# Low expression of *GABARAPL1* is associated with a poor outcome for patients with hepatocellular carcinoma

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Received October 5, 2013; Accepted December 27, 2013

DOI: 10.3892/or.2014.3096

**Abstract.** Autophagy is an evolutionarily conserved cellular process that degrades cytoplasmic materials through the lysosomal pathway. The deregulation of autophagy is associated with several diseases, particularly cancer. Hepatocellular carcinoma (HCC) is one of the most aggressive cancers with a poor prognosis. The expression of autophagy-related genes in HCC and their relationships with HCC are largely unknown. In the present study, we analyzed the expression of autophagy-related genes based on the Oncomine database and quantitative PCR of HCC and adjacent liver tissues. We found that the mRNA and protein expression of *GABARAPL1* was significantly decreased in HCC tissues compared with their adjacent liver tissues. In HCC cancer cell lines, overexpression of *GABARAPL1* inhibited cell growth, while knockdown of *GABARAPL1* expression via siRNA promoted cell growth. In addition, we found a significant correlation of low *GABARAPL1* expression with poor differentiation of HCC cells (P=0.018), and with the absence of tumor capsules (P=0.047). Kaplan-Meier survival analysis showed a significant association between low *GABARAPL1* expression and poor prognosis of HCC patients (P=0.0094). Our data showed for the first time that *GABARAPL1* expression is associated with poor prognosis of HCC patients.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and malignant cancers in the world (1). Over 600,000 new HCC cases are diagnosed annually (2). Despite the improvements in diagnosis and treatment, the prognosis of patients with HCC remains poor. Therefore, the exploration of promising therapies and prognostic factors for HCC is of great clinical significance.

Autophagy is an evolutionarily conserved cellular pathway which degrades and recycles cytoplasmic components via the lysosomal system (3,4). Deregulated autophagy is related to several physiological defects, including liver injury, muscular disorder, neurodegeneration, pathogen infections and cancer (5,6). The relationship between autophagy and cancer development has been studied in various types of cancer (7-10). Autophagy-related genes are reported to be cancer repressor genes (11-14). Specifically, in HCC, it was reported that the expression of *ATG5*, *ATG7* and *BECN1*, and the autophagic activity was decreased in HCC cell lines (15). The expression of *BECN1* was decreased in HCC tissues compared to the adjacent liver tissues, and it was shown to be a prognostic factor in Bcl-xL$^+$ patients (15). However, the expression of other autophagy-related genes in HCC and their correlation with HCC development remain largely unknown.

*GABARAPL1* (also known as *GEC1* or *ATG8L*) was first identified as an early estrogen-induced gene in quiescent guinea-pig endometrial glandular epithelial cells (16,17). Previous studies showed that GABARAPL1 is one of the six human Atg8 family proteins which locate in autophagic vesicles after post-translational modification. They mediate the cargo recognition and the autophagosome formation (18,19). Recently, *GABARAPL1* was reported to be downregulated in breast adenocarcinoma and the expression of *GABARAPL1* is associated with the risk of metastasis, specifically for lymph node-positive patients (20).

In the present study, we examined the mRNA expression of autophagy-related genes in the Oncomine database and dissected tissue samples from HCC patients. We found that both the mRNA and protein expression of the *GABARAPL1* gene was decreased in HCC tissues. Overexpression or knockdown of *GABARAPL1* in HCC cell lines affected their growth rates. In addition, we found a significant association between low *GABARAPL1* expression and poor prognosis in HCC patients.

## Materials and methods

*Patients and tissue specimens*. Seventy-three pairs of HCC tissues and adjacent liver tissues were collected from patients

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*Abbreviations:* HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR

*Key words:* hepatocellular carcinoma, *GABARAPL1*, prognosis, autophagy

undergoing resection from 2006 to 2009 at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai. Tumor specimens were obtained from the areas of the tumor, necrotic tissues were avoided. The specimens were then snap frozen in liquid nitrogen and stored at -80˚C. Patients were monitored after surgery until March 2010. Overall survival was defined as the interval between surgery and mortality or the last observation. The histological grade of tumor differentiation was determined according to the classification proposed by Edmondson and Steine, as described by Wittekind (21). TNM stage was determined according to the 6th edition of Tumor-node-metastasis classification of the International Union against Cancer. Ethics approval for the present study was obtained from the Research Ethics Committee of Zhongshan Hospital, and informed consent was obtained from each patient.

