Pathology - Research and Practice 215 (2019) 152403

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

# Decreased expression levels of DAL-1 and TOB1 are associated with clinicopathological features and poor prognosis in gastric cancer

Haonan Guo[a], Rui Zhang[a,b], Justice Afrifa[a], Yuanyuan Wang[a], Jingcui Yu[a,*]

[a] Scientific Research Centre, The Second Affiliated Hospital of Harbin Medical University, Harbin, 150081, China
[b] Prenatal Diagnosis Center, The Second Affiliated Hospital of Harbin Medical University, Harbin, 150081, China

ARTICLE INFO

*Keywords:*
Gastric cancer
DAL-1
TOB1
Gene expression
Immunohistochemistry

ABSTRACT

*Purpose:* We previously demonstrated that the functional inactivation of DAL-1 and TOB1 promotes an aggressive phenotype in gastric cancer cells, but the links between both genes and the survival of patients with gastric cancer are unknown. Here, we investigated the correlations of the expression levels of DAL-1 and TOB1 with the progression of gastric cancer.

*Methods:* A total of 270 patients who underwent resectable gastrectomy were included. The expression of DAL-1 and TOB1 was detected by immunohistochemistry.

*Results:* Low expression of DAL-1 in cancer tissue was significantly associated with tumor site ($p < 0.05$), histological grade ($p < 0.01$), depth of invasion ($p < 0.05$), lymph node metastasis status ($p < 0.05$), Lauren classification ($p < 0.001$), and clinical stage ($p < 0.01$). A lower level of TOB1 was observed in gastric cancer patients with diffuse type disease compared to patients with either intestinal or mixed type disease ($p < 0.001$). Additionally, Spearman's correlation analysis revealed that decreased expression of DAL-1 was positively correlated with low TOB1 expression ($r = 0.304$, $p < 0.001$). The survival analysis showed that low levels of DAL-1 and TOB1 were significantly associated with poor survival of gastric cancer patients ($p < 0.001$ and $p < 0.05$, respectively).

*Conclusion:* The downregulation of DAL-1 and TOB1 expression is associated with shorter survival of gastric cancer patients. Hence, DAL-1 and TOB1 may be considered potential novel markers for predicting the outcomes of patients with gastric cancer.

## 1. Introduction

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide [1]. Recent advances in the treatment and management of gastric tumors have incorporated various regimens, including chemotherapy, radiotherapy, and surgical techniques; however, the overall gastric cancer survival rate is still less than 30% [2]. Like other malignant tumors, gastric carcinogenesis involves multiple factors, multiple genes, and multiple steps. The activation of oncogenes and inactivation of tumor suppressor genes are the main molecular mechanisms of gastric cancer.

In previous studies using allelotyping for the loss of heterozygosity (LOH) with microsatellite markers on chromosomes 17 and 18 in 45 patients with primary gastric cancer, we first described the chromosomal LOH regions at 17q21.33 (TOB1 locus) and 18p11.3 (DAL-1 locus), which suggested that both genes may be involved in gastric carcinogenesis [3–5]. In subsequent studies, we demonstrated that the functional inactivation triggered by the abnormal expression of TOB1 (downregulation/deletion or phosphorylationaccumulation) promotes the development of gastric cancer and that TOB1 exerts antiproliferative effects in the nucleus [6,7]. Furthermore, the polymorphisms in the TOB1 gene were also closely associated with the risk of gastric cancer in the Han population of northeastern China [8]. Aberrant expression of DAL-1 mediated by the hypermethylation of its promoter region resulted in tumor suppressor gene behavior that plays important roles in the malignancy of gastric cancer [9].

DAL-1 (the differentially expressed in adenocarcinoma of the lung-1) belongs to the protein 4.1 superfamily, a large family with functional roles in cell adhesion and structure as well as in regulation of the membrane skeleton [10]. Studies have shown decreased and/or missing DAL-1 expression in cancer tissue from patients with brain [11], kidney [12], breast [13], prostate [14], ovarian [15], and esophageal tumors [16], and the expression of DAL-1 was earlier revealed to be downregulated in lung cancer [17]. In the digestive system, DAL-1 not only

* Corresponding author at: 246 Xuefu Road, Nangang District, Harbin, 150081, China.
*E-mail address:* yujingcui@ems.hrbmu.edu.cn (J. Yu).

https://doi.org/10.1016/j.prp.2019.03.031
Received 7 November 2018; Received in revised form 19 March 2019; Accepted 31 March 2019

maintains the normal structure of cells but also regulates the proliferation and adhesion of normal cells, thereby inhibiting the malignant transformation of epithelial cells [18]. DAL-1 gene regulatory factors, such as long chain noncoding RNAs (LINC00052), have been found to inhibit the migration and invasion of hepatocellular carcinoma (HCC) cells through the upregulation of DAL-1 expression mediated by the binding of miR-452-5p [19]. MiR-223 reduced DAL-1 expression by targeting the 3′-UTR of DAL-1 in the gastric cancer cell lines XGC-9811 L and NUGC-3. The overexpression of DAL-1 could inhibit the migration and invasion of XGC-9811 L cells, and the downregulation of DAL-1 expression promoted the migration and invasion of NUGC-3 cells [20].

The TOB1 (transducer of ErbB-2,1) gene encodes the TOB1 protein, which is a member of the BTG/TOB antiproliferative protein family [21]. TOB1 is known for its reduced expression in various cancerous tissues from patients, including in lung [22], breast [23], thyroid [24], and skin tumors [25], and Yoshida Y et al. found that mice lacking TOB1 are prone to the spontaneous formation of tumors [26]. Recently, the downregulation of TOB1 expression was also found in chronic myeloid leukemia (CML) [27]. The overexpression of TOB1 induced apoptosis and inhibited proliferation, migration, and invasion of gastric cancer cells by activating Smad4 and inhibiting βcatenin-mediated signaling pathway [28]. Additionally, miR-25 repressed TOB1 expression by directly binding to the TOB1 3′-UTR, thus explaining the inverse relationship observed between the expression levels of miR-25 and TOB1 in primary gastric cancer tissue [29]. In addition, the clinicopathological significance of cytoplasmic TOB1 expression was noted in gastric cancer tissue [30].

