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Expression of Multidrug Resistance Protein 1 in Human Hepatocellular Carcinoma

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Background: Multidrug resistance protein 1 (MDR1) encoded by ATP-binding cassette, sub-family B (Mdr/Tap), member 1 (*ABCB1*) mediates cross-resistance to antineoplastic drugs, and its expression is related to tumor aggressiveness. **Methods:** MDR1 expression was investigated in 100 hepatocellular carcinomas (HCCs) by immunohistochemical staining. The epigenetic mechanisms underlying *ABCB1* transcriptional regulation were investigated in cell lines. **Results:** MDR1 was normally localized in the bile canalicular surface of the hepatocytes. Among 100 HCCs, 45 showed canalicular/luminal (CL) staining similar to the normal pattern, another 45 displayed membranous/cytoplasmic (MC) overexpression, and the remaining 10 revealed loss of expression. MC pattern or null staining of HCCs correlated with a higher histological grade and had a poorer prognosis than HCCs with a CL pattern ($p < 0.05$). They also tended to have a poor prognosis by multivariate survival analysis. The *ABCB1* promoter was hypomethylated regardless of MDR1 expression or *ABCB1* mRNA levels in 10 HCC cell lines. Histone deacetylase inhibitor treatment induced *ABCB1* upregulation in 4 cell lines with low or moderate *ABCB1* levels. **Conclusions:** Our findings suggest that either an increase or a loss of MDR1 expression may contribute to the poor outcome of HCCs; histone deacetylation may be one of the epigenetic mechanisms directing the *ABCB1* expression in HCCs.

Key Words: Carcinoma, hepatocellular; MDR1 protein, human; Immunohistochemistry; Survival analysis; Epigenesis, genetic

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the 5th most common cancer in Korea.[1,2] Surgical resection improves survival and offers the chance of a cure in localized HCC, but chemotherapy is often adopted as a primary treatment in inoperable cases, as an adjuvant therapy after surgical resection or as a preoperative procedure. Unfortunately, response rates and the benefits of chemotherapy are generally unsatisfactory.[3]

Intrinsic or acquired development of multidrug resistance to antitumor agents can severely impede the efficacy of the cancer treatment. Multidrug resistance protein 1 (MDR1), a 170-kDa transmembrane ATP-dependent drug transport pump encoded by the ATP-binding cassette, sub-family B (Mdr/Tap), member 1 (*ABCB1*) gene, is a well-established mediator of multidrug resistance. A number of cancer types show MDR1 upregulation after chemotherapy or other exogenous stimuli, and thus are resistant to multiple anticancer agents.[4-7] On the other hand, MDR1 is endogenously expressed in several organs such as the liver, kidneys, and intestines. These organs are the sites from which cytotoxic natural products or their metabolites are removed from the body. Thus, MDR1 may also have a protective function in the context of mutagenesis and carcinogenesis.[4,8] Significant progress has been made in terms of delineating the function and regulation of MDR1. However, the molecular mechanisms that underlie its expression and silencing in tumor cells have not been fully defined, although several studies have indicated that the transcriptional activation of the *ABCB1* gene is controlled by both genetic and epigenetic modifications.[9-14]

Expression of MDR1 has been studied in human liver and HCCs.[15-23] Some investigators have reported that MDR1 expression is correlated with carcinogenesis and tumor progression, whereas others have produced contradictory results.[18-23] Moreover, the mechanisms of *ABCB1* gene activation/inactivation have not been elucidated in HCCs.

In the present study, MDR1 expression was investigated in HCCs, and the relationships between expression, clinicopathologic parameters and survival were examined. In addition, the mechanisms underlying *ABCB1* gene transcriptional regulation, especially in terms of the epigenetic control, were investigated.

MATERIALS AND METHODS

Tissue samples and array

One hundred HCC tissue samples resected between 1991 and 2001, and 20 nonneoplastic liver tissues obtained by resection for diseases other than HCC were obtained from Inje University Seoul Paik Hospital. All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. The preparation of tissue array was carried out as detailed previously.[24] Briefly, core tissue biopsies (2 mm in diameter) were taken from individual donor blocks and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea). For a cell line tissue array, which contained 10 human HCC cell lines, we adopted the protocol recommended by http://www.tissue-array.com/ver3 after harvesting cells either by trypsinization or scraping.

Experimental protocols involving human tissue specimens were reviewed and approved by the Institutional Review Board of Seoul Paik Hospital.

