the incidence of PVT after splenectomy. Therefore, the preoperative decrease in ATIII activity and its additional reduction during the early postoperative phase contribute to the development of PVT. In liver cirrhosis, hemostatic balance is fragile and easily tips to either a hypo- or hypercoagulable state.[7-9] After surgery, the decrease in hepatic synthesis of anticoagulants, such as ATIII, and their increased consumption due to intravascular coagulation can lead to a hypercoagulable state in the splanchnic and systemic circulation. Prophylactic administration of ATIII concentrates can return a hypercoagulable status to equilibrium between pro- and anticoagulants, but not to a hypocoagulable status. Therefore, despite being administered the day after surgery, ATIII concentrates do not contribute to bleeding complications.

In the testing cohort, we based the need for PVT prophylaxis on a cutoff level of preoperative ATIII activity. As ATIII activity decreases postoperatively, postoperative ATIII activity might be more accurate for predicting PVT than preoperative activity. However, ATIII activity could not be monitored on weekends in our hospital, and administration of ATIII concentrates should be started as soon as possible after splenectomy. Therefore, it might be more convenient to measure pre- than postoperative ATIII activity. Calculating ROC curves for the 25 patients included in the previous study and increasing the sensitivity to reduce the likelihood of false negatives resulted in our initial criterion, in which ATIII concentrates were administered to patients with ATIII activity ≤60%. Of the 53

patients in our testing cohort, 37 had ATIII ≤60% and received prophylactic treatment with ATIII concentrates, with PVT developing in only 3 (8.1%). However, of the 16 patients with ATIII >60%, considered at lower risk for PVT, PVT developed in 7 (43.8%) in the absence of prophylactic treatment. Of the 25 patients in the previous study, 12 had ATIII >60%, PVT developed without ATIII prophylaxis in only 1 (8.3%). The discrepancy between the current and previous studies might result from differences in potential risk levels, except for ATIII activity, in the 2 patient populations. Our other previous study showed that large SVD, which was associated with a decrease in portal venous flow after splenectomy, and low white cell counts (<2,000/μL) were risk factors for PVT after splenectomy.[19] In the current and previous studies, none of the patients with ATIII activity >60% had low white cell counts (<2,000/μL), and the mean spleen weight in patients with ATIII >60% was significantly greater in this study than in our previous study (473 ± 197 g vs 297 ± 160 g; p = 0.014), indicating that patients with ATIII >60% in this study had larger SVDs than in the previous study. Additionally, 6 of the 37 patients with ATIII ≤60% had low white cell counts (<2,000/μL), but PVT did not develop after ATIII prophylaxis in any of these patients. Therefore, this study assessed combinations of ATIII activity and SVD, but not white cell counts as risk factors for PVT. Using ATIII activity <70% or SVD ≥10 mm as a threshold to predict the incidence of PVT yielded a sensitivity of 100% and a specificity of 77.8%, and using ATIII activity <70% or SVD ≥9 mm had a sensitivity of 100% and a specificity of 55.6% (Fig. 2). These findings are consistent with our previous results, showing that SVD ≥9 mm is a risk factor for PVT after splenectomy,[19] although the cutoff level in this study is likely between 9 and 10 mm. We therefore defined high risk for PVT as ATIII activity <70% or SVD >10 mm.

Before the start of this study, we did not consider administering ATIII concentrates to all splenectomized patients, regardless of their potential risks of PVT, but wanted to identify patients who did not require ATIII prophylaxis, because of the potential biologic hazards and expense of ATIII concentrates. Although we failed to detect patients at lower risk for PVT in the testing cohort, none of the 14 patients in the validation cohort considered as at low risk for PVT according to risk stratification had PVT. Risk stratification showed that 7 (13%) of the 53 patients in the testing cohort, compared with 14 (25%) of the 57 patients in the validation cohort, were at low risk for PVT. Even in different patient populations, risk stratification of PVT can accurately identify patients who do or do not require ATIII prophylaxis.

Of the 37 patients in our testing cohort with ATIII ≤60% who received ATIII concentrates, PVT developed after splenectomy in only 3 (8.1%). In addition, PVT developed in only 1 of 30 (3.3%) patients with SVD <15 mm, compared with 2 of 3 patients with SVD ≥15 mm (Fig. 3). This result indicated that, in patients with SVD <15 mm, correction of a hypercoagulable state with ATIII concentrates can overcome the effects of decreased portal venous flow. Of the 3 patients with postoperative PVT despite prophylactic ATIII monotherapy, 2 had supermassive splenomegaly with SVD ≥15 mm, and 1 had an SVD of 13.5 mm and huge hepatofugal collateral vessels of 14 mm in diameter. Doppler US showed that postoperative portal venous flow was <50% of preoperative flow in these patients, suggesting that patients with SVD ≥15 mm or huge hepatofugal collateral vessels are at highest risk for PVT because ATIII monotherapy cannot overcome the great decrease in portal venous flow (Table 2). However, the diameter of collateral vessels predictive of PVT can be difficult to determine because the extent of portal venous flow through collateral vessels can depend on their location, number, size, and/or intrahepatic portal vascular resistance. Patients with huge collateral vessels, ≥10 mm in diameter, require systematic screening with repeated Doppler US and/or CT (Table 2). In the validation cohort, PVT developed in only 2 of 32 (6.3%) patients classified as at high risk for PVT (Fig. 4). Stratification of PVT risk level using 2 indicators, ATIII activity, which is associated with a hypercoagulable state, and SVD, which is related to decreased portal venous flow, was more specific and more correct than stratification by ATIII activity alone. In addition, ATIII monotherapy can prevent PVT in most patients at high risk.

We found that 3 patients in the testing cohort who were treated early, from April 2008 to December 2009, had PVT develop despite ATIII monotherapy (Fig. 3). These patients were started on intravenous danaparoid sodium (2,500 U/day) just after detection of PVT. Treatment for 14 days abolished PVT in the portal trunk, although thrombosis remained in the splenic vein. Warfarin was subsequently administered for 3 months until the thrombosis in the splenic vein was eliminated, preventing the recurrent extension of splenic vein thrombosis to the portal trunk. Patients with SVD ≥15 mm were considered at highest risk for PVT, with ATIII monotherapy not always sufficient to prevent PVT. The 15 patients at highest risk for PVT, who were treated from January 2010 to September 2013, received ATIII combined therapy, followed by danaparoid sodium and warfarin (Figs. 3 and 4). Eight of these patients showed partial PVT transiently extending from the splenic vein thrombosis to the splenoportal confluence by CT on POD 7, but disappearing within 3 months after splenectomy. Portal vein thrombosis never developed in the

remaining 7 patients. These findings suggest that prophylaxis for patients at highest risk for PVT is more therapeutic than prophylactic. Although better methods might prevent PVT, our prophylactic regimen for highest risk for PVT might be acceptable, eradicating PVT without any bleeding complications. Additionally, this combined regimen successfully eradicated PVT in 7 patients who received no prophylactic treatment and in 5 who received ATIII prophylaxis. Antithrombin III combined therapy with danaparoid sodium and warfarin is promising for the prevention or treatment of PVT after splenectomy, with an excellent safety profile.

Danaparoid sodium, rather than a continuation of ATIII concentrates, was used for prophylaxis of patients at highest risk for PVT or to treat PVT. Our previous study showed that administration of ATIII concentrates for 3 days maintained ATIII activity at a near-normal level (70%) up to POD 7.[18] Higher-dose ATIII concentrates and/or continued treatment, resulting in supranormal activity, did not have beneficial effects in patients with sepsis or disseminated intravascular coagulation.[21,22] As administration of ATIII concentrates to supranormal levels is associated with a potential bleeding risk and is very costly, we used the low-molecular weight heparinoid danaparoid sodium as an anticoagulant. Three classes of heparins were also available: unfractionated heparin (UFH), low-molecular weight heparins (LMWHs), such as enoxaparin and the synthetic pentasaccharide fondaparinux. The anticoagulant effects of these drugs depend on anti-Xa and anti-thrombin activities. The ratios of anti-Xa to anti-thrombin activity are about 1 for UFH; about 4 for enoxaparin; $\geq$22 for danaparoid sodium; and about 7,400 for fondaparinux. The anticoagulation effects of danaparoid sodium and fondaparinux are characterized by higher selectivity for factor Xa compared with UFH and enoxaparin. Direct inhibition of thrombin by UFH and enoxaparin inhibits thrombus formation and can increase bleeding risk. Low-molecular weight heparins and especially UFH have potential risks of heparin-induced thrombocytopenia, and danaparoid sodium and fondaparinux do not. Although there is limited evidence on the use of these drugs in patients with liver cirrhosis and PVT, LMWHs such as enoxaparin appear to be safe and effective in prophylactic or therapeutic treatment of PVT, even in patients with liver cirrhosis.[23-25] However, these heparins require ATIII to exert their anticoagulant effects and their efficacy can be unpredictable in cirrhotic patients with decreased ATIII activity.[7,9] Using in vitro thrombin generation assays of plasma from patients with liver cirrhosis, LMWHs were found to amplify anticoagulant effects, despite reductions in ATIII activity, especially in patients with Child-Pugh class C.[26,27] Additionally, a reduced anticoagulant response to fondaparinux was observed in plasma from patients with live cirrhosis.[26] The ratios of anti-Xa to anti-thrombin activity and the results presented here suggest that danaparoid sodium can be as effective as LMWHs, as well as safer, in preventing or treating PVT in patients with liver cirrhosis.

Patients with highest risk for PVT or those with substantial PVT require long-term treatment with anticoagulants until the thrombosis in the splenic vein disappears. Because long-term intravenous administration of danaparoid sodium is inconvenient, patients were switched to oral warfarin for up to 3 months. Although long-term administration of warfarin eradicated PVT without bleeding complications, warfarin has a narrow therapeutic window and requires frequent monitoring and dose adjustments. In addition, warfarin has been associated with a higher bleeding risk than LMWHs in patients with liver cirrhosis, especially in those with thrombocytopenia (platelet counts $<50 \times 10^3/\mu L$).[24] The optimal warfarin dose in patients with high prothrombin time and INR is unclear.[9] Warfarin can reduce the concentrations of vitamin K-dependent anticoagulant factors, such as protein C, potentially increasing the risk for thrombosis.[8] Fortunately, in our patient population, platelet counts were $>100 \times 10^3/\mu L$ on POD 14,[28] and end-stage liver cirrhosis did not develop in any patient, as indicated by prothrombin time and INR >2.0. Two novel oral anticoagulants have recently been approved for clinical use: rivaroxaban, a direct inhibitor of factor Xa; and dabigatran, a direct inhibitor of factor IIa.[9] Both drugs are independent of ATIII, do not require dose adjustment, and have a wider therapeutic range in the general population. To date, only 2 case reports have described the successful treatment of PVT with rivaroxaban of 1 cirrhotic and 1 noncirrhotic patient.[29,30] In vitro thrombin generation assays using plasma from cirrhotic patients showed a reduced anticoagulant response to rivaroxaban and a substantially increased anticoagulant response to dabigatran.[26] Although both agents are promising pharmacologic drugs, large cohorts are necessary to assess their efficacy and safety in cirrhotic patients with coagulation disorders.