Currently, the link between the DAL-1 and TOB1 expression levels and the survival of gastric cancer patients is unknown, although we have previously demonstrated that the functional inactivation of DAL-1 and TOB1 promotes an aggressive phenotype in gastric cancer cells.Hence, it is likely that there may be a relationship between DAL-1 and TOB1 in the progression of gastric cancer. In this paper, we performed a retrospective cohort study to evaluate the prognostic value of DAL-1 and TOB1 expression in 270 clinical patients with gastric cancer by analyzing their expression patterns using immunohistochemical methods.

**Table 1**
Clinicopathological characteristics of the gastric cancer patients (n = 270).

| Characteristics | Number of cases (%) |
|---|---|
| **Age(y)** | |
| < 70 | 184(68.1) |
| ≥70 | 86(31.9) |
| **Sex** | |
| Male | 177(65.6) |
| Female | 93(34.4) |
| **Tumor size(cm)** | |
| < 5 | 103(38.1) |
| ≥5 | 167(61.9) |
| **Tumor site** | |
| Upper | 42(15.6) |
| Middle | 96(35.6) |
| Lower | 132(48.8) |
| **Histological grade** | |
| G1/G2 | 109(40.4) |
| G3 | 161(59.6) |
| **Depth of invasion** | |
| T1-T2 | 40(14.8) |
| T3-T4 | 230(85.2) |
| **Lymph node metastasis** | |
| No | 69(25.6) |
| Yes | 201(74.4) |
| **Metastasis** | |
| No | 263(97.4) |
| Yes | 7(2.6) |
| **Lymphvascular invasion** | |
| No | 199(73.7) |
| Yes | 71(26.3) |
| **Lauren classification** | |
| Intestinal | 144(53.3) |
| Diffuse | 81(30.0) |
| Mixed | 45(16.7) |
| **Clinical stage** | |
| I-II | 105(38.9) |
| III-IV | 165(61.1) |

## 2. Materials and methods

### 2.1. Patient samples

Gastric cancer tissue and matched adjacent noncancerous tissue (at least 5 cm away from the tumor) from 270 patients who had surgery for gastric cancer were collected between February 2008 and November 2009, which were used to construct tissue microarrays(HStm-Ade180Sur-03, HStm-Ade180Sur-06, and HStm-Ade180Sur-07)and purchased from Shanghai Superchip Co., Ltd. (Shanghai, China). No patients received chemotherapy, neoadjuvant chemotherapy or immunotherapy before surgery. The clinical information for each patient was obtained from their original pathology report. The detailed clinicopathologic characteristics of the patients are listed in Table 1. The size of the tumor was determined according to the longest diameter in the sample, and the pathological histology was classified as G1, G2, or G3 [31]. The Lauren classifications were defined as intestinal type, diffuse type, and mixed type [32]. Gastric cancer was staged using the TNM staging system of the 7th Union for International Cancer Control (UICC) / American Joint Committee on Cancer (AJCC) manual [33]. Overall survival was measured from the time of the operation to death or the last follow–up date (September 2014).

### 2.2. Immunohistochemistry

A Two-Step immunohistochemistry (IHC) Detection Reagent (PV-6001) kit (ZhongShan Golden Bridge Biological Technology Inc., Beijing, People's Republic of China) was used to perform the IHC staining for DAL-1 and TOB1 according to the manufacturer's protocol. Briefly, TMA slides were dewaxed in xylene and a series of graded alcohols, after which they were hydrated and washed in phosphate-buffered saline (PBS). Endogenous peroxidase was then blocked by immersing the slides in 3% $H_2O_2$ for 30 min, and then the slides were washed three times with PBS for 5 min each time. The slides were then autoclaved for 5 min in citrate buffer (pH 6.0) for antigen retrieval and blocked to prevent nonspecific binding. This was followed by the addition of polyclonal anti-bodies recognizing DAL-1 and TOB1 (DAL-1, ab154071 and TOB1, ab168947; both from Abcam; expression in the cytoplasm and nucleus was detected) at dilutions of 1:400 and 1:1000, respectively. The slides were incubated overnight in a humid chamber at 4 °C. The slides were then incubated with a secondary antibody (Zhongshan Golden Bridge Biological Technology Inc) at a dilution of 1:500 at 37 °C for 20 min. Each slide was treated with diaminobenzadine (DAB) reagent for 2 min and then counterstained with hematoxylin. Negative controls were treated identically but without the use of primary antibodies.

Three pathologists who had no prior knowledge of the clinical information assessed the slides blindly after IHC staining. Five visual fields were randomly selected, and 300 cells were counted in each view. The staining intensity of tumor cells was scored as negative (0), weak (1), moderate (2), and strong (3).The extent of the staining was scored according to the percentage of positive tumor cells in the field: 0% (0),1–25% (1), 26–50% (2), 51–75% (3), and 76–100% (4)." The

percentage of positive cells and staining intensity scores were multiplied to calculate an immunoreactive score (IRS = SI × PP). The product of the intensity and percentage scores was considered the overall immunohistochemistry score. The IRS ranged from 0 to 12 (0, 1, 2, 3, 4, 6, 8, 9, and 12).The median value of the IRS was chosen as the cut-off for high and low DAL-1 and TOB1 expression levels.IRS ≤ 6 was classified as a low level of expression, whereas IRS > 6 was classified as a high level of expression [34].

### 2.3. Ethical considerations

The present study was approved by the ethics committee of Taizhou Hospital of Zhejiang Province (Grant no.2005DKA21300).Written informed consent was obtained from all patients prior to their enrollment in the study. The ethical principles of human medical research were based on Helsinki Declaration of 1964.

### 2.4. Statistical analysis

SPSS 20.0 software (Version 20.0, Chicago, IL, USA), GraphPad Prism 5.0 software and R software (version 3.2.2) were used for all statistical analyses. The differential expression of DAL-1 and TOB1 in the gastric cancer tissue and the matched adjacent noncancerous tissue was evaluated by the Wilcoxon matched pairs test. The correlation between DAL-1 and TOB1 expression and the clinicopathologic features of gastric cancer patients was analyzed by the $\chi^2$ test or Fisher's exact test. Spearman's rank correlation analysis was used to assess the correlation between DAL-1 and TOB1 expression levels. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to examine the statistical significance. A Cox proportional hazards regression model was performed for univariate and multivariate survival analyses. All statistics were two-tailed, with $p < 0.05$ being considered statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