Immunohistochemical (IHC) staining

Serial 4 μm-thick sections of tissue array blocks were subjected to IHC staining. Sections were deparaffinized and endogenous peroxidase activity was blocked with 3% H_2O_2. After antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes at 95ºC, slides were labeled with MDR1 monoclonal antibody (1 : 100 , clone G-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. After washing with phosphate-buffered saline, primary antibody enhancer and HRP polymer (UltraVision LP, Lab Vision Corporation, Fremont, CA, USA) were applied and incubated for 30 minutes. 3-Amino-9-ethylcarbazole was used as a chromogen and sections were counterstained with Mayer's hematoxylin. A negative control with omitted primary antibody was run simultaneously.

Cell culture

The human HCC cell lines used in this experiment were; SNU-182, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, Hep-3B, and PLC/PRF/5. The SNU series were obtained from the Korean Cell Line Bank (Seoul, Korea) and Hep-3B and PLC/PRF/5 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) at 37ºC in a humidified CO_2 incubator.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIZOL reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). To generate cDNA, mRNA (5 ng) was reverse-transcribed using MMLV-reverse transcriptase (Takara, Shiga, Japan) with random-primer. PCR amplification was performed over 28 cycles (95ºC for 30 seconds, 50ºC for 30 seconds, 72ºC for 1 minute), and lastly, a final extension at 72ºC for 10 minutes in a thermal cycler (Bioneer, Seoul, Korea). To normalize the expression levels, *GAPDH* was amplified under the same conditions. The sequences of the primers used are listed in Table 1.

Methylation-specific PCR (MSP) and bisulfite genomic sequencing

MSP and bisulfite genomic sequencing of the CpG sites in exon 1 of *ABCB1* were carried out as detailed previously[10] with some modification. Briefly, 2 μg of genomic DNA was denatured with NaOH (final concentration, 0.2 M). Next, 3 M sodium-bisulfite (Sigma, St. Louis, MO, USA) and 10 mM hydroquinone (pH 5.0, Sigma) were added and incubated at 50ºC for 16 hours. DNA was purified using a Wizard DNA purification kit (Promega, Madison, WI, USA), treated with NaOH, recovered in ethanol, and resuspended in 20 μL of distilled water. PCR was conducted in a thermal cycler at 95ºC for 5 minutes, followed by 35 amplification cycles (95ºC for 30 seconds, 58-64ºC for 30 seconds, 72ºC for 1 minute), and a final extension at 72ºC for 10 minutes. PCR products of MSP and unmethylation-specific PCR (USP) were subjected to electrophoresis in 2.5% agarose. The products were directly sequenced using a BigDye Terminator cycle sequencing ready reaction kit and an ABI 3100 sequencer (Applied BioSystem, Foster City, CA, USA). The primer sequences of MSP, USP, and sequencing are shown in Table 1.

5-Aza-2′-deoxycytidine (Aza-dC) and trichostatin A (TSA) treatment

To elucidate the epigenetic mechanisms by which *ABCB1* gene expression are regulated, cells were treated or not treated with DNA demethylating agent Aza-dC (5 μM, Sigma), his-

Table 1. Primer sequences

Genes		Sequences	Product size (bp)
ABCB1			
RT-PCR	S	5′- GAATCTGGAGGAAGACATGACC -3′	259
	AS	5′- TCCAATTTTGTCACCAATTCC -3′	
MSP	S	5′- CGAGGAATTAGTATTTAGTTAATTCGGGTCGG -3′	95
	AS	5′- ACTCAACCCACGCCCCGACG -3′	
USP	S	5′- TGAGGAATTAGTATTTAGTTAATTTGGGTTGG -3′	95
	AS	5′- ACTCAACCCACACCCCAACA -3′	
Bisulfite sequencing	S	5′ -GGAAGTTAGAATATTTTTTTTGGAAAT -3′	223
	AS	5′- ACCTCTACTTCTTTAAACTTAAAAAAACC -3′	
ChIP	S	5′- CCTCCTGGAAATTCAACCTG -3′	154
	AS	5′- TGTGGCAAAGAGAGCGAAG -3′	
GAPDH			
RT-PCR	S	5′- GAAGGTGAAGGTCGGAGTC -3′	226
	AS	5′- GAAGATGGTGATGGGATTTC -3′	

ABCB1, ATP-binding cassette, sub-family B (Mdr/Tap), member 1; RT-PCR, reverse transcription-polymerase chain reaction; S, sense; AS, anti-sense; MSP, methylation-specific PCR; USP, unmethylation-specific PCR; ChIP, chromatin immunoprecipitation.

tone deacetylase (HDAC) inhibitor TSA (300 nM, Sigma), or a combination of Aza-dC and TSA for 24 hours. The cultured cells were harvested 4 days later. Differences in expression level of *ABCB1* mRNA transcript and MDR1 protein before and after the treatment were analyzed by RT-PCR and IHC staining.