CONCLUSIONS

We optimized risk stratification of PVT after splenectomy in patients with liver cirrhosis and portal hypertension and developed prophylactic treatments of PVT centered on ATIII concentrates. Classification of risk level can reduce the incidence of PVT after splenectomy and accurately identifies patients who do not require prophylaxis of PVT. Antithrombin III, as monotherapy or combined therapy, followed by danaparoid sodium and warfarin, is

safe and effective for both primary prophylaxis of PVT and treatment of PVT after splenectomy. These regimens warrant clinical trials for prevention of PVT after splenectomy or treatment of de novo PVT in patients with liver cirrhosis.

Author Contributions

Study conception and design: Kawanaka, Akahoshi, Shirabe, Takenaka, Maehara

Acquisition of data: Kawanaka, Akahoshi, Itoh, Iguchi, Harimoto, Uchiyama, Yoshizumi, Shirabe

Analysis and interpretation of data: Kawanaka, Akahoshi, Itoh, Iguchi, Harimoto, Uchiyama, Yoshizumi, Shirabe

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Critical revision: Kawanaka, Akahoshi, Shirabe, Takenaka, Maehara

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Role of per-rectal portal scintigraphy in long-term follow-up of congenital portosystemic shunt

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BACKGROUND: Congenital portosystemic shunt (CPSS) has the potential to cause hepatic encephalopathy and thus needs long-term follow-up, but an effective follow-up method has not yet been established. We aimed to evaluate the importance of per-rectal portal scintigraphy (PRPS) for long-term follow-up of CPSS.

METHODS: We retrospectively examined shunt severity time course in patients (median: 9.6 y, range: 5.2–16.6 y) with intrahepatic (n = 3) or extrahepatic (n = 3) CPSS by using blood tests, ultrasonography or computed tomography, and PRPS. Per-rectal portal shunt index (cutoff: 10%) was calculated by PRPS.

RESULTS: PRPS demonstrated that the initial shunt index was reduced in all intrahepatic cases (from 39.7±9.8% (mean ± SD) to 14.6±4.7%) and all extrahepatic cases (from 46.2±10.9 to 27.5±12.6%) during the follow-up period. However, ultrasonography and computed tomography disclosed different shunt diameter time courses between intrahepatic and extrahepatic CPSSs. Initial shunt diameter (5.8±3.5 mm) reduced to 2.0±0.3 mm in intrahepatic cases, but the initial diameter (6.3±0.7 mm) increased to 10.6±1.0 mm in extrahepatic cases. All patients had elevated serum total bile acid or ammonia levels at initial screening, but these blood parameters were insufficient to assess shunt severity because the values fluctuate.

CONCLUSION: PRPS can track changes in the shunt severity of CPSS and is more reliable than ultrasonography and computed tomography in patients with extrahepatic CPSS.

Congenital portosystemic shunt (CPSS), which is a major cause of neonatal hypergalactosemia without galactose-metabolizing-enzyme deficiency (1), causes brain manganese deposition, pulmonary hypertension, and hyperammonemia leading to hepatic encephalopathy (2–8). CPSS is generally suspected if serum total bile acid (TBA) and ammonia levels are elevated, and it is diagnosed by using color Doppler ultrasonography, dynamic contrast-enhanced computed tomography (CT), and per-rectal portal scintigraphy (PRPS) (7,9–12). Some shunts close spontaneously, whereas others need to be closed surgically or with embolization because of hyperammonemia leading to severe hepatic encephalopathy (7,8,13–15). Therefore, it is important to follow up CPSS patients carefully with color Doppler ultrasonography, dynamic contrast-enhanced CT, and blood tests. Despite this, there is no gold standard for accurately assessing the degree of shunt. Color Doppler ultrasonography and dynamic contrast-enhanced CT are useful for detecting shunt location and for assessing shunt diameter and flow, but these imaging modalities cannot be used to evaluate shunt severity quantitatively. By contrast, PRPS can be used to calculate a shunt index (SI) for quantifying shunt severity, as previously reported (9). Currently, the use of PRPS is limited to the diagnosis of CPSS, and its application to long-term follow-up of CPSS is uncertain. Here, we aimed to clarify the role for PRPS in the long-term follow-up of patients with CPSS by retrospectively evaluating changes in shunt severity over time as assessed with PRPS.

RESULTS

Patients

Six patients (mean age: 9.6 y; range: 5.2–16.6 y) were diagnosed as having CPSS during the first year of life. On the basis of ultrasonography and dynamic contrast-enhanced CT, three of the six patients were identified as having intrahepatic CPSS, and the remaining three patients were found to have extrahepatic CPSS (**Table 1**). None of the six patients had abnormalities in the abdominal cavity, including hepatic tumors. Two of the patients with intrahepatic CPSS had shunts between the left portal vein and the central hepatic vein, and one had a shunt between the left portal vein and left hepatic vein (**Table 1**). Of the patients with extrahepatic CPSS, two had splenorenal shunts (**Table 1**; **Figure 1a,b**), and one had a mesocaval shunt (**Table 1**; **Figure 1c**; **Supplementary Video S1** online). All six patients had normal mental development without hepatic encephalopathy and showed absence of pulmonary hypertension.

Shunt Diameter

Ultrasonography or dynamic contrast-enhanced CT, or both, disclosed a difference between intrahepatic and extrahepatic CPSSs in terms of changes in shunt diameter over time. All three

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Table 1. Profiles of six children with congenital portosystemic shunt

Patient no.	Age at initial examination (mo)	Sex	Shunt location	Initial laboratory findings				
				Galactose[a] (<8 mg/dl)	TBA (<10 μmol/l)	NH_3 (30–80 μg/dl)	AST (20–70 IU/l)	ALT (10–70 IU/l)
1	0.5	Male	Intrahepatic (LPV–LHV)	14.97	37	178	23	18
2	1	Male	Intrahepatic (LPV–CHV)	8.9	44	89	41	28
3	1	Male	Intrahepatic (LPV–CHV)	11.6	56	141	30	19
4	1	Male	Extrahepatic (SV–LRV)	8.0	92.2	52	24	13
5	1	Male	Extrahepatic (SV–LRV)	8.0	184	94	55	40
6	1	Male	Extrahepatic (IMV–IIV)	7.4	58	80	38	23

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHV, central hepatic vein; IIV, internal iliac vein; IMV, inferior mesenteric vein; IU, international units; LHV, left hepatic vein; LPV, left portal vein; LRV, left renal vein; SV, splenic vein; TBA, total bile acid.
[a]Galactose was evaluated within 45 d after birth by newborn mass screening.

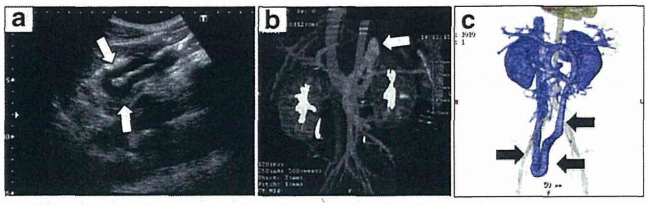

Figure 1. Ultrasonography and computed tomography (CT) portal venography of extrahepatic congenital portosystemic shunt patients. (**a**) Splenorenal shunt (arrow) was clearly visualized in the sagittal view on ultrasonography (16 y). (**b**) Splenorenal shunt (arrow) on maximum intensity projection image of CT portal venography (1.5 y). (**c**) Mesocaval shunt (arrow) on three-dimensional volume-rendered image of CT portal venography (5 y).

intrahepatic CPSS patients showed spontaneous shunt regression. The initial shunt diameter of 5.8 ± 3.5 mm (mean ± SD) at 0.7 ± 0.5 y of age was reduced to 2.0 ± 0.3 mm at 6 ± 2.6 y of age—a reduction of 59.6 ± 16.3% over 5.2 ± 2.8 y (**Figure 2a–c**). By contrast, the shunt diameter increased in all three extrahepatic CPSS patients: the initial shunt diameter of 6.3 ± 0.7 mm at 0.6 ± 0.2 y of age increased to 10.6 ± 1.0 mm at 11.3 ± 5.6 y of age—an increase of 70.0 ± 25.8% over 10.6 ± 5.6 y (**Figure 2d–f**).

Shunt Index

PRPS was performed in each patient two to four times at a median interval of 4.5 y (range: 1.4–6.7 y). For all six patients, the SI derived from PRPS decreased from 42.9 ± 9.9% (mean ± SD, cutoff: 10%) at 0.9 ± 0.8 y of age to 21.1 ± 11.1% at 9.3 ± 4.9 y of age (**Figure 2**; **Supplementary Figure S1** online)—a decrease of 51.8 ± 18.9% over 8.3 ± 5.2 y. In the patients with intrahepatic CPSS, the initial SI of 39.7 ± 9.8% at 1.2 ± 1.1 y of age decreased to 14.6 ± 4.7% at 6.6 ± 2.6 y of age (**Figure 2a–c**; **Supplementary Figure S1** online)—a reduction of 60.7 ± 19.9% over 5.4 ± 3.1 y. In the patients with extrahepatic CPSS, the initial SI of 46.2 ± 10.9% at 0.6 ± 0.4 y of age decreased to 27.5 ± 12.6% at 11.8 ± 5.6 y of age (**Figure 2d–f**; **Supplementary Figure S1** online)—a reduction of 42.9 ± 16.3% over 11.3 ± 5.7 y.

Blood Tests

Hypergalactosemia was identified in all six patients by newborn screening (**Table 1**). At the initial examination at our hospital, serum levels of TBA or ammonia were elevated in all six patients (**Table 1**). No elevations of aspartate aminotransferase and alanine aminotransferase levels were found in any of these patients. During the follow-up period, galactose levels decreased to within the normal range by 2 y of age despite persistent elevation of TBA and ammonia levels (**Figure 3**). The persistently high values of TBA or ammonia suggested the presence of a shunt, but the values fluctuated because of intestinal motility and changes in the types of meals consumed before fasting, limiting the use of these blood parameters in the quantitative assessment of shunt severity.

DISCUSSION

We retrospectively evaluated the long-term clinical course of CPSS patients, focusing on the degree of shunt as determined with PRPS. Various imaging modalities and blood tests indicated that the natural histories of intrahepatic and extrahepatic CPSSs differed. Intrahepatic CPSS without hepatic tumor has been reported to spontaneously close or regress, whereas extrahepatic CPSS does not spontaneously regress (3,7,8,16–18). Ultrasonography and dynamic contrast-enhanced CT evaluation showed spontaneous reductions in the shunt size in all of our patients with intrahepatic CPSS but in none with extrahepatic CPSS, strengthening the previous findings. Furthermore, all of our patients with extrahepatic CPSS showed marked enlargement in shunt diameter over time. The reason for this difference between intrahepatic and extrahepatic CPSSs is unclear; however, one possible explanation is the difference in the environments surrounding intrahepatic and extrahepatic CPSSs. An intrahepatic CPSS is tightly surrounded by liver parenchyma and may be under pressure during growth, perhaps leading to spontaneous closure. One case report of a child with intrahepatic CPSS who died of pulmonary hypertension described an enlarged portal tract with multiple thin-walled angiomatous vessels; these may be a feature of shunts that tend to close or regress under pressure (5). By contrast, an extrahepatic CPSS is under less pressure from the surrounding tissues and thus may retain its size, or enlarge, as the patient grows.

