Axl and autophagy LC3 expression in tumors is strongly associated with clinical prognosis of hepatocellular carcinoma patients after curative resection

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Abstract

**Background:** The role of Axl and LC3 as predictors of tumor recurrence and overall survival (OS) after hepatocellular carcinoma (HCC) resection remains unclear.

**Methods:** We retrospectively included 535 HCC patients who underwent hepatectomy from 2010 to 2014 in this study. Axl and the autophagy-related marker LC3 were immunohistochemically assessed in tumors.

**Results:** Axl expression was significantly associated with advanced clinicopathological features, including cirrhosis, microvascular invasion, macrovascular invasion, tumor size, BCLC stage, recurrence, and mortality. HCC recurrence occurred in 245 patients, and 219 patients died. The 5-year cumulative incidences of HCC recurrence and OS rate after HCC resection were 53.3% and 58.8%, respectively. In the Cox proportional analyses, high Axl expression and high LC3 expression were significantly associated with a poor clinical outcome.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the third common cause of cancer-related death in the world.\(^1\)\(^,\)\(^2\) In Taiwan, viral- and alcohol-related cirrhosis frequently result in HCC.\(^3\) Moreover, HCC is hard to diagnose at the very early and early stages, resulting in higher mortality rates worldwide.\(^1\)\(^,\)\(^2\) Although the 5-year survival rate of individuals diagnosed in the early stage exceeded 50% after curative resection, these patients still had a high recurrence rate of approximately 60% after curative resection.\(^4\)\(^-\)\(^8\) The highly sensitive marker alpha-feto-protein (AFP) predicts clinical outcome in HCC patients after hepatectomy, but the result is still unsatisfactory.\(^9\) Hence, the identification of biomarkers for HCC recurrence and overall survival (OS) could help improve the clinical prognosis of HCC patients undergoing hepatectomy.

Axl, a member of the Tyro3, Mer, and Axl family of tyrosine kinase receptors, regulates some aspects of cancer biology.\(^10\) The Axl-mediated signaling pathway is frequently affected in the development and progression of various cancers, including breast cancer, pancreatic cancer.\(^11\)\(^-\)\(^13\) Recently, Reichl et al reported that high serum levels of soluble Axl are correlated with vascular involvement and lymph node metastasis, and the serum level of soluble Axl is a potential biomarker for the early diagnosis of HCC and the early prediction of HCC recurrence.\(^14\) Moreover, Wu et al demonstrated that Axl overexpression/hyperactivation plays a major role in epithelial-to-mesenchymal transition, cancer chemotherapy resistance, and increased metastasis, all of which implicate Axl as an important target.\(^15\) Several studies showed that Axl may be a negative predictor for HCC patients, and high Axl expression was positively associated with differentiation, lymph node metastasis, higher recurrence rates, and lower survival rates in HCC patients.\(^16\)\(^,\)\(^17\) Furthermore, Axl expression was associated with increased tumor invasion and predicted a worse prognosis for HCC patients undergoing resection.\(^18\) Our previous studies showed that high LC3 expression in the liver and tumor microenvironments is strongly correlated with higher OS and lower HCC recurrence.\(^7\)\(^,\)\(^8\) However, whether Axl expression is associated with clinical prognosis in HCC patients remains largely unknown. In addition, the role of Axl and the autophagy-related marker LC3 in OS and HCC recurrence is not clear. Hence, we investigate the impact of Axl and LC3 expression on tumor recurrence and OS in a large cohort of HCC patients who underwent curative resection.

2 MATERIALS AND METHODS

2.1 Patients and follow-up

We retrospectively collected the data from 535 HCC patients who underwent resection from 2010 to 2014 at Changhua Christian Hospital, Changhua, or E-Da Hospital, I-Shou
University, Kaohsiung, Taiwan. All participants were regularly followed-up every three to six months after surgery. The last recorded follow-up was on 31 December 2016. OS was the duration from the date of hepatectomy to the date of death or the last visit. Survival data were censored on the end-date of follow-up. Time to recurrence was the duration from the date of hepatectomy to the date of recurrence. HCC recurrence was based on histology or at least two typical HCC imaging methods according to the HCC guidelines of the American Association for the Study of Liver Disease.19