## 3. Results

### 3.1. Decreased expression of DAL-1 and TOB1 in gastric cancer

To assess the expression status of the two genes (DAL-1 and TOB1) in gastric cancer, immunohistochemical methods were used on 270 samples of gastric cancer(GC) and adjacent noncancerous gastric tissue (NG). DAL-1 protein was mainly distributed in the cytoplasm (Fig. 1A). The cytoplasm-positive and membrane-positive rates for DAL-1 protein expression were (85.6%, 231/270) and (14.4%, 39/270) in GCs, while cytoplasm-positive and membrane-positive rates in NG tissues were (65.6%, 177/270) and (34.4%, 93/270) (P = 0.000).In contrast, TOB1 was expressed mainly in the nucleus (Fig. 1B).The nucleus-positive and cytoplasm-positive rates for TOB1 protein expression were (70.7%, 191/270) and (29.3%, 79/270) in GCs, while nucleus-positive and cytoplasm-positive rates in NG tissues were (92.2%, 249/270) and (7.8%, 21/270) (P = 0.000).The rates of DAL-1 low expression in the gastric cancer tissue and noncancerous tissue samples were 38.9% (105/270) and 21.5% (58/270), respectively. Additionally, the rates of low expression of TOB1 in the gastric cancer tissue and noncancerous tissue samples were 69.6% (188/270) and 13.7% (37/270), respectively. There was significantly reduced expression of DAL-1 and TOB1 in gastric cancer tissue compared with noncancerous tissue (both $p < 0.001$). These results indicate that the expression of suppressor genes DAL-1 and TOB1 is down-regulated in gastric cancer.

To evaluate the association between the expression levels of the two genes (DAL-1 and TOB1) and clinicopathological features in gastric cancer, a correlation analysis was used on the data from the 270 patients. Among all gastric cancer specimens examined (Table 2), low expression of DAL-1 was significantly associated with a tumor site in the lower stomach (Lower vs. Middle and Upper, $p = 0.030$), poor histological grade (G3, $p = 0.002$), deep tumor invasion (T3 and T4, $p = 0.021$), lymph node metastasis ($p = 0.011$), Lauren classification of diffuse type (Diffuse vs. Intestinal and Mixed, $p < 0.001$), and high clinical stage (III and IV, $p = 0.006$). Lower TOB1 expression was observed in gastric cancer patients with diffuse type disease compared to patients with intestinal type/mixed type disease ($p < 0.001$).In order to clarify the correlation between the expression of DAL-1 protein and TOB1 protein in 270 cases of gastric cancer, the expression of two proteins in each of 270 cases of gastric cancer were enumerated. The expression trend was the same in 53.7% of 270 patients with gastric cancer(Supplementary Table).Spearman's correlation analysis showed that the low expression of DAL-1 positively correlated with the low TOB1 expression ($r = 0.304$, $p < 0.001$) (Fig. 2).Our data demonstrated that the decreased expression of DAL-1 and TOB1 are associated with various clinicopathological features in gastric cancer.

### 3.2. Decreased expression levels of DAL-1 and TOB1 are associated with poor prognosis in gastric cancer

To reveal the association between the abnormal expression levels of DAL-1 and TOB1 and prognosis in gastric cancer, survival analysis was used on the data from the 270 patients. The survival analysis revealed a 5-year overall survival (OS) rate of 38.9% and a median survival of 71 months. In addition, the 5-year survival rates were 20.6% and 34% in patients with low DAL-1 and TOB1 expression levels, respectively, while rates of 50.3% and 50% were recorded for patients with high DAL-1 and TOB1 expression levels, respectively. Kaplan–Meier survival curves revealed that patients with low expression levels of DAL-1 ($p < 0.001$) and TOB1 ($p = 0.03$) achieved worse survival than patients with high expression levels of DAL-1 (Fig. 3A) and TOB1 (Fig. 3B).

The data for the univariate Cox proportional hazards analysis for the major clinicopathologic features and the expression levels of DAL-1 and TOB1 in gastric cancer tissue are presented in Table 3. Low expression of both DAL-1 (low expression vs. high expression) [hazard ratio (HR) 2.2, (95% confidence interval (CI): 1.617–2.992, $p < 0.001$)] and TOB1 (low expression vs. high expression) [HR 1.476(95% CI: 1.037–2.102, $p = 0.030$)], tumor size (≥5 vs. < 5) [HR 2.010(95% CI: 1.435–2.816, $p < 0.001$)], poor histological grade G3 [HR 1.822(95% CI: 1.310–2.533, $p < 0.001$)], deep tumor invasion (T3 and T4) [HR 3.556(95% CI: 1.927–6.562, $p < 0.001$)], lymph node metastasis (Yes vs. No) [HR 6.788(95% CI: 3.840–11.998, $p < 0.001$)], metastasis (Yes vs. No) [HR 5.143(95% CI: 2.367–11.173, $p < 0.001$)], lymphvascular invasion(Yes vs. No) [HR 1.874(95% CI: 1.355–2.591, $p < 0.001$)], Lauren classification(diffuse type/mixed type vs. intestinal type) [HR 1.950(95% CI: 1.383–2.749, $p < 0.001$);HR 1.976(95% CI: 1.304–2.992, $p < 0.001$)], and high clinical stage (III and IV)[HR 4.594(95% CI: 3.109–6.788, $p < 0.001$)] were factors associated with an increased risk of death among gastric cancer patients. Meanwhile, low expression of DAL-1 in well and poorly differentiated histologically graded tumors was associated with worse survival than high expression of DAL-1 in the same tumor types($p < 0.001$and $p = 0.02$, respectively) (Fig. 4A). With respect to the depth of invasion, low expression of DAL-1 correlated with worse survival than high expression of DAL-1 ($p < 0.001$) (Fig. 4B). Among patients with lymph node metastasis, low expression of DAL-1 was associated with worse survival than high expression of DAL-1 ($p < 0.001$) (Fig. 4C). In the different clinical stages, the prognosis associated with low expression of DAL-1 was worse than that of high expression of DAL-1 ($p < 0.001$ and $p = 0.006$, respectively) (Fig. 4D). In the intestinal type subgroup, the prognosis of patients with decreased expression of DAL-1 was worse than that of patients with increased DAL-1 expression ($p < 0.001$) (Fig. 4E).

