RESEARCH ARTICLE

High Expression of ABCC1 Indicates Poor Prognosis in Intrahepatic Cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is a serious health problem in Thailand. To reach a cure, radical resection is the gold standard but most patients are not candidates because of delayed first presentation. Palliative surgery and/or combined chemotherapy are alternatives; however, outcomes are still unsatisfactory. A low response to multiple anticancer drugs might be due to a multidrug resistance (MDR) phenotype of ICC. In this study, we investigated the expression profile of selected adenosine triphosphate binding cassette (ABC) transporter superfamily members, the major contributors to cancer MDR, and determined the clinical significance of certain examples in ICC. Expression of 9 ABC transporters; *ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC11* and *ABCG2*, was determined in 55 ICC tissues using real-time RT-PCR. The results showed that *ABCC1, ABCC2, ABCC3* and *ABCC4* were differentially expressed in ICC tissues. Only *ABCC1* expression was significantly higher in ICC tissues than those of the corresponding non-tumor tissues (P<0.001), significantly correlating with shortened overall survival time (P<0.05). Multivariate analysis indicated that expression is an independent clinicopathological factor (adjusted HR=5.689; 95%CI=1.042-31.076; P<0.05). These results suggested that *ABCC1* is a candidate prognostic marker for ICC.

Keywords: Bile duct cancer - intrahepatic cholangiocarcinoma - ICC - ABC transporter expression - ABCC1

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Introduction

The highest incidence rates of intrahepatic cholangiocarcinoma (ICC) in the world have been reported in Northeastern part of Thailand (Shin et al., 2010). It is the important health problem in this area. The diagnosis of ICC in an early stage of the disease is extremely difficult. Patients are mostly presented in the late stage of disease. The gold standard treatment for ICC is curative surgery, but this is only effective in an early stage of the disease (Khan et al., 2002). Therefore, most of the patients are not candidates. Palliative surgery and/or combined chemotherapy were required for these patients (Morise et al., 2010). Several attempts with multiple regimens of combined chemotherapy have been tried for ICC treatment; however, the results were unacceptable. In the advanced ICC, combined chemotherapy reached a response rate of 30-40% with a median survival time of only approximately one year (Andre et al., 2004; Cho et al., 2005; Nehls et al., 2008). A low response of ICC to multiple anti-cancer drugs might be due to development of multidrug resistance (MDR) phenotypes in cancer cells.

The adenosine triphosphate-binding cassette (ABC) transporter superfamily is a group of well-known multidrug resistant transporters. Several reports demonstrated the correlations between over-expressions of ABC transporter superfamily members and tumor progression or patient's outcomes (Plasschaert et al., 2005; Haber et al., 2006; Vander Borght et al., 2008; Guo et al., 2009). The *ABCB, ABCC* and *ABCG* subfamilies of the ABC transporter superfamily have several members known to confer cellular resistance to clinically important chemotherapeutic agents. A better understanding of expression and roles of ABC transporters, which have been detected in liver tissues and involved in 5-fluorouracil (5-FU; a drug that has been widely used in ICC treatment) resistance (Oguri et al., 2007) is very critical.

Little is known about ABC transporters in ICC, especially in liver fluke associated ICC. The correlations of *ABCC3* expression and multidrug resistances were reported in CCA cell lines (Tepsiri et al., 2005) and the loss of *ABCG2* expression was an independent poor

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prognostic factor in patients with moderately or poorly differentiated ICC (Larbcharoensub et al., 2011). In this study, we hypothesized that aberrant expressions of certain ABC transporters might confer MDR phenotypes to ICC; therefore it might be useful for predicting prognosis of patients. Nine ABC trasnsporter proteins selected for the study were *ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC11* and *ABCG2*. These ABC transporters are either expressed in liver tissues or confer resistance to 5-FU. The mRNA expression profiles of those ABC transporter genes in ICC tissues were determined. Differentially expressed ABC transporters were chosen for further clinicopathological analysis. The results of this study would demonstrate a potential role of using ABC transporter expression in prediction of ICC outcome.

