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Correlation Between Low Cytoplasmic Expression of XBP1 and the Likelihood of Surviving Hepatocellular Carcinoma

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Abstract. *Background/Aim: Our objectives in this study were to (i) evaluate the clinical significance of X-box-binding protein 1 (XBP1) expression in cases of hepatocellular carcinoma (HCC) and (ii) assess the potential of XBP1 to be used as a prognostic biomarker. Patients and Methods: The expression of XBP1 protein in 267 HCC tissue specimens was measured using immunohistochemistry in order to characterize the associations among XBP1 expression, clinicopathological factors and survival outcomes. Survival analysis using follow-up data was used to assess the prognostic value of XBP1 in cases of HCC. Immunohistochemistry revealed a significant decrease in cytoplasmic XBP1 protein expression in HCC tumor tissue. Results: Immunoreactivity results showed that low cytoplasmic XBP1 expression was significantly associated with vascular invasion, as well as poor 5-year overall survival and long-term disease-specific (DSS) and disease-free (DFS) survival rates. Kaplan-Meier survival curves further confirmed a significant association between low cytoplasmic XBP1 protein expression and poor DSS and DFS. Univariate and multivariate analyses revealed that XBP1 expression, tumor differentiation, vascular invasion, tumor stage, and the rate of recurrence were linked to DSS, while low cytoplasmic XBP1 expression remained an independent predictor of poor DSS. Our analysis also revealed that XBP1 expression, tumor differentiation, vascular invasion, and T classification were linked to DFS, while low cytoplasmic XBP1 expression remained an independent predictor of poor DFS. Conclusion: Low cytoplasmic XBP1 protein expression may play an important role in the pathogenesis of HCC, which suggests that XBP1 could potentially be targeted to benefit therapeutic strategies for HCC.*

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Hepatocellular carcinoma (HCC) is currently the sixth most common cancer in the world, the second most fatal cancer among men, and the sixth most fatal cancer among women (1). Indeed, the 5-year HCC survival rate is around 10 to 20% (2), and worldwide, the disease has been estimated to result in more than 550,000 deaths per year (3). Sub-Saharan Africa and Southeast Asia are high-risk areas for HCC. In Taiwan, HCC has been the leading cause of cancer death since 1984, with roughly 6,000-8,000 deaths attributable to HCC every year (4). Risk factors for HCC include excessive alcohol consumption, obesity-induced steatohepatitis, and chronic exposure to aflatoxin B1. In most cases, HCC is caused by infection with hepatitis B or C virus (5-7). The early stages

of HCC are asymptomatic; thus, in most patients, HCC has reached advanced stages by the time it is diagnosed, and at this point the disease is largely incurable (8).

Hepatocarcinogenesis is highly complex and presents extraordinary molecular heterogeneity. Despite advances in chemotherapy and targeted therapies over the past three decades, therapeutic strategies have had little impact on survival rates. Thus, early identification of molecular markers is particularly important for effective drug therapy planning (9, 10).

Human X-box-binding protein (XBP1) is an important transcription factor in numerous signal transduction processes. Post-transcriptional *XBP1* mRNA can be processed or left unprocessed to generate XBP1 isoforms which are spliced (XBP1s) or unspliced (XBP1u) (11, 12). The unspliced isoform has been implicated in a variety of human physiological and pathological processes, including lipogenesis, adipogenesis, atherosclerosis, ischemia, and liver cancer (13-17). XBP1 is involved in the unfolded protein response (UPR) pathway, a pathway that is activated to restore cellular homeostasis following endoplasmic reticulum (ER) stress. However, in cases where ER stress cannot be resolved, the UPR pathway induces cellular apoptosis (18, 19).