In addition, multivariate analysis by Cox regression and correction for all histopathologic features revealed that the low expression of DAL-1[HR 1.799(95% CI:1.278 − 2.532, $p = 0.001$)] as well as tumor size (≥5) [HR 1.649(95% CI: 1.154–2.359, $p = 0.006$)], lymph node

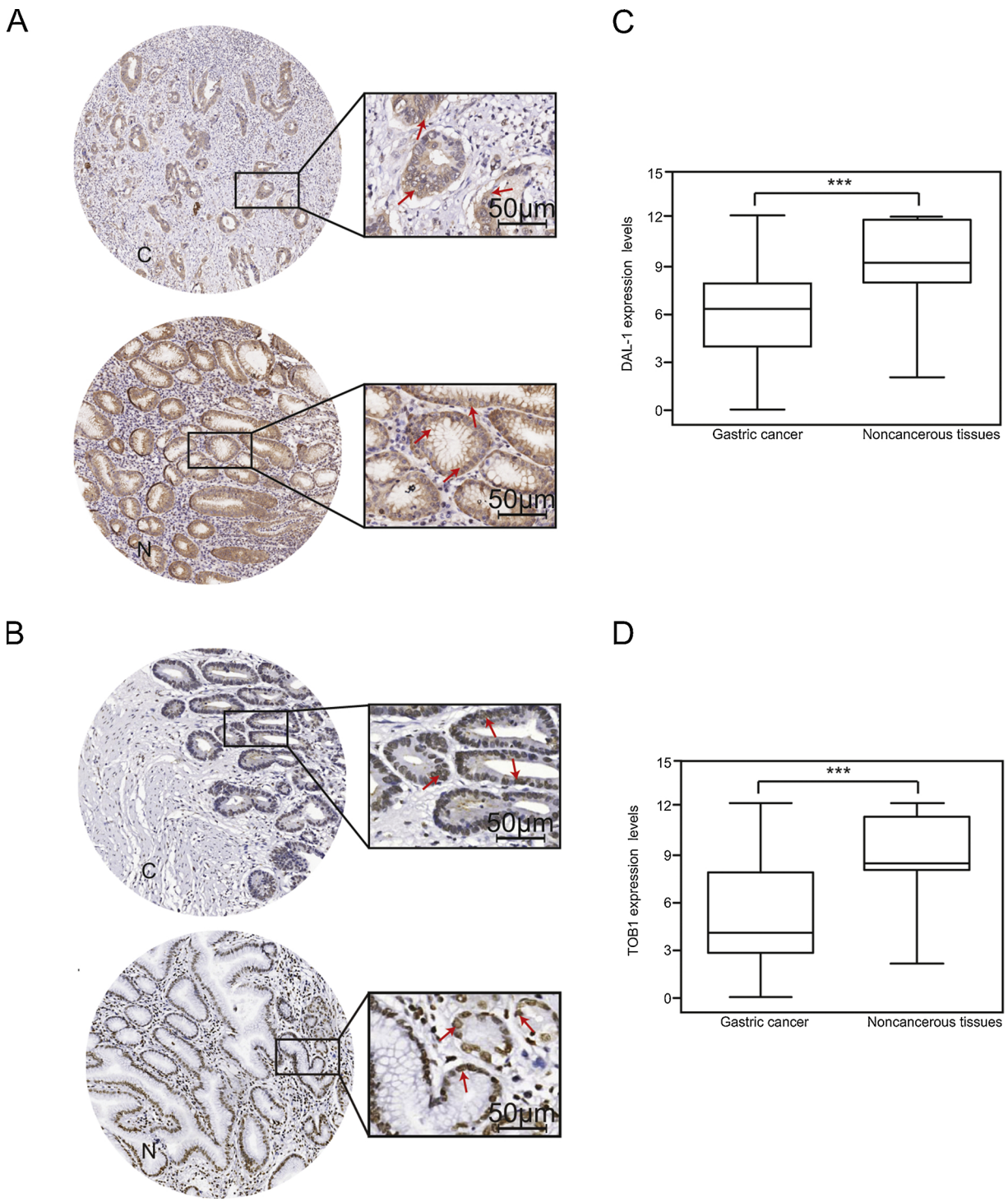


**Fig. 1.** Representative pictures of immunohistochemical staining for DAL-1 and TOB1 in gastric cancer and adjacent noncancerous gastric tissue. (A) DAL-1 protein. (B) TOB1 protein. (C–D) The expression levels of DAL-1 and TOB1 in gastric cancer and adjacent noncancerous gastric tissue were compared by IRS. C, Gastric cancer tissue; N, Noncancerous tissue. Red arrows indicate the position. The scale bar corresponds to 50 μm.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

metastasis status [HR 3.308(95% CI: 1.616–6.769, $p = 0.001$)], metastasis status [HR 2.795(95% CI: 1.270–6.154, $p = 0.011$)], and clinical stage (III and IV)[HR 1.712(95% CI: 1.001–2.926, $p = 0.049$) were independent prognostic factors for OS, whereas histological grade ($p = 0.228$), depth of invasion ($p = 0.420$), lymphvascular invasion ($p = 0.565$), Lauren classification ($p = 0.320$), and TOB1($p = 0.092$) expression level were not statistically significant. These data indicate that the decreased expression levels of DAL-1 and TOB1are associated with poor prognosis in gastric cancer.

## 4. Discussion

In the present study, we showed significant variations in the expression levels of DAL-1 and TOB1 between gastric cancer tissue and adjacent noncancerous gastric tissue, indicating that DAL-1 and TOB1 may be involved in the progression of gastric cancer. Furthermore, low expression of DAL-1 and TOB1 was associated with worse survival outcomes in patients with gastric cancer. Conversely, high expression of DAL-1 and TOB1 was associated with better survival in gastric cancer patients.