Chromatin immunoprecipitation (ChIP)

ChIP was performed on SNU-398 cells after TSA treatment following the instructions issued by the supplier of anti-acetyl H3 and anti-acetyl H4 (Upstate Biotechnology, Lake Placid, NY, USA). Briefly, cells were harvested, proteins were cross-linked to DNA by adding 1% formaldehyde, quenched with glycine (0.125 M), resuspended in sodium dodecyl sulfate lysis buffer containing protease inhibitor, and sonicated to fragment chromatin to less than 400 bp. Immunoprecipitation was carried out overnight at 4ºC with 10 μg of anti-acetyl H3 or 5 μg of anti-acetyl H4. Immunoprecipitated DNA was collected, purified and eluted in TE buffer. PCR was performed using primers (Table 1) targeting the region between -164 and -11 upstream of the transcription start site. Amplified input DNA before antibody addition served as a control.

Statistical analysis

Statistical analyses were performed using SPSS ver. 11.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was used to examine whether a correlation existed between the variables. Survival was analyzed using the Kaplan-Meier method, and survival curves were compared using the log rank test. For multivariate analyses, the prognostic significance was assessed using the Cox proportional hazards regression model. p-values less than 0.05 were considered to be statistically significant and p-values between 0.05 and 0.1 were interpreted as being borderline significant.

RESULTS

MDR1 expression in tissue array

In nonneoplastic liver tissue, MDR1 was localized to the bile canalicular surface of hepatocytes with moderate to strong staining intensity (Fig. 1A). Bile duct epithelial cells exhibited strong cytoplasmic positivity, whereas stromal cells were entirely negative.

Among 100 HCCs, 90 exhibited MDR1 positivity, and two different expression patterns were noted: canalicular/luminal (CL) (n = 45) and membranous/cytoplasmic (MC) (n = 45) (Fig. 1B, C). The staining intensity was not very different between the two groups; however, the staining area was higher in the MC pattern than the CL pattern. Within individual tissue array cores, the staining intensity and pattern were homogeneous with little variation in the different fields. Ten HCCs revealed complete loss of MDR1 expression (Fig. 1D).

The clinicopathologic profiles and their relationships with MDR1 expression are summarized in Table 2. In HCCs with macrotrabecular or compact type, the MC pattern was more frequently noted than the CL pattern, while HCCs of microtrabecular or pseudoglandular type mostly showed the CL pattern (p = 0.058). Both the MC and negative HCC groups were asso-

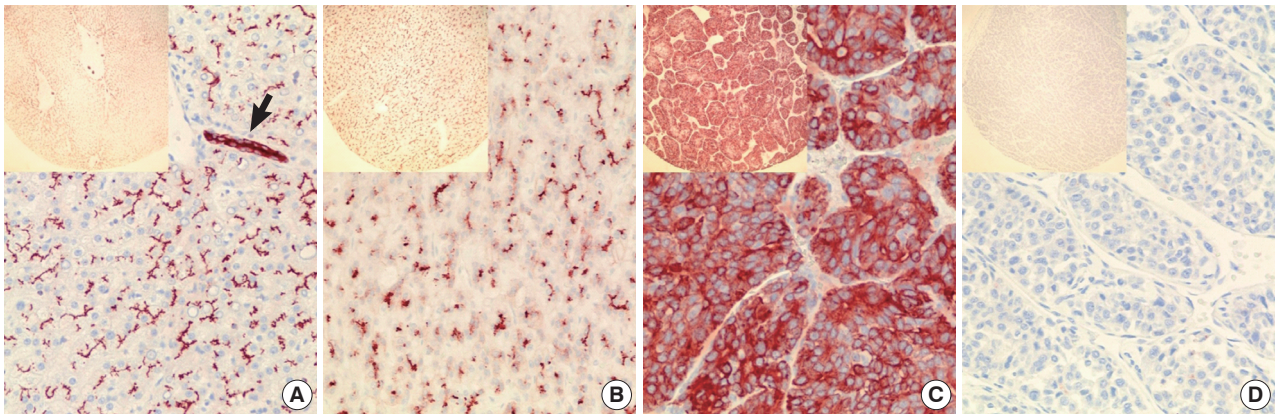

Fig. 1. Immunohistochemical analysis of multidrug resistance protein 1 (MDR1) expression in the tissue array. (A) Nonneoplastic liver tissue showing positive staining along the bile canalicular surface. Bile duct epithelial cells (arrow) display supranuclear cytoplasmic positivity. (B-D) Hepatocellular carcinomas with a canalicular/luminal staining pattern (B) membranous/cytoplasmic staining pattern (C) and complete loss of MDR1 expression (D).