*Oncomine data analysis*. Oncomine (http://www.oncomine.com) is an integrated cancer microarray database which contains unified bio-informatics resources from 715 datasets (version 4.4.4.3 after Q2 update 2013) (22). We compared the mRNA expression of autophagy-related genes from liver cancer datasets which contain data from both HCC tissues and normal liver tissues. Four datasets were included in our study, Chen *et al* (23), Roessler *et al* (24), Wurmbach *et al* (25) and Mas *et al* (26). The differentiated expression for each gene between HCC tissues and normal liver tissues was analyzed and their fold-change values and statistical significance determined by P-value were collected.

*RNA extraction and quantitative real-time PCR (qRT-PCR)*. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen). Two micrograms of total RNA were applied for reverse transcription using oligo(dT) primer and reverse transcriptase (Invitrogen). qRT-PCR was performed with SYBR-Green Supermix kit (Takara) and LightCycler® 480 system (Roche). The primer for each gene was designed with Beacon Designer (Bio-Rad). The amplification conditions for each gene were optimized by using melting curve analysis and gel electrophoresis. Relative gene expression was calculated using the formula $2^{-\Delta Ct}$ and *GAPDH* was used as internal gene for normalization. ΔCt (critical threshold) = Ct of genes of interest - Ct of *GAPDH*. Relative gene expression between HCC tissues and adjacent liver tissues was calculated using the $2^{-\Delta\Delta Ct}$ as previously described (27).

*Western blot analysis*. Protein samples were subjected to 12% SDS-PAGE followed by standard western blotting protocols. The anti-GABARAPL1 antibody was purchased from Proteintech. Anti-myc antibody and anti-β-actin antibody were purchased from Sigma-Aldrich.

*Stable cell line and siRNA knockdown*. The *GABARAPL1* gene was cloned from HeLa cell cDNA. The GABARAPL1-G116A point mutation was prepared using a mutated primer. Then, *GABARAPL1* and the G116A mutant were subcloned into a pcDNA3.1 vector. Stable cell lines which overexpress GABARAPL1 or the G116A mutant protein and control cell lines were prepared as previously described (28).

The siRNA duplexes were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The siRNA sequences