There is evidence which suggests that the involvement of XBP1 in tissue-specific transcriptional networks is linked to many types of cancer (20-23). XBP1 has also been identified as a survival factor in a variety of human cancer types, including mesenchymal and epithelial cancer (18, 24-27). There is mounting evidence to support the role of XBP1 in tumor progression and invasion. There is also evidence to indicate that XBP1 helps trigger epithelial–mesenchymal transition (EMT) by promoting the expression of TWIST family basic helix-loop-helix transcription factors and SNAIL family transcriptional repressor proteins in tumor cells (28-32). However, the link between XBP1 and HCC prognosis has yet to be fully elucidated. In this study, we performed analysis by immunohistochemistry (IHC) to investigate XBP1 protein expression levels in a large number of HCC tissue specimens. Our primary objective was to identify the correlation between XBP1 expression and a variety of clinicopathological features. However, we also sought to determine whether XBP1 could be used as a prognostic biomarker for patients with HCC.

Patients and Methods

Patients with HCC. The current study analyzed samples from 267 patients with HCC treated at Changhua Christian Hospital, Taiwan. Samples were collected between January 1996 and October 2008. Approval for this research was obtained from the Ethics Committees of Changhua Christian Hospital (Changhua, Taiwan, ROC), and all patients provided written, informed consent. Analysis was conducted according to guidelines approved by the Institutional Review Board (IRB number: 151019, approval date: January 19, 2016). The HCC patient population included 200 males and 67 females. Pathological evaluation (with tumor staging and histologic differentiation grading) followed the guidelines outlined in the American Joint Commission on Cancer (seventh edition). Patients data were used to classify tumor stages and grades according to the TNM staging system guidelines (33). Age, differentiation grade, T classification, N status, metastasis, tumor stage, tumor recurrence, disease-free survival (DFS), and disease-specific survival (DSS) were derived from histopathological and clinical data.

Tissue microarrays (TMAs). To create TMAs (5-μm), representative HCC specimens were selected, sectioned, and stained using hematoxylin and eosin. Tissue cylinders (2 mm in diameter) were punched from the marked regions of paraffin blocks using a semi-automated device (34). The punched cores contained a large number of viable tumor cells with minimal necrosis in the peripheral or central regions. Punches of tumor specimens were arranged in new paraffin blocks. Following hematoxylin and eosin staining of the TMAs, a senior pathologist (Dr. Hui-Ting Hsu) confirmed that these TMAs contained morphologically representative lesions of the original cancer.

IHC analysis of XBP1 protein level. IHC analysis was performed in accordance with the protocol outlined in a previous study (34). Briefly, we used rabbit polyclonal antibodies raised against amino acids 76-263 of mouse XBP1 (catalog number: sc-7160; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect XBP1 protein. TMA sections were incubated with anti-XBP1 at 4˚C overnight. LASB 2 kit (Dako, Carpinteria, CA, USA) was then used to detect the resulting immune complex, and activity was visualized using aminoethyl carbazole as a substrate. Finally, sections were counterstained using hematoxylin and mounted using Glycergel mounting medium (Dako, Glostrup, Denmark). Appropriate positive and negative controls were included in the same IHC program. The intensity of cytoplasmic staining was defined according to the following scores: Negative staining: 0; weak staining: 1+; moderate staining: 2+; and strong staining: 3+. The percentage of immunoreactive tumor cells was also recorded. Scores were assessed by two senior independent pathologists (Drs. Hui-Ting Hsu and Yueh-Min Lin) under blinded conditions (34).

Bioinformatics and statistical analysis. Survival analysis in this study was based on data related to the mRNA expression of *XBP1*, which are available in the Kaplan-Meier plotter database (http://kmplot.com/analysis/index.php?*p*=background). The Kaplan-Meier plotter system features gene chips and RNA-seq data sources, whereas the database includes data from the Gene Expression Omnibus, the European Genome-phenome Archive, and The Cancer Genome Atlas. The main purpose of the tool is to discover and validate biomarkers based on meta-analysis (35).