The most important finding of our study is the reduction in SI in extrahepatic CPSS, despite the increase in shunt diameter. PRPS is a noninvasive method that results in little exposure to

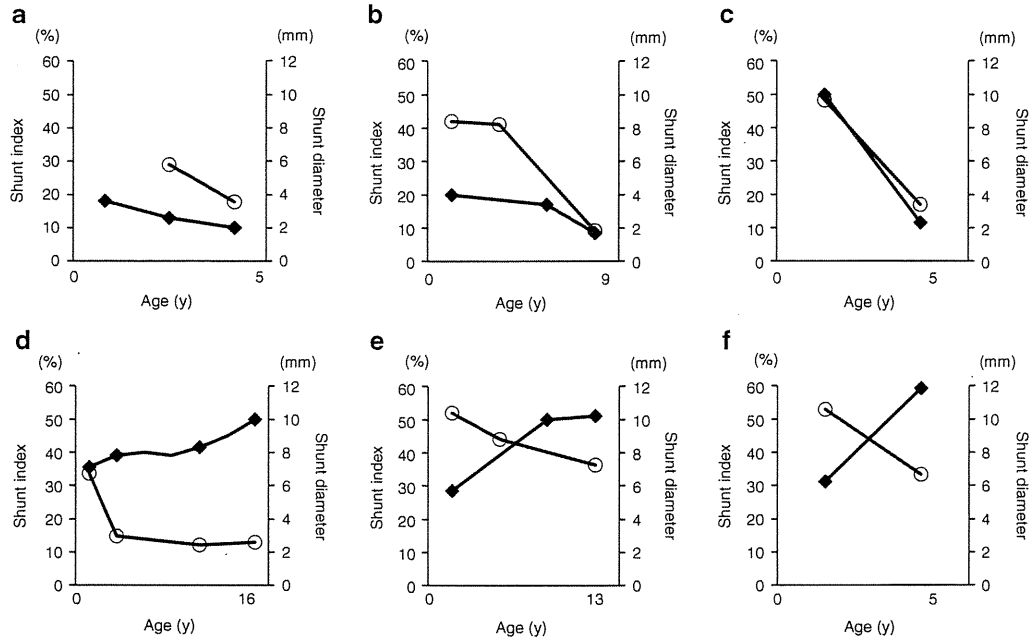

Figure 2. Time course of shunt index and shunt diameter in patients with congenital portosystemic shunt (CPSS). (**a**) Case 1 with intrahepatic CPSS between LPV and LHV. (**b**) Case 2 with intrahepatic CPSS between LPV and CHV. (**c**) Case 3 with intrahepatic CPSS between LPV and CHV. (**d**) Case 4 with extrahepatic CPSS between SV and LRV. (**e**) Case 5 with extrahepatic CPSS between SV and LRV. (**f**) Case 6 with extrahepatic CPSS between IMV and IIV. Case number is same as the patient number. Shunt index is shown as open circle, and shunt diameter is shown as solid diamond. CHV, central hepatic vein; IMV, inferior mesenteric vein; IIV, internal iliac vein; LHV, left hepatic vein; LPV, left portal vein; LRV, left renal vein; SV, splenic vein.

radionuclide and is an effective method for evaluating portal circulation; it is therefore used to diagnose or evaluate CPSS in children and to assess the severity of cirrhosis in adults (9,19–21). Shiomi *et al.* (19) reported the usefulness of PRPS in providing detailed information about changes in portal hemodynamics; they reported that SI in adults increases as liver cirrhosis progresses. Uchino *et al.* (3) reported that the risk of hepatic encephalopathy increases with the degree of portosystemic shunting, as indicated by the PRPS SI. The SI has also been shown to be useful for evaluating the postoperative course in dogs with extrahepatic CPSS (22). In children, a recent study demonstrated that PRPS is complementary to ultrasonography and endoscopy in the assessment of portal hypertension associated with chronic cholestasis (23). We found that SI decreased in all of our subjects with CPSS during long-term follow-up; this may be a previously unrecognized feature of the natural course of CPSS in humans. However, the change in SI paralleled a reduction in shunt diameter in the children with intrahepatic CPSS but contrasted with the increase in shunt diameter in patients with extrahepatic CPSS. CPSS without spontaneous closure or regression is considered to reflect an increase in the degree of shunt severity and is associated with complications (16). In addition, the enlargement of shunt diameter in our patients with extrahepatic CPSS may have led physicians to consider that the severity of the shunt has worsened. However, our study demonstrated that an increase in shunt diameter, as shown by imaging modalities such as ultrasonography and dynamic contrast-enhanced CT, is not an indicator of the severity of the shunt in CPSS, whereas PRPS can be used to quantify shunt severity by using the SI, regardless of changes in shunt appearance.

Imaging modalities such as ultrasonography and dynamic contrast-enhanced CT are useful for detecting the location and size of CPSS but cannot provide the degree of shunt severity as quantitatively as can PRPS. In addition, the image quality of ultrasonography for extrahepatic CPSS is often influenced by abdominal conditions (e.g., intestinal contents) because the shunt is surrounded by the stomach or the small or large intestine, whereas PRPS is not influenced by abdominal conditions because the radiological agent is instilled through the rectum. The concentrations of TBA and ammonia are useful for monitoring the presence of PSS, but because these values can fluctuate with changes in gut conditions, their use in accurately assessing progress toward shunt closure is problematic. Therefore, it is important and effective to use PRPS in addition to ultrasonography, dynamic contrast-enhanced CT, and blood tests to assess shunt severity in the diagnosis and follow-up of CPSS.

Surgical repair or embolization may be recommended for extrahepatic CPSS without closure because of the high risk of hepatopulmonary syndrome, pulmonary hypertension, and hepatic encephalopathy (4,7,8,13–16). Stringer (8) described that CPSS-affected individuals are at risk of developing hepatic encephalopathy and/or an intrahepatic tumor depending largely on the volume and duration of the shunt. The risk of hepatic encephalopathy is related to the degree of portosystemic shunting, as measured by PRPS (3); therefore, it is rational to take the SI into consideration when deciding whether surgical treatment or embolization is indicated. On the other hand, Bernard *et al.* (7) recommended that, even when no complication is detected, closure of shunt should be considered early to prevent complications

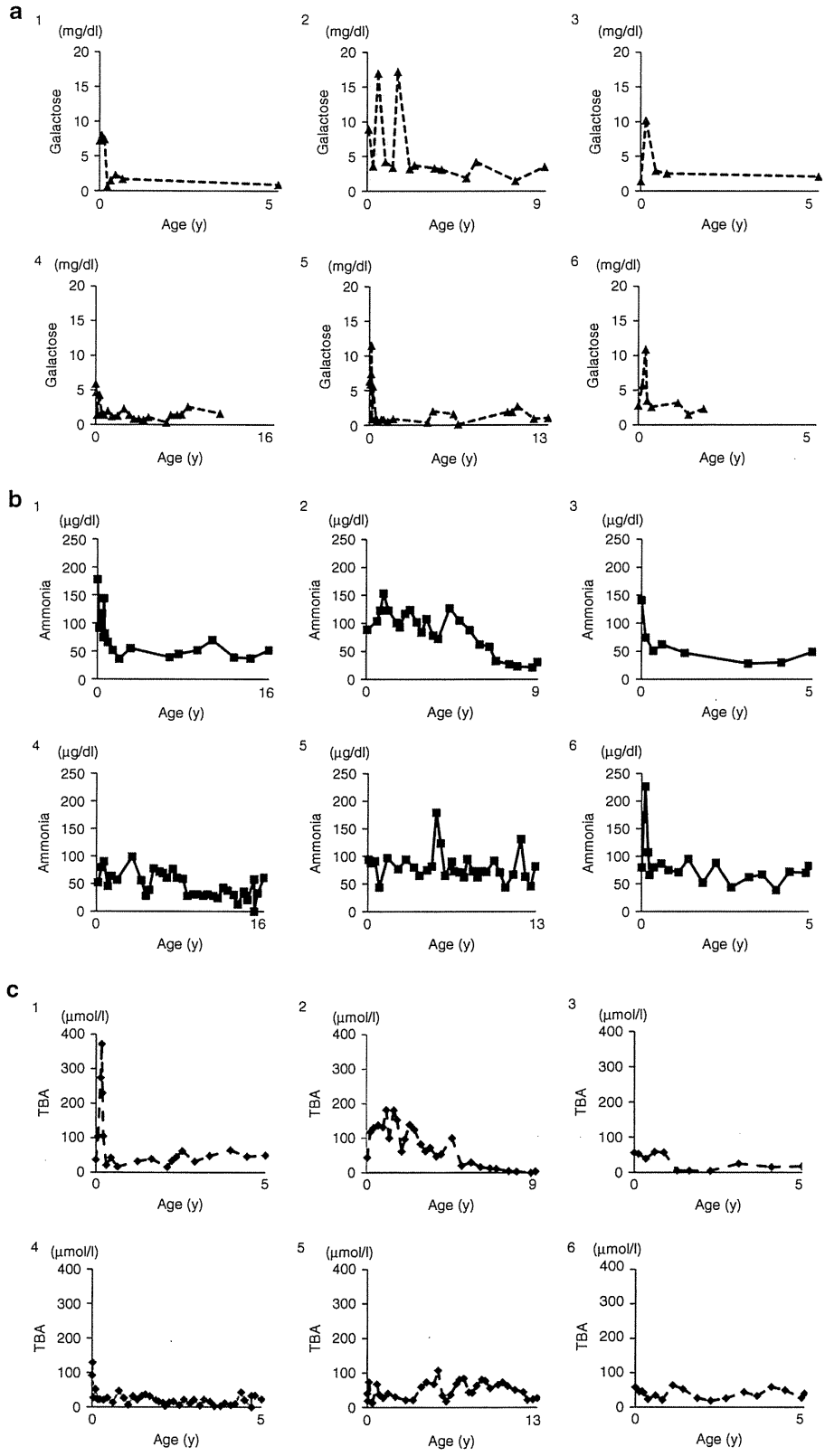

Figure 3. Time course of (**a**) galactose, (**b**) TBA, and (**c**) ammonia levels in children with congenital portosystemic shunt (CPSS). (1) Case 1 with intrahepatic CPSS between LPV and LHV. (2) Case 2 with intrahepatic CPSS between LPV and CHV. (3) Case 3 with intrahepatic CPSS between LPV and CHV. (4) Case 4 with extrahepatic CPSS between SV and LRV. (5) Case 5 with extrahepatic CPSS between SV and LRV. (6) Case 6 with extrahepatic CPSS between IMV and IIV. Case number is the same as patient number. CHV, central hepatic vein; IIV, internal iliac vein; IMV, inferior mesenteric vein; LHV, left hepatic vein; LPV, left portal vein; LRV, left renal vein; SV, splenic vein; TBA, total bile acid.

in cases of CPSS except for small intrahepatic shunt. Therefore, it remains unclear whether SI affects the indications for closure of shunt and there are no criteria for initiating treatment based on PRPS. In our study, the SI of extrahepatic CPSS decreased even though the shunt diameter increased during the long-term natural course of the condition. Taking the current and previous findings together suggests that it is important to determine the SI to evaluate the risk of hepatic encephalopathy in patients with extrahepatic CPSS; in addition, SI may be considered an important parameter in addition to hyperammonemia, portal pressure, shunt size, and clinical symptoms suggesting hepatic encephalopathy when deciding whether surgical treatment or embolization is warranted in patients with extrahepatic CPSS, although further clinical study is necessary to support this hypothesis.