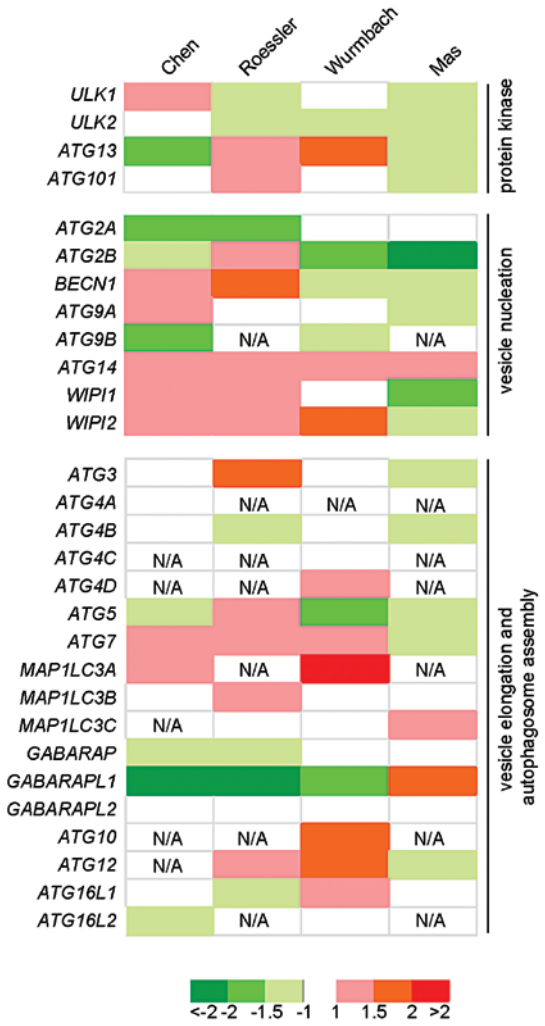


Figure 1. Oncomine analysis of 29 autophagy-related genes in 4 liver datasets. Gene expression data from the Oncomine database were analyzed for HCC tissues and normal liver tissues. The fold-change of each gene in HCC tissues compared with normal liver tissues is indicated by different colors. Blank cell stands for not statistically significant. N/A, no data. The author for each data set is listed above. Genes functioning in the same step of the autophagy process are grouped and indicated on the right.

targeting *GABARAPL1* were 1, 5'-GGACCAUCCCUUUGAGUAUUU-3' and 2, 5'-GAAAAGAUCCGGAAGAAAUUU-3'. Control siRNA was 5'-UAAGGCUAUGAAGAGAUACUU-3'. siRNAs were transfected into the cells using oligofectamine (Invitrogen) according to the manufacturer's instructions.

*Cell proliferation assay*. Control cells and cells stably expressing GABARAPL1 or the G116A mutant protein were plated in 96-well plates at a density of 1,500 cells/well in the Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 800 μg/ml G418 (Invitrogen). At the indicated time, cell proliferation was determined by using the Cell Counting Kit-8 assay according to the manufacturer's instructions. For the cell proliferation assay after siRNA treatment, cells were first transfected with siRNA duplexes. After 24 h, cells were plated in 96-well plates as day 0. At days 2 and 6, cells were again subjected to siRNA treatment in 96-well plates. Cell proliferation was determined by the Cell Counting Kit-8 assay at the indicated time.