The association between XBP1 expression levels and the clinicopathological parameters of HCC was analyzed using Fisher's exact test. The prognostic significance of variables related to XBP1 expression was evaluated using the Cox regression model and hazard ratio analysis. Differences in DSS or DFS survival curves were derived using the Kaplan-Meier method and the log-rank test. The Cox proportional hazards model based on univariate and multivariate analyses was used to identify factors independently associated with DFS and DSS (34). All statistical

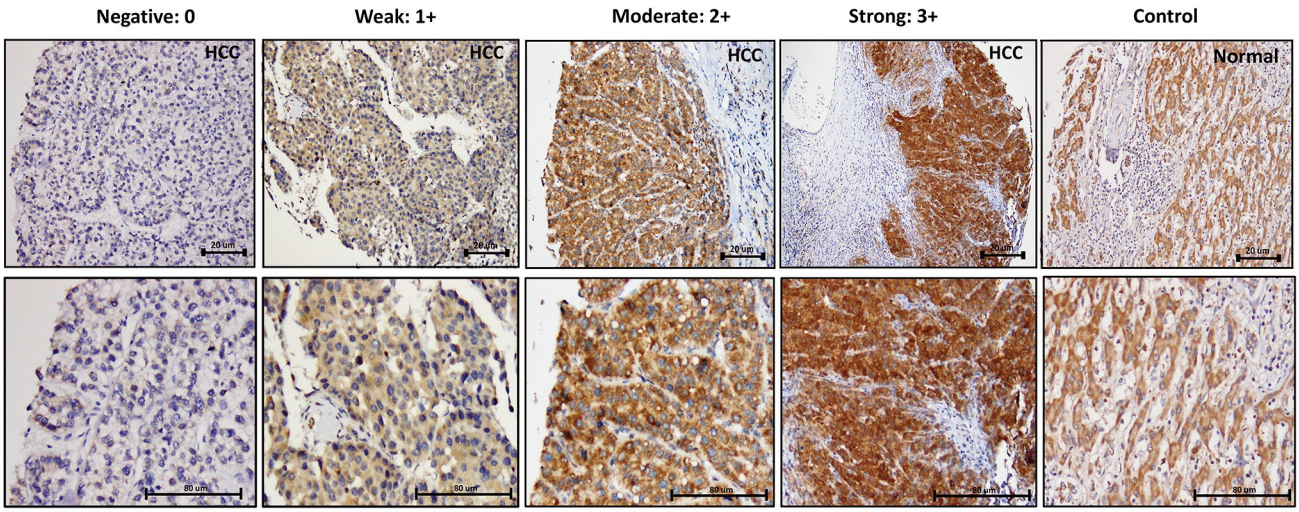

Figure 1. *Immunohistochemical staining and ranking of X-box-binding protein 1 (XBP1) expression in HCC and normal tissues. The intensity of cytoplasmic staining was defined according to the following scores: Negative staining: 0; weak staining: 1+; moderate staining: 2+; strong staining: 3+. Normal control tissue is also shown. Magnification: top panel, 200×; lower panel, 400×. Scale bars=20 μm and 80 μm.*

analysis was conducted using SPSS statistical software version 17 (SPSS, Inc., Chicago, IL, USA). Differences with a *p*-value of less than 0.05 were considered statistically significant.

Results

Patient characteristics. This study included a total of 267 cases of HCC from a sample population that comprised 200 males and 67 females. The patients ranged from 17 to 87 years old, whereby the mean age was 59.3 years, and the median age was 62 years. A total of 159 (59.6%) patients suffered from hepatitis B infection, 100 (37.5%) patients had hepatitis C infection, and 21 (7.9%) patients had concurrent coinfections with hepatitis B and hepatitis C. Cirrhosis was clinically diagnosed in 117 (43.8%) patients. Moderately-differentiated (G2) tumors were observed in 57.3% of patients (n=153), whereas poorly-differentiated tumors were observed in 31.1% of patients, and well-differentiated tumors were observed in 10.5% of patients. The distribution of disease stages was as follows: Stage I (123 patients; 46.1%), stage II (72 patients; 27.0%), stage III (64 patients; 24.0%), and stage IV (8 patients; 3.0%). A total of 9 patients (3.0%) presented lymph node metastasis, and 6 patients (2.2%) initially presented metastatic disease. A total of 167 patients (62.5%) suffered from tumor recurrence, during the mean follow-up period of 4.6 years.

