RESEARCH ARTICLE

# Identification of Homer1 as a Potential Prognostic Marker for Intrahepatic Cholangiocarcinoma

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## Abstract

**Background: The aim of the present study was to analyze whether Homer1 is a potential prognostic marker for intrahepatic cholangiocarcinoma (ICC). Materials and Methods: The expression of Homer1 in ICC tissue was detected with immunohistochemistry and levels of protein in ICC and paratumor tissues were evaluated by Western blotting. Survival analysis by the Kaplan-Meier method was performed to assess prognostic significance. Results: Homer1 expression was high in 67.4% (58/86) of ICC samples, and there was significant difference between ICC and adjacent noncancerous tissues ($p$<0.001); high expression was associated with poor histologic differentiation ($p$=0.019), TNM stage ($p$=0.014), lymph node metastasis ($p$=0.040), and lymphatic invasion ($p$=0.025). On Kaplan-Meier analysis, a comparison of survival curves of low versus high expressors of Homer1 revealed a highly significant difference in OS ($p$=0.001) and DFS ($p$=0.006), indicating that high expression of Homer1 was linked with a worse prognosis. Multivariate analyses showed that Homer1 expression was an independent risk factor predicting overall survival[Hazard ratio(HR), 7.52; 95% confidence interval (CI), 2.63-21.47; $p$=0.002] and disease-free survival (HR, 11.56; 95%CI, 5.17-25.96; $p$<0.001) in ICC. Conclusions: Homer1 promotes lymphatic invasion and associates with lymph node metastasis and poor prognosis of ICC. The current study shows that Homer1 may be an independent prognostic factor for ICC patients after curative resection, and it provides an important basis for screening/treating high-risk patients.**

**Keywords:** Intrahepatic cholangiocarcinoma - Homer1 - prognosis - survival

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## Introduction

Cholangiocarcinoma is a malignant tumor originating from the bile duct epithelium (Alpini et al., 2002). It can be classified anatomically into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) (Khan et al., 2005). However, ICC is difficult to diagnose at an early stage, almost all patients with ICC present with advanced, incurable disease (Shaib et al., 2007). Even ICC patients who have undergone complete surgical resection, the recurrence rate remains quite high and the 5-year survival rate is unfavorable (Kawarada et al., 2002; Anderson et al., 2004). Therefore, ICC is a slow growing but highly metastatic cancer, which is the major cause of death in ICC patients. A global increase in ICC related mortality and incidence of ICC have been reported (Khan et al., 2002; Patel, 2002). The prognosis for patients with ICC is still not good despite better diagnostic techniques and treatment innovations. Improved survival of patients with ICC requires better methods for prediction of prognosis.

Previous studies showed that Homer1 expression was negligible or very low even not detected by PCR amplification in various tissues other than brain and heart (Soloviev et al., 2000), but in various tumor cells (e. g., HeLa, HCT116, HEK 293, DU145, and A549) originated from tissues of cervix, colon, kidney, and lung express the significant amounts of Homer1 protein, suggesting that Homer1 may play some roles in these tumor cells. However, the correlations between Homer1 expression and clinicopathologic significance and predicting clinical outcome in ICC patients have not yet been investigated.

In the current study, we examined expression patterns of Homer1 in ICC tissues and investigated the relationship between Homer1 expression and clinicopathological factors and prognosis of ICC.

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## Materials and Methods

*Patient selection and tissue collection*

The study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University, and written informed consent was obtained from all subjects. Between March 2005 and November 2006, 86 patients with ICC who underwent successful tumor resection at the Department of General Surgery, Zhongnan Hospital of Wuhan University, were enrolled in this study. There were 47 men and 39 women with a median age of 57 years (range 37-75years). Preoperative diagnostic imaging examinations, including ultrasonography, computed tomographic scan, and angiography, were conducted for all patients. All patients underwent curative resection with pathologically confirmed negative margins and regional lymph node dissection. The tumor-node-metastasis (TNM) classification was based on the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC).