Materials and Methods

Patient samples

Fifty-five frozen ICC tissues and 28 corresponding frozen normal liver tissues were selected from non-tumor areas of the same surgical specimens as for the ICC tissues were obtained from the specimen bank of the Liver Flukes and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. The samples were collected from February 2007 to September 2010 and selected from 624 CCA cases under the following criteria: 1) the tumors were ICC; 2) the patients had undergone hepatic resection; 3) complete clinicopathological data were available, and 4) patients had no known history of other cancers or previous history of chemotherapy administration. Peri-operative deaths or patients with overall survival<30 days were excluded. Informed consent was obtained from each subject for the study that followed the protocol approved by the Ethics Committee for Human Research, Khon Kaen University (HE471214 and HE521209).

RNA isolation and reverse transcription

Total RNA was isolated from frozen tissues using the TRIzol® Reagent (Invitrogen, CA, USA) as the manufacturer's recommendation. In brief, tumor tissues were homogenized in TRIzol® reagent and the RNA was extracted and precipitated by using chloroform and isopropanol, respectively. The obtained RNA was washed with 75% ethanol, dried and dissolved in diethylpyrocarbonate (DEPC)-treated water. Possible contaminated genomic DNA was digested using DNase I treatment (Roche Diagnostics, Mannheim, Germany). RNA quality was checked by conventional denaturation agarose gel electrophoresis and spectrophotometry. The RNA was reverse transcribed by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Briefly, the reaction mixture containing 25 μM random hexamer, 4 mM of each dNTPs, 1xRT buffer, 50 U of Multiscribe™ MMLV Reverse Transcriptase, and 2 μg of total RNA was incubated for 10 minutes at 25°C, followed by 2 hours at 37°C, and 5 minutes at 85°C. cDNA were stored at -80°C until used.

Real-time reverse-transcriptase polymerase chain reaction (real-time RT-PCR)

The expressions of 9 ABC transporter genes (*ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC11* and *ABCG2*) were detected using the LightCycler 480® real-time PCR system (Roche Diagnostics, Mannheim, Germany). Each PCR reaction contained LightCycler 480®SYBR Green I master mix, 4 μM of each primers, and 50 ng of cDNA. The amplification was initiated by denaturation at 95°C for 5 min, followed by 40 repeated cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 3 sec. Concomitantly, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression of each sample was determined and served as an internal control for normalization. Mean and SD values of the crossing point (Cp) cycle, and normalized gene expression values were calculated using LightCycler480® Relative Quantification software (Roche Diagnostics, Mannheim, Germany). Gene expression levels were determined and categorized into low and high expressions by dichotomizing at the median $2^{-\Delta Cp}$ values where $\Delta Cp=Cp_{target}- Cp_{GAPDH}$. All PCR primers and the expected PCR product sizes are listed in Table 1.

Statistical analysis

Statistical analysis was performed using a SPSS software package (version 16.0; SPSS, IL, USA). Associations between categorical clinicopathological variables and ABC transporter expressions were assessed using either the chi-square test or Fisher's exact test. Survival curves were constructed according to Kaplan-Meier and compared using a log rank test. In case of mean comparisons between two groups, the independent two-tailed t test was used. P<0.05 was considered statistically significant.