The clinicopathological features of the patients, such as demographic data and tumor characteristics, were recorded. Antiviral treatment was defined as patients with hepatitis B virus (HBV), hepatitis C virus (HCV), or dual HBV/HCV infection who received therapy with oral nucleosides, pegylated interferon-based therapy or direct-acting agents according to the guidelines of the Taiwan Association for the Study of the Liver.17,18 Interferon-based therapy or direct-acting agents were administered according to the clinicopathological features, including cirrhosis (111 [36.4%] vs 62 [27.0%], P = 0.021), microvascular invasion (119 [39.0%] vs 127 [55.2%], P < 0.001), macrovascular invasion (47 [15.4%] vs 64 [27.8%], P < 0.001), tumor size ≥ 5 cm (89 [29.2%] vs 94 [40.9%, P = 0.005), BCLC stage B/C (92 [47.7%] vs 88 [52.3%], P = 0.001), recurrence (102 [33.4%] vs 143 [62.2%, P < 0.001), and death (73 [23.9%] vs 146 [63.5%], P < 0.001).

### 3.1 Baseline demographic data

The demographic and clinicopathological features of the 535 patients are presented in Table 1. First, 46.7% of the HCC patients had HBV, and 28.4% had HCV. One-third of the patients had liver cirrhosis. Regarding tumor stage, 36.1% and 16.4% of the patients were Barcelona clinic liver cancer (BCLC) stage B-C and TNM stage III-IV, respectively. In addition, 91.6% of the HCC tissues showed high LC3 expression.

### 3.2 Axl expression is associated with advanced clinicopathological features

Within the cohort, 43.0% (230) and 57.0% (305) of the 535 HCC tissues had high and low Axl expression, respectively, as presented in Table 1. Axl expression was significantly correlated with advanced clinicopathological features, including cirrhosis (111 [36.4%] vs 62 [27.0%], P = 0.021), microvascular invasion (119 [39.0%] vs 127 [55.2%], P < 0.001), macrovascular invasion (47 [15.4%] vs 64 [27.8%], P < 0.001), tumor size ≥ 5 cm (89 [29.2%] vs 94 [40.9%, P = 0.005), BCLC stage B/C (92 [47.7%] vs 88 [52.3%], P = 0.001), recurrence (102 [33.4%] vs 143 [62.2%, P < 0.001), and death (73 [23.9%] vs 146 [63.5%], P < 0.001).

### 3.3 Predictive factors associated with tumor recurrence in HCC patients who underwent hepatectomy

HCC recurrence occurred in 245 patients, and the HCC recurrence rate at 1, 3, 5, and 7 years after hepatectomy was 9.7%, 33.9%, 53.3% and 66.3%, respectively. In the univariate analysis, macrovascular invasion, liver cirrhosis, high Axl expression in tumors and low LC3 expression in tumors were significantly correlated with increased HCC recurrence (Table 2).

The multivariate Cox regression analysis revealed that high Axl expression in tumors was significantly correlated with increased HCC recurrence (hazard ratio [HR]: 3.85, 95% confidence interval [CI]: 2.95-5.02, P < 0.001), but high LC3 expression in tumors was not significantly correlated with decreased HCC recurrence (HR: 0.38, 95% CI: 0.26-0.55, P < 0.001), as shown in Table 2.