*Immunohistochemistry analysis*

After review of the hematoxylin-eosin stained slides of all surgical specimens, one representative paraffin block was selected from each case. Typical tumor tissues were present in all selected blocks. Informed consent for the use of the specimens was obtained from all patients. Successive sections at 4 μm intervals from each block were used for evaluation. Anti-Homer1 rabbit polyclonal antibody (Synaptic Systems) was used for Homer1 immunohistochemical staining, respectively. Immunohistochemical staining was carried out with the streptavidin peroxidase complex method. Briefly, sections were dewaxed and rehydrated with xylene and graded alcohol. Endogenous peroxidases were inactivated by incubating the sections in 3% hydrogen peroxide for 10 min. Optimal antigen retrieval was carried out in citrate buffer (pH6.0) for 10 min with a microwave oven to enhance immunoreactivity, and then incubated in blocking serum for 30 min at 37°C to reduce nonspecific binding. The sections were incubated overnight at 4°C in a humidified chamber with the primary antibody against Homer1 in a dilution of 1:100. Subsequently, biotinylated secondary antibodies and streptavidin peroxidase complex reagent were applied. The 3, 3'-diaminobenzidine solution was then added and incubated until desired staining was achieved. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. Each stained section was blindly evaluated by two investigators unaware of the clinical information. In each immunohistochemistry run, the positive section provided by reagent company served as positive control and omission of the primary antibody served as negative control.

*Western blotting analysis*

The homogenized ICC samples, including cancerous and noncancerous tissues , were lysed in RIPA lysis buffer, and the lysates were harvested by centrifugation (12, 000 rpm) at 4°C for 30 min. Protein samples of approximately 20 μg were the separated by electrophoresis in a 12% sodium dodecyl sulfate polyacrylamide gel and were transfer red onto a polyvinylidene fluoride membrane. After blocking the nonspecific binding sites for 60 min with 5% nonfat milk, the membranes were incubated overnight at 4°C with a mouse monoclonal antibody

**Table 1. The Relationship between Homer1 Expression Levels of Tumors and Clinicopathological Feature**

| Variable | Cases | Homer 1 expression | | *p* value |
|---|---|---|---|---|
| | | High | Low | |
| Age | | | | |
| <65years | 41 | 26 | 15 | 0.447 |
| ≥65years | 45 | 32 | 13 | |
| Gender | | | | |
| Male | 47 | 35 | 12 | 0.127 |
| Female | 39 | 23 | 16 | |
| Tumor diameter(mm) | | | | |
| ≤35 | 42 | 28 | 14 | 0.881 |
| > 35 | 44 | 30 | 14 | |
| Histology type | | | | |
| Tubular type | 24 | 14 | 10 | 0.427 |
| Papillary type | 52 | 36 | 16 | |
| Mixed type | 10 | 8 | 2 | |
| Differentiation | | | | |
| Well/Moderate | 63 | 47 | 16 | 0.019* |
| Poor | 23 | 11 | 12 | |
| Tumor number | | | | |
| Solitary | 65 | 43 | 22 | 0.654 |
| Multiple | 21 | 15 | 6 | |
| TNM Stage | | | | |
| I-II | 20 | 9 | 11 | 0.014* |
| III-IV | 66 | 49 | 17 | |
| Jaundice | | | | |
| Negative | 53 | 37 | 16 | 0.552 |
| Positive | 33 | 21 | 12 | |
| Serum CEA(ng/ml) | | | | |
| ≤ 5 | 34 | 23 | 11 | 0.974 |
| >5 | 52 | 35 | 17 | |
| AST(IU/L) | | | | |
| ≤ 34 | 28 | 20 | 8 | 0.584 |
| >34 | 58 | 38 | 20 | |
| ALT(U/L) | | | | |
| ≤ 36 | 29 | 22 | 7 | 0.235 |
| >36 | 57 | 36 | 21 | |
| ALP(U/L) | | | | |
| ≤ 94 | 12 | 8 | 4 | 0.951 |
| >94 | 74 | 50 | 24 | |
| Total bilirubin(mg/dl) | | | | |
| ≤ 1.3 | 26 | 16 | 10 | 0.442 |
| >1.3 | 60 | 42 | 18 | |
| Albumin(ng/dl) | | | | |
| ≤ 3.5 | 38 | 25 | 13 | 0.771 |
| >3.5 | 48 | 33 | 15 | |
| Lymph node metastasis | | | | |
| Negative | 65 | 40 | 25 | 0.040* |
| Positive | 21 | 18 | 3 | |
| Nerve invasion | | | | |
| Negative | 67 | 47 | 20 | 0.314 |
| Positive | 19 | 11 | 8 | |
| Venous invasion | | | | |
| Negative | 20 | 13 | 7 | 0.790 |
| Positive | 66 | 45 | 21 | |
| Lymphatic invasion | | | | |
| Negative | 16 | 7 | 9 | 0.025* |
| Positive | 70 | 51 | 19 | |