Results

Demographic characteristics of patients

From a total of 55 ICC cases, 34 were males and 21

Table 1. Primers for Real-Time RT-PCR

Gene	Primers	Primer sequence (5´-3´)	Expected sizes (bp)	References
ABCB1	1	TGTGGGTTGCTGAACATCGA	288	*
	2	GCTTTCTGTCTTGGGCTTGT		
ABCB11	1	TGTGGGTTGCTGAACATCGA	263	*
	2	TGGTCAGCTATGGCATCATTG		
ABCC1	1	CTGGGCTTATTTCGGATCAA	163	**
	2	TGAATGGGTCCAGGTTCATT		
ABCC2	1	TCGGAATGTGAATAGCCTGAAG	157	*
	2	CGCAAGGATGATGAAGAATATCG		
ABCC3	1	CCTGCTCTCCTTCATCAATC	156	*
	2	ATGTAGTGGTAATAGTGTTGTAAG		
ABCC4	1	TACAAGTGGTTGGTGTGGTCTCTG	143	*
	2	TGTAGATTCCAGGCGCTTCACA		
ABCC6	1	CTGGAGACGGTGCAGCTCAAA	229	*
	2	ACTGTGCAAACCAGCTCCCGA		
ABCC11	1	TAGCTGAAAGAATTGGCAGGAACT	242	*
	2	TCATGGTTCTCAAGGCAGCATC		
ABCG2	1	CACCTTATTGGCCTCAGGAA	206	**
	2	CCTGCTTGGAAGGCTCTATG		
GADPH	1	CGCTCTCTGCTCCTCCTG	100	*
	2	ACTCCGACCTTCACCTTCC		

*Primers were newly designed for current study, **Hu et al., 2008

were females with a male to female ratio of 3.1: 1.9. The ages of the patients ranged from 38-73 with a median age of 58 years. The demographic characteristics of patients are summarized in Table 2. Survival time was calculated from the time of surgery to death. More than 50% of patients presented with the advanced stage (stage IV) tumors and had a survival time of less than 1 year. A majority of ICC in this study was non-papillary type with tumor masses larger than 5 cm. Approximately 60% of the patients received post-operative adjuvant chemotherapy, of which 26% got complete 6-courses treatment and 33% received partial treatment.

ABC transporter Gene Expression Profile

Expression of 9 ABC transporter genes, *ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC11* and *ABCG2*, were determined in 55 ICC using real-time RT-PCR analysis. Expression of these genes was categorized into low and high level by median $2^{-\Delta Cp}$ values. Only 4 genes, *ABCC1*, *ABCC2*, *ABCC3*, and *ABCC4*, were differentially expressed and selected for further analysis.

Table 2 Clinical Characteristics of Patients

Characteristics		values	%
Age (years)	Mean	57.8 (38-73)	
Sex	Male	34	61.8
	Female	21	38.2
Overall survival (days)	Mean	384 (41-1465)	
Tumor stage*	I	1	1.8
	II	5	9.1
	III	19	34.5
	IVa	28	50.9
	IVb	2	3.6
Surgical margin	R0	30	54.5
	R1/R2	25	45.5
Tumor size (cm)	≤5	14	25.5
	>5	41	74.5
Histological types	Papillary	18	32.7
	Non-papillary	37	67.3
	Well differentiated	25	45.5
	Moderately differentiated	8	14.5
	Poorly differentiated	1	1.8
	Others	3	5.5
Local invasion:	Presence	42	76.4
	Absence	13	23.6
Vascular invasion:	Presence	18	32.7
	Absence	37	67.3
Lymph node invasion:	Presence	26	47.3
	Absence	29	52.7
Distant invasion:	Presence	2	3.6
	Absence	53	96.4
Adjuvant chemotherapy:	No	29	40.7
	Partial	15	33.3
	Complete	11	26
Tumor markers	Alpha feto-protein (AFP)†		
	≤10 IU/mL	40	81.6
	>10 IU/mL	9	18.4
Carbohydrate antigen 19-9	(CA19-9)‡		
	≤37 U/mL	21	38.9
	>37 U/mL	33	61.1
Carcinoembryonic antigen	(CEA)‡‡		
	≤2.5 ng/mL	9	17.3
	>2.5 ng/mL	43	82.7

*(Nathan and Pawlik, 2010), †n=49; ‡n=54, ‡‡n=52

Correlation of ABC transporter Expression and Clinicopathological features

Univariate analysis was performed to investigate the correlation between expression levels of *ABCC1, ABCC2, ABCC3* and *ABCC4* and clinicopathological characteristics of patients as described in Table 2. The expression levels of *ABCC1, ABCC2, ABCC3* and *ABCC4*, however, were not correlated with any clinical variables (Table 3).