Table 2. Relationship between MDR1 expression and clinicopathological characteristics

Parameters		MDR1 expression		Negative	p-value
		CL pattern	MC pattern		
Age (yr)[a]	< 53	19	23	5	0.398
	≥ 54	26	22	5	0.654
Sex	Male	42	39	5	0.292
	Female	3	6	5	0.003**
Tumor size (cm)	≤ 5	25	22	4	0.527
	> 5	20	23	6	0.373
Histologic type	Compact	17	21	6	0.058*
	Macrotrabecular	19	23	3	0.495
	Microtrabecular	4	0	1	
	Pseudoglandular	5	1	0	
TNM stage	I/II	26	18	3	0.092*
	III/IV	19	27	7	0.112
Edmondson grade	2	20	8	1	0.006**
	3/4	25	37	9	0.043**
Growth pattern	Expansile	35	31	7	0.340
	Infiltrative	10	14	3	0.601
Intrahepatic metastasis (n = 99)[b]	Negative	30	27	5	0.602
	Positive	15	17	5	0.322
Portal vein thrombi	Negative	23	13	4	0.031**
	Positive[c]	22	32	6	0.525
TAE	Not done	35	29	6	0.163
	Done	10	16	4	0.243
Surgical margin (cm)	≤ 1	29	34	6	0.250
	> 1	16	11	4	0.792
Recurrent HCC (n = 92)[b]	Absent	16	12	3	0.537
	Present	28	28	5	0.951
Serum HBsAg	Negative	12	13	3	0.814
	Positive	33	32	7	0.830
Serum anti-HCV (n = 97)[b]	Negative	41	39	9	0.458
	Positive	3	5	0	0.420

The two consecutive p-values for each parameter represent the comparison between the CL and MC groups and the CL and negative groups, respectively.
*Borderline significant, or p-values between 0.05 and 0.1; **Statistically significant, or $p < 0.05$.
[a]Mean patient age is 53.4 yr (range, 24 to 79 yr); [b]Parameters with uninformed cases; [c]Positive portal vein thrombi include both macroscopic/microscopic (n = 27) and microscopic only (n = 33) involvement of vessels.
MDR1, multidrug resistance protein 1; CL, canalicular/luminal; MC, membranous/cytoplasmic; TNM, tumor, node and metastasis; TAE, transarterial chemoembolization; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

ciated with a higher histological grade than the CL group ($p < 0.05$). Within the MDR1 positive HCCs, the MC group had a more frequent portal vein thrombi and higher tumor, node and metastasis (TNM) stage[25] than the CL group ($p = 0.031$ and $p = 0.092$, respectively). The comparison between the MDR1 positive and negative groups indicated that MDR1 negativity was more frequently noted in the female patients ($p < 0.05$). No significant difference in other clinicopathologic parameters including the treatment by preoperative transarterial chemoembolization (TAE) was observed among the MDR1 expression groups.

Survival analysis

The median survival period of the patients was more than 60 months for the CL group, 48 months for the MC group, and 23 months for null group. By univariate analysis, the HCCs with MC pattern staining and MDR1 null staining had a significantly shorter survival than the CL group ($p = 0.0183$ and $p = 0.0148$, respectively) (Fig. 2). No difference in survival was noted between the MDR1 negative and positive groups taken as a whole (data not shown). The clinicopathologic variables with significant prognostic value for survival were; TNM stage, Edmondson's grade, portal vein tumor thrombi formation, recurrent HCC, and tumor growth pattern ($p < 0.05$).

A multivariate analysis including MDR1 expression status, TNM stage, and histological grade revealed that TNM stage had the most significant prognostic value. MDR1 expression status had borderline significant values: Both MC and null groups tended to have a poorer prognosis than HCCs with the CL pattern (hazard ratio, 2.058 and 2.852; $p = 0.079$ and $p = 0.044$, respectively) (Table 3).

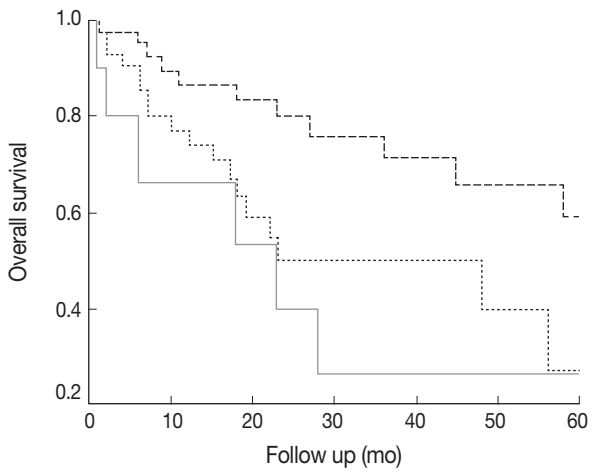

Fig. 2. Overall survival in patients with hepatocellular carcinomas according to multidrug resistance protein 1 (MDR1) expression. The dashed line represents a canalicular/luminal (CL) pattern; the dotted line, membranous/cytoplasmic (MC) pattern; the continuous line, null expression group. p-values are 0.0183 when comparing the CL and MC groups and 0.0148 when comparing the CL and null groups.

