ABCG2 Overexpression Confers Poor Outcomes in Hepatocellular Carcinoma of Elderly Patients

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Abstract. *Background/Aim: Breast cancer resistance protein [BCRP/ATP-binding cassette subfamily G member 2 (ABCG2)] is a member of the ATP-binding cassette transporter family, used as a maker of cancer stem cells (CSCs) and thought to be responsible for drug resistance by pumping them out of cells. However, its precise role in various cancer types has been controversial, and the aim of this study was to investigate the expression of ABCG2 in hepacellular carcinoma (HCC) and relate the results to established prognostic factors. Patients and Methods: We conducted analysis of 181 HCC and paired-match adjacent normal liver tissue by immunohistochemistry from tissue array of slides. Results: The mean score for ABCG2 expression was higher in tumor than in adjacent normal liver tissue of HCC patients (p<0.001). There was a statistically significant correlation between ABCG2 expression and age, differentiation status and hepatitis B surface antigen (p=0.031, p=0.015 and p=0.033, respectively). Additionally, increased expression of ABCG2 in HCC and its statistically significant correlation with hepatitis B surface antigen was found in elderly (p=0.039), not in younger patients (p=0.518). Importantly, by using Kaplan-Meier and Cox regression analysis, overall survival in patients with high expression of ABCG2 was found reduced in elderly patients (p=0.029 and p=0.081, respectively). Conclusion: ABCG2 can be used as a target for the development of novel therapies in HCC.*

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed cancer in male and ninth most commonly diagnosed cancer in female patients, and the second leading cause of cancer-related death worldwide, and generally more common in non-white populations (1). The risk factors have been recognized as associated with hepatocellular carcinoma, including HBV, HCV, alcoholic liver disease, and most probably non-alcoholic fatty liver disease (2, 3). However, systemic therapies used to treat HCC patients are facing many obstacles including drug resistance and drug toxicity (4).

Human ATP-binding cassette subfamily G member 2 (ABCG2), also known as the breast cancer resistance protein (BCRP) is an efflux protein shown be involved in tumor resistance to many therapeutic agents (5, 6). The normal function of ABCG2 is highly expressed in placental trophoblastic cells to pump toxic metabolites from the fetal to the maternal blood vessels (7). Particularly, BCRP/ABCG2 inhibitor greatly augmented the cytotoxicity of multikinase inhibitors such as sorafenib and gefitinib, especially in HCC, suggesting that blockage of BRCP/ABCG2 may be a potential strategy to increase the sensitivity of targeted therapy drugs and reverse ABCG2-mediated drug resistance (8, 9).

In addition, ABCG2 overexpression has been associated with cancer progression by promoting proliferation and anti-apoptosis *via* MAPK singnaling pathway in laryngeal squamous cell carcinoma (10). Conversely, in renal carcinoma, aberrant promoter methylation-dependent is a repressor complex in the CpG island contributes to down-regulation of ABCG2 (11). In addition to membranous form

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of ABCG2, it is found inside the nucleous that promote lung colonization of circulating cancer cells (12).

Recently, the theory of cancer stem cells (CSCs) and cancer-initiating cells are proposed, and ABCG2 is also widely used as a marker in various cancers, including HCC, suggesting that those cells with ABCG2 expression cause tumorigenicity, proliferation, drug resistance, and metastasis ability (13-16). However, the expression of ABCG2 in hepatocellular carcinogenesis remains elusive.

In this study, we analyzed 181 tumor specimen and their paired adjacent normal tissue without chemotherapy or targeted therapy drugs before surgery, and examined ABCG2 levels by immunohistochemistry. We further investigated whether ABCG2 and clinicopathological parameters can be an independent prognostic value in hepatocellular carcinoma by Kaplan-Meier and cox regression analysis.

Patients and Methods

Patients. Primary tumor tissues were obtained from 181 HCC patients receiving surgical resection in Changhua Christian Hospital from July 2011 to November 2013. The initial characteristics and clinical outcomes were collected until death, censorship or loss of follow-up. For each patient, representative tissue cores of the HCC tumor parts and the adjacent normal part were carefully collected and made into tissue microarray. This study was approved by the Ethics Committee of the Institutional Review Board of Changhua Christian Hospital. Informed consents were obtained from all sample donors in accordance with the Declaration of Helsinki and were obtained at the time of their donation. The age of all patients was between 29 and 81 years (mean±SD 63.1±11.7). Clinical parameters and overall survival data were collected from chart review. The survival time was defined to be the period of time from the date of primary surgery to the date of death. The median followup time after surgery was 884 months and the median overall survival of all patients was 935 days. During this survey, 41 patients died. On the basis of the follow-up data, 7 patients relapsed.