In conclusion, our study clearly revealed that PRPS can provide information about the severity of CPSS beyond that provided by biochemical tests, ultrasonography, and dynamic contrast-enhanced CT. SI decreased over time, both in patients with intrahepatic CPSS, whose shunts decreased in diameter, and in patients with extrahepatic CPSS, whose shunts increased in diameter. We recommend following up patients with CPSS by using PRPS.

METHODS

Six patients with CPSS were retrospectively analyzed. Informed consent was obtained, and the study was approved by the Institutional Review Board of Osaka City University Hospital. All six patients were found to have hypergalactosemia during newborn screening but did not have galactose-metabolizing-enzyme deficiency. Each patient was diagnosed as having CPSS by blood tests, including aspartate aminotransferase, alanine aminotransferase, galactose, TBA, and ammonia levels, in addition to ultrasonography, dynamic contrast-enhanced CT, and PRPS. All patients were followed using the same modalities. Shunt location, shunt flow, and shunt diameter were evaluated by using ultrasonography and dynamic contrast-enhanced CT (**Table 1**). Chest roentgenography, electrocardiography, and echocardiography were undertaken to assess pulmonary hypertension. PRPS was performed as previously described (9). Briefly, a polyethylene tube (Nélaton's catheter, French 8–12, Terumo Cooperation, Tokyo, Japan) was inserted 10 cm deep into the rectum, reaching the upper part. A large-field scintillation camera (Vertex Plus; Adac Laboratories, Milpitas, CA) was used to generate time–activity curves. The camera had a low-energy, multipurpose, parallel-hole collimator and was interfaced with a digital computer (Pegasys; Adac Laboratories). The camera was positioned over the patient's abdomen so that the field of view included the heart, liver, and spleen. First, 111 MBq (megabecquerels) of Tc-99m–pertechnetate (1 ml) was given through the tube, followed by 10–20 ml of air. Thereafter, time–activity curves for the areas of the liver and heart were obtained every 4 s. At the end of the 5-min examination, the 5-min summed image, displayed in color, was recorded. To evaluate the extent of the portosystemic shunt in terms of an SI, we calculated the ratio of counts for the liver to counts for the heart integrated for 24 s immediately after the appearance of the liver time–activity curve.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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Phosphorylated Sp1 is the regulator of DNA-PKcs and DNA ligase IV transcription of daunorubicin-resistant leukemia cell lines ☆

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ABSTRACT

Multidrug resistance (MDR) is a serious problem faced in the treatment of malignant tumors. In this study, we characterized the expression of non-homologous DNA end joining (NHEJ) components, a major DNA double strand break (DSB) repair mechanism in mammals, in K562 cell and its daunorubicin (DNR)-resistant subclone (K562/DNR). K562/DNR overexpressed major enzymes of NHEJ, DNA-PKcs and DNA ligase IV, and K562/DNR repaired DSB more rapidly than K562 after DNA damage by neocarzinostatin (MDR1-independent radiation-mimetic). Overexpressed DNA-PKcs and DNA ligase IV were also observed in DNR-resistant HL60 (HL60/DNR) cells as compared with parental HL60 cells. Expression level of DNA-PKcs mRNA paralleled its protein level, and the promoter activity of DNA-PKcs of K562/DNR was higher than that of K562, and the 5′-region between −49 bp and the first exon was important for its activity. Because this region is GC-rich, we tried to suppress Sp1 family transcription factor using mithramycin A (MMA), a specific Sp1 family inhibitor, and siRNAs for Sp1 and Sp3. Both MMA and siRNAs suppressed DNA-PKcs expression. Higher serine-phosphorylated Sp1 but not total Sp1 of both K562/DNR and HL60/DNR was observed compared with their parental K562 and HL60 cells. DNA ligase IV expression of K562/DNR was also suppressed significantly with Sp1 family protein inhibition. EMSA and ChIP assay confirmed higher binding of Sp1 and Sp3 with DNA-PKcs 5′-promoter region of DNA-PKcs of K562/DNR than that of K562. Thus, the Sp1 family transcription factor affects important NHEJ component expressions in anti-cancer drug-resistant malignant cells, leading to the more aggressive MDR phenotype.

1. Introduction

Anthracyclines, as exemplified by daunorubicin (DNR), are a class of anti-neoplastic agents widely used for the treatment of malignancy. Their cytotoxic mechanism involves the production of DNA damage through intercalating with DNA and the inhibition of topoisomerase (topo) II, finally causing DNA-double-strand breaks (DSBs) and inducing apoptosis [1]. Drug resistance is a major obstacle in the successful treatment of leukemia and solid tumors and is still a major cause of death in leukemia and cancer patients [2]. The development of a resistance mechanism in response to doxorubicin-induced apoptosis includes P-glycoprotein and Bcl-2 overexpression, altered topo II activity, and loss of p53 function, etc. [3]. For example, overexpression of P-glycoprotein confers resistance to a variety of structurally and functionally unrelated anti-cancer drugs, a function known as multidrug resistance (MDR).

DNA damage represents a persistent threat to genomic stability. A critical link exists between DNA mutation, chromosomal rearrangement and cancer development. In myeloid malignancies, various chromosomal translocations and/or mutations increased cellular reactive oxygen species (ROS), followed by DSBs [4]. The majority of tumor cells have defects in maintaining genomic stability due to the loss of an appropriate response to DNA damage. Mutations in the genes that encode DNA damage response proteins are responsible for a variety of genomic instability syndromes. Thus, DNA repair is an important mechanism for maintaining genetic stability.

Abbreviations: DNR, daunorubicin; NHEJ, non-homologous end-joining; MDR, multidrug resistance; DSB, DNA-double-strand break; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation

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Non-homologous end-joining (NHEJ) is the most important DNA repair mechanism in mammalian cells. NHEJ repairs DSBs by ligating two free DNA ends with little or no homology. Several components involved in NHEJ have been reported, such as Ku70, Ku80, DNA-dependent protein kinase (DNA-PK), and DNA ligase IV. A DSB is first recognized by Ku70/Ku80 complex and DNA-PK catalytic subunit (DNA-PKcs). DNA-PKcs is a 470 kDa serine/threonine protein kinase catalytic subunit (DNA-PKcs), and Ku proteins are its DNA-binding regulatory components [5,6]. Finally, DNA ligase IV heterodimerized with XRCC4, a nuclear phosphoprotein, is recruited to the DSB site and executes the final rejoining step.

DNA-PKcs mutant mice exhibit congenital bone marrow failure associated with deficiencies in DNA repair [7]. DNA-PKcs activation is an essential step in the repair process of DSB [8], and it has been proposed that DNA-PKcs is a molecular sensor for DNA damage that enhances cellular response signaling via phosphorylation of many downstream targets. Compared with another error-free DSB repair mechanism, homologous recombination (HR) repair, NHEJ, is error-prone, leading to more complicated genetic mutations and more malignant phenotypes, including anti-cancer drug resistance [9,10].

The modulation of NHEJ components is expected to affect anti-cancer drug sensitivity. The relationship between DNA ligase IV expression and etoposide resistance has been reported in a human leukemia cell line, CEM [11]. In the case of DNA PKcs, increased expression of DNA ligase IV confers resistance to adriamycin [12], and inhibition of DNA-PKcs by wortmannin, an inhibitor of phosphatidylinositol 3-kinase, has been shown to potentiate the chemosensitivity of multidrug-resistant human leukemia, CEM cells [13]. However, the mechanism of DNA-PKcs expression remains to be elucidated, especially its overexpression in malignant tumors or leukemia cells. Furthermore, the relationship between DNA ligase IV and the MDR phenotype has not been fully disclosed.

The present study was performed to characterize the multidrug-resistant phenotype of daunorubicin (DNR)-resistant leukemia cell lines by focusing on NHEJ, and revealed (1) an overexpression of DNA-PKcs and DNA ligase IV in DNR-resistant cells compared with the parental leukemia cell lines, and (2) the importance of Sp family transcription factors for anti-cancer drug resistance by regulating DNA-PKcs, DNA ligase IV and MDR1, but not Bcl-xL transcription. Our finding from this study that increased or activated transcription factor Sp1 is the main regulator of various aspects of the MDR phenotype provides an informative basis for future therapeutic strategies to overcome MDR in chemoradiotherapy.

2. Materials and methods

2.1. Cell lines and reagents

Human erythroleukemia cell lines, K562 and its daunorubicin-resistant subline (K562/DNR), have been reported previously [14]. Human leukemia cell lines, HL60 and its daunorubicin-resistant HL60/DNR, were obtained from Prof. T. Okazaki (Kanazawa Medical University, Kanazawa, Japan) [15]. Anti-MDR1 (H-241: sc-8313), anti-Ku70 (A-9: sc5309), anti-Ku86 (H-300: sc9034), anti-DNA ligase IV (H-300: sc-28232), anti-XRCC4 (c-20: sc-8285), anti-Sp1 (PEP 2: sc-59,) anti-Sp3 (D-20: sc-644), rabbit anti-goat IgG-HRP (sc-2768), and goat anti-mouse IgM-HRP (sc-2973) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-DNA-PKcs (Ab-4: MS-423-P1) antibody was obtained from NeoMarkers (Fremont, CA, USA). Anti-Bcl-xL (#51-9001913), anti-Bcl2 (#51-9001912), anti-Bax (#51-9001914), and anti-Mcl-1 (#M54020) antibodies were from BD Transduction Laboratories (San Jose, CA, USA). Anti-H2AX (A300-083A) was purchased from Bethyl Laboratories (Montogomery, AL, USA). Anti-γH2AX (05-636) antibody was purchased from Upstate Biotechnology (NY, USA). Anti-β actin antibody (3598-100) was from BioVision. Mithramycin A (M6891-1MG), NU7026 (DNA-PKcs inhibitor), and neocarzinostatin (N9162-100UG) were from Sigma (St. Lois, MO, USA). The MDR1 (downstream) promoter luciferase vector used in some preliminary experiments was obtained from Prof. K. Scotto (The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, NJ, USA).

2.2. Quantitative RT-PCR of DNA-PKcs mRNA

Quantitative RT-PCR was performed as reported before [16,17]. The primer sequences were as follows: DNA-PKcs forward 5′-CATCCAGA GTAGCGAATACTTCC-3′, reverse 5′-TTGTTTCGCAACCAGTTCAC-3′; DNA Ligase IV forward 5′-AACCATCAAGATCTCATTTTACAGC-3′, reverse 5′-GTGATGAATCTTCTCGTTTAACTGG-3′; MDR1 forward 5′-GAATTGGGAT AAAGAAAGCTATTAC-3′, reverse 5′-CCCAATTAATACAGAAAAGAATAC AG-3′; BCL xL forward 5′-GGAGATGCAGGTATTGGTGAG-3′ reverse 5′-CATAGAGTTCCACAAAAGTATCCC-3′; Glucosidase β (GUSB) forward 5′-GCGTGGAGCAAGACAGTGGGC-3′, and reverse 5′-GGTCCCAGTCCC ATTCGCCA-3′. The relative expression level was expressed by the ratio of respective mRNA/GUSB mRNA. The data of control K562 and HL60 cells were regarded as 1.0, respectively.