Abbreviations: L, low expression; H, high expression; Statistically significant *$P$<0.05, **$p$ <0.01

against Homer1 (1:3,000 dilution). Then the membranes were incubated for 1 hour with IRDye800CW- conjugated goat anti-rabbit IgG and IRDye680-conjugated goat anti-mouse IgG secondary antibodies (LI-COR Biosciences) diluted in Odyssey Blocking Buffer. The blots were then washed three times with PBST and rinsed with PBS. Proteins were visualized by scanning the membrane on an Odyssey Infrared Imaging System (LI-COR Biosciences) with both 700- and 800-nm channels. An antibody against β-actin (Sigma-Aldrich) served as a loading control. Relative blot intensity was quantified with the Gauge Image software, ver. 4.0. All standards and samples were analyzed in triplicate.

*Quantification of IHC staining*

Assessment of the staining was scored independently by two investigators (JQY and DBW) who were blinded to all clinical data. The allocation of tumors and scoring staining by the two investigators was similar. In cases of disagreement, slides were reevaluated and discussed until a consensus was achieved. Homer1 staining was considered positive if there was cytoplasm expression. The staining intensity and percentage of tissue staining were recorded (staining intensity: 1=no staining or weak staining, 2=intermediate staining, 3=strong staining; percentage:1=<10%, 2=10-30%, 3=>30%). Both scores multiplied, If more than 3 points that case was considered to be high expressed, otherwise it is low expressed. The final results were subjected to statistical analysis.

*Follow-up*

None of the patients suffered major perioperative complications, and all were discharged from the hospital. Survival time is defined as the date of last follow-up since surgery or the date of death. All patients were followed up for 5 years.The closing date for follow-up was May 31st, 2013, and if the follow-up was incomplete, patients or their families were contacted by telephone thereafter. The median follow-up time was 42.6 months (range 5.2-67.3 months).

*Statistical analysis*

Associations among categorical variables were assessed using Fisher's exact probability test or the ×2 test. Overall survival (OS) and disease-free survival (DFS) was measured by the Kaplan-Meier method. The prognostic value of the 13 variables was tested by univariate analysis using the log-rank test. Multivariate Cox proportional hazard models were used to define the potential prognostic significance of individual parameter. A P-value of less than 0.05 was considered significant. All statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

## Results

*Correlation of Homer1 expression and clinicopathological features in ICC*

The Homer1 staining was as performed in 86 ICC patients by immunohistochemistry. The Homer1 high expression was detected in 67.4% (58/86) ICC. The

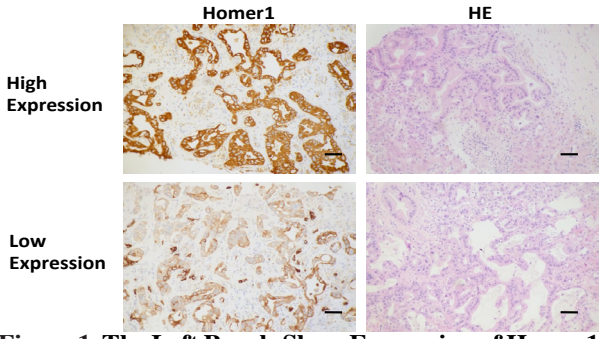


**Figure 1. The Left Panels Show Expression of Homer1 by Immunohistochemistry in the ICC Tissues with High and Low Expression.** The scores for Homer1 in tissue with high and low expression were 9 and 2, respectively. The right panels show the sections stained with H&E. Bar indicates 50 μm