Patients with high Axl expression in tumors had a significantly higher HCC recurrence rate than those with low Axl expression in tumors by Kaplan-Meier analysis. In patients with high and low Axl expression in tumors, the 1-, 3-, 5- and 7-year HCC recurrence rates were 19.3%, 58.6%, 77.9% and 85.9%, respectively.
| Characteristics                        | All patients (n = 535) | Axl-low (n = 305) | Axl-high (n = 230) | P-value* |
|---------------------------------------|------------------------|-------------------|--------------------|----------|
| **Gender**                            |                        |                   |                    |          |
| Female                                | 144 (26.9)             | 70 (23.0)         | 74 (32.2)          | 0.017    |
| Male                                  | 391 (73.1)             | 235 (77.0)        | 156 (67.8)         |          |
| **Age (y)**                           | 63.1 ± 11.5            | 62.9 ± 12.1       | 63.4 ± 10.6        | 0.580    |
| **HCC etiology**                      |                        |                   |                    |          |
| HCV                                   | 152 (28.4)             | 98 (32.1)         | 54 (23.5)          | 0.010    |
| HBV                                   | 250 (46.7)             | 143 (46.9)        | 107 (46.5)         |          |
| Non-HBV/HCV                           | 112 (20.9)             | 50 (16.4)         | 62 (27.0)          |          |
| HBV + HCV                             | 21 (3.9)               | 14 (4.6)          | 7 (3.0)            |          |
| **AFP (ng/dL)**                        | 2797 ± 13215           | 3019 ± 13076      | 2504 ± 13418       | 0.410    |
| **Liver cirrhosis**                   |                        |                   |                    |          |
| Negative                              | 362 (67.7)             | 194 (63.6)        | 168 (73.0)         | 0.021    |
| Positive                              | 173 (32.3)             | 111 (36.4)        | 62 (27.0)          |          |
| **Antiviral therapy**                 |                        |                   |                    |          |
| Negative                              | 185 (43.7)             | 103 (40.4)        | 82 (48.8)          | 0.088    |
| Positive                              | 238 (56.3)             | 152 (59.6)        | 86 (51.2)          |          |
| **Edmondson-steiner grade**           |                        |                   |                    |          |
| I-II                                  | 51 (9.5)               | 30 (9.8)          | 21 (9.1)           | 0.783    |
| III-IV                                | 484 (90.5)             | 275 (90.2)        | 209 (90.9)         |          |
| **Macrovascular invasion**            |                        |                   |                    |          |
| Negative                              | 424 (79.3)             | 258 (84.6)        | 166 (72.2)         | <0.001   |
| Positive                              | 111 (20.7)             | 47 (15.4)         | 64 (27.8)          |          |
| **Microvascular invasion**            |                        |                   |                    |          |
| Negative                              | 289 (54.0)             | 186 (61.0)        | 103 (44.8)         | <0.001   |
| Positive                              | 246 (46.0)             | 119 (39.0)        | 127 (55.2)         |          |
| **Tumor count**                       |                        |                   |                    |          |
| One                                   | 438 (81.9)             | 247 (81.0)        | 191 (83.0)         | 0.540    |
| Multiple                              | 97 (18.1)              | 58 (19.0)         | 39 (17.0)          |          |
| **Tumor size**                        |                        |                   |                    |          |
| <5 cm                                 | 352 (65.8)             | 216 (70.8)        | 136 (59.1)         | 0.005    |
| ≥5 cm                                 | 183 (34.2)             | 89 (29.2)         | 94 (40.9)          |          |
| **TNM stage**                         |                        |                   |                    |          |
| I-II                                  | 447 (83.6)             | 261 (85.6)        | 186 (80.9)         | 0.146    |
| III-IV                                | 88 (16.4)              | 44 (14.4)         | 44 (19.1)          |          |
| **BCLC stage**                        |                        |                   |                    |          |
| 0-A                                   | 342 (63.9)             | 213 (62.3)        | 129 (37.7)         | 0.001    |
| B-C                                   | 193 (36.1)             | 92 (27.7)         | 88 (52.3)          |          |
| **Recurrence**                        |                        |                   |                    |          |
| No                                    | 290 (54.2)             | 203 (66.6)        | 87 (37.8)          | <0.001   |
| Yes                                   | 245 (45.8)             | 102 (33.4)        | 143 (62.2)         |          |
| **Death**                             |                        |                   |                    |          |
| No                                    | 316 (59.1)             | 232 (76.1)        | 84 (36.5)          | <0.001   |
| Yes                                   | 219 (40.9)             | 73 (23.9)         | 146 (63.5)         |          |
| **LC3 in tumors**                     |                        |                   |                    |          |
| Low                                   | 45 (8.4)               | 16 (5.2)          | 29 (12.6)          | 0.002    |
| High                                  | 490 (91.6)             | 289 (94.8)        | 201 (87.4)         |          |

Data are shown as the mean ± standard deviation or number (%).
Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona clinic liver cancer; HBV, Hepatitis B virus; HCV, hepatitis C virus.