2.3. Western blotting

Western blotting was performed according to the method described before [17]. Protein samples were dissolved in the lysis buffer and separated on 6%, 10% or 15% of SDS-PAGE depending on the molecular weight, and transferred to PVDF membrane. Membranes were blocked with 1–5% dry milk in phosphate-buffered saline (PBS), with 0.05% Tween 20 at room temperature for 1 h. Concentrations of primary antibodies used were as follows: anti-DNA-PKcs (1:1000), anti-MDR1 (1:500), anti-Ku70 (1:1000), anti-Ku86 (1:1000), anti-DNA ligase IV (1:1000), anti-Bcl-xL (1:500), anti-Bcl2 (1:500), anti-Bax (1:500), anti-Sp1 (1:1000), anti-Sp3 (1:1000), anti-phosphoserine (1:1000), and anti-β actin (1:8000). Secondary antibodies were anti-rabbit IgG HRP-linked antibody or anti-mouse IgG HRP-linked antibody (1:1000–2000). Proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA).

2.4. siRNA and transfection

siRNAs of Sp1 and Sp3 were purchased from Sigma Genosys (Hokkaido, Japan). Sequences of human Sp1 and Sp3 siRNA were 5′-GGAUGGUUCUGGUCAAAUATT-3′, and 5′-GUUGGGGAGGUGGAGCC UTT-3′, respectively [18]. Allstars Negative Control siRNA (QIAGEN) was used as the control scramble siRNA. Twenty nmol of siRNA was transfected using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer.

2.5. Cloning of the 5′ promoter regions of DNA-PKcs, DNA ligase IV, and the upward 5′ promoter of MDR1

Based on an original report [19] and online information, the 5′ promoter of DNA-PKcs was cloned by PCR method using the primer set described below. Forward: 5′-GCTCAGTCACGTGCAGAGGGGATGCTTTAG GCTTTAGGCGTTAG-3′, reverse: 5′-GGGAAGCTTGGACCCGGAAATACCCC TACGCGCGGA-3′. The PCR product was digested by EcoRV and HindIII, inserted into the pGL4.10 [luc2] vector, which was also treated with EcoRV and HindIII, and named −1622 bp/luc. Truncated promoter was prepared by digesting suitable restriction enzymes (described below) followed by blunting and self-ligation. Their DNA sequences were confirmed, and were named −1033 bp/luc (SacI and MluI), −771 bp/luc (SacI and EcoNI), −214 bp/luc (SacI and AvrI), and −49 bp/luc (SacI and SmaI), respectively. Reporter vectors, −161 bp/luc, −145 bp/luc, −113 bp/luc, −76 bp/luc and +1 bp/luc, were prepared by PCR using a primer set as shown in Table 1. A mutation insertion into the GC-rich

Table 1
Primer sets for DNA-PKcs promoter.

DNA-PKcs promoter region	Forward primer	Reverse primer
−161 bp	5′-AACACCAAGTAGCCCAAACTAC CTC-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−145 bp	5′-CAAACTACCTCCGCAGGTCAGACG TTTTTC-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−113 bp	5′-TTAGGTTTCCATGTTGATTCGGGC CA-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−76 bp	5′-TACTGGCCAGGCCTTCCCGCA-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
1 bp	5′-GGGGCATTTCCGGGTCCAAGC-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−49 bp/mutation1	5′-GGGAAAGTTCCT GCttCCGCGCCCCGC-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−49 bp/mutation2	5′-GGGAAAGTTCCTGCCG CCtataCCCGCA-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′
−49 bp/mutation3	5′-GGGAAAGTTCCTGCCGCCGCGCCC CGCAGCaaaaCCTCCGCG-3′	5′-CTGGGCCCTTCTTAAT GTTTTTGGCATCT-3′

Small letters denote the mutated sequences.

region of the 5′ promoter was performed using primer sets as shown in Table 1.

The 5′ promoter region of DNA ligase IV (variant 3) was cloned by PCR method using the primer set described below. Forward: 5′-AAAA CTCGAGTGGTGCAATGGAAAGGAGACAGTG-3′, reverse: 5′-AAAAAAAG ATCTTCCGGCACCTCTTCACCACG-3′. The PCR product was digested by XhoI and BglII, inserted into pGL4.10 [luc2] vector, which was also treated with XhoI and BglII, and named −1834 bp/luc. Reporter vectors, −1426 bp/luc, −744 bp/luc, −68 bp/luc, and +1 bp/luc, were prepared by PCR using respective primer set as shown in Table 2.

We also performed 5′ RACE experiments with MDR1, because recent online information suggests the possibility that another exon 1 might exist far upstream from the conventional exon 1. We did confirm its presence in the preliminary experiments. The downstream MDR promoter luciferase vector was derived from Dr. Scotto (University of Medicine and Dentistry of New Jersey). For the upstream promoter, we cloned this promoter region using the primer set described below. Forward: 5′-GGGGGTACCTAAAGAATTACTCATCCCCATGT-3′, reverse: 5′-GGGAAGCTTAGTGTTTATCCCAGTACCAGAGG-3′. The PCR product was digested by KpnI and HindIII. It was inserted into the pGL4.10 [luc2] vector, which was also treated with KpnI and HindIII, and was named −1112 bp UP/luc, as shown in Supplementary Fig. 3.

2.6. Promoter analysis

Promoter analysis was performed as described previously with minor modifications [20]. One microgram of various lengths of firefly luciferase vectors and 0.2 μg of renilla luciferase control reporter vectors were cotransfected using Lipofectamine 2000 (Invitrogen) and Dual luciferase reporter system (Promega). The relative promoter activity was calculated as firefly luciferase/renilla luciferase activity, and the relative promoter activity of +1 bp/luc (DNA-PKcs, DNA ligase IV and MDR1 upstream and downstream promoters, respectively) was regarded as 1.0. The mean +/− SD was then calculated from three independent experiments.

Table 2
Primer sets for DNA ligase IV promoter.

DNA ligase IV promoter region	Forward primer	Reverse primer
−1426 bp	5′-AAAACTCGAGTCCTGCCACC CATTCCACATTC-3′	5′-AAAAAAAGATCTTCCG GCWordBreak> ACCTCTTCACCACG-3′
−744 bp	5′-AAAACTCGAGTTTACTTTTC ATGTCTGTGTGGGAGGG-3′	5′-AAAAAAAGATCTTCCGGC ACCTCTTCACCACG-3′
−68 bp	5′-AAAACTCGAGCCTGTTCTCC GTCTCCGCTCC-3′	5′-AAAAAAAGATCTTCCGGC ACCTCTTCACCACG -3′
1 bp	5′-AAAACTCGAGTGCCAGTGAG CCCCCGC 3′	5′-AAAAAAAGATCTTCCGGC ACCTCTTCACCACG -3′

2.7. Phosphorylated Sp1 detection

Whole cell lysate was prepared with RIPA buffer (150 mM NaCl, 1% NP-40, 0.05% deoxycholic acid, 0.1% SDS, and 50 mM Tris (pH 8.0)). Dynabeads® Protein A (Invitrogen) was added to anti-Sp1 antibody (1 μg) diluted in 200 μl phosphate-buffered saline (PBS(−)) with 0.05% Tween 20 and rotated for 10 min at room temperature. After washing, the sample (500 μg/500 μl RIPA) was added to the beads–antibody complex and rotated for 10 min at room temperature. After three washes using PBS(−), the sample was eluted by 20 μl of sample buffer and heated for 5 min at 95 °C. Western blotting was performed using anti-phosphoserine antibody (1:1000) as described above.

2.8. Sp1 transcription factor activity assay

To examine the different potentials of Sp transcription factors between K562 and K562/DA cell lysates, EMSA was performed as described previously [20] using the consensus Sp1 EMSA probe (Panomics, CA, USA) according to the manufacturer's recommendations or the probe containing the 5′-proximal GC-rich region of the DNA-PKcs genome. The labeled probe used was 5′-GTTCCTGCCGCCGCGCCCCGCAGC CCCGCCTCCG-3′. A cold competitor was used 10, 100 and 300 times as much as the labeled probe.

2.9. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously [17]. For the immunoprecipitation, anti-Sp1 or Sp3 antibody (Santa Cruz, 1 μg/sample) was added to Dynabeads® Protein A (Invitrogen, Carlsbad, CA, USA) and rotated overnight at 4 °C. Normal rabbit IgG was used as a control IgG. After cross-linking cells with formaldehyde and sample preparation, the antibody and beads complex was mixed with samples and rotated for 6 h at 4 °C. After DNA extraction, the promoter region containing Sp1 motifs was amplified by the PCR method using the primer set described below. The primer sequences were as follows: DNA-PKcs following primers, forward 5′-CCAAGTCCAACACCAAGTAGCCACCCA-3′; and reverse 5′-CCGCCATGCCGCCGAGTCCC-3′. DNA ligase IV forward 5′-CTGTTCTCCGTCTCCGCTCCC-3′; reverse 5′-CCGCCGAGGTATCTTTTC CGT-3′.

2.10. Statistical analysis

Statistical significance was analyzed using Student's *t*-test, or one-way factorial analysis of variance and multiple comparison test (Fisher's method) using Excel software (Microsoft).

3. Results

3.1. DNA-PKcs, DNA ligase IV, and Bcl-xL in daunorubicin-resistant leukemia cell lines

Daunorubicin (DNR)-resistant subclones of K562 and HL60 cells were highly resistant to DNR as compared with parental cell lines (Supplementary Fig. 1). Both MDR1 protein and mRNA were overexpressed in K562/DNR cells (Supplementary Fig. 1a right). We analyzed multidrug-resistant phenotypes in these cell lines mainly focusing on the components of NHEJ. Fig. 1 shows that DNA-PKcs and DNA ligase IV, but not Ku70 and Ku86, were overexpressed in DNR-resistant subclones. Furthermore, Bcl-xL was overexpressed in both DNR-resistant cells. We found that mRNA levels of DNA-PKcs and DNA ligase IV in K562/DNR and HL60/DNR were significantly higher than those of the parental

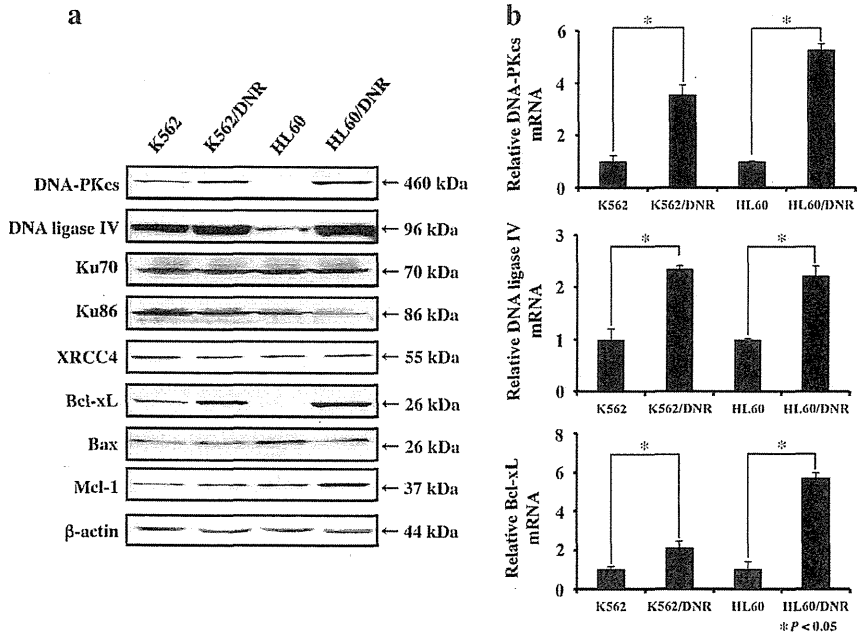

Fig. 1. NHEJ and apoptosis-related protein expression of DNR-resistant cell lines.(a) Western blotting of NHEJ components (Ku70, Ku80, DNA PKcs, and DNA ligase IV, XRCC4) and apoptosis-related proteins (Bcl-xL, Bax and Mcl-1) of K562, K562/DNR, HL60 and HL60/DNR cells; Western blotting was performed using respective antibody described in Materials and methods. Bcl2 could not be detected in our assay condition. β-Actin was illustrated as the internal control. (b) Quantitative RT-PCR of DNA-PKcs, DNA ligase IV, and Bcl-xL mRNA; quantitative RT-PCR was performed as described in Materials and methods. The relative mRNA levels of K562 and HL60 cells were regarded as 1.0, respectively. The mean +/− SD was calculated from three different samples. Statistical significance was evaluated. Experiments were repeated three times with similar results.