Association between cytoplasmic XBP1 expression and patient characteristics. IHC analysis revealed strong XBP1 expression in the cytoplasm of non-tumor or normal hepatocytes. The staining intensity of XBP1 in non-tumor hepatocytes was used as an internal positive control and scoring baseline for XBP1 staining. XBP1 staining patterns in the cytoplasm of tumor cells were relatively homogeneous. There were no indications of nuclear staining. Based on the relative staining intensity of XBP1 in the cytoplasm, we subdivided XBP1 immunostaining results as follows: Low: scores 0 and 1+; high: 2+ and 3+; Figure 1, normal control tissue is also shown. Immunostaining identified 83 patients (31.1%) with low XBP1 expression and 184 patients (68.9%) with high XBP1 expression. As shown in Table I, Fisher's exact test was used to assess the clinical significance of cytoplasmic XBP1 protein expression levels in HCC tissues; XBP1 protein expression was significantly correlated with a number of clinicopathological characteristics, including vascular invasion (p=0.037), 5-year survival (p=0.021), and DSS (p=0.009). No significant differences in cytoplasmic XBP1 expression were observed when results were stratified according to age (p=0.31), sex (p=0.06), tumor differentiation (p=0.521), T classification (p=0.333), tumor stage (p=0.094), distant metastasis (p=0.311), tumor recurrence (p=0.096), or cirrhosis (p=0.664).

The expression of XBP1 mRNA and XBP1 proteins was associated with shorter survival times. The Kaplan-Meier plotter database was first mined to determine whether *XBP1* mRNA expression levels were associated with DSS in patients with HCC. Survival analysis was based on data obtained from 362 patients listed in the Kaplan-Meier plotter database (35). The Kaplan-Meier plotter function separated the patients into two groups, namely with a high or low gene-expression signature for *XBP1*. The Kaplan-Meier

Table I. *Characteristics of patients with hepatocellular carcinoma and the status of cytoplasmic X-box-binding protein 1 (XBP1) protein expression.*

Variable		Overall	XBP1 expression		*p*-Value*
			Low	High	
Number (%)	Total	267 (100)	83 (31.1)	184 (68.9)	
Age, years	Mean±SD	267 (100)	60.86±13.8	58.6±13.7	0.310
Sex, n (%)	Female	67 (25.1)	27 (32.5)	40 (21.7)	0.060
	Male	200 (74.9)	56 (67.5)	144 (78.3)	
Tumor differentiation, n (%)	G1-G2	181 (67.8)	54 (65.1)	127 (69.0)	0.521
	G3	86 (32.2)	29 (34.9)	57 (31.0)	
T Classification, n (%)	T1-T2	195 (74.9)	59 (71.1)	140 (76.6)	0.333
	T3-T4	67 (25.1)	24 (28.9)	43 (23.4)	
Vascular invasion, n (%)	No	125 (46.8)	31 (37.3)	94 (51.1)	**0.037**
	Yes	142 (53.2)	52 (62.7)	90 (48.9)	
Tumor stage, n (%)	I-II	150(78.5)	55 (66.3)	140 (76.1)	0.094
	III-IV	72(21.5)	28 (33.7)	44 (23.9)	
Status at 5 years, n (%)	Dead	149 (55.8)	55 (66.3)	94 (51.1)	**0.021**
	Alive	118 (44.2)	28 (33.7)	90 (48.9)	
Tumor recurrence, n (%)	No	100 (37.5)	25 (30.1)	75 (40.8)	0.096
	Yes	167 (62.5)	58 (69.9)	109 (59.2)	
Distant metastasis, n (%)	No	261 (97.8)	80 (96.4)	181 (98.4)	0.311
	Yes	6 (2.2)	3 (3.6)	3 (1.6)	
Disease-specific survival, n (%)	Alive	107 (47.8)	24 (34.8)	83 (53.5)	**0.009**
	Dead	117 (52.2)	45 (65.2)	72 (46.5)	
Cirrhosis, n (%)	No	150 (56.2)	45 (54.2)	105 (57.1)	0.664
	Yes	117 (43.8)	38 (45.8)	79 (42.9)	

*By Fisher's exact test. Statistically significant *p*-values are shown in bold.