*P-value for the comparison between the Axl-low and Axl-high groups.
| Characteristics               | Univariate analyses | Multivariate analyses |
|------------------------------|---------------------|-----------------------|
|                              | Without recurrence (n = 290) | With recurrence (n = 245) | P-value | HR (95% CI) | P-value |
| Gender                       |                     |                      |          |             |         |
| Female                       | 69 (23.8)           | 75 (30.6)            | 0.076    |              |          |
| Male                         | 221 (76.2)          | 170 (69.4)           |          |              |          |
| Age (y)                      |                     |                      |          |             |         |
| <65                          | 104 (35.9)          | 68 (27.8)            | 0.056    |              |          |
| ≥65                          | 186 (64.1)          | 177 (72.2)           |          |              |          |
| HCC etiology                 |                     |                      |          |             |         |
| HCV                          | 85 (29.3)           | 67 (27.3)            | 0.826    |              |          |
| HBV                          | 132 (45.5)          | 118 (48.2)           |          |              |          |
| Non-HBV/HCV                  | 60 (20.7)           | 52 (21.2)            |          |              |          |
| HBV + HCV                    | 13 (4.5)            | 8 (3.3)              |          |              |          |
| AFP (ng/dL)                  |                     |                      |          |             |         |
| <400                         | 236 (81.4)          | 202 (82.4)           | 0.749    |              |          |
| ≥400                         | 54 (18.6)           | 43 (17.6)            |          |              |          |
| Liver cirrhosis              |                     |                      |          |             |         |
| Negative                     | 211 (72.8)          | 151 (61.6)           | 0.006    | 1            | 0.306   |
| Positive                     | 79 (27.2)           | 94 (38.4)            | 0.86 (0.65-1.14) |          |         |
| Antiviral therapy            |                     |                      |          |             |         |
| Negative                     | 92 (40.0)           | 93 (48.2)            | 0.091    |              |          |
| Positive                     | 138 (60.0)          | 100 (51.8)           |          |              |          |
| Edmondson-steiner grade      |                     |                      |          |             |         |
| I-II                         | 34 (11.7)           | 17 (6.9)             | 0.060    |              |          |
| III-IV                       | 256 (88.3)          | 228 (93.1)           |          |              |          |
| Macrovascular invasion       |                     |                      |          |             |         |
| Negative                     | 240 (82.8)          | 184 (75.1)           | 0.030    | 1            | 0.192   |
| Positive                     | 50 (17.2)           | 61 (24.9)            | 1.21 (0.91-1.64) |          |         |
| Microvascular invasion       |                     |                      |          |             |         |
| Negative                     | 158 (54.5)          | 131 (53.5)           | 0.815    |              |          |
| Positive                     | 132 (45.5)          | 114 (46.5)           |          |              |          |
| Tumor count                  |                     |                      |          |             |         |
| One                          | 238 (82.1)          | 200 (81.6)           | 0.896    |              |          |
| Multiple                     | 52 (17.9)           | 45 (18.4)            |          |              |          |
| Tumor size                   |                     |                      |          |             |         |
| <5 cm                        | 191 (65.9)          | 161 (65.7)           | 0.971    |              |          |
| ≥5 cm                        | 99 (34.1)           | 84 (34.3)            |          |              |          |
| TNM stage                    |                     |                      |          |             |         |
| I-II                         | 241 (83.1)          | 206 (84.1)           | 0.761    |              |          |
| III-IV                       | 49 (16.9)           | 39 (15.9)            |          |              |          |
| BCLC stage                   |                     |                      |          |             |         |
| 0-A                          | 185 (63.8)          | 157 (64.1)           | 0.945    |              |          |
| B-C                          | 105 (36.2)          | 88 (35.9)            |          |              |          |

Axl in tumors

(Continues)
In addition, patients with low LC3 expression in tumors had a significantly higher HCC recurrence rate than those with high LC3 expression in tumors. In patients with low and high LC3 expression in tumors, the 1-, 3-, 5- and 7-year HCC recurrence rates were 15.6%, 39.2%, 73.3% and 94.9% and 8.4%, 26.7%, 37.9% and 69.5%, respectively (Figure 1B).

### 3.4 Predictive factors associated with OS in HCC patients who underwent hepatectomy

The median follow-up time was 42 months, and 219 patients died. The OS rates at 1, 3, 5 and 7 years after resection were 91.0%, 72.3%, 58.8%, and 27.7%, respectively. In the univariate analysis, the following factors were significantly correlated with OS: sex, HBV-related HCC, cirrhosis, antiviral treatment, the presence of macrovascular and microvascular invasion, primary tumor size, BCLC stage, HCC recurrence, Axl expression in tumors and LC3 expression in tumors.

The multivariate Cox regression analysis showed that patients with HCC recurrence had the highest mortality (HR: 2.87, 95% CI: 2.01-4.01, \( P < 0.001 \)), followed by those with microvascular invasion (HR: 1.85, 95% CI: 1.08-3.19, \( P = 0.026 \)), HBV-related HCC (HR: 1.77, 95% CI: 1.21-2.56, \( P = 0.003 \)), and high Axl expression in tumors (HR: 1.66, 95% CI: 1.41-1.97, \( P < 0.0001 \)); these data are summarized in Table 3. Mortality was also significantly decreased in patients receiving antiviral treatment (HR: 0.54, CI: 0.38-0.76, \( P < 0.001 \)) and in those with high LC3 expression in tumors (HR: 0.41, 95% CI: 0.28-0.58, \( P < 0.001 \)), as shown in Table 3.