MDR1 and *ABCB1* mRNA expression in HCC cell lines

MDR1 expression was examined in the 10 human HCC cell lines by IHC staining. Five cell lines (SNU-182, SNU-354, SNU-368, Hep-3B, and PLC/PRF/5) showed diffuse strong cytoplasmic staining; while 2 cell lines (SNU-387 and SNU-398) were completely negative (Fig. 3A, B). The 3 remaining cell lines (SNU-423, SNU-449, and SNU-475) revealed focal or intercellular staining in cells harvested by scraping, and punctate intracellular staining in the cells harvested by trypsinization (Fig. 3C).

ABCB1 mRNA levels measured by RT-PCR are shown in Fig. 4A. *ABCB1* mRNA expression was observed in 7 of the 10 HCC cell lines; six cell lines (SNU-182, SNU-354, SNU-368, SNU-423, SNU-449, and Hep-3B) expressed high levels of *ABCB1*, and one (SNU-475) expressed a moderate *ABCB1* level. The SNU-387, SNU-398, and PLC/PRF/5 cell lines displayed a very low level of or no detectable *ABCB1*. The MDR1 staining and the *ABCB1* mRNA expression in the HCC cell lines showed a good correlation.

Methylation and histone acetylation status of *ABCB1* promoter

To understand the mechanistic basis of *ABCB1* expression, the promoter methylation status of *ABCB1*was examined. MSP produced positive bands for all cell lines when primers for the unmethylated sequence were used; and no visible band when primers for the methylated sequence were used (Fig. 4A). Bi-

Table 3. Multivariate survival analysis for predictive factors of overall survival using the Cox regression model

Variable	Hazard ratio (95% confidence interval)	p-value
MDR1 expression		0.089
MC vs CL positive	2.058 (0.920-4.604)	0.079
Null vs CL positive	2.852 (1.031-7.888)	0.044
TNM stage		
III/IV vs I/II	7.246 (2.573-20.410)	0.000
Edmondson grade		
3/4 vs 2	1.187 (0.377-3.742)	0.769

MC, membranous/cytoplasmic; CL, canalicular/luminal; TNM, tumor, node and metastasis.