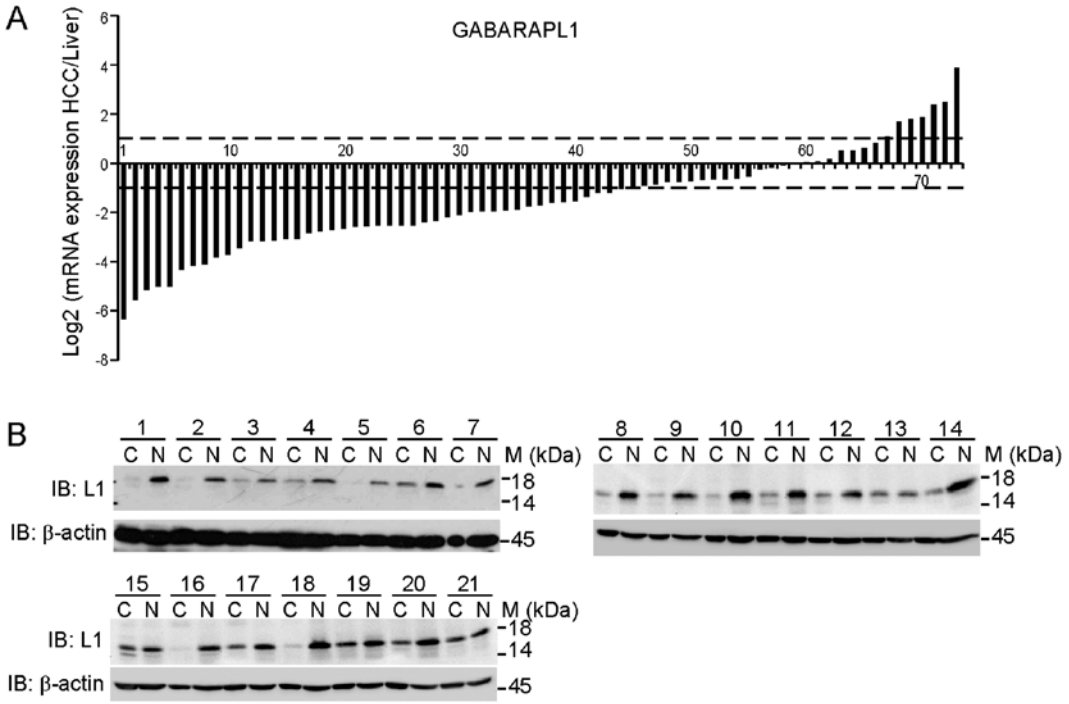


Figure 2. *GABARAPL1* is downregulated in HCC samples. (A) *GABARAPL1* mRNA expression was detected in 73 pairs of HCC tissues and adjacent liver tissues by qRT-PCR. Values were expressed as the log2 transformed relative expression ratio of HCC/adjacent liver tissues after normalization to the housekeeping gene *GAPDH*. +1 and -1 were marked as cut-off values for upregulation or downregulation. (B) Protein samples from 21 randomly selected pairs of tissues were subjected to western blot analysis with anti-GABARAPL1 and anti-β-actin antibodies. Protein standards are marked in the right. C, tumor; N, adjacent liver tissue; M, protein markers; L1, GABARAPL1.

*Statistical analysis*. Statistical analyses were performed using SPSS 12.0 for Windows. The P-values and gene fold-change values from Oncomine were previously described (22,29). The $\chi^2$ test, Fisher's exact probability, and Student's t-test were used for comparison between groups. Cumulative survival time was calculated by the Kaplan-Meier method and analyzed by the log-rank test.

## Results

*Oncomine datasets suggest GABARAPL1 is downregulated in HCC tissues*. To explore the potential differentially expressed autophagy-related genes in HCC, we firstly analyzed Oncomine, the integrated cancer microarray database. Four liver cancer data sets (23-26), which contain both normal and HCC tissue data, were selected to analyze the mRNA expression of 29 autophagy-related genes. These genes function in different steps of the autophagy process, including protein kinase initiation step, vesicle nucleation, vesicle elongation and autophagosome assembly. We compared their mRNA expression between HCC tissues and normal liver tissues, and collected their fold-change values and P-value. As shown in Fig. 1, *ATG14*, *WIPI2* and *ATG7* were upregulated in HCC tissues in 3 of these 4 data sets. *ULK2*, *ATG2B* and *GABARAPL1* were downregulated in HCC tissues also in 3 of these 4 data sets. Among these genes, the fold-change values of *GABARAPL1* were -2.65 and -2.646 in Chen_Liver and Roessler_Liver data sets, respectively, suggesting that *GABARAPL1* was greatly downregulated in HCC tissues compared to the normal liver tissues.

*GABARAPL1 is downregulated in HCC tissues*. To corroborate the gene expression patterns experimentally, we randomly selected 24 pairs of HCC tissues and adjacent liver tissues and detected the mRNA expression of 21 autophagy-related genes by qRT-PCR. We found that *GABARAP*, another member of the human Atg8 family, was slightly but significantly decreased in HCC tissues (averagely 20% decrease in HCC tissues as compared to adjacent liver tissues, P=0.003), and the expression of *GABARAPL1* was largely decreased in HCC tissues (78.2% decrease in HCC tissues as compared to adjacent liver tissues P<0.0001).

To confirm this result in a larger sample size, we detected the GABARAPL1 transcript expression in 73 pairs of HCC tissues and adjacent liver tissues. Fig. 2A presents the log2 transformed fold-change of *GABARAPL1* mRNA expression ratio of tumor/adjacent liver tissue. Forty-five of 73 cases (61.6%) showed significant reduction of *GABARAPL1* expression in HCC tissues (log2 transformed fold-change ≤-1); 21/73 cases (28.8%) showed no alteration (-1<log2 transformed fold-change <1); and only 7/73 cases (9.6%) showed upregulation (log2 transformed fold-change ≥1). The average expression of *GABARAPL1* mRNA in HCC was 33.7% of adjacent liver tissues. To detect the protein expression of GABARAPL1, we randomly selected 21 pairs of samples and carried out immunoblotting analysis. As shown in Fig. 2B, the protein expression of GABARAPL1 was largely decreased in HCC tissues as compared to adjacent liver tissues, consistent with the mRNA expression pattern.