Patients with high Axl expression in tumors had significantly lower OS than those with low Axl expression in tumors. In patients with high and low Axl expression in tumors, the 1-, 3-, 5- and 7-year OS rates were 83.0%, 51.3%, 28.8% and 15.6% and 97.0%, 87.8%, 79.3% and 38.4%, respectively (Figure 1A).

Patients with low LC3 expression in tumors had significantly lower OS than those with high LC3 expression in tumors. In patients with low and high LC3 expression in tumors, the 1-, 3-, 5- and 7-year OS rates were 84.4%, 60.8%, 26.7% and 5.1% and 91.6%, 73.3%, 62.1% and 30.5%, respectively (Figure 2B).
### TABLE 3
Univariate and multivariate analyses of factors associated with mortality of hepatocellular carcinoma patients who underwent curative resection

| Characteristics | Univariate analyses | Multivariate analyses |
|-----------------|---------------------|----------------------|
|                 | No mortality (n = 316) | Mortality (n = 219) | P-value | HR (95% CI) | P-value |
| Gender          |                      |                      |         |             |         |
| Female          | 74 (23.4)            | 70 (32.0)            | 0.030   | 1           | 0.143   |
| Male            | 242 (76.6)           | 149 (68.0)           | 0.78 (0.55-1.09) |         |         |
| Age (y)         |                      |                      |         |             |         |
| <65             | 107 (33.9)           | 65 (29.7)            | 0.309   |             |         |
| ≥65             | 209 (66.1)           | 154 (70.3)           |         |             |         |
| HCC etiology    |                      |                      |         |             |         |
| HCV             | 102 (32.3)           | 50 (22.8)            | 0.015   | 1           |         |
| HBV             | 141 (44.6)           | 109 (49.8)           | 1.77 (1.21-2.56) | 0.003 |         |
| Non-HBV/HCV     | 57 (18.0)            | 55 (25.1)            | 0.99 (0.38-2.58) | 0.990 |         |
| HBV + HCV       | 16 (5.1)             | 5 (2.3)              | 0.92 (0.62-1.23) | 0.753 |         |
| AFP (ng/dL)     |                      |                      |         |             |         |
| <400            | 260 (82.3)           | 178 (81.3)           | 0.768   |             |         |
| ≥400            | 56 (17.7)            | 41 (18.7)            |         |             |         |
| Liver cirrhosis |                      |                      |         |             |         |
| Negative        | 227 (71.8)           | 135 (61.6)           | 0.013   | 1           | 0.316   |
| Positive        | 89 (28.2)            | 84 (38.4)            | 1.20 (0.84-1.69) |         |         |
| Antiviral therapy |                   |                      |         |             |         |
| Negative        | 98 (37.8)            | 87 (53.0)            | 0.002   | 1           | <0.001  |
| Positive        | 161 (62.2)           | 77 (47.0)            | 0.54 (0.38-0.76) |         |         |
| Edmondson-steiner grade |       |                      |         |             |         |
| I-II            | 28 (8.9)             | 23 (10.5)            | 0.551   |             |         |
| III-IV          | 288 (91.1)           | 196 (89.5)           |         |             |         |
| Macrovacular invasion |              |                      |         |             |         |
| Negative        | 261 (82.6)           | 163 (74.4)           | 0.022   | 1           | 0.528   |
| Positive        | 55 (17.4)            | 56 (25.6)            | 0.85 (0.52-1.40) |         |         |
| Microvascular invasion |         |                      |         |             |         |
| Negative        | 185 (58.5)           | 104 (47.5)           | 0.012   | 1           | 0.026   |
| Positive        | 131 (41.5)           | 115 (52.5)           | 1.85 (1.08-3.19) |         |         |
| Tumor count     |                      |                      |         |             |         |
| One             | 250 (79.1)           | 188 (85.8)           | 0.052   |             |         |
| Multiple        | 66 (20.9)            | 31 (14.2)            |         |             |         |
| Tumor size      |                      |                      |         |             |         |
| <5 cm           | 220 (69.6)           | 132 (60.3)           | 0.026   | 1           | 0.475   |
| ≥5 cm           | 96 (30.4)            | 87 (39.7)            | 1.21 (0.72-2.01) |         |         |
| TNM stage       |                      |                      |         |             |         |
| I-II            | 262 (82.9)           | 185 (84.5)           | 0.722   |             |         |
| III-IV          | 54 (17.1)            | 34 (15.5)            |         |             |         |
| BCLC stage      |                      |                      |         |             |         |
| 0-A             | 214 (67.7)           | 128 (58.4)           | 0.035   | 1           | 0.613   |
| B-C             | 102 (32.3)           | 91 (41.6)            | 0.86 (0.47-1.56) |         |         |