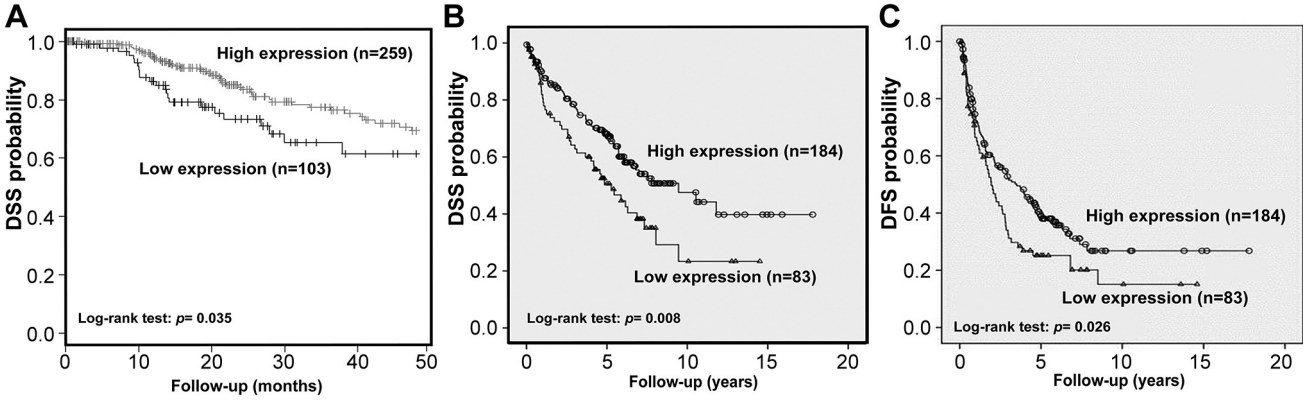

Figure 2. *Kaplan-Meier curves for survival of stratified by expense of X-box-binding protein 1 (XBP1) mRNA or protein in hepatocellular carcinoma (HCC) with differences established using log-rank test. Kaplan-Meier curves for disease-specific survival (DSS) according to XBP1 mRNA expression level (A) using HCC samples from the Kaplan-Meier plotter database. B and C: Kaplan-Meier analysis of DSS and disease-free survival (DFS) among the study patients with HCC according to XBP1 protein expression level.*

plotter was then employed to perform log-rank tests in which Kaplan-Meier plots were generated. These plots were used to derive survival rates for the two groups. In Figure 2, data indicative of high and low *XBP1* mRNA expression are shown in red and black, respectively. Note that there was a correlation between low expression of XBP1 and DSS (p=0.035) (Figure 2A).

Kaplan-Meier analysis was also used to estimate the correlation between XBP1 protein expression and DSS and DFS in our cohort of patients with HCC. Clinicopathological and outcome information (available for all 267 patients) revealed that 117 of the patients died of HCC during the follow-up period (until June 2015) (note that the mean follow-up period was 4.6 years). Kaplan-Meier survival

Table II. *Univariate and multivariate analyses of disease-specific survival in patients with hepatocellular carcinoma.*

		Univariate			Multivariate		
Variable		HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
XBP1 expression	Low	1.00			1.00		
	High	0.65	0.42-0.88	**0.008**	0.64	0.44-0.94	**0.021**
Tumor differentiation	G1-2	1.00			1.00		
	G3-4	1.52	1.27-1.83	**<0.001**	1.45	1.20-1.75	**<0.001**
Vascular invasion	No	1.01			1.00		
	Yes	2.62	1.79-3.85	**<0.001**	1.50	0.95-2.38	0.085
Tumor stage	I-II	1.00			1.00		
	III-IV	3.59	2.47-5.23	**<0.001**	2.78	1.77-4.36	**<0.001**
Tumor recurrence	No	1.00			1.01		
	Yes	2.36	1.52-3.68	**<0.001**	1.92	1.22-3.00	**0.005**

CI: Confidence interval; HR: hazard ratio; XBP1: X-box-binding protein 1. Statistically significant *p*-values are shown in bold.