Immunohistochemistry and scoring. Immunohistochemistry (IHC) was used to detect ABCG2 protein expression. The ABCG2 antibody (sc-377176) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Paraffin-embedded HCC tissue sections (4-μm thick) on poly-1-lysine-coated slides were deparaffinized and rinsed with 10mM Tris-HCl (pH 7.4) and 150mM sodium chloride. Peroxidase was quenched with methanol and 3% hydrogen peroxide. Slides were then placed in 10mM citrate buffer (pH 6.0) at 100˚C for 20 min in a pressurized heating chamber. After incubation with 1:200 dilution of ABCG2 antibody (sc-377176) for 1 h at room temperature, slides were thoroughly washed three times with phosphate-buffered salinen (PBS). Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin. At last, the slides were photographed with the microscope (BX50, OLYMPUS, Japan). Negative controls were obtained by performing all IHC steps, but leaving out the primary antibody. The immunohistochemical staining scores were defined as described previously (17) and the intensities of signals were evaluated by a board-certified pathologist. Immunostaining scores were defined as the cell staining intensity (0=nil; 1=weak; 2=moderate; and 3=strong) multiplied by the percentage of labeled cells (0-100%), leading to scores from 0 to 300. A score higher than mean were defined as 'high' immunostaining, while a score equal to or lower than mean was categorized as 'low' in tumor.

Statistical analysis. Chi-square analysis and paired-samples *t*-test were conducted using SPSS software (Version 13.0 SPSS Inc, Chicago, IL, USA) for statistical analysis. Statistical differences for survival data were analyzed using the log-rank test. Survival curves were plotted using the Kaplan–Meier method and variables related to survival were analyzed using Cox's proportional hazards regression model using SPSS software. A *p*-Value less than 0.05 was considered to be statistically significant.

Results

ABCG2 expression is higher in tumor than in adjacent normal liver tissue. A total of 181 hepatocellular carcinoma patients were enrolled in the study. ABCG2 expression was detected by immunohistochemistry in 181 paired hepatocellular tumor and adjacent normal liver tissue. Representative results are shown in Figure 1. ABCG2 expression level in hepatocellular carcinoma was significantly higher than the paired adjacent normal liver tissues ($p<0.001$) (Figure 1). The mean score of ABCG2 in tumors and their adjacent normal liver tissues was 149.02±64.71 *versus* 90.40±48.39. A score higher than mean were defined as 'high' immunostaining, while a score equal to or lower than mean was categorized as 'low' in tumor, and then as the following analysis.

ABCG2 is significantly correlated with age and hepatitis B virus infection. To verify whether ABCG2 was linked with clinico-pathological parameters, further stastitical analysis was performed. Among the clinico-pathological parameters studied, including age, gender, differentiation grade, tumor stage, hepatitis B surface antigen and hepatitis C virus, the age was negatively correlated with ABCG2 and hepatitis C virus was positively correlated with ABCG2 significantly ($p=0.031$ and $p=0.033$, respectively) (Table I). Interestingly, hepatocellular carcinoma patients with poor tumor differentiation had a higher frequency of ABCG2 (55%) than other groups ($p=0.015$). Our results suggest that significance of ABCG2 expression was found on age, tumor differentiation and hepatitis B virus.

Effects of hepatitis B virus on ABCG2 expression according to age. Participants were stratified according to age to determine whether hepatitis B virus had differing effects on ABCG2 expression. The hepatitis B virus was not associated with ABCG2 expression in the younger (Table II). However, as shown in Table II, hepatitis B virus was associated with ABCG2 expression in the elderly ($p=0.039$).