K562 and HL60 cells. We also measured Bcl-xL mRNA. DNR-resistant cell lines also exhibited higher mRNA levels than the parental K562 and HL60 cells (Fig. 1b).

3.2. Recovery from DNA double strand breaks (DSBs)

Fig. 2 illustrates the recovery of a DSB from a short-term neocarzinostatin treatment, a radiomimetic which is not transported from cells with ABC transporters, in K562 and K562/DNR cells. Similar DSBs (as detected by γ-H2AX) were observed when treated with the same dose of neocarzinostatin (NCZ) for a 1 h exposure, but K562/DNR cells' recovery from DSB (decreased γ-H2AX) was faster than that of K562 cells (Fig. 2a). siRNA treatment for DNA-PKcs and NU7026, an inhibitor of DNA-PKcs, enhanced DNR-induced cell death of K562/DNA (Fig. 2b and c), suggesting the involvement of DNA-PKcs in the recovery phase from DSB and the sensitivity of DNR cytotoxicity.

3.3. Importance of GC-rich promoter region of DNA PKcs

DNA-PKcs 5′-promoter was analyzed using various lengths of truncated promoter luciferase vectors. We focused on K562 and K562/DNR from the transfection efficiency. In both K562 and K562/DNR cell lines, the promoter activity increased depending on the promoter lengths (detailed data not shown), indicating involvement of various transcription factors. Online search of this region revealed several transcription binding motifs. It has been reported that a drug-resistant human breast cancer cell line, MCF-7, exhibits increased AP-1 activity [21]. Furthermore, NFκB has been shown to stimulate MDR1 transcription [22]. Interestingly, in all promoter vectors used, higher promoter activities were observed in K562/DNR cells (Fig. 3b and data not shown).

We were interested in the most proximal 49 bp region, because the higher promoter activity of K562/DNR was still preserved in this small region. This region between −49 bp and the first exon of DNA-PKcs was highly GC-rich, and was expected to be the binding site of the Sp1 family transcription factor and related transcription factors (Fig. 3a). Insertion of three mutations into three GC-rich regions diminished the promoter activity considerably in both K562 and K562/DNR cells, but some variations were seen in their inhibition levels (Fig. 3c). K562 cells possess BCR/ABL translocation, which might affect the MDR phenotype. Thus, we established BaF3 cells stably transfected with p210 BCR/ABL, and also T315I-mutated p210 BCR/ABL, which is imatinib-resistant. Supplementary Fig. 2 showed that increased DNA-PKcs expression was observed in T315I–P210-transformants but not in P210 (BCR/ABL)-overexpressed cells, suggesting that the overexpressed DNA-PKcs observed in K562/DNR were not due to BCR/ABL translocation.

3.4. Activated Sp1 in K562/DNR cells

Sp family members have been regarded as the housekeeping genes and their expression has been well observed in various cell lines and tissues. Sp1 and Sp3 protein levels did not differ between parental and doxorubicin-resistant K562 and HL60 cells (Fig. 4a). Sp proteins have been reported to be activated by phosphorylation [23]. It has also been noted that Sp1 of K562/DNR and HL60/DNR was more phosphorylated than in their parental cell lines (Fig. 4b). In case of phosphorylated Sp3, we could not detect a significant difference between K562 and K562/DNR cells under our experimental condition. Using three-tandem repeats of Sp1 consensus sequence as the EMSA probe (Promega), a higher Sp1 transcription activity was detected in K562/DNR cells rather than K562 cells (data not shown).

3.5. Sp family protein modulates DNA PKcs, DNA ligase IV, and MDR1 expression

We tried to modulate Sp1 and Sp3 protein function and expression levels by Sp family-specific inhibitor, mithramycin A (MMA), and siRNAs for Sp1 and Sp3. Both MMA and siRNAs for Sp1 and Sp3 reduced DNA-PKcs protein expression (Fig. 5a and b). We also examined the effect of Sp1 and Sp3 on DNA ligase IV, MDR1 and Bcl-xL protein expression. Interestingly, MMA and siRNA for Sp1/Sp3 inhibited DNA ligase IV

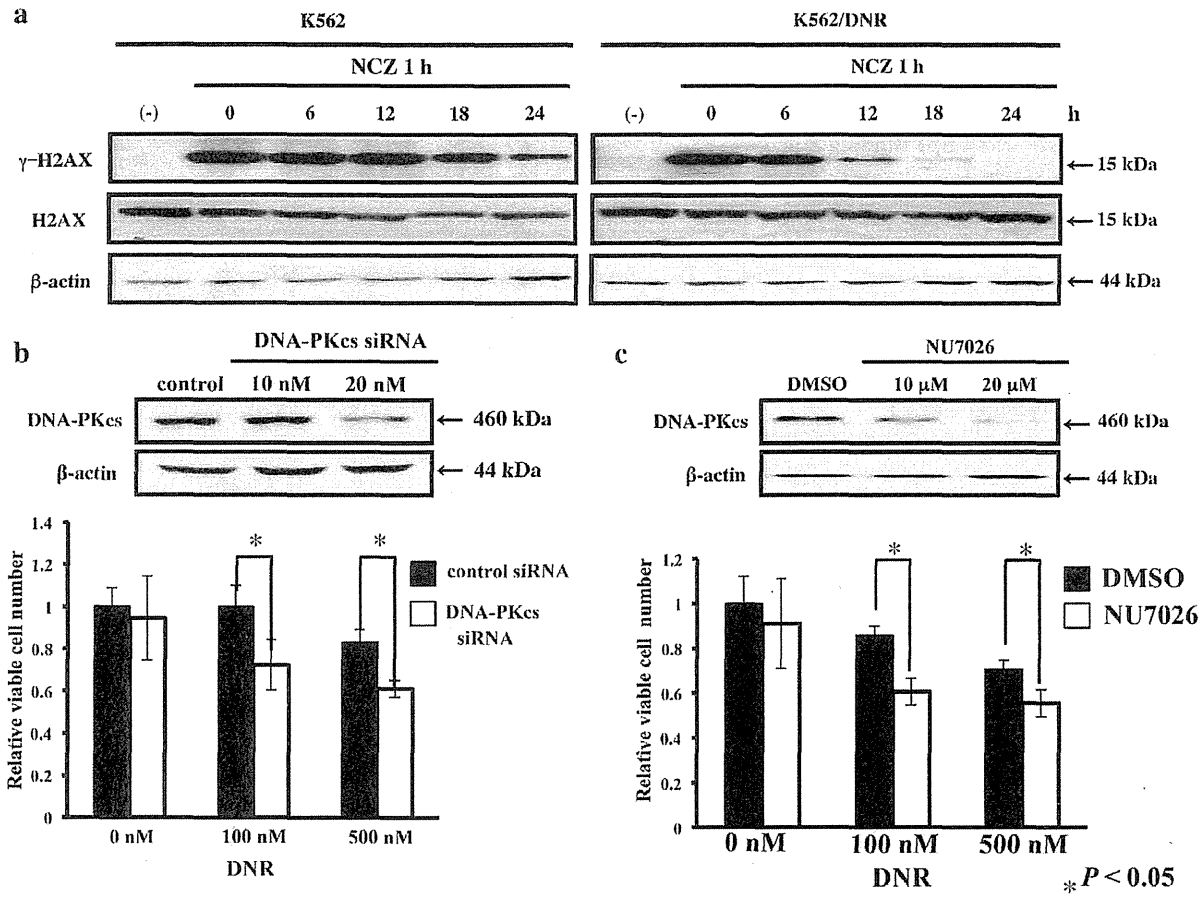

Fig. 2. Recovery from DNA damage by neocarzinostatin of K562 and K562/DNR cells and effects of DNA-PKcs modulation of DNR sensitivity. (a) K562 and K562/DNR cells were treated with 100 ng/ml of neocarzinostatin (NCZ) for 1 h, and then NCZ was removed from the culture medium. Cells were collected sequentially, and Western blotting was performed using anti-γ-H2AX or anti H2AX antibody, respectively. β-Actin was used as the internal control. (b) Upper part denotes the effect of DNA-PKcs siRNA (10 and 20 nM) on DNA-PKcs protein expression. Lower part illustrated the effect of siRNA on NDR-treated K562/ADR cell viability. K562/DNR was transfected with either control siRNA (control) or DNA-PKcs siRNA (20 nM) for 24 h, and treated with or without DNR for another 24 h. Viable cells were counted by trypan blue dye exclusion test. Viable cell number of control K562/DNR cells treated with control siRNA was regarded as 1.0. (c) In a similar way as (b), effect of NU7026 was examined. Upper part shows the effect of NU7026 on DNA-PKcs protein expression. Lower part; K562/DNR cells were treated with either DMSO (vehicle) or NU7026 (20 μM) for 2 h; and then DNR was added for another 24 h. Viable cells were counted. DMSO-treated control K562/DNR cells were regarded as 1.0.

and MDR1, but not Bcl-xL expression of K562/DNR cells (Fig. 6a and b), suggesting a similar regulatory mechanism of DNA ligase IV and MDR1 but, not Bcl-xL, in K562/DNR cells. An online search indicated that a GC-rich region, where Sp1 family can bind, was also present in the promoter region of DNA ligase IV and MDR1. In the case of Bcl-xL, NFkB, GATA and ETS have been reported as the responsible transcription factors in different tumor types [24–26]. However, the involvement of Sp1 in Bcl-xL has been reported to be relatively small [27], supporting our present results. At present, we cannot explain the reason why MDR1 of HL60/DNR was not overexpressed, but it could be possible that another important factor other than Sp1 for MDR1 transcription is lacking in HL60/DNR cells.