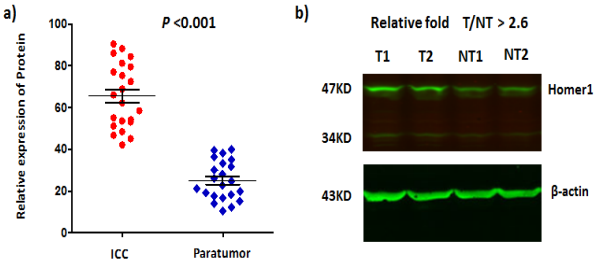


**Figure 2. a) Homer1 Relative Expression in ICC and Paratumor were Analyzed by Western blot. The Relative Amount of Homer1 Protein in ICC was Higher than in the corresponding Paratumor, the Difference were Statistically Significant (p<0.001). b) The Electrophoretogram of Western Blot of Homer1 from Paird ICC Tissues(T) and Non-neoplastic Tissues(NT), β-actin was used as an internal Control**

expression of Homer1 was found in cytoplasm (Figure 1).

When comparing the Homer1 status with clinicopathological variables, we found significant positive correlations between Homer1 expression and histologic differentiation ($p$=0.019), TNM stage ($p$=0.014), lymph node metastasis ($p$=0.040), and Lymphatic invasion ($p$=0.025) (Table1).

*Analysis of Homer1 protein expression*

To investigate whether Homer1 was also elevated at the protein level, Western blotting was performed on the same specimens what were used in the detection of Homer1 protein. The Western blot analysis of Homer1 protein also showed that the expression of Homer1 protein was significantly higher in ICC tissues compared to that in adjacent noncancerous tissues ($p$<0.001, Figure 2a). We found that Homer1 protein expression in ICC tissues are higher than Homer1 protein expression in non-neoplastic tissues (at least a 2.6-fold increase, Figure 2b).

*Relationship between Homer1 and OS and DFS of ICC patients*

To investigate the prognostic value of Homer1 for ICC, we performed univariate survival analysis of Homer1 protein expression and factors including age, gender, tumor size, histology type, tumor number, serum CEA, TNM stage, venous invasion, nerve invasion,

differentiation, lymph node metastasis, and Lymphatic invasion in patients with ICC. In the univariate Cox proportional hazard regression model analysis shown in Table 2, histologic differentiation ($p$=0.004), TNM stage ($p$=0.025), node status ($p$=0.022), lymphatic invasion ($p$=0.001), and expression intensity of Homer1 ($p$=0.001) were significantly associated with OS (Figure 3a). The histologic differentiation ($p$=0.028), TNM stage ($p$=0.009), node status ($p$=0.004), lymphatic invasion ($p$=0.001), and expression intensity of Homer1 ($p$=0.006) were significantly associated with DFS (Figure 3b). Consequently, patients with tumors with negative or low expression of Homer1 had a better prognosis than those with tumors having high Homer1 expression. The log-rank test showed that the survival time of patients with ICC was significantly different between the group with high Homer1 expression and the low Homer1 group ($p$<0.001). In patients with ICC, the high Homer1 expression group had shorter survival time, whereas the low Homer1 expression group had better survival (Figure 3a ).

To further investigate whether the expression of Homer1 is an independent prognostic factor for ICC, we performed a multivariate Cox regression analysis of OS and DFS (Table 3). The expression intensity of Homer1 ($p$=0.0002), TNM stage ($p$=0.001), node status ($p$=0.003) , and Lymphatic invasion ($p$=0.043)showed a significant association with OS. The expression intensity of Homer1 ($p$<0.001), histologic differentiation ($p$=0.011), TNM stage ($p$=0.002), node status ($p$=0.041), and lymphatic invasion ($p$=0.003) showed a significant association with DFS. These results showed that the expression of Homer1 protein was an independent prognostic factor for ICC.

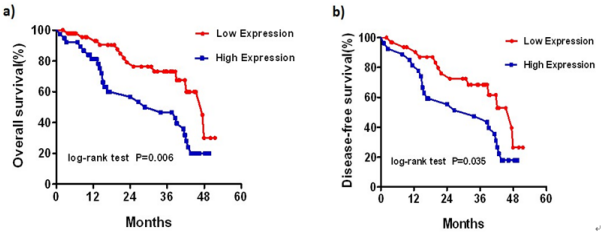


**Figure 3. Survival Curves of 86 Patients with ICC. a)** Overall survival; **b)** Disease-free survival of patients with tumors low (L) or high (H) expression levels of Homer1