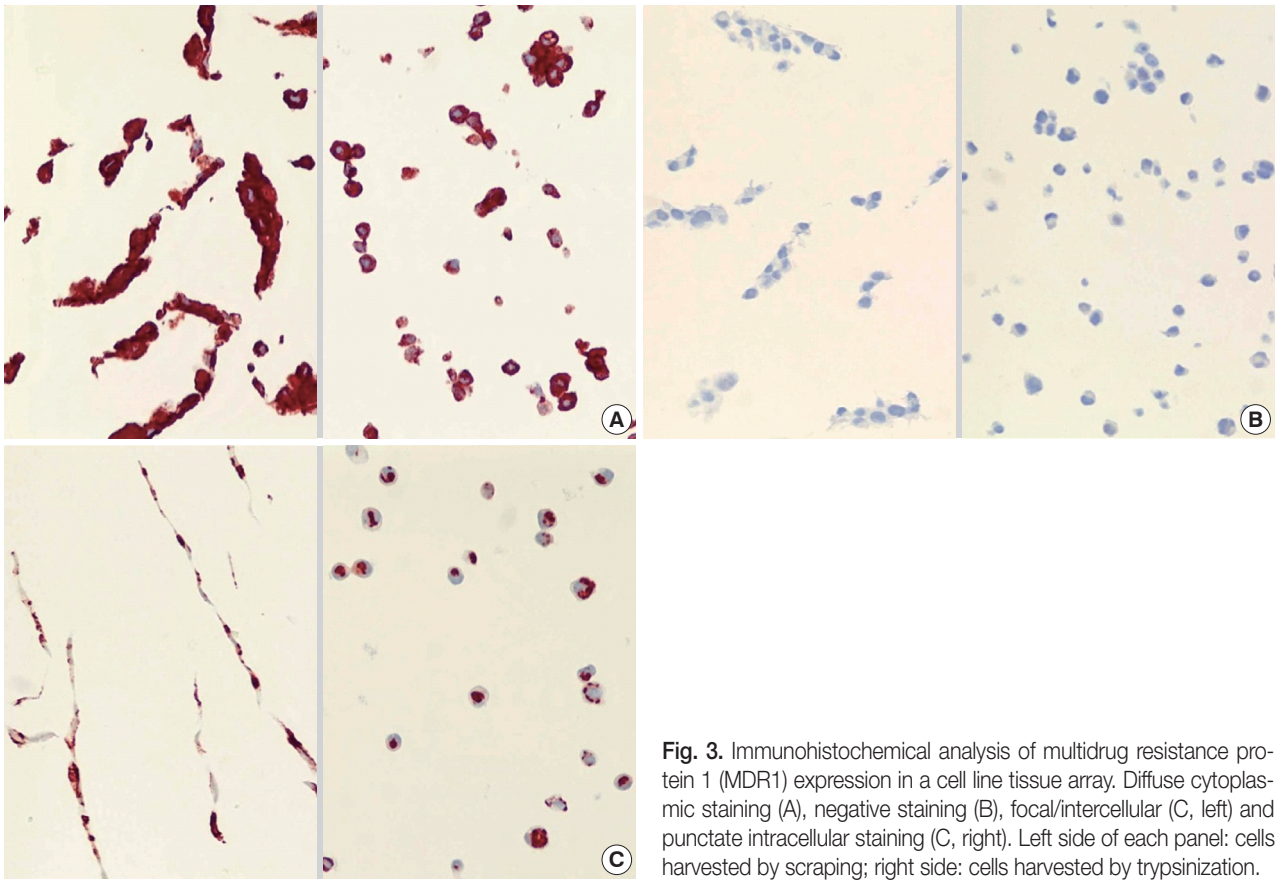

Fig. 3. Immunohistochemical analysis of multidrug resistance protein 1 (MDR1) expression in a cell line tissue array. Diffuse cytoplasmic staining (A), negative staining (B), focal/intercellular (C, left) and punctate intracellular staining (C, right). Left side of each panel: cells harvested by scraping; right side: cells harvested by trypsinization.

sulfite genomic sequencing revealed the absence of CpG methylation in the examined *ABCB1* promoter region (Fig. 4B). In addition, we tested HCC cell lines for the response to treatment with the DNA demethylating agent Aza-dC or HDAC inhibitor TSA. RT-PCR reaction revealed that TSA treatment induced the *ABCB1* upregulation; however, Aza-dC treatment did not (Fig. 5A). This upregulation was noted in 4 cell lines with very low to moderate expression of *ABCB1* mRNA. In 6 cell lines with high levels of *ABCB1* mRNA expression, no further upregulation was identified. Moreover, treatment with Aza-dC did not induce *ABCB1* upregulation in any cell line, and combined TSA and Aza-dC treatment did not enhance the level of *ABCB1* mRNA level over that achieved by TSA alone (Fig. 5B). Concordant gain of MDR1 protein expression in the same cell lines were noticed after the treatment of TSA (Fig. 5B).

To determine whether the chromatin structure remodeling by posttranslational modification of nucleosome structure plays a role in the activation of the *ABCB1*, we assessed the acetylation status of the core histone proteins H3 and H4 by ChIP assays. Treatment of SNU-398 cells with TSA induced histone H4 acetylation in the *ABCB1* promoter (Fig. 5C).

DISCUSSION

We investigated the expression of MDR1 in 100 HCCs and evaluated its relationship with clinicopathologic findings. Ninety percent of the HCCs expressed MDR1 in two distinct staining patterns. HCCs with CL expression presented a pattern similar to that of a nonneoplastic liver, as they maintained bile canalicular localization. The MC staining pattern, which differed from the normal pattern, may represent a high level of MDR1 expression. Earlier studies have described canalicular, luminal, membranous, or cytoplasmic MDR1 localization in HCCs with expression rates ranging from 65 to 92%.[16-21,23] The majority of these studies described the overall positivity of tumor cells and did not discriminate the discrete staining patterns. Ng *et al.*[21] noticed the MC pattern in about 30% of the MDR1 positive HCCs, but did not further clarify the clinicopathologic relationship of the staining pattern. In the present study, two MDR1 staining patterns were described as well as the different clinicopathologic characteristics (histological type and grade, tumor stage, and presence of intravenous tumor thrombi formation) between the groups. Moreover, 10 HCCs