(Continues)
Tumor recurrence and OS according to combined Axl and LC3 expression

Next, the combination of Axl and LC3 expression in tumors was analyzed. For HCC recurrence, compared to patients (n = 289) with low Axl and high LC3 expression in tumors, patients (n = 29) with high Axl and low LC3 expression had the highest risk of HCC recurrence (HR: 6.53, 95% CI: 4.11-10.4, P < 0.001), followed by those (n = 201) with high Axl and high LC3 expression (HR: 4.04, 95% CI: 3.05-5.35, P < 0.001) and those (n = 16) with low Axl and low LC3 expression (HR: 2.99, 95% CI: 1.60-5.59, P = 0.001). Patients with low Axl and high LC3 expression in tumors had 1-, 3-, 5- and 7-year HCC recurrence rates of 3.2%, 15.9%, 34.9% and 50.4%, respectively. Compared with this group, the low Axl/low LC3 group (0%, 40.0%, 77.1% and 100%, respectively), high Axl/high LC3 group (18.8%, 56.9%, 75.7% and 81.5%, respectively) and high Axl/low LC3 group (23.1%, 70.1%, 90.0% and 100%, respectively) had significantly higher HCC recurrence rates (Figure 3A).

Moreover, compared with patients with low Axl and high LC3 expression in tumors, patients with high Axl and low LC3 expression had the highest risk of mortality (HR: 6.66, 95% CI: 4.07-10.9, P < 0.001), followed by those with high Axl and high LC3 expression (HR: 5.48, 95% CI: 3.98-7.54, P < 0.001) and those with low Axl and low LC3 expression (HR: 4.45, 95% CI: 2.39-8.29, P < 0.001).

The results showed that patients with low Axl and high LC3 expression in HCC tissues had 1-, 3-, 5- and 7-year OS rates of 96.9%, 88.2%, 82.2% and 41.6%, respectively.
Compared with this group, the low Axl/low LC3 group (100%, 80.4%, 34.7% and 0%, respectively), high Axl/high LC3 group (84.1%, 51.7%, 30.0% and 16.8%, respectively) and high Axl/low LC3 group (75.9%, 49.2%, 16.8% and 8.4%, respectively) had significantly lower OS rates (Figure 3B).

**4 | DISCUSSION**

In this study, 535 HCC patients who underwent curative resection were analyzed to identify predictive factors for HCC recurrence and OS. High Axl expression in tumors was significantly associated with advanced clinicopathological features, high HCC recurrence rates, and low OS rates. In addition, low LC3 expression in tumors was also significantly correlated with high HCC recurrence and low OS rates. Furthermore, the combined expression of Axl and LC3 in tumors was analyzed. Patients with high Axl and low LC3 expression in tumors had the highest HCC recurrence (HR: 6.53; \( P < 0.001 \)) and mortality rates (HR: 6.66; \( P < 0.001 \)). These findings suggest that Axl and LC3 expression levels in tumors may serve as predictors of HCC recurrence and OS after hepatectomy for HCC.

Several studies showed that high Axl expression in HCC patients was positively associated with more aggressive tumor invasiveness, a high risk of tumor recurrence, lymph node metastasis, and lower survival.\(^{16-18} \) Our study demonstrated that Axl expression was significantly correlated with advanced clinicopathological features, including cirrhosis, microvascular invasion, macrovascular invasion, tumor size, BCLC stage, recurrence, and mortality in the univariate analysis. In the multivariate analysis, Axl expression was significantly correlated with tumor recurrence and mortality (data not shown). In addition, high Axl expression in tumors was strongly correlated with high HCC recurrence and low OS rates. The 5-year cumulative incidences of HCC recurrence and OS were 77.9% and 28.8%, respectively, in patients with high Axl expression. Our study results are consistent with those of previous reports.\(^ {16-18} \) High Axl expression in tumors corresponded to more aggressive tumor behavior and a worse clinical prognosis. Axl expression in tumors may be a negative predictor of HCC recurrence and OS and may predict a worse clinical prognosis in HCC patients.