*The expression of GABARAPL1 in HCC cell lines affects cellular growth rates*. Based on the pronounced down-

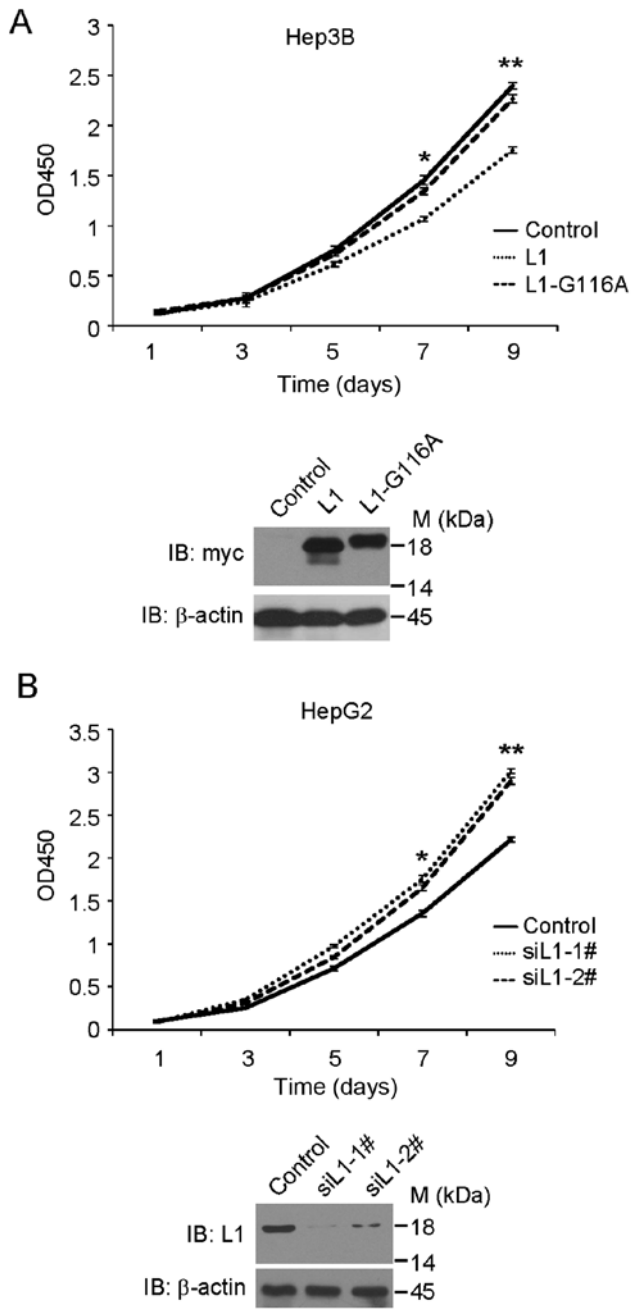


Figure 3. GABARAPL1 overexpression and siRNA knockdown affect the cellular growth. (A) Cells stably expressing the indicated proteins were subjected to cell proliferation assay. The protein expression was shown with anti-myc and anti-β-actin antibodies. (B) Cells treated with indicated siRNA were subjected to a cell proliferation assay. GABARAPL1 protein expression at day 9 is shown with anti-GABARAPL1 and anti-β-actin antibodies. Data are from three independent experiments and are shown as mean ± SD. *P<0.05, **P<0.01 from Student's t-test. M, protein markers; L1, GABARAPL1.