**Table 2**
Associations between DAL-1 and TOB1 expression levels and the clinicopathological factors of the gastric cancer patients.

| Variables | DAL-1 expression | | | TOB1 expression | | |
|---|---|---|---|---|---|---|
| | Low (%) | High(%) | *P* value | Low(%) | High(%) | *P* value |
| **Age(y)** | | | 0.234 | | | 0.751 |
| < 70 | 76(41.3) | 108(58.7) | | 127(69) | 57(31) | |
| ≥70 | 29(33.7) | 57(66.3) | | 61(71) | 25(29) | |
| **Sex** | | | 0.314 | | | 0.082 |
| Male | 65(36.7) | 112(63.3) | | 117(66.1) | 60(33.9) | |
| Female | 40(43) | 53(57) | | 71(76.3) | 22(23.7) | |
| **Tumor size(cm)** | | | 0.597 | | | 0.150 |
| < 5 | 38(36.9) | 65(63.1) | | 77(74.8) | 26(25.2) | |
| ≥5 | 67(40.1) | 100(59.9) | | 111(66.5) | 56(33.5) | |
| **Tumor site** | | | 0.030 | | | 0.624 |
| Upper | 21(50) | 21(50) | | 27(64.3) | 15(35.7) | |
| Middle | 43(44.8) | 53(55.2) | | 66(68.8) | 30(31.2) | |
| Lower | 41(31.1) | 91(68.9) | | 95(72) | 37(28) | |
| **Histological grade** | | | 0.002 | | | 0.063 |
| G1/G2 | 30(27.5) | 79(72.5) | | 69(63.3) | 40(36.7) | |
| G3 | 75(46.6) | 86(53.4) | | 119(73.9) | 42(26.1) | |
| **Depth of invasion** | | | 0.021 | | | 0.956 |
| T1-T2 | 9(22.5) | 31(77.5) | | 28(70) | 12(30) | |
| T3-T4 | 96(41.7) | 134(58.3) | | 160(69.6) | 70(30.4) | |
| **Lymph node metastasis** | | | 0.011 | | | 0.356 |
| No | 18(26.1) | 51(73.9) | | 45(65.2) | 24(34.8) | |
| Yes | 87(43.3) | 114(56.7) | | 143(71.1) | 58(28.9) | |
| **Metastasis** | | | 0.074 | | | 0.348 |
| No | 100(38) | 163(62) | | 182(69.2) | 81(30.8) | |
| Yes | 5(71.4) | 2(28.6) | | 6(85.7) | 1(14.3) | |
| **Lymphvascular invasion** | | | 0.337 | | | 0.441 |
| No | 74(37.2) | 125(62.8) | | 136(68.3) | 63(31.7) | |
| Yes | 31(38.9) | 40(61.1) | | 52(73.2) | 19(26.8) | |
| **Lauren classification** | | | < 0.001 | | | < 0.001 |
| Intestinal | 33(22.9) | 111(77.1) | | 89(61.8) | 55(38.2) | |
| Diffuse | 54(66.7) | 27(33.3) | | 71(87.7) | 10(12.3) | |
| Mixed | 18(40) | 27(60) | | 28(62.2) | 17(37.8) | |
| **Clinical stage** | | | 0.006 | | | 0.398 |
| I-II | 30(28.6) | 75(71.4) | | 70(66.7) | 35(33.3) | |
| III-IV | 75(45.5) | 90(54.5) | | 118(71.5) | 47(28.5) | |

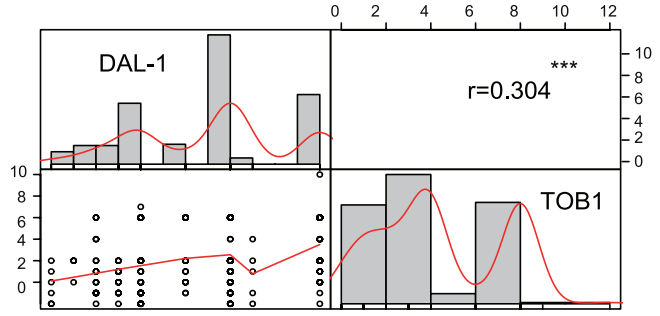


**Fig. 2.** Spearman's rank correlation analyses of DAL-1 and TOB1 expression in gastric cancer. The low expression of DAL-1 positively correlated with the low expression of TOB1 (r = 0.304, $p < 0.001$).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Our immunohistochemistry findings in the gastric cancer tissue samples from 270 patients revealed that the DAL-1 protein was predominantly distributed in the cytoplasm, although the DAL-1 protein was previously reported to be mainly distributed on the cell membrane and concentrated in the contact area between intestinal epithelial cells [18]. In our previous description, the absence of or notable decrease in DAL-1 mRNA and protein was highly correlated with CpG hypermethylation of the DAL-1 promoter in primary gastric cancer tissue and in gastric cancer cell lines; abnormalDAL-1 subcellular localization was also observed in the gastric cancer cell line HGC-27, whereDAL-1 proteins were observed throughout the cytoplasm except along the cell membrane [9]. The present finding in gastric cancer tissue from clinical patients confirmed our observation in the gastric cancer cell line, indicating that the abnormal localization of DAL-1 is critical for the progression of gastric cancer [9]. Proper membrane localization of 4.1B/DAL-1 might be required for growth suppression because the localization of the U2 domain of Protein 4.1B at the membrane has been found to be necessary and sufficient for growth suppression in meningioma cells [35]. It was also suggested that a similar aberrant pattern of subcellular distribution in renal clear cell carcinoma is associated with the impairment of the potential tumor suppressor function of DAL-1 [12]. Additional studies are necessary to prove the validity of this idea in gastric cancer.

We also found that a lower level of DAL-1 in gastric cancer tissue was an independent prognostic factor for OS after correction for all the histopathologic features of gastric cancer patients. The patients whose gastric cancers had decreased DAL-1 expression had significantly worse survival than the patients whose cancers had high expression of DAL-1 ($p < 0.001$). The current data from clinical patients with gastric cancer supported our previous results in gastric cancer cell lines that demonstrated that exogenous DAL-1 effectively promoted apoptosis of AGS cells and inhibited the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of AGS cells and that the knockdown of endogenous DAL-1 in HGC-27 cells produced gastric tumor cells that appeared highly aggressive [9].

TOB1 was previously demonstrated to be a gastric cancer-related tumor suppressor by our team [3,4]. We showed that the functional inactivation caused by the downregulation or phosphorylation of TOB1 protein may promote gastric cancer progression. Furthermore, single nucleotide polymorphisms (SNPs) in TOB1 were closely associated with the risk of gastric cancer in the Han population of northeastern China [8]. Here, we showed that the expression of TOB1 in gastric cancer