Correlation of clinicopathological variables and ABC expression and overall survival

ABCC1, ABCC2, ABCC3 and *ABCC4* expressions were tested for their correlations with the patient's overall survival. No correlation between selected ABC transporter expressions and overall survival was observed. Nonetheless, among clinical variables of patients tested, positive correlation was detected with good survival in patients who got complete courses of adjuvant chemotherapy with longer survival times than those who did not receive or was partially received chemotherapy (P<0.001) (Figure 1).

Expression of ABCC1 was higher in ICC than in normal adjacent tissues

The expressions of *ABCC1, ABCC2, ABCC3* and *ABCC4* of tumors and the corresponding normal liver tissues from 28 ICC patients were determined using real-time RT-PCR (Figure 2). It was found that only *ABCC1*

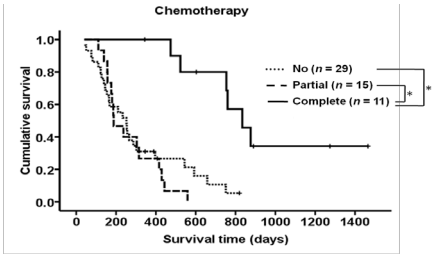

Figure 1. Kaplan-Meier Survival Curves with Log Rank Test for Groups of Patients with or without Adjuvant Chemotherapy. The results indicated that patients who received partial courses of adjuvant chemotherapy or no subsequent chemotherapy had shorter overall survival times than those who received complete courses of chemotherapy (*P<0.001)

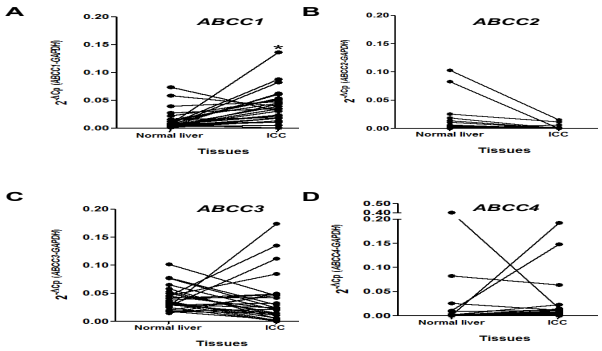

Figure 2. Comparison of *ABCC1*, *ABCC2*, *ABCC3* and *ABCC4* Expressions in 28 Pairs of Matched ICC and Normal Liver Tissues. A: *ABCC1*; B: *ABCC2*; C: *ABCC3*; D: *ABCC4*. *ABCC1* expression was significantly higher in tumor than in normal tissues (*P<0.001)

Table 3. Univariate Analysis of *ABCC1*, *ABCC2*, *ABCC3* and *ABCC4* Expressions with Clinicopathological Variables

Variables		N	*ABCC1*			*ABCC2*			*ABCC3*			*ABCC4*		
			Low	High	P*	Low	High	P*	Low	High	P*	Low	High	P*
Age (years)	≤55	25	15	10	0.218	11	14	0.349	14	11	0.491	12	13	0.694
	>55	30	13	17		17	13		14	16		16	14	
Sex	Male	34	15	19	0.2	18	16	0.701	19	15	0.348	19	15	0.348
	Female	21	13	8		10	11		9	12		9	12	
Tumor stage#	I-III	25	13	12	0.883	15	10	0.218	11	14	0.422	14	11	0.491
	IV	30	15	15		13	17		17	13		14	16	
Margin	R0	30	16	14	0.694	15	15	0.883	15	15	0.883	16	14	0.694
	R1/R2	25	12	13		13	12		13	12		12	13	
Size (cm)	≤5	14	5	9	0.188	6	8	0.485	6	8	0.485	7	7	0.937
	>5	41	23	18		22	19		22	19		21	20	
Histotype	Papillary	18	8	10	0.504	6	12	0.069	9	9	0.925	9	9	0.925
	Non-papillary	37	20	17		22	15		19	18		19	18	
Local invasion	Presence	42	22	20	0.695	24	18	0.121	23	19	0.304	22	20	0.695
	Absence	13	6	7		4	9		5	8		6	7	
Vascular invasion	Presence	18	9	9	0.925	10	8	0.631	6	12	0.069	8	10	0.504
	Absence	37	19	18		18	19		22	15		20	17	
Lymph node invasion	Presence	26	10	16	0.08	11	15	0.227	14	12	0.68	11	15	0.227
	Absence	29	18	11		17	12		14	15		17	12	
Chemotherapy	No/partly	44	21	23	0.503	24	20	0.329	23	21	0.686	23	21	0.686
	Complete	11	7	4		4	7		5	6		5	6	