We next examined the MDR1 promoter to know whether the Sp1 transcription factor in K562/DNR stimulates MDR1 transcription. The transcription mechanism of human MDR1 gene has been previously studied extensively. However, it has recently been reported that another transcription start site exists [28]. Thus, two different promoters (upstream and downstream) are present depending on the cellular context. Previous studies mostly analyzed downstream promoter (DSP) [29,30]. Our preliminary 5′ RACE analysis of K562/DNR cells confirmed these two transcription start sites described above (data not shown). Thus, we examined the upstream promoter (USP) and DSP of K562/DNR separately, and showed that mithramycin A inhibited both DSP and USP promoter activities, suggesting that Sp1 binding motifs are important in both USP and DSP of MDR1 (Supplementary Fig. 3).

3.6. EMSA and ChIP analysis of the 5′ promoter region of DNA-PKcs

In vitro binding of Sp family proteins from nuclear lysates with the 5′ promoter of DNA-PKcs was analyzed by EMSA using the region covering this GC-rich sequence (Fig. 3) as the probe (Fig. 7a). K562/DNR cells exhibited higher binding activity against the 5′ GC-rich promoter region of DNA-PKcs (Fig. 7b left). Because the supershift assay using anti-Sp1 and anti-Sp3 antibodies did not exhibit reproducibility in our assay, we analyzed the effect of siRNA for Sp1 and SP3. Nuclear extracts from cells treated with either siRNA for Sp1 and Sp3 or a Sp1 inhibitor, mithramycin A, reduced all the shifted bands 1, 2 and 3 (Fig. 7b right). Fig. 7c shows the results of the ChIP assay covering this GC-rich region. Increased binding of Sp1 and Sp3 was observed in K562/DNR cells. siRNA for Sp1 decreased the band intensity (data not shown).

We also examined DNA ligase IV, which has been shown to possess three different transcription start sites. We focused on one small region having a sequence conserved among species and also possessing one GC-rich region (Supplementary Fig. 4). Promoter analysis revealed the importance of a Sp1 site of the 5′promoter region of DNA ligase IV

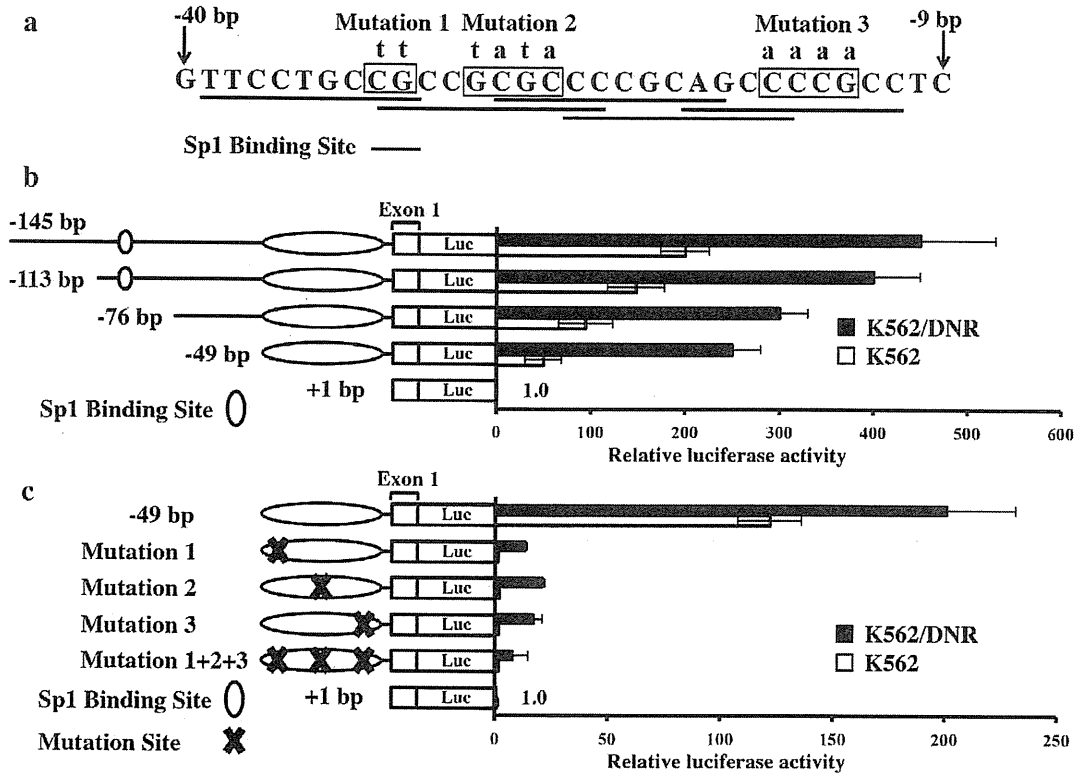

Fig. 3. Promoter analysis of the 5′-promoter region of DNA-PKcs genome. (a) GC-rich promoter region located within −49 bp/luc was illustrated. Inserted mutations were also described in a small letter. (b) and (c) The 5′-promoter region of DNA PKcs was illustrated in the left part. Circles and ovals mean putative Sp1 binding sites. Various lengths of promoter vectors were used to measure promoter activity. Cross mark (X) indicates the mutation insertion (in (c)). Primer sets used in experiments were shown in Table 1. Transfection into K562 cells and luciferase assay were described in Materials and methods. The solid column denotes K562/DNR cells and the open column represents K562 cells. The mean +/− SD was shown from three independent experiments.

(Fig. 8a). MMA inhibited the promoter activity of −68 bp/luc, which contains one Sp1 binding site (Supplementary Fig. 4 lower part). ChIP analysis of this region revealed increased Sp1 and Sp3 (although less distinct) binding in K562/DNR cells (Fig. 8b), suggesting the positive role of the Sp family transcription factor in DNA ligase IV transcription.

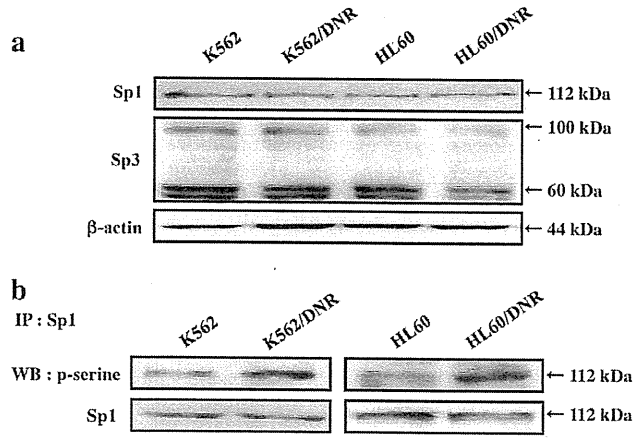

Fig. 4. Western blotting of Sp1 family proteins. (a) Sp1 and Sp3 proteins of K562, K562/DNR, HL60 and HL60/DNR were shown. β-Actin was used as the internal control. (b) Serine phosphorylation of Sp1 in K562 and K562/DNR was illustrated. Each cell lysate was immunoprecipitated with anti-Sp1 antibody followed by anti-phosphoserine antibody or anti-Sp1 antibody according to Materials and methods.

4. Discussion

It has been reported that a correlation between DNA-PK and radiosensitivity exists in lung carcinoma cell lines and cervical cancer cells [9,31]. Cytotoxic drug resistance such as that of daunorubicin (DNR) depends on the loss or recovery from DNA damage and it at least partially results from overexpression of DNA-PKcs [12,32,33]. It has also been reported that DNA-PK is a therapeutic target and an indicator of poor prognosis in B-cell chronic lymphocytic leukemia [34]. The tumor/normal tissue expression ratio of ATM and DNA-PKcs is useful for evaluating non-small cell lung cancer patients [35]. Moreover, down-regulation of DNA-PKcs and Ku70 increased chemo-sensitization of Hela cells [36]. It has been reported that CML stem cell and progenitor cells exhibit higher DNA-PKcs expression [37] than their normal counterparts, suggesting that they have better error-prone DNA repair. Rapid repair from DNA damage by error-prone NHEJ as shown in Fig. 2 is expected to make these cells more resistant to anti-cancer drugs and also to induce further genomic changes such as complex chromosomal abnormality.

In the present study, we mainly analyzed the CML-derived K562 cell and its subline, K562/DNR. Overexpression of NHEJ components, DNA-PKcs and DNA ligase IV, was observed in two different DNR-resistant leukemia sublines, K562/DNR and HL60/DNR, suggesting that these overexpressions were commonly induced during their establishing process by the continuous exposure of anthracyclines.

The promoter of human DNA-PKcs has not been fully analyzed. Although the presence of a GC-rich region was reported, the direct analysis of DNA-PKcs promoter activity has not been performed [19,38]. Our promoter analysis revealed that various factors and promoter areas are involved in its transcription and that the proximal short GC-rich

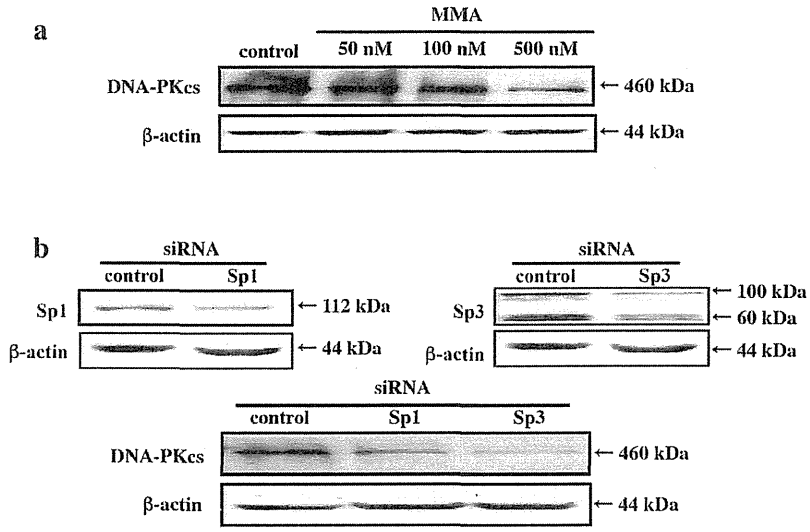

Fig. 5. Effects of Sp family transcription factor inhibition on DNA-PKcs expression. (a) Effect of mithramycin A (MMA) on DNA-PKcs was examined using K562/DNR cells. Cells were treated with various concentrations of MMA for 24 h. DNA-PKcs and β-actin were analyzed by Western blotting. (b) K562/DNR cells were treated with siRNA for Sp1 and Sp3 (20 nM) for 24 h respectively as described in Materials and methods. Control siRNA was also used as the negative control. Sp1, Sp3, DNA-PKcs and β-actin expression were examined by Western blotting.

promoter region plays an important role in DNR-resistance. Although it has been reported that CML cells have altered DNA repair processes, including error-prone HR and different NHEJ mechanisms [39], overexpression of NHEJ components was also observed in HL60/DNR, suggesting that this overexpression could be induced regardless of initial genomic changes. Our analysis using p210 (BCR/ABL) and T315I mutated p210-overexpressed BaF3 cells (Supplementary Fig. 2) supports this interpretation.