Previous studies of LC3 expression in terms of clinical outcomes have reported controversial results.\(^ {21-23} \) Our previous work showed that high LC3 levels in both the liver and tumor microenvironments are significantly correlated with decreased HCC recurrence and increased OS after surgical resection. High LC3 expression seems to have a protective effect on clinical outcomes, including HCC recurrence and OS after hepatectomy.\(^ {7,8} \) Our present study demonstrated that low LC3 expression in tumors is correlated with high HCC recurrence and low OS rates. The 5-year cumulative incidences of HCC recurrence and OS were 73.3% and 26.7%, respectively, in patients with low LC3 expression. LC3 expression in tumors can predict the clinical outcomes of HCC patients after hepatectomy and may exert a protective effect against HCC recurrence, thus improving OS. The impact of Axl and LC3 expression in tumors on HCC recurrence and OS has never been reported in the literature. Hence, we revealed that the combination of high Axl expression and low LC3 expression in tumors had additional deleterious effects on HCC recurrence and OS. Our results show the important role of Axl and LC3.

**FIGURE 3** Cumulative incidence of tumor recurrence and overall survival according to the combination of Axl and LC3 expression in tumors. Patients with +/+ or −/+ Axl/LC3 expression had a significantly higher incidence of recurrence than patients with −/+ Axl/LC3 expression (A). Patients with +/−, −/− or +/+ Axl/LC3 expression had significantly lower overall survival than patients with −/+ Axl/LC3 expression (B). −, low; +, high; CI, confidence interval; HCC, hepatocellular carcinoma, HR, hazard ratio.
expression in tumors in the prognosis of HCC recurrence and OS. The study is the first to demonstrate that Axl and LC3 expression in tumors is significantly associated with HCC recurrence and OS. The combination of high Axl and low LC3 expression in tumors significantly increases the risk of HCC recurrence and mortality in HCC patients after hepatectomy.

Our study showed that high Axl expression in tumors increased HCC recurrence (HR: 3.85) and mortality (HR: 1.66) compared with low Axl expression and that high LC3 expression in tumors decreased HCC recurrence (HR: 0.38) and mortality (HR: 0.41) compared with low LC3 expression. Furthermore, high Axl and low LC3 expression in tumors increased HCC recurrence (HR: 6.53) and mortality (HR: 6.66) compared with low Axl and high LC3 expression. We aimed to find better markers to precisely predict HCC recurrence and mortality. Axl expression in tumors was a better predictor than LC3 expression of HCC recurrence. The combination of Axl and LC3 expression in tumors by IHC staining significantly predicted HCC recurrence and mortality compared with the expression of either marker alone. The combination of two markers was a better predictor of HCC recurrence and mortality than a single marker. In clinical practice, tumor tissues may be subjected to IHC staining for Axl and LC3 after hepatectomy. Patients with high Axl and low LC3 expression in tumors will be identified as having a higher risk of HCC recurrence and mortality than those with low Axl and high LC3 expression in tumors. Close postoperative follow-up is suggested for this patient population to increase the detection rate of early-stage HCC, and further aggressive management may prolong the survival of these high-risk patients.

This study had some limitations. First, this study was retrospective, which could have introduced unintended bias. Second, cirrhotic patients composed only one-third of our cohort, while other studies have included a majority of cirrhosis patients (80%). This difference in prevalence of cirrhosis may have affected the generalizability of the outcomes. Finally, the underlying mechanism of Axl and LC3 expression related to carcinogenesis and clinical prognosis needs to be further investigated in vivo and in vitro.

In summary, high Axl expression in tumors is significantly associated with advanced clinicopathological features, high HCC recurrence rate, and low OS rate. In addition, low LC3 expression in tumors is also significantly associated with high HCC recurrence and low OS rates. Furthermore, patients with a combination of high Axl expression and low LC3 expression in tumors had higher HCC recurrence and lower OS rates. This study is the first to demonstrate that the combined Axl and LC3 expression pattern in the tumor microenvironment is critical for predicting HCC recurrence and OS. Analysis of Axl and LC3 expression in tumors, in conjunction with clinicopathological features, could identify predictors of HCC recurrence and OS after curative hepatectomy. Our results indicated that the combination of high Axl and low LC3 expression significantly correlated with HCC recurrence and OS and that Axl and LC3 may serve as potential biomarkers for predicting HCC recurrence and OS.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Hsu CC performed the experiments. Chen YS, Lo GH, Hsieh PM, Lin HY, and Chen YL collected the materials, reagents, and patient data. Dai CY, Huang JF, Chuang WL, and Yu ML analyzed the data. Lin CW designed the study and wrote the manuscript with Hsu CC. All of the authors made equally important recommendations for the manuscript and approved the final version of the manuscript.