*P-values were determined by chi-square test, #Tumor stage were classified according to the 7th edition of the AJCC cancer staging manual

Table 4. Multivariate Analysis using Cox Proportional Hazard Regression Model of Clinicopathological Factors and ABCC1 Expression in 28 pairs of ICC and Histological Normal Liver Tissues

Variables		N	Adjusted HR	95% CI	P
Age (years)					
	≤55	12	1		
	>55	16	1.428	0.484-4.218	0.519
Sex	Male	19	1		
	Female	9	0.501	0.177-1.417	0.192
Stage	I-III	16	1		
	IV	12	2.166	0.740-6.339	0.158
Histotype	Papillary	8	1		
	Non-papillary	20	1.928	0.510-7.292	0.333
Chemotherapy					
	No/partial	24	1		
	Complete	4	0.097	0.010-0.907	0.041*
ABCC1 expression					
	Tumor ≤Normal	5	1		
	Tumor >Normal	23	5.689	1.042-31.076	0.045*

*P<0.05

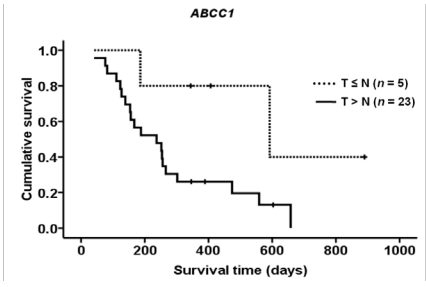

Figure 3. Kaplan-Meier Survival Curves with Log Rank Test of *ABCC1* Expression in 28 Pairs of ICC and Adjacent Normal Lliver Tissues. The higher ABCC1 expression in tumor than in normal liver tissue (T>N) was correlated with the shorter survival time, P=0.042

was significantly higher in ICC than normal liver tissues (Figure 2A, P<0.001). The comparison of *ABCC2*, *ABCC3* and *ABCC4* expressions between pairs of ICC and normal tissues without significant difference were demonstrated in figure 2B, 2C and 2D, respectively.

ABCC1 is a poor prognostic marker for ICC

The clinical significance of the increased expression of *ABCC1* in ICC patients was further determined. The ratios of *ABCC1* expression in ICC tissue to corresponding normal tissue were determined. The median ratio of *ABCC1* at 1.2 was used to dichotomize the samples into low and high *ABCC1* expression; samples with *ABCC1* ratios more than 1.2 were considered as high *ABCC1* expressing tumors (T>N) while those with ratios equal to or less than 1.2 were considered as low *ABCC1* (T≤N). The Kaplan-Meier survival curve with log rank test was constructed. This detected the significant correlation of the higher *ABCC1*expression in ICC (T>N) with a shortened overall survival (P=0.042) (Figure 3). Multivariate analysis using Cox proportional hazards model indicated that the high ABCC1 expression (T>N) was an independent indicator for predicting overall survival of patients (P=0.045) as shown in Table 4. Moreover, the chemotherapeutic treatment option was also an independent prognostic factor in which the patients who got complete courses of adjuvant chemotherapy had longer survivals than those who got partial or had no adjuvant treatment (P=0.041).