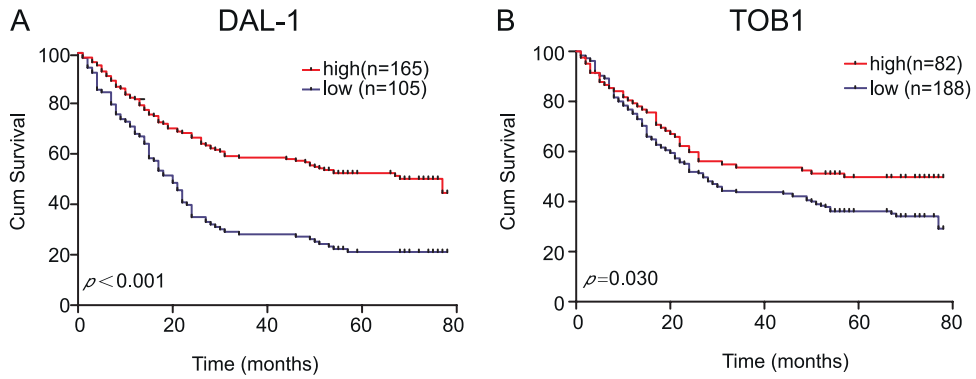


**Fig. 3.** Kaplan-Meier analyses of the overall survival of 270 gastric cancer patients. (A) Correlation between DAL-1 expression and overall survival (B) Correlation between TOB1 expression and overall survival. The low expression levels of DAL-1($p < 0.001$) and TOB1 ($p < 0.05$) were closely correlated with poor survival.

tissue was significantly lower than that in adjacent noncancerous gastric tissue, which is consistent with the data in cancerous tissue from other patients, including those with gastric cancer [6,7] and several different pathological types of cancers [23,24,29,30]. We also found that TOB1 protein was mainly concentrated in the nucleus of gastric cancer cells. Furthermore, compared with high expression of TOB1, decreased expression of TOB1 was associated with a worse Lauren classification (diffuse type). Tsuzuku et al. showed that the nuclear localization of TOB1 was important for its antiproliferative effects in NIH3T3 cells [36]. We previously showed that TOB1 exerts antiproliferative effects in the nuclei of AGS gastric cancer cells [7]. Thus, the lower concentration of TOB1 in the nucleus might impair the antiproliferative activity of TOB1 and contribute to the development of gastric cancer.

**Table 3**
Univariate and multivariate analyses of the prognostic factors in 270 with gastric cancer.

| Variables | Univariate | | Multivariate | |
|---|---|---|---|---|
| | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
| Age(y) | | 0.583 | | |
| < 70 | REF | | | |
| ≥70 | 1.095(0.791-1.517) | | | |
| Sex | | 0.115 | | |
| Male | REF | | | |
| Female | 1.287(0.940-1.760) | | | |
| Tumor size(cm) | | < 0.001 | | 0.006 |
| < 5 | REF | | REF | |
| ≥5 | 2.010(1.435-2.816) | | 1.649(1.154-2.359) | |
| Tumor site | | 0.633 | | |
| Upper | REF | | | |
| Middle | 0.840(0.538-1.310) | | | |
| Lower | 0.814(0.531-1.248) | | | |
| Histological grade | | < 0.001 | | 0.228 |
| G1/G2 | REF | | REF | |
| G3 | 1.822(1.310-2.533) | | 1.306(0.846-2.016) | |
| Depth of invasion | | < 0.001 | | 0.420 |
| T1-T2 | REF | | REF | |
| T3-T4 | 3.556(1.927-6.562) | | 1.333(0.663-2.680) | |
| Lymph node metastasis | | < 0.001 | | 0.001 |
| No | REF | | REF | |
| Yes | 6.788(3.840-11.998) | | 3.308(1.616-6.769) | |
| Metastasis | | < 0.001 | | 0.011 |
| No | REF | | REF | |
| Yes | 5.143(2.367-11.173) | | 2.795(1.270-6.154) | |
| Lymphvascular invasion | | < 0.001 | | 0.565 |
| No | REF | | REF | |
| Yes | 1.874(1.355-2.591) | | 1.105(0.786-1.554) | |
| Lauren classification | | < 0.001 | | 0.320 |
| Intestinal | REF | | REF | |
| Diffuse | 1.950(1.383-2.749) | | 0.803(0.492-1.311) | |
| Mixed | 1.976(1.304-2.992) | | 1.126(0.692-1.833) | |
| Clinical stage | | < 0.001 | | 0.049 |
| I-II | REF | | REF | |
| III-IV | 4.594(3.109-6.788) | | 1.712(1.001-2.926) | |
| DAL-1 expression | | < 0.001 | | 0.001 |
| High | REF | | REF | |
| Low | 2.2(1.617-2.992) | | 1.799(1.278-2.532) | |
| TOB1 expression | | 0.030 | | 0.092 |
| High | REF | | REF | |
| Low | 1.476(1.037-2.102) | | 1.379(0.949-2.004) | |

HR hazard ratio, 95% CI 95% confidence interval, REF reference value.