Table III. *Univariate and multivariate analysis of disease-free survival in patients with hepatocellular carcinoma.*

		Univariate			Multivariate		
Variable		HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
XBP1 expression	Low	1.00			1.00		
	High	0.70	0.51-0.96	**0.027**	0.72	0.52-0.99	**0.043**
Tumor differentiation	G1-2	1.00			1.00		
	G3-4	1.83	1.33-2.50	**<0.001**	1.81	1.32-2.49	**<0.001**
Vascular invasion	No	1.00			1.00		
	Yes	1.69	1.24-2.30	**0.001**	1.24	0.86-1.73	0.260
T-Classification	T1-T2	1.00			1.00		
	T3-T4	1.46	1.23-1.74	**<0.001**	1.34	1.09-1.64	**0.005**

CI: Confidence interval; HR: hazard ratio; XBP1: X-box-binding protein 1. Statistically significant *p*-values are shown in bold.

curves revealed that the DSS and DFS of patients with low cytoplasmic XBP1 expression were significantly worse than among patients with high cytoplasmic XBP1 expression (p=0.008 and p=0.026, Figure 2B and C, respectively), and this finding was further confirmed by log-rank test results.

Prognostic value of clinicopathological characteristics and XBP1 in patients with HCC based on the Cox regression model and hazard ratio analysis. The prognostic value of XBP1 in HCC was evaluated using univariate and multivariate analyses based on the Cox regression model and hazard ratios. In this evaluation, our aim was to characterize the relationships among DSS, DFS and various clinicopathological variables, including XBP1 expression (high *vs*. low), tumor differentiation (G3-G4 *vs*. G1-G2), vascular invasion (yes *vs*. no), tumor stage (III-IV *vs*. I-II), tumor recurrence (yes *vs*. no), and T classification (T3-T4 *vs*. T1-T2) in patients with HCC. Multivariate analyses confirmed univariate findings that poor DSS was significantly associated with low XBP1 expression (p=0.008 and p=0.021, respectively), G3-G4 tumor differentiation (p<0.001 and p<0.001, respectively), vascular invasion (p<0.001), stage III-IV tumor (p<0.001 and p<0.001, respectively), and tumor recurrence (p<0.001; p=0.005, respectively) (Table II). Poor DFS was also significantly associated with low XBP1 expression (p=0.027 and p=0.043, respectively), G3-G4 tumor differentiation (p<0.001 and p<0.001, respectively), vascular invasion (p=0.001), and T3-T4 classification (p<0.001 and p=0.005, respectively) (Table III).

Discussion

Despite improvements in the diagnosis and treatment of HCC, the prognosis for patients with liver cancer remains poor (36). In the clinical diagnosis of HCC, early identification of primary metastatic malignancies is crucial to achieving effective treatment and good prognosis for HCC and metastatic tumors (37). Numerous candidate biomarkers for HCC are currently being evaluated (38-40), including

genetic and epigenetic changes, gene expression, and genome-based markers (41-43).

Previous studies discussed the possibility that XBP1 is involved in tumorigenesis. XBP1 is an important aspect of UPR and has been studied as a novel protein associated with various forms of cancer (44). However, the cytoplasmic and nuclear expression of XBP1 in HCC has not previously been investigated as far as we are aware. Nonetheless, one study reported that the unspliced form (XBP1u) is located mainly in the cytoplasm and may be triggered by a subset of genes implicated in the progression of cancer and transcriptional programs (45). In esophageal squamous cell carcinoma and oral squamous cell carcinoma (OSCC), overexpression of XBP1 in the cytoplasm and nucleus has been linked to tumor invasion and poor prognosis (29, 31). Other research noted that HepG2 cells and HCC tissue had significantly greater *XBP1s* mRNA and protein expression than did normal tissue. This study also found that XBP1s was localized in the hepatocyte nucleus and that the expression of XBP1s was closely related to distant metastasis and poor HCC prognosis (32). In the current study, XBP1 proteins were located in the cytoplasm of HCC tumor tissue (Figure 1). The same XBP1 antibody has been used to detect cancer of the breast, colon, mouth, and prostate, and other research has reported the presence of XPB1 proteins in the cytoplasm of tumor cells (34, 46-48). The difference in results may be attributable to the effects of fixation on its apparent subcellular distribution (*e.g.* c-MYC proteins), and antibodies recognizing the cytoplasmic repertoire of the XBP1 protein prior to activation (49). XBP1u has a nuclear-exclusion domain that allows proteins to shuttle in and out of the nucleus (50). In the nucleus of lymphoid tissue cells, there is a higher ratio of XBP1s/XBP1u, and the predominant subtype is the spliced form. The XBP1u transcript (restricted to the cytoplasmic fraction) is representative of the cytoplasmic storage pool of XBP1u proteins, which are the dominant negative inhibitors of UPR (51).