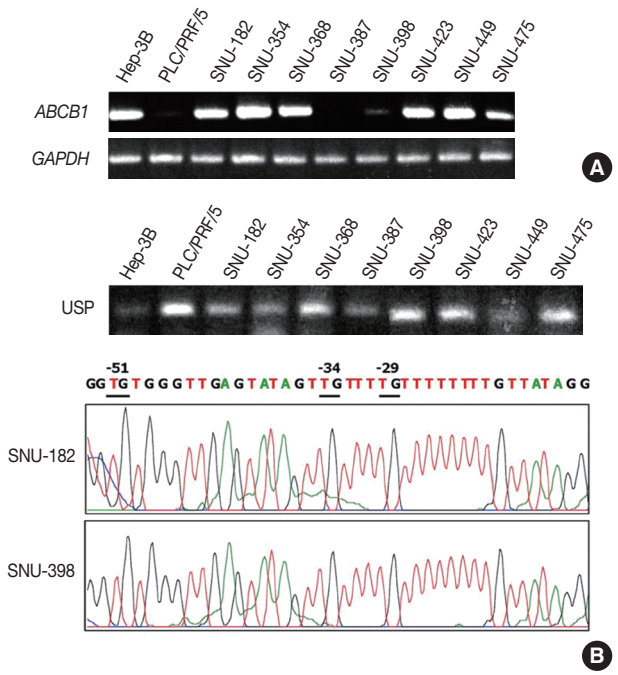

Fig. 4. Methylation status of ATP-binding cassette, sub-family B (Mdr/Tap), member 1 (*ABCB1*) promoter. (A) *ABCB1* mRNA levels in hepatococellular carcinomas cell lines measured by reverse transcription-polymerase chain reaction (PCR). Three cell lines showing nearly absent *ABCB1* mRNA. (B, upper) Presence of unmethylation-specific PCR (USP) bands in all hepatococellular carcinomas cell lines. (B, lower) Bisulfite genomic sequencing showing that all three CpG sites of -51, -34, and -29 are not methylated in both SNU-182 and SNU-398.

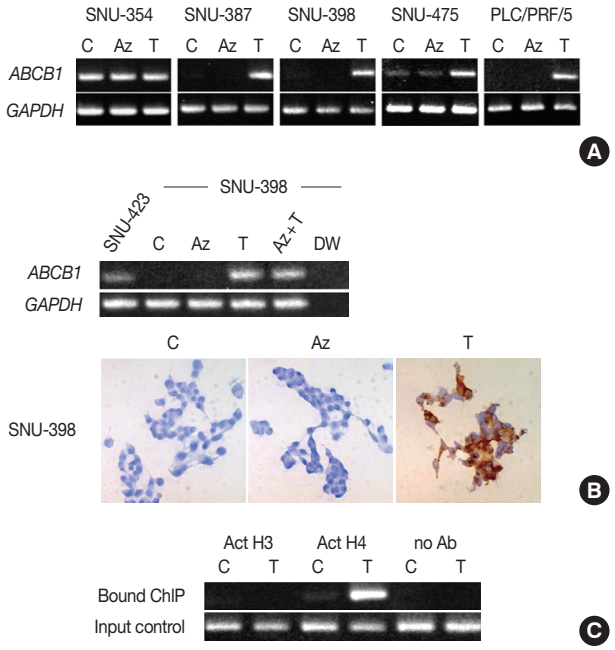

Fig. 5. Histone acetylation status of the multidrug resistance protein 1 (*MDR1*) promoter. (A) Reverse transcription-polymerase chain reaction of ATP-binding cassette, sub-family B (Mdr/Tap), member 1 (*ABCB1*) after treatment with 5-aza-2′-deoxycytidine (Aza-dC) or trichostatin A (TSA). TSA inducing the expression of *ABCB1* in cell lines with low or no expression. (B) *ABCB1* expression (upper panel) and MDR1 expression (lower panel) in SNU-398 cells following treatment with Aza-dC, TSA, or both. (C) Chromatin immunoprecipitation (ChIP) assay targeting the *ABCB1* promoter in SNU-398 cells revealing TSA treatment inducing the acetylation of H4.
C, control without treatment; Az, Aza-dC; T, TSA; DW, distilled water; Act, anti-acetyl; Ab, antibody.

revealed negative MDR1 staining and were associated with a higher histological grade. As in a previous study[26] we observed a trend between the pseudoglandular type HCC and CL pattern MDR1 expression. While the study described many trabecular type HCCs lost MDR1 expression, no such finding was noticed in our study. MDR1 negativity was also frequently noted in the female patients; nonetheless this association needs more verification because only 14 female patients were included in this study. We postulated from the above results that both higher level MC expression patterns and loss of MDR1 expression represent altered MDR1 expression in HCCs compared to the normal staining pattern in nonneoplastic liver.

Several prior studies have reported a patchy distribution for MDR1 positive carcinoma cells and variable intensity of immunoreactivity within HCC.[20,21] In the present study, we performed IHC staining using the tissue array and observed homogeneous MDR1 expression within the individual array cores of neoplastic and non-neoplastic tissues.