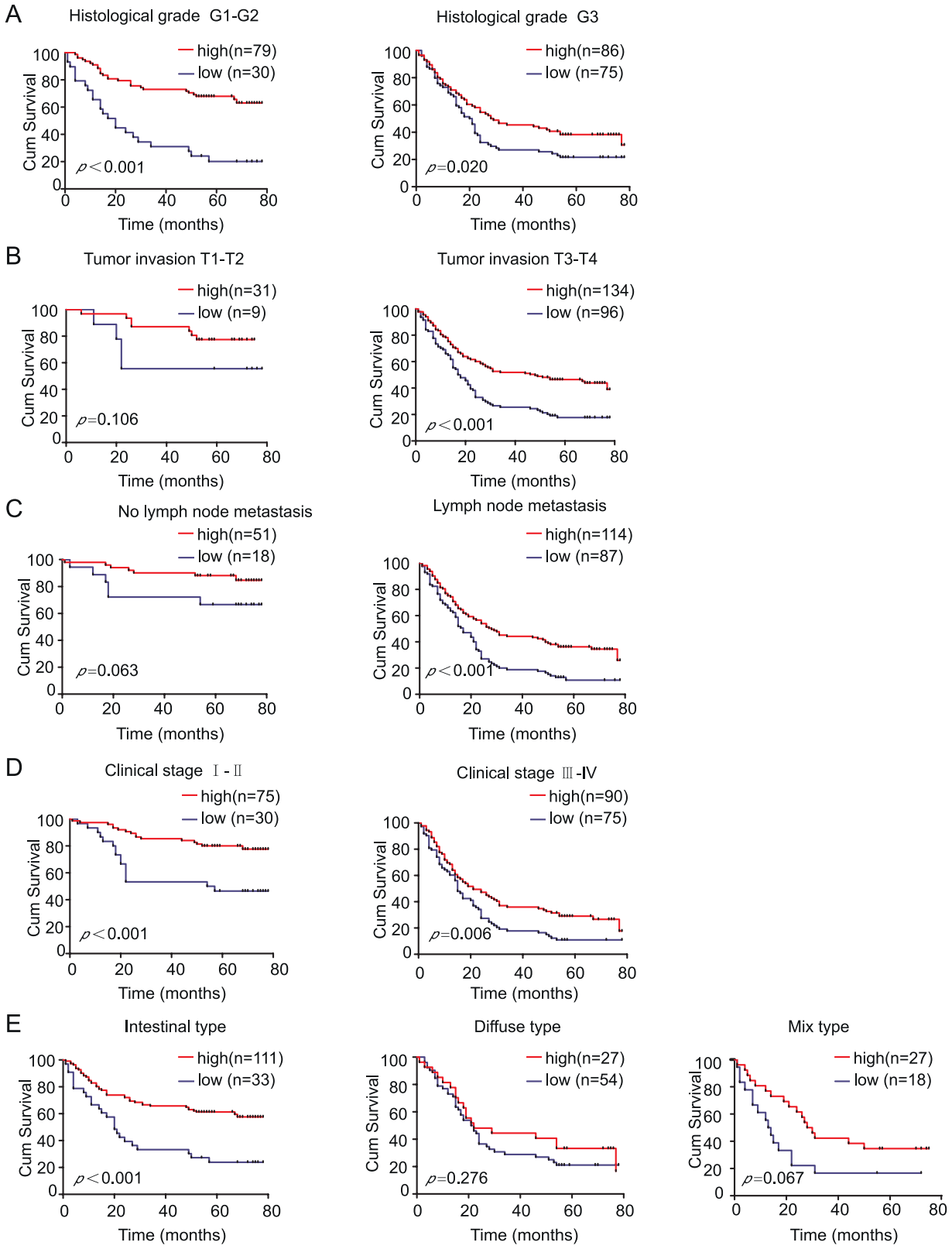


**Fig. 4.** Kaplan-Meier analyses of overall survival stratified by clinicopathological factors. Patients were classified according to (A) histological grade, (B) depth of invasion, (C) lymph node metastasis status, (D) clinical stage, and (E) Lauren classification. Low expression of DAL-1 was associated with poor survival outcomes in patients with gastric cancer ($p < 0.05$).

ErbB2 (also called HER2) belongs to the erbB/HER type 1 family of receptor tyrosine protein kinase [37]. An earlier study revealed TOB1 can directly interact with ErbB2 to exert its anti-proliferation effect, but the effect of TOB1 can be blocked by the presence of kinase-active ErbB2 [38]. ErbB2 interacts indirectly with DAL-1 by binding with EGFR and inhibits the abnormal proliferation of breast epithelial cells by inhibiting ErbB2 phosphorylation in breast cancer MCF-7 cells [39,40]. In order to clarify the correlation between the expression of

DAL-1 protein and TOB1 protein in 270 cases of gastric cancer, the expression of two proteins in each of 270 cases of gastric cancer were enumerated. The expression trend was the same in 53.7% of 270 patients with gastric cancer. We revealed a significant positive correlation between decreased DAL-1 expression and decreased TOB1 expression, suggesting that together the genes may play a synergistic role in gastric cancer.TOB1 and DAL-1 are gastric cancer-related tumor suppressors revealed by our group for the first time [3,4].In the present study, through survival analysis, we demonstrated that the downregulation of these two anti-oncogenes (DAL-1 and TOB1) exerted a negative effect on the survival of gastric cancer patients. The epigenetic inactivation of DAL-1 and CADM1 plays a synergistic role in the pathogenesis of NL and affects the invasion and metastasis abilities of cancer cells [41].Based on the ideas of this study, we will further verify the synergistic effect of DAL-1 and TOB1 in gastric cancer cells. The malignant phenotype changes of gastric cancer cells were observed by interfering with the expression of either gene and exogenous co-overexpression of the two genes. To verify that the co-expression of two genes can inhibit the proliferation, migration and invasion of gastric cancer cells more effectively than any of them.

With the aid of a univariate Cox proportional hazards analysis assessing the expression of DAL-1 and TOB1 in gastric cancer tissue, our findings revealed that low expression of the two anti-oncogenes correlated with an increase in the risk of death. A multivariate analysis by Cox regression with corrections for all the histopathologic features revealed that the low expression of DAL-1 was an independent predictor of poor prognosis. In essence, our data suggested that DAL-1 may be considered a potential prognostic biomarker for overall survival and a potential therapeutic target for intervention in patients with gastric cancer.

In conclusion, our study demonstrated that the low expression levels of DAL-1 and TOB1 are significantly associated with poor survival in patients with gastric cancer. To the best of our knowledge, DAL-1 and TOB1 are tumor suppressor genes, and the low expression of both genes in gastric cancer cell lines and tissue is primarily caused by DNA methylation and protein phosphorylation, respectively. The decreased expression of DAL-1 and TOB1 may play a synergistic role in the pathogenesis of gastric cancer, affecting tumor growth, metastasis, and invasion. The low expression of DAL-1 and TOB1 in clinical patients with gastric cancer suggested that they can be used as ideal molecular markers for the diagnosis of this particular tumor. More studies with a larger number of cases are necessary to validate our findings.

## Conflict of interest

The author(s) declare that they have no potential conflicts of interest.

## Author contributions

HaonanGuo and Rui Zhang: Study design, data analysis, and writing of the original draft; Justice Afrifa: Writing of the draft and data analysis and interpretation; Yuanyuan Wang: Collection and assembly of the data, data analysis and laboratory work. Jingcui Yu: Designed the study, provided financial support, and wrote the manuscript.

## Acknowledgements

The authors are grateful to all sample donors enrolled in this study. We also appreciate the excellent technical assistance of Zhanfeng Jin and Sen Li. We thank Tie Zhao and Mengxi Wang for their fruitful help. This work was supported by the National Natural Science Foundation of China (Grant no.81372174).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.03.031.