Our IHC analysis of tissue specimens from 267 patients with HCC revealed a strong correlation between low cytoplasmic XBP1 protein expression and poor prognostic factors, such as vascular invasion and reduced overall 5-year survival rates. However, we did not observe a significant correlation between low cytoplasmic XBP1 protein expression and age, tumor differentiation, T classification, tumor stage, tumor recurrence, distant metastasis, or cirrhosis (Table I). This led us to the initial conclusion that XBP1 expression is independent of these clinicopathological factors. These findings also suggest that low cytoplasmic protein expression of XBP1u strongly down-regulates expression of XBP1s protein (which normally triggers apoptosis) and that low XBP1u expression is indicative of poor prognosis and reduced overall survival. Our Kaplan-Meier survival database analysis revealed that the clinical outcomes, in terms of DSS and DFS, of patients with reduced cytoplasmic *XBP1* mRNA levels or protein expression in the case of our cohort were worse than those of patients with pronounced cytoplasmic XBP1 expression (Figure 2). Reduced XBP1 expression has previously been shown to be associated with poor prognosis, overall survival, and progression-free survival in patients with OSCC, multiple myeloma, and prostate cancer (34, 47, 52). However, findings reported by Wu *et al.* pertaining to the expression of XBP1s in HCC (32) differed from our findings in the current study, which suggests that additional unknown mechanisms may underly HCC and require further investigation.

In univariate and multivariate analyses, XBP1 was found to be the main predictor of DSS and DFS. Low cytoplasmic XBP1 expression also had a statistically significant association with factors, such as vascular invasion, tumor stage, and tumor recurrence (Table II) and tumor differentiation, vascular invasion, and T classification (Table III). In other words, XBP1 expression was shown to be an independent predictor of DSS and DFS. One previous study employed Cox regression analysis and reported that, in patients with OSCC or HCC, XBP1 was a prognostic factor for tumor differentiation, tumor stage, and tumor classification (34). In another study, the inositol-requiring enzyme 1 (IRE1)–XBP1s pathway was found to promote EMT in pulmonary fibrosis by mediating SNAIL expression (53) and the XBP1s–TWIST–SNAIL axis that mediates EMT in HCC cells and invasion and metastasis of HCC (32). The overexpression of lysyl oxidase-like 2 (LOXL2) promotes the accumulation of LOXL2 proteins in the ER and the interaction of these proteins with heat-shock protein family A member 5 (HSPA5). These interactions activate the IRE1-XBP1 signaling pathway of the ER-stress response in tumor cells (54). Still other research noted that low *miR-199* expression was associated with HCC and was directly involved with the inhibition of XBP1 (55). These results may help explain why, in the current study, low cytoplasmic XBP1 protein expression was associated with worse DSS and DFS outcomes among patients with HCC. We recommend that future studies further explore this potential mechanism.

Conclusion

In conclusion, our results revealed that (i) the presence of XBP1 in the cytoplasm is an important biomarker associated with poor survival in patients with HCC and (ii) a decrease in the cytoplasmic expression of XBP1 might indicate poor HCC prognosis. It is still too early to claim that XBP1 is an independent prognostic factor for HCC. Nonetheless, evidence does suggest that XBP1 may be an independent prognostic indicator for DSS and DFS in patients with HCC.

Moreover, the strong correlation between XBP1 and HCC indicates that, not only is XBP1 protein expression a potential prognostic indicator of HCC, but it might also be useful in developing improved therapeutic strategies for patients with HCC.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

HTH, YML, MTH, KTY and JWL analyzed and drafted the article. HTH, YML, MTH, KTY and JWL assisted with data interpretation. HTH, YML, JWL and SFY wrote the article. The Authors critically revised the article and approved the final version.

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