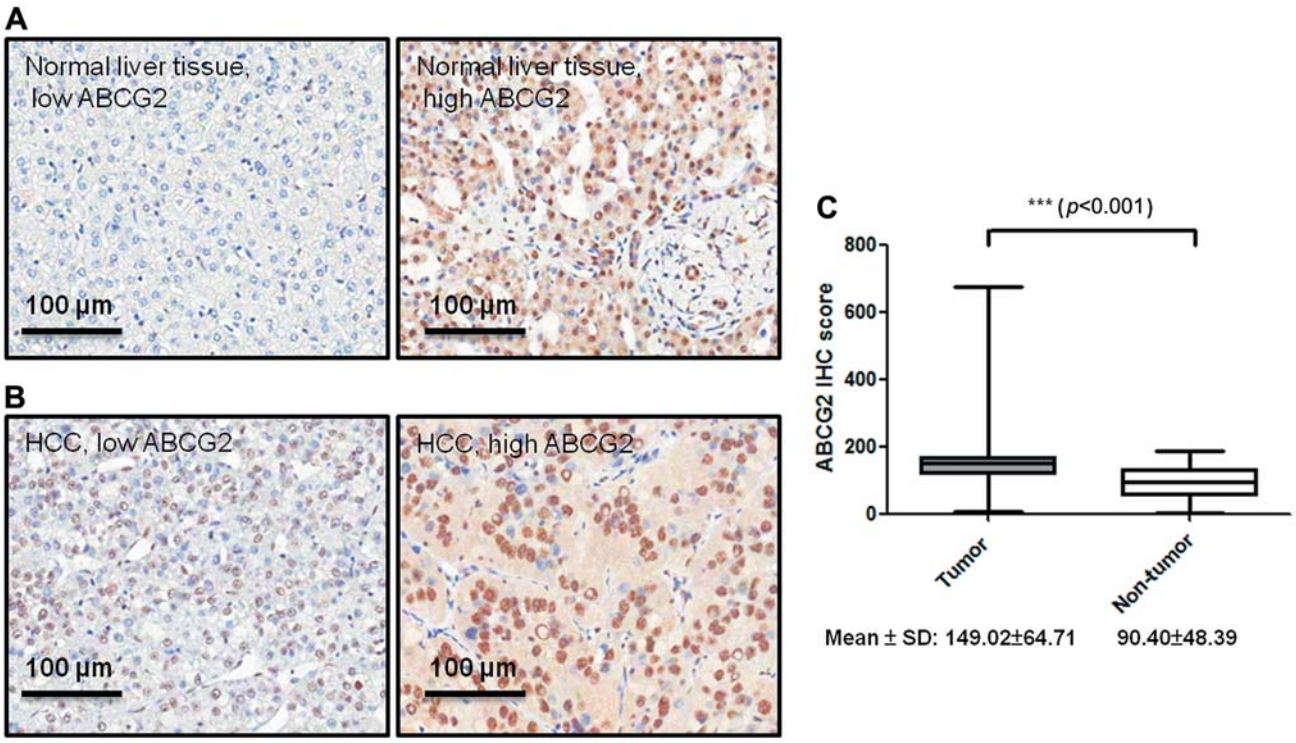


Figure 1. *Comparison of ABCG2 expression in paired tumor and adjacent normal liver tissue of HCC patients. (A) Representative low or high ABCG2 immunostaining results in adjacent normal liver tissue (×100). (B) Representative low or high ABCG2 immunostaining results in tumor (×100). (C) ABCG2 levels were compared in tumor and pair matched non-tumor liver tissues of HCC patients (×100).*

ABCG2 expression is associated with overall survival (OS) in hepatocellular carcinoma patients. The association of ABCG2 and clinico-pathological parameters with patients' survival was statistically investigated by using univariate analysis. Results showed that several characteristics including age, gender, differentiation, stage, hepatitis B surface antigen, hepatitis C virus and ABCG2. (OS: p=0.021 for age, p=0.981 for gender, p=0.358 for differentiation, p<0.001 for stage, p=0.491 for hepatitis B surface, p=0.377 for hepatitis C virus and p=0.271 for ABCG2; Table III). Based on the results of Table II, we next examined whether ABCG2 expression could be associated with clinical outcomes stratified by age in HCC patients. In the elderly but not in the younger, we found that patients with a high level of ABCG2 expression had shorter survival days than those with low level of ABCG2 expression by using univariate analysis (Table IV). Kaplan-Meier analysis showed that patients with a high level of ABCG2 expression had shorter OS periods when compared to patients with low level of ABCG2 expression in the elderly (Figure 2C). Cox regression analysis further indicated a prognostic significance of these three molecules on OS in the elderly population (Table III). The hazard ratios of ABCG2 were 2.130 for OS, when low ABCG2 was used as a reference (Table V). Additionally, the hazard ratios of stage III and IV were 3.076 for OS, when stage I and II was used as the reference (Table V). These results suggest that ABCG2 expression was higher in the elderly than the younger and may be due to persistent hepatitis B virus infection and consequently result in poor outcomes in hepatocellular carcinoma patients.