Table I. Correlation between *GABARAPL1* mRNA expression and clinicopathological variables.

| | *GABARAPL1* mRNA expression | | |
|---|---|---|---|
| Variables | Low | High | P-value |
| Age (years) | | | |
| ≤57 | 18 | 20 | 0.555 |
| >57 | 19 | 16 | |
| Gender | | | |
| Female | 7 | 8 | 0.727 |
| Male | 30 | 28 | |
| Hepatitis history | | | |
| No | 17 | 14 | 0.542 |
| Yes | 20 | 22 | |
| AFP (ng/ml) | | | |
| ≤20 | 19 | 17 | 0.724 |
| >20 | 18 | 19 | |
| Liver cirrhosis | | | |
| No | 33 | 31 | 0.736[a] |
| Yes | 4 | 5 | |
| Tumor size (cm) | | | |
| ≤5 | 11 | 16 | 0.193 |
| >5 | 26 | 20 | |
| Tumor multiplicity | | | |
| Single | 31 | 28 | 0.515 |
| Multiple | 6 | 8 | |
| Differentiation | | | |
| I+II | 19 | 28 | 0.018 |
| III+IV | 18 | 8 | |
| TNM stage | | | |
| I | 3 | 1 | 0.615[a] |
| II+III | 34 | 35 | |
| Tumor capsule | | | |
| Present | 13 | 21 | 0.047 |
| Absent | 24 | 15 | |

AFP, α-fetoprotein; TNM, tumor-node-metastasis. P-values were calculated from the $\chi^2$ test or [a]Fisher's exact test.

regulated expression of *GABARAPL1* in HCC tissues, we next investigated whether the expression of *GABARAPL1* affects cell growth. We first detected the mRNA and protein expression of *GABARAPL1* in 9 HCC cell lines. We found that Hep3B, SMMC-7721, Focus and PLC/PRF/5 cell lines have low *GABARAPL1* expression, while HepG2, Huh7, QGY-7703, YY-8103 and SK-Hep1 have moderate to high expression of GABARAPL1 (data not shown). We then established stable Hep3B cell lines that expressed pcDNA3.1 empty vector, pcDNA3.1-myc-GABARAPL1, or pcDNA3.1-myc-GABARAPL1-G116A mutant. For each vector, we chose three single clones which expressed indicated protein and mixed them as a pool. As shown in Fig. 3A, cell growth assay suggested that overexpression of *GABARAPL1* inhibited the cellular growth rate in Hep3B cells. However, overexpression of GABARAPL1-G116A mutant, which cannot be functionally located to the autophagosomes (18), did not inhibit the cellular growth rate in Hep3B cells, suggesting that GABARAPL1 inhibits cellular growth via the autophagy-related pathway. Similar results were observed in SMMC-7721 cells (data not shown).

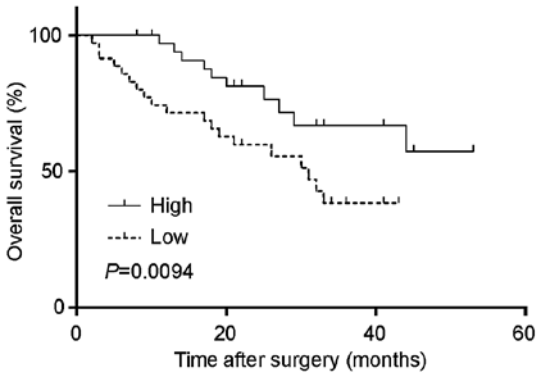


Figure 4. Association between low *GABARAPL1* expression and poor prognosis of HCC patients. Kaplan-Meier analysis of overall survival for the *GABARAPL1* high expressing group (n=36) and low expressing group (n=37). P=0.0094 from log-rank test.

We chose HepG2 cells to carry out siRNA treatment with control or *GABARAPL1* siRNAs. Two different siRNAs were designed to knock down *GABARAPL1* expression. As shown in Fig. 3B, after siRNA treatment, cells treated with *GABARAPL1* siRNA grew faster than those treated with control siRNA. Similar data were observed in Sk-Hep1 cells (data not shown). These results suggest that *GABARAPL1* expression may affect the cellular growth rate in HCC cell lines.