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REFERENCES

1. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012;379:1245-1255.
2. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology. 2012;142(1264–1273):e1.
3. Lin CW, Lin CC, Mo LR, et al. Heavy alcohol consumption increases the incidence of hepatocellular carcinoma in hepatitis B virus-related cirrhosis. J Hepatol. 2013;58:730–735.
4. Roayaie S, Obeidat K, Sposito C, et al. Resection of hepatocellular cancer ≤2 cm: results from two western centers. Hepatology. 2013;57:1426-1435.
5. Forner A, Vilana R, Ayuso C, et al. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. Hepatology. 2008;47:97-104.
6. Tabrizian P, Jibara G, Shraga B, Schwartz M, Roayaie S. Recurrence of hepatocellular cancer after resection: patterns, treatments, and prognosis. Ann Surg. 2015;261:947-955.
7. Lin CW, Chen YS, Lin CC, et al. Autophagy-related gene LC3 expression in tumor and liver microenvironments significantly predicts recurrence of hepatocellular carcinoma after surgical resection. Clin Transl Gastroenterol. 2018;9:166.
8. Lin CW, Chen YS, Lin CC, et al. Significant predictors of OS in patients with hepatocellular carcinoma after surgical resection. *PLoS ONE*. 2018;13:e0202650.

9. Toyoda H, Kumada T, Tada T, et al. Changes in highly sensitive alpha-fetoprotein for the prediction of the outcome in patients with hepatocellular carcinoma after hepatectomy. *Cancer Med*. 2014;3:643-651.

10. Paccez JD, Vogelsang M, Parker MI, Zerbini LF. The receptor tyrosine kinase Axl in cancer: biological functions and therapeutic implications. *Int J Cancer*. 2014;134:1024-1033.

11. Koorstra J-B, Karikari C, Feldmann G, et al. The Axl receptor tyrosine kinase confers an adverse prognostic influence in pancreatic cancer and represents a new therapeutic target. *Cancer Biol Ther*. 2009;8:618-626.

12. Holland SJ, Pan A, Franci C, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res*. 2010;70:1544-1554.

13. Vajkoczy P, Knayzev P, Kunkel A, et al. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. *Proc Natl Acad Sci USA*. 2006;103:5799-5804.

14. Reichl P, Fang M, Starlinger P, et al. Multicenter analysis of soluble Axl reveals diagnostic value for very early stage hepatocellular carcinoma. *Int J Cancer*. 2015;137:385-394.

15. Wu X, Liu X, Koul S, Lee CY, Zhang Z, Halmos B. Axl kinase as a novel target for cancer therapy. *Oncotarget*. 2014;5:9546-9563.

16. Xu J, Jia L, Ma H, Li Y, Ma Z, Zhao Y. Axl gene knockdown inhibits the metastasis properties of hepatocellular carcinoma via PI3K/Akt-PAK1 signal pathway. *Tumour Biol*. 2014;35:3809-3817.

17. Reichl P, Dengler M, van Zijl F, et al. Axl activates autocrine transforming growth factor-beta signaling in hepatocellular carcinoma. *Hepatology*. 2015;61:930-941.

18. Liu J, Wang K, Yan Z, et al. Axl expression stratifies patients with poor prognosis after hepatectomy for hepatocellular carcinoma. *PLoS ONE*. 2016;11:e0154767.

19. Bruix J, Sherman M. Practice guidelines committee AAFsSLOD. *Management of hepatocellular carcinoma*. *Hepatology*. 2005;42:1208-1236.

20. Lin C-W, Lin C-C, Lee P-H, et al. The autophagy marker LC3 strongly predicts immediate mortality after surgical resection for hepatocellular carcinoma. *Oncotarget*. 2017;8:91902-91913.

21. Lee YJ, Hah YJ, Kang YN, et al. The autophagy-related marker LC3 can predict prognosis in human hepatocellular carcinoma. *PLoS ONE*. 2013;8:e81540.

22. Chen KD, Wang CC, Tsai MC, et al. Interconnections between autophagy and the coagulation cascade in hepatocellular carcinoma. *Cell Death Dis*. 2014;5:e1244.

23. Wu DH, Jia CC, Chen J, et al. Autophagic LC3B overexpression correlates with malignant progression and predicts a poor prognosis in hepatocellular carcinoma. *Tumour Biol*. 2014;35:12225-12233.

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