Discussion

We presented evidence that patients with HBV-related HCC have higher ABCG2 expression than those without HBV infection, and that ABCG2 may be the powerful predictor of prognostic value in hepatocellular carcinoma of elderly patients. Interestingly, HBV-associated HCC has been characterized by high chemotherapy resistance due to hepatitis B virus x protein (HBx)-mediated ABCG2 up-regulation and HBV-integrated HCC (18). More interestingly, a study showed that the extremely elderly patients with stage I/II HCC had significantly worse overall survival compared to non-elderly patients (19). Presumably, these results are due to elevated ABCG2 expression in HBV-mediated HCC to pump virus-related drugs such as interferon-α treatment. On the other hand, our immunohistochemical results showed

Table I. *Relationship of clinical parameters with ABCG2 expression in hepatocellular carcinoma patients.*

Variables	N	ABCG2		*p*-Value
		Low	High	
Age (years)				
<65	108	52 (48)	56 (52)	0.031
≥65	73	47 (64)	26 (46)	
Gender				
Female	53	27 (51)	26 (49)	0.514
Male	128	72 (56)	56 (44)	
Differentiation				
Undifferentiation	5	3 (60)	2 (40)	0.015
Well	9	9 (100)	0 (0)	
Moderate	92	53 (58)	39 (42)	
Poor	75	34 (45)	41 (55)	
Stage				
I, II	147	84 (57)	63 (43)	0.125
III, IV	33	14 (42)	19 (58)	
Hepatitis B surface antigen				
Negative	76	48 (63)	28 (37)	0.033
Positive	98	46 (47)	52 (53)	
Hepatitis C virus				
Negative	117	60 (51)	57 (49)	0.615
Positive	56	31 (55)	25 (45)	

p-Value was obtained from χ^2 test.

Table II. *Relationship of hepatitis B surface antigen with ABCG2 expression stratfied by age in hepatocellular carcinoma patients.*

Variables	N	ABCG2		*p*-Value
		Low	High	
<65 aged patients				
Hepatitis B surface antigen				
Negative	39	20 (51)	19 (49)	0.518
Positive	67	30 (45)	37 (55)	
≥65 aged patients				
Hepatitis B surface antigen				
Negative	37	28 (76)	9 (23)	0.039
Positive	31	16 (52)	15 (48)	

p-Value was obtained from χ^2 test.

Table III. *Univariate analysis of influence of clinical characteristics on overall survival in hepatocellular carcinoma patients.*

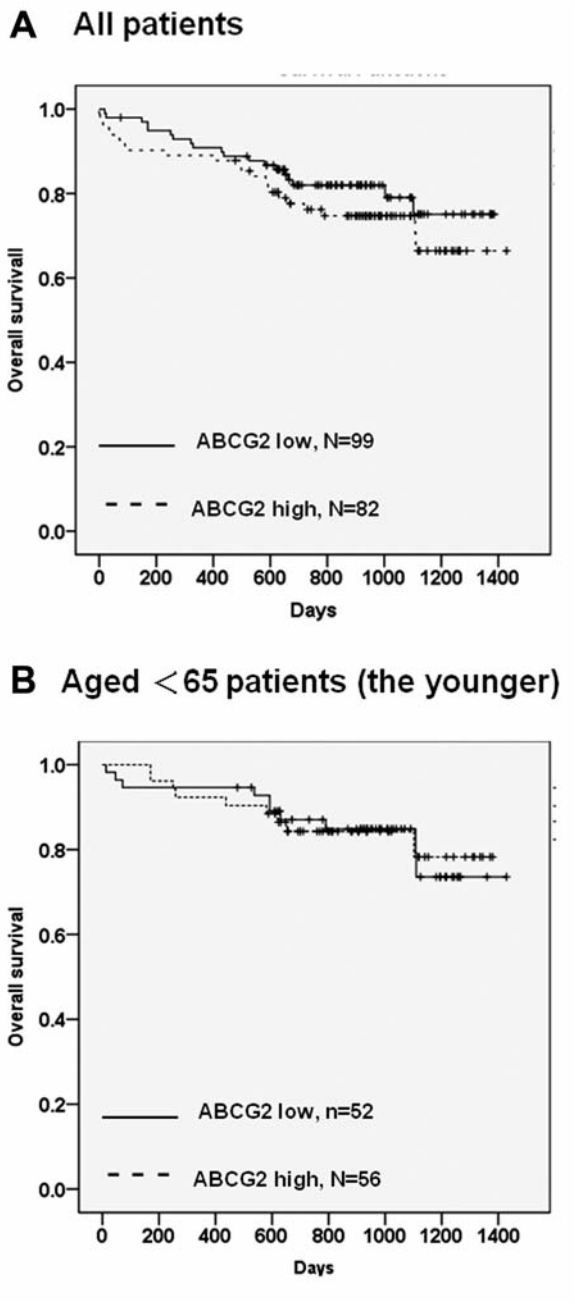
Characteristic	N	OS		Log-rank
		Median survival (days)	Survival (%)	
Age (years)				
<65	108	937	82.4%	0.021
>65	73	768	69.9%	
Gender				
Female	53	879	77.4%	0.981
Male	128	902	77.3%	
Differentiation				
Undifferentiation				
Moderate, well	106	903	79.2%	0.358
Poor	75	829	74.7%	
Stage				
I, II	147	917	82.3%	<0.001
III, IV	33	654	54.5%	
Hepatitis B surface antigen				
Negative	76	858	75.0%	0.491
Positive	98	913	78.6%	
Hepatitis C virus				
Negative	117	879	75.2%	0.377
Positive	56	832	82.1%	
ABCG2				
Low	99	830	80.8%	0.271
High	82	934	73.2%	