MDR1 participates in the resistance to anticancer agents, and its expression is often induced after chemotherapeutic drug treatment. However, intrinsic MDR1 expression in the absence of drug treatment has also been noted in a number of malignancies.[6,7,12,14] In our study, 30 patients received preoperative TAE along with a combination cisplatin (60 mg/m^2) and epirubicin (60 mg/m^2); the MDR1 expression in HCC was not significantly affected by this preoperative TAE. This result as well as the high incidence of MDR1 expression supports the intrinsic nature of MDR1 expression in HCC, which also concurs with the findings of the previous studies.[16,17,19,22]

A few studies have conducted a survival analysis on HCCs with respect to MDR1 and have reported MDR1 positivity with a poor prognostic[18,22] or no prognostic correlation.[21] We further evaluated a possible correlation between MDR1 expression and clinical outcome. HCCs with an MC pattern for MDR1 expression or null staining were found to have an unfavorable prognosis compared to the HCCs with a CL pattern. The differences in survival between the groups were statistically signifi-

cant by univariate analysis. MDR1 expression remained a meaningful factor after the tumor stage as per a multivariate survival analysis. However, MDR1 negative or positive groups, taken as a whole, did not reveal any differences in survival. Taken together, our data suggest that altered MDR1 expression as an MC pattern and loss of expression may be important in the progression of HCCs rather than MDR1 expression *per se*.

MDR1 has been described as a double-edged sword. Its expression is viewed as a major obstacle to successful cancer chemotherapy. However, its action as a drug efflux pump protects cells against noxious substances (including mutagenic anticancer drugs) and prevents or reduces mutagenesis and carcinogenesis.[4,8] Therefore, loss of MDR1 expression may have adverse effects, e.g., it may induce further mutagenesis, even to the neoplastic cells and thus increase aggressiveness in HCC, as postulated in our study. In this respect, the pharmaceutical trial of MDR1 inhibitor aiming to overcome the resistance to chemotherapeutic treatment might not be always beneficial in the treatment of HCCs.

Earlier studies have indicated that transcriptional activation tends to be a more common event in *ABCB1* gene regulation than amplification.[6,11] Recently, studies on *ABCB1* transcriptional regulation have been widened in scope to examine the role of epigenetic components, such as promoter methylation or histone acetylation status, which both modify the chromatin accessibility of transcription factors.[9-14] Since the mechanism of *ABCB1* gene expression has not been precisely studied in human HCCs, we tried to elucidate the means by which *ABCB1* gene expression is regulated by epigenetic analysis using HCC cell lines. MDR1 expression was noted in 8 out of 10 HCC cell lines and also exhibited two staining patterns, which possibly corresponds to our HCC tissue array findings. *ABCB1* mRNA levels were frequently high and correlated well with MDR1 immunohistochemical expression. In addition, the result of *ABCB1* RT-PCR correlated well with the mRNA levels evaluated in the same 10 HCC cell lines as determined by oligonucleotide microarray analysis using the GeneChip® Human Genome U133A 2.0 array (Affymetrix, Santa Clara, CA, USA) (data not shown). In the methylation analysis, the *ABCB1* promoter was hypomethylated regardless of *ABCB1* mRNA transcript levels, and thus was a competent factor in transcriptional activation. In addition, treatment of HCC cell lines with the DNA demethylating agent Aza-dC did not increase the *ABCB1* transcript level. These results suggest that CpG methylation might not be a contributing factor associated with the *ABCB1* transcriptional silencing in HCC. The above findings also suggest that promoter hypomethylation is necessary, but that it is probably not sufficient to induce *ABCB1* activation in HCCs. Alternatively, the transcriptional activity of the hypomethylated *ABCB1* promoter could be regulated by histone modification.[11-14] Therefore we next explored the acetylation status of histones H3 and H4 within the *ABCB1* promoter region. TSA treatment stimulated *ABCB1* expression in cells with no or low expression, and the ChIP analysis using antibodies against acetyl-H3 or acetyl-H4 displayed acetylation of histone H4 in SNU-398 cells after TSA treatment. These findings suggest that *ABCB1* gene silencing and activation in a subset of HCCs is responsive to histone H4 acethylation status. Since the cell lines used in this study were hepatitis B virus (HBV)-positive cells (http://cellbank.snu.ac.kr), histone deacetylation might be one of the epigenetic mechanisms that regulate the transcription of the *ABCB1* gene in HBV-related HCC.

In summary, the present study showed that MDR1 expressional pattern might be a useful predictor of tumor progression and survival in HCCs. The results also suggest that histone deacetylation might be one of the epigenetic mechanisms that direct *ABCB1* gene transcriptional regulation in HCCs. Further study to delineate the biological and functional significance of MDR1 expressional pattern and *ABCB1* regulation in HCC is warranted.

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