## Discussion

The Homer protein family (known as the family of cytoplasmic scaffolding proteins) include Homer1, Homer2, and Homer3. Homer protein family was firstly discovered as a new protein family being upregulated in response to brain seizures (Brakeman et al., 1997), and the role of the Homer proteins has been extensively investigated (Li et al., 2013). During the decade Homer proteins have been identified not only exist in thenervous system but also exist in various peripheral tissues, including cardiac and skeletal muscles (Xiao et al., 1998; Sandona et al., 2000). Apart from their known role at central synapses, Homers are important physiological determinants in differentiation, development, and adaptation in skeletal muscle and the neuromuscular system, and thus integrating motor neuron control, for example, with downstream calcium signaling pathways in muscle fibers (Salanova et al., 2013). Homer1 expressed differentially between benign and malignant pheochromocytomas.The differentially expressed with known function belonged to 8 biologic process categories:

**Table 2. Univariate Cox Regression Analysis of OS and DFS in ICC**

| Parameter | Overall survival | | Disease-free survival | |
|---|---|---|---|---|
| | Log-rank test | $p$ value | Log-rank test | $p$ value |
| Age (<60, ≥60 y) | 2.113 | 0.146 | 0.171 | 0.68 |
| Gender | 0.651 | 0.420 | 2.501 | 0.475 |
| Tumor size(<3.5cm, ≥3.5cm) | 0.344 | 0.558 | 0.136 | 0.712 |
| Histology type(tubular, papillary, mixed) | 0.186 | 0.666 | 1.890 | 0.169 |
| Tumor number(solitary,mutilple) | 7.376 | 0.007** | 0.478 | 0.490 |
| Serum CEA(≤ 5ng/ml,>5ng/ml,) | 1.344 | 0.246 | 0.374 | 0.541 |
| TNM Stage (I,II,III,IV) | 5.029 | 0.025* | 6.875 | 0.009** |
| Venous invasion(N0, N(+)) | 1.103 | 0.294 | 2.368 | 0.124 |
| Nerve invasion(N0, N(+)) | 1.085 | 0.297 | 1.304 | 0.309 |
| Differentiation(well, moderate, poor ) | 8.075 | 0.004** | 4.843 | 0.028* |
| Lymph node metastasis (N0, N(+)) | 5.255 | 0.022* | 8.234 | 0.004** |
| Lymphatic invasion | 15.664 | 0.001** | 13.502 | 0.001** |
| Homer1 expression(low, high) | 12.932 | 0.001** | 7.596 | 0.006** |

NOTE. Statistically significant *$p$<0.05, **$p$ <0.01.Abbreviations: N0, no nodal metastasis; N(+), nodal metastasis.

**Table 3. Multivariate Cox Regression Analysis of OS and DFS in ICC**

| Parameter | Overall survival | | Disease-free survival | |
|---|---|---|---|---|
| | HR (95%CI ) | $p$ value | HR (95%CI ) | $p$ value |
| Histologic differentiation(well, moderate, poor) | 1.64 (0.82-3.37) | 0.200 | 1.33 (1.26-2.19) | 0.011* |
| TNM Stage (I, II, III, IV) | 4.89 (1.87-12.81) | 0.001** | 3.95 (1.68-9.36) | 0.002** |
| Lymph node metastasis (N0, N(+)) | 2.91 (1.54-6.08) | 0.003** | 2.73 (1.12-7.16) | 0.040* |
| Lymphatic invasion | 2.26 (1.09-5.17) | 0.046* | 2.69 (1.46-5.06) | 0.003** |
| Homer1 expression ( low, high) | 7.52 (2.63-21.47) | 0.002** | 11.56 (5.17-25.96) | <0.001** |