## References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (2015) 359–386.
[2] K.D. Miller, R.L. Siegel, C.C. Lin, A.B. Mariotto, J.L. Kramer, J.H. Rowland, K.D. Stein, Cancer treatment and survivorship statistics, 2016, CA Cancer J. Clin. 66 (2016) 271–289.
[3] J. Yu, H. Zhou, Y. Jin, J. Bai, Y. Yu, J. Geng, J. Qi, S. Fu, Three distinct regions of allelic deletion on chromosome 17 involved in sporadic gastric cancer, Hepatogastroenterology 55 (2008) 1487–1491.
[4] J. Yu, H. Zhou, J. Bai, Y. Yu, J. Geng, J. Qi, S. Fu, Human gastric adenocarcinoma allelotype on chromosomes 17 and 18, J. Int. Med. Res. 36 (2008) 279–288.
[5] J. Yu, K. Sun, B. Liu, S. Fu, Allelotyping for loss of heterozygosity on chromosome 18 in gastric cancer, World J. Gastroenterol. 10 (2004) 1964–1966.
[6] J. Yu, P. Liu, X. Cui, Y. Sui, G. Ji, R. Guan, D. Sun, W. Ji, F. Liu, A. Liu, Identification of novel subregions of LOH in gastric Cancer and analysis of the HIC1 and TOB1 tumor suppressor genes in these subregions, Mol. Cells 32 (2011) 47–55.
[7] R. Guan, L. Peng, D. Wang, H. He, D. Wang, R. Zhang, H. Wang, H. Hao, J. Zhang, H. Song, Decreased TOB1 expression and increased phosphorylation of nuclear TOB1 promotes gastric cancer, Oncotarget 8 (2017) 75243–75253.
[8] H. Wang, H. Hao, H. Guo, Y. Wang, X. Zhang, L. Xu, J. Yu, Association between the SNPs of theTOB1gene and gastric cancer risk in the Chinese Han population of northeast China, J. Cancer 9 (2018) 1371–1378.
[9] W. Hao, X. Man, X. Cui, Y. Liu, Z. Yi, S. Yu, W. Dong, P. Lei, D. Wang, J. Yu, Aberrant expression of the candidate tumor suppressor gene DAL-1 due to hypermethylation in gastric cancer, Sci. Rep. 6 (2016) 21755–21765.
[10] D.H. Gutmann, A.C. Hirbe, Z.Y. Huang, C.A. Haipek, The protein 4.1 tumor suppressor, DAL-1, impairs cell motility, but regulates proliferation in a cell-type-Specific fashion, Neurobiol. Dis. 8 (2001) 266–278.
[11] D.H. Gutmann, J. Donahoe, A. Perry, N. Lemke, K. Gorse, K. Kittiniyom, S.A. Rempel, J.A. Gutierrez, I.F. Newsham, Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas, Hum. Mol. Genet. 9 (2000) 1495–1500.
[12] D. Yamada, S. Kikuchi, Y.N. Williams, M. Sakurai-Yageta, M. Masuda, T. Maruyama, K. Tomita, D.H. Gutmann, T. Kakizoe, T. Kitamura, Promoter hypermethylation of the potential tumor suppressor DAL-1/4.1B gene in renal clear cell carcinoma, Int. J. Cancer 118 (2010) 916–923.
[13] G. Heller, J. Geradts, B. Ziegler, I. Newsham, M. Filipits, E.M. Markis-Ritzinger, D. Kandioler, W. Berger, W. Stiglbauer, D. Depisch, Downregulation of TSLC1 and DAL-1 expression occurs frequently in breast cancer, Breast Cancer Res. Treat. 103 (2007) 283–291.
[14] D.B. Bernkopf, E.D. Williams, Potential role of EPB41L3 (Protein 4.1B/DAL-1) as a target for treatment of advanced prostate cancer, Expert Opin. Ther. Targets 12 (2008) 845–853.
[15] D. Dafou, B. Grun, J. Sinclair, K. Lawrenson, E.C. Benjamin, E. Hogdall, S. Krugerkjaer, L. Christensen, H.M. Sowter, A. Alattar, Microcell-mediated chromosome transfer identifies EPB41L3 as a functional suppressor of epithelial ovarian cancers, Neoplasia 12 (2010) 579–589.
[16] R. Zeng, J.P. Huang, X.F. Li, W.B. Xiong, G. Wu, Z.J. Jiang, S.J. Song, J.Q. Li, Y.F. Zheng, J.R. Zhang, Epb41l3 suppresses esophageal squamous cell carcinoma invasion and inhibits MMP2 and MMP9 expression, Cell Biochem. Funct. 34 (2016) 133–141.
[17] Y.K. Tran, O. Bögler, K.M. Gorse, I. Wieland, M.R. Green, I.F. Newsham, A novel meber of the NF2/ERM/4.1 superfamily with growth suppressing properties in lung cancer, Cancer Res. 59 (1999) 35–43.
[18] N. Ohno, N. Terada, S. Murata, H. Yamakawa, I.F. Newsham, R. Katoh, O. Ohara, S. Ohno, Immunolocalization of protein 4.1B/DAL-1 during neoplastic transformation of mouse and human intestinal epithelium, Histochem. Cell Biol. 122 (2004) 579–586.
[19] L. Zhu, N. Yang, J. Chen, T. Zeng, S. Yan, Y. Liu, G. Yu, Q. Chen, G. Du, W. Pan, LINC00052 upregulates EPB41L3 to inhibit migration and invasion of hepatocellular carcinoma by binding miR-452-5p, Oncotarget 8 (2017) 75132–75143.
[20] X. Li, Y. Zhang, H. Zhang, X. Liu, T. Gong, M. Li, L. Sun, G. Ji, Y. Shi, Z. Han, miRNA-223 promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3, Mol. Cancer Res. 9 (2011) 824–833.
[21] G.S. Winkler, The mammalian anti-proliferative BTG/Tob protein family, J. Cell. Physiol. 222 (2010) 66–72.
[22] K. Iwanaga, N. Sueoka, A. Sato, T. Sakuragi, Y. Sakao, M. Tominaga, T. Suzuki, Y. Yoshida, J. Ktsuzuku, T. Yamamoto, Alteration of expression or phosphorylation status of tob, a novel tumor suppressor gene product, is an early event in lung cancer, Cancer Lett. 202 (2003) 71–79.
[23] C. Klebig, S. Seitz, E. Korsching, G. Kristiansen, D. Gustavus, S. Scherneck, I.J.G. Petersen, Chromosomes, cancer, profile of differentially expressed genes after transfer of chromosome 17 into the breast cancer cell line CAL51, Genes Chromosomes Cancer 44 (2010) 233–246.
[24] Y. Ito, T. Suzuki, H. Yoshida, C. Tomoda, T. Uruno, Y. Takamura, A. Miya