Table IV. *Univariate analysis of influence of clinical characteristics on overall survival stratfied by age in hepatocellular carcinoma patients.*

Characteristic	N	OS		Log-rank
		Median survival (days)	Survival (%)	
<65 years				
ABCG2				
Low	52	830	82.7%	0.957
High	56	934	82.1%	
≥65 years				
ABCG2				
Low	47	830	78.7%	0.029
High	26	695	53.8%	

that ABCG2 was not only localized to the cytoplasm but also in the nucleus of HCC or adjacent normal liver tissue (Figure 1A and B). Recently, in an A549 of lung cancer cell model it was shown that an increased level of ABCG2 induces E-cadherin by binding E-box of its promoter, and attenuates invasiveness but promotes colonization of circulating cancer cells (12). Although ABCG2 location is still not fully understood thus far, our results reveal that ABCG2 overexpression may be correlated with poor outcome, especially in elderly patients.

Additionally, as shown in Table I, ABCG2 was also positively correlated with poor differentiation status of HCC, and this relationship seems to mimic the role of ABCG2 in CSCs. Recent studies revealed that cancer stem cells are



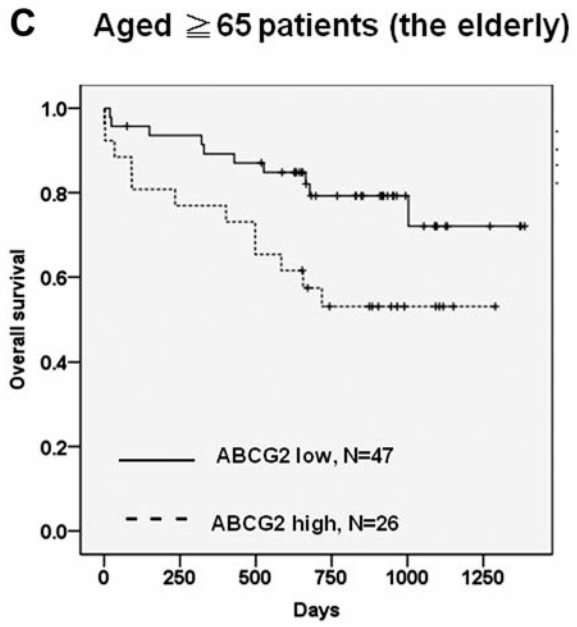


Figure 2. *Kaplan–Meier analysis of the influence of ABCG2 expression on overall survival in patients with HCC.*

Table V. *Cox regression analysis for the influence of stage and ABCG2 on overall survival in the elderly of hepatocellular carcinoma patients.*

	OS			
	HR	Unfavorable/Favorable	*p*-Value	(95% CI)
ABCG2	2.130	High/Low	0.081	0.910-4.985
Stage	3.076	III, IV/I, II	0.017	1.217-7.770

RR was adjusted for ABCG2 and tumor stage.

critically dependant on the hypoxia inducible factors (HIFs) for survival, self-renewal, and tumour growth, and consequently ABCG2 is up-regulated (20, 21, 22, 23). Up-regulation of ABCG2 enhanced the rate of CSCs, proliferation, doxorubicin resistance, and metastasis in HCC, while down-regulation of ABCG2 manifestly decreased these malignant behaviors (16).

Collectively, our findings reveal that ABCG2 could confer poor outcome in elderly patients, potentialy due to chronic hepatitis B virus infection. Additionally, our immunohistochemical results show ABCG2 location to be either cytoplasmic or nucleic, and its mechanism is still a mystery in HCC. However, the blockage of ABCG2 is a beneficial strategy for chemotherapy, target therapy and HBV treatment in HCC patients.

Conflicts of Interest

The Authors declare they have no conflicts of interest.

Acknowledgements

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