NOTE. Statistically significant *$p$<0.05, **$p$ <0.01.Abbreviations: N0, no nodal metastasis; N(+), nodal metastasis; HR, hazard ratio;CI, confidence interval

signal transduction, transcription, protein transport, protein synthesis, smooth muscle contraction, ion transport, chemotaxis, and electron transport. Gene set enrichment analysis revealed significant correlation between the microarray profiles of malignant pheochromocytomas and several known molecular pathways associated with carcinogenesis and dedifferentiation. Ten differentially expressed genes had high diagnostic accuracy, and 5 of these genes (CFC1, FAM62B, HOMER1, LRRN3, TBX3, ADAMTS) in combination had an area under the receiver operating characteristic (ROC) curve of 0.96 for distinguishing benign versus malignanttumors (Suh et al., 2009). In the present study, we have demonstrated for the first time that the expression of Homer1 is associated with clinicopathologic features and poor prognosis in ICC.We found Homer1 expression in ICC tissues is significantly higher than the corresponding paratumor tissues (at least a 2.6-fold increase), and there was statistically significant ($p$<0.001). Furthermore, high levels of Homer1 in ICC correlated with tumor differentiation, TNM stage, lymph node metastasis, and lymphatic invasion (Table 1). Our results demonstrated that Homer1 was closely correlated to ICC progression and metastasis.

Furthermore, the OS and DFS of patients with high Homer1 expression was significantly worse than that of patients with low Homer1 expression.The univariate survival analysis revealed Homer1 expression was a significant prognostic factor as well as histologic differentiation, TNM Stage, lymph node metastasis, and lymphatic invasion. The status of Homer1 expression might be dependent on the status of lymph node metastasis or other variables. So the multivariate Cox regression analysis for OS and DFS was undertook, and multivariate analysis found that Homer1 expression had its independent prognostic significance. The Homer1 expression level plays important functions in the biology of ICC and shows a more aggressive tumor phenotype of ICC. Preoperative adjuvant therapy in ICC is desired to improve survival and reduce distant metastasis. Our results also showed that the tumors with a high expression of Homer1 were associated with an increased lymph node metastasis and lymphatic invasion, which suggests that patients with high Homer1 expression may be prone to metastasis. So, Homer1 high expression was closely related to poor prognosis, and Homer1 may serve as a marker for poor prognosis. The previous report implied that up-regulation of Homer3 expression might be an important event of pathogenesis and prognosis in AML (Li et al., 2013).

The conceivable mechanisms responsible for these correlations maybe as follow.Firstly, Homer1 may be implicated in tumor apoptosis process (Shin et al., 2009). TRAIL is anapoptotic cell death-inducing ligand that belongs to a TNF superfamily. TRAIL is the "professional killer" with a limited activation of NF- k B activation, and it induces the apoptotic cell death through its cognate death receptors DR4/DR5 (Nagata et al., 1999; Kim et al., 2003; LeBlanc et al., 2003; Wang et al., 2003). Studies have demonstrated that once TRAIL activated, it transmits the apoptotic death signals through Fas Associated Death Domain (FADD), caspase-8, Bid, and Bax, that are utilized in the apoptotic death signaling of TNF-α. Studies using FADD-, caspase-8-, or Bax-deficient cells have demonstrated that DR4/DR5 also utilizes FADD, caspase-8 and Bax as essential mediators for TRAIL-induced cell death (Kischkel et al., 2000; Kuang et al., 2000; Bodmer et al., 2002; Deng et al., 2002; LeBlanc, 2002). It would be interesting to note that TRAIL has been known as a tumor selective killer but limited or no killing activity on normal cells; however, the underlying mechanism of the tumor selectivity by TRAIL remains to be unsolved. The findings demonstrated that TRAIL only kills the Homer1-positive cells and that Homer1 is limitedly expressed in normal tissues (Soloviev et al., 2000). Thus, Homer1 may confer tumor cells susceptible to TRAIL and play a critical role in determining the apoptotic susceptibility to TRAIL. Secondly, Homer1 may involve in promoting lymph node metastasis.It is well known that metastatic spread of cancer cells from a primary lesion to distant organs is a critical event in the progression of disease and is associated with poor prognosis (Li et al., 2013; Senol et al., 2013; Yin et al., 2013). Among the various patterns, lymphatic metastasis is one of the major processes of distant metastasis in most solid tumors. Lymph node metastasis, which is used for staging and therapeutic decisions, is the most important prognostic factor for ICC. Most importantly, the underlying molecular mechanism may ultimately help in the development of innovative therapeutic strategies against ICC.

Taken together, the findings of the present study show that Homer1 expression may be helpful to prognosis judgment for ICC patients after curative resection. Homer1 may serve as a new prognostic factor and a therapeutic target for patients with ICC in the future.

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