Discussion

In this study, the mRNA expression of 9 ABC transporter genes in 55 ICC tissues was investigated. The *ABCC1, ABCC2, ABCC3* and *ABCC4* genes were differentially expressed in ICC regardless of the

clinicopathological variables of patients. *ABCC1* showed significantly higher expression in tumor areas than the non-tumor areas, and patients with high *ABCC1* ratios (T>N) had shorter survival times than those with low *ABCC1* ratios (T≤N). It is noteworthy that all the ICC patients in this study had no chemotherapy prior to resection; hence, the increased expression of *ABCC1* observed in ICC tissue should be the intrinsic and independent of chemotherapeutic treatment. The similar observation of increased *ABCC1* in tumor areas has been reported in hepatocellular carcinoma (HCC) (Vander et al., 2008; Hoffmann et al., 2011). The increased expression might imply the tumor cell origin or the involvement of an upstream regulator of *ABCC1* expression. In HCC, the increased expression of *ABCC1* was observed in tumors with hepatoblast phenotype (Vander Borght et al., 2008). The induction of *ABCC1* expression by mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) or the suppression by miRNA, particularly miR-199a/b, miR-296, was previously reported in HCC (Hoffmann et al., 2011; Borel et al., 2012).

Little is known regarding ABC transporter expression in ICC. There is no previous report regarding the ABC transporter gene expression profile and comparison of gene expression between ICC and surrounding tissues. Overexpression of *ABCC1* and *ABCC3* and their correlations with chemotherapeutic drug resistances in CCA cell lines were reported (Tepsiri et al., 2005). Larbcharoensub et al., (2011) investigated ABC transporter protein expression in 60 Thai ICC cases, and detected *ABCC1* in 33% and *ABCG2* in 60% of ICC. In contrast, in current study, *ABCC1* were detected in most of the samples whereas *ABCG2* was barely detectable in these samples. This discrepancy is possibly due to the differences in clinical specimens and the detection methods. In this study, we determined the expression of ABC transporters using real-time RT-PCR, whereas the Larbcharoensub's study used immunohistochemistry. The sensitivity of the technique and stability of the target molecules are probably responsible for these differences. Due to limited numbers of samples, the correlation of increased *ABCC1* and the degree of ICC differentiation was not observed in the present samples as in HCC (Vander et al., 2008). Even though this study did not determine the MAPK pathway which is the upstream regulator of *ABCC1*; however, up-regulation of MAPK pathway was demonstrated in CCA (Wang et al., 2009) and aberrant expression of miRNA in ICC was also reported (Chen et al., 2009). Further study in the regulation of *ABCC1* expression might be useful for a better understanding of ABCC1 control in ICC.

The role of *ABCC1* as a predictor of patients' poor prognosis was evident in several cancers including HCC (Vander et al., 2008). Enhanced expression of *ABCC1* may increase xenobiotic efflux pump and confer an MDR phenotype of cancer cells (Chang, 2007). Nevertheless, functions of *ABCC1* other than xenobiotic efflux pump should also be considered. Xenobiotic efflux-independent functions of *ABCC1* have been demonstrated in several studies. In Abcc1-/- mice, migration of peripheral dendritic cells to lymph nodes is greatly reduced (Robbiani et al., 2000). Consistently, siRNA-mediated depletion of *ABCC1* or inhibition of *ABCC1* with Reversan in neuroblastoma cell lines impaired cell motility and clonogenic capacity independently to chemotherapeutic agent usage (Henderson et al., 2011). Therefore, functions of *ABCC1* in ICC should be explored for a better understanding of the clinical significance of *ABCC1* in ICC pathogenesis.

The current study indicated that a complete course of post-operative adjuvant chemotherapy significantly increased patient's overall survival. These data emphasized the clinical role of adjuvant chemotherapy in ICC. In addition, the shorter survival time observed in patients who received incomplete courses of chemotherapy may partly due to MDR cancers, as chemotherapy schedule is generally terminated in patients with disease progression or treatment failure. These findings may be useful for physicians to select chemotherapeutic drugs for ICC patients. *ABCC1* transporter substrates (e.g., doxorubicin, vincristine, etoposide (VP-16), and some heavy metal anions) should be avoided in ICC patients with high *ABCC1* expression (Chang, 2007). Moreover, reversing MDR through the inhibition of specific ABC transporters may be of benefit for ICC.

In conclusion, this study reported herein the increased expressions of *ABCC1* in ICC and the correlations with shorter overall survival of the patients. This correlation was independent to other clinicopathological factors. It is therefore proposed that *ABCC1* might be used as a predictor of the prognosis of ICC patients.

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