Sp family protein has been regarded as the housekeeping gene and is ubiquitously expressed. Although Sp family expression is regulated with some stimuli [17,20], the total Sp family protein levels are not changed in most cases, as observed in our present study (Fig. 4). Constitutive binding of the low mobility forms of Sp1 and AP-1 has been observed in araC-resistant HL60 cells [40], and the involvement of Sp1 in response to DNA damage has been reported before [41,42]. In fact, DNA-PKcs inhibition with NU7026 and siRNA for DNA-PKcs enhanced the sensitivity to DNR in K562/DNR cells (Fig. 2). Moreover, genes actively transcribed by DNA damage such as Gadd45, DDB1/2 and XRCC1 have been shown to possess Sp1 sites in their promoter region [43–45]. We and others have reported that doxorubicin influences

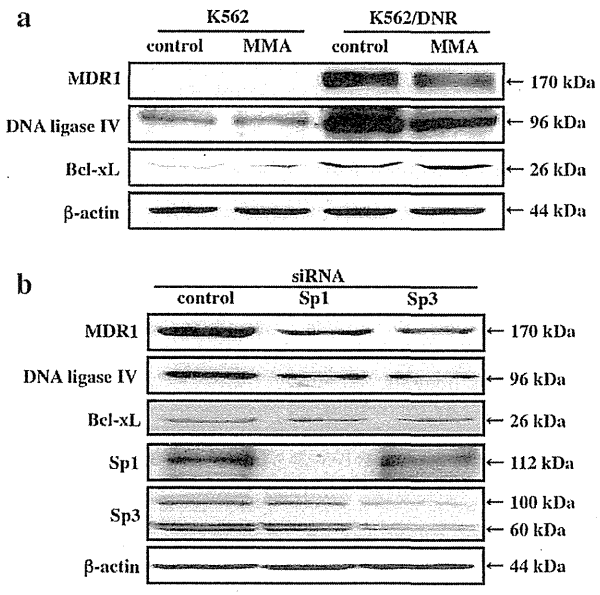

Fig. 6. Effect of MMA and siRNAs for Sp1 and Sp3 on DNA ligase IV, MDR1 and Bcl-xL expression of K562 and K562/DNR cells. (a) K562 and K562/DNR cells were treated with or without 0.5 μM of MMA for 24 h, and MDR1, DNA ligase IV, and Bcl-xL protein levels were analyzed by Western blotting. (b) siRNAs for Sp1 and Sp3 siRNA (20 nM) were used to suppress respective transcription factor as described in Materials and methods. Control siRNA was also used as the negative control. Twenty-four h after transfection, MDR1, DNA-ligase IV, Bcl-xL, Sp1, Sp3, and β-actin expression were examined by Western blotting.

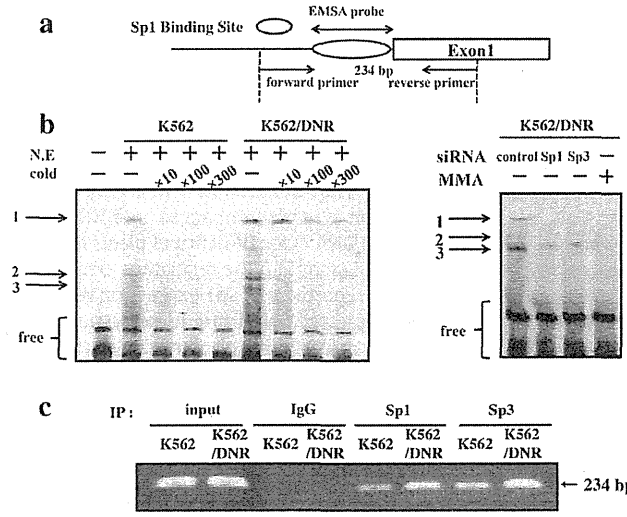

Fig. 7. EMSA and ChIP assay. (a) The schematic presentation of EMSA and ChIP assay probe. Localization of EMSA probe and the PCR primer set used for ChIP assay were illustrated. (b) Left: EMSA was shown using the probe illustrated in (a). N.E. denotes nuclear extract of K562 and K562/DNR. Cold means cold competitor. Free means free labeled probe. Right: effects of siRNA and Sp1 inhibitor, mithramycin A (MMA), were shown. K562/DNR cells were treated with siRNA for Sp1, Sp3, control siRNA or MMA (0.5 μM) for 24 h. ESMA was performed for these nuclear extract. (c) ChIP assay was performed as described in Materials and methods. After immuneprecipitation of K562 and K562/DNR cells with either non-specific IgG, anti Sp1 or anti-Sp3 antibody followed by protein digestion and DNA extraction, PCR analysis was performed using the primer set described in Materials and methods. The PCR product was 234 bp long.

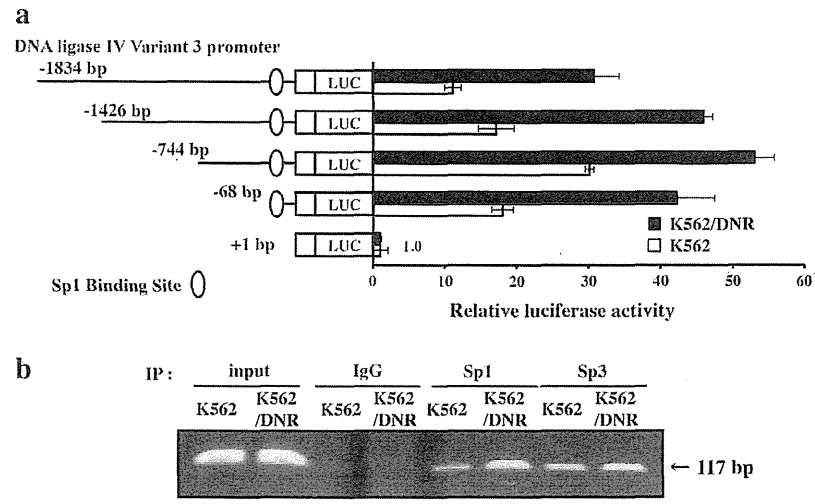

Fig. 8. 5′ promoter analysis and ChIP assay of DNA ligase IV. Upper part; the promoter analysis (variant 3 type illustrated in Supplementary Fig. 4) of DNA ligase IV was performed as described in Materials and methods. Solid column denotes K562/DNR, whereas open column represents K562 cells. The mean +/− SD was shown. The lower part shows ChIP assay using anti-Sp1 or anti-Sp3 antibody, respectively. Primer set was described in Materials and methods and in Supplementary Fig. 4. The PCR product was 117 bp in length.

the expression of neutral sphingomyelinase 2 (NSMase2) and glucosylceramide synthase (GCS) through the Sp1 site of respective promoters [17,46]. Up-regulation of both DNA-PKcs and Sp1 protein of colorectal cancer has been demonstrated only by a clinical immunohistochemical study [47].

Sp1 is a zinc-finger protein constitutively activating housekeeping genes, however, it has been recently reported that Sp1 mediates the inducible gene regulation [48]. Furthermore, Sp1 is phosphorylated in response to DNA damage by Ataxia Teleangiectasia-Mutated kinase [49] or other kinases. Phosphorylation of Sp1 serine 101 residue was reported by UV-irradiation or hydroxyurea treatment [50]. Although it has recently been reported that Sp1 facilitates DSB repair through a non-transcriptional mechanism [51], the regulatory mechanism of DNA-PKcs by activated Sp1 transcription factor was, for the first time, described in the present study. Considering the inhibitory effect of MMA and siRNAs for Sp1 and Sp3 on DNA-PKcs expression, increased Sp1 phosphorylation in K562/DNR was at least partially responsible for the observed increase of DNA-PKcs expression. Similarly, we have previously reported that activated Sp1 but not total Sp1 is responsible for NSMase2 transcription in ATRA-treated MCF-7 cells [52].

DNA ligase IV expression was also enhanced in DNR-resistant subclones (Fig. 1). KU80- and DNA ligase IV-deficient plants are reportedly sensitive to ionizing radiation [53]. The regulatory mechanism of DNA ligase IV has not been reported yet. Sp1 expression reduced by siRNA and Sp1 inhibitor suppressed DNA ligase IV expression (Fig. 6). Online information indicates at least three transcription start sites, but the relative contribution of these transcription start sites has not been elucidated. One promoter type we focused on contained one Sp1 binding site and its promoter analysis revealed the positive involvement of this site on DNA ligase IV transcription (Fig. 8a and Supplementary Fig. 4). Furthermore, in our ChIP assay, increased direct binding of Sp1 to the Sp1 binding site of the 5′ promoter region of the variant 3 DNA ligase IV was shown in K562/DNR cells (Fig. 8). However, further analysis is needed to elucidate the DNA ligase IV transcription mechanism in chemo-resistant cells.

MDR1 and Bcl-xL overexpressions were also observed in K562/DNR cells (Fig. 1). DNR has been demonstrated to induce MDR1 expression in K562 cells [54]. As shown in Fig. 6b, modulation of Sp1 and Sp3 affects MDR1 but not Bcl-xL expression of K562/DNR cells. The importance of Sp1 in MDR1 transcription has been reported previously [29]. Regulation of the MDR1 promoter has been shown to be dependent on the cyclic AMP-dependent protein kinase and the transcription factor, Sp1 [55]. We also demonstrated higher promoter activity of K562/DNR cells against both MDR1 USP and DSP (Supplementary Fig. 3), supporting the theory that the Sp transcription factor is responsible for MDR1 overexpression of K562/DNR cells. In addition to Sp1, other transcription factors including FOXO3a and STAT5 have been demonstrated to cause MDR1 overexpression of K562 cells [54,56,57], suggesting the presence of heterogeneous pathways that induce MDR1 transcription.

The involvement of FOXO3 and STAT5 in DNA-PKcs and DNA ligase IV has not been reported before, and the search by the ECR browser (http://ecrbrowser.decode.org) revealed that 1 kb of the 5′ promoter of DNA-PKcs possesses 4 STAT (−908 bp to −901 bp, −676 bp to −656 bp, −533 bp to −526 bp and −66 bp to −59 bp) and no FOXO binding site, whereas the 5′ promoter of DNA ligase IV we analyzed possesses no STAT or FOXO binding sites. The effect of STAT binding site (−66 bp to −59 bp) was negligible (Fig. 3b), however, our preliminary analysis revealed that other two sites (−676 bp to −656 bp, and −533 bp to −526 bp) were responsible for some promoter activity (data not shown). However, the important point is that the difference in the DNA-PKcs promoter activity between K562 and K562/DNR was derived from the region between −49 bp and the first exon. The reason for the absence of MDR overexpression in HL60/DNR cells is not known but the absence of an important transcription factor would be suspected.

Inhibitors against DNA-PKcs, ATM and MDR1 protein have been reported to overcome fludarabine resistance in CLL cells [57]. Thus, it seems likely that components of NHEJ including DNA-PKcs and DNA ligase IV or their common transcription factor, Sp1, are the medicinally treatable target for overcoming the MDR phenotype. However, further work is needed to rule out unexpected adverse effects by inhibiting housekeeping transcription factors such as Sp1.

Taken together, our analysis revealed that NHEJ is one of the plausible mechanisms of drug resistance and that Sp1 transcription factor induces several important gene transcriptions leading to the MDR phenotype.

5. Conclusion

In this study, Enzymes of NHEJ, DNA-PKcs and DNA ligase IV were overexpressed in MDR-phenotype leukemia cell lines, which increased double strand break repair, and also their overexpression was due to the activated Sp1 transcription factor bound with respective 5′ promoter region. These overexpressed enzymes play a role in the MDR phenotype

and might enhance further genetic abnormality by repeated anti-cancer drug treatment.

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