*Low expression of GABARAPL1 in HCC tissues is associated with poor outcome of HCC patients*. To explore the clinicopathological correlation of *GABARAPL1* downregulation in HCC, we analyzed the *GABARAPL1* mRNA expression with various clinical parameters in 73 HCC patients. The patients were divided into low or high expression groups according to their *GABARAPL1* transcript expression levels. As shown in Table I, we found that *GABARAPL1* was significantly associated with pathological differentiation (P=0.018) and tumor encapsulation (P=0.047). No correlation was found with other variables. These correlations suggest that the downregulation of *GABARAPL1* may serve as a prognosis indicator of HCC.

Furthermore, we analyzed the survival time with the *GABARAPL1* expression. As shown in Fig. 4, the low expression of *GABARAPL1* was significantly associated with a poorer outcome, while patients with a higher expression of *GABARAPL1* were more likely to have a longer overall survival time (P=0.0094).

## Discussion

Previous studies revealed that defective autophagy was related to poor prognosis in various cancers, including HCC (15,30), breast cancer (31) and bladder cancer (32). In the present study, we combined both meta-analysis and experimental data to search differentially regulated autophagy-related genes in HCC and liver tissues. We found that the expression of *GABARAPL1* was significantly downregulated in HCC tissues, both in mRNA and protein expression levels. Also, *GABARAPL1* might be a potential biomarker for HCC patients, as the low expression of *GABARAPL1* in HCC tissue correlated the poor survival of HCC patients. However, the sample size of the present study was relatively small (n=73), and these patients were mainly from South and East China. Therefore, in a future study, a lager sample size should be used to evaluate the extent of *GABARAPL1* as a predictive biomarker of patient survival.

In addition to *GABARAPL1*, *GABARAP* was also found to be downregulated from both meta-analysis and experimental data. These two proteins are members of the GABARAP sub-family and their functions in autophagy were reported to be distinct from those of LC3 sub-family proteins (including *MAP1LC3A*, *MAP1LC3B* and *MAP1LC3C*), although they have high sequence similarity. Specifically, in the initiation step of autophagy, the GABARAP sub-family proteins are much preferred in the ULK complex assembly (33). LC3 sub-family proteins are involved in the following elongation of the phagophore membrane. Then, at the late stage of autophagosome formation, the GABARAP sub-family proteins are essential for autophagosome completion (34). Thus, downregulation of these two members may decrease the cellular autophagic activity, and then affect the tumorigenesis in HCC development. The same regulation defect was reported in breast cancer (31) and neuroblastoma (35) for *GABARAP,* and in breast cancer (20) for *GABARAPL1.*

Another notable finding was that the expression of *GABARAPL1* was related to the cell growth rate in HCC cell lines. This function of *GABARAPL1* was dependent on its role in the autophagy process. Recent research identified that GABARAPL1 may negatively regulate the Wnt signaling pathway by mediating Dvl2 degradation through autophagy (36). Thus, it is possible that downregulation of *GABARAPL1* may inhibit the selective autophagy mediated by GABARAPL1, thereby affecting the cell growth or tumorigenesis process. The detailed mechanism of GABARAPL1 in cell growth control requires further investigation.

Collectively, our data suggest that *GABARAPL1*, one of the proteins functioning during the autophagosome formation, is downregulated in HCC and its expression is associated with the survival of HCC patients and can be used as a prognostic factor in HCC.

## Acknowledgements

We thank Dr Jie Zuo, Dr Haijie Ma and Dr He-Xi Ge Sai-Yin (Fudan University) for technical assistance. The authors are grateful to Matthew D. Pauly (University of Michigan Medical School) for proofreading. The present study was supported by the National Natural Science Foundation of China for Creative Research Groups (30024001 to L.Y.), the National Key Sci-Tech Special Project of China (2013ZX10002010 and 2008ZX10002-020 to L.Y.), the Project of the Shanghai Municipal Science and Technology Commission (03dz14086 to L.Y.), and the National Natural Science Foundation of China (31071193 to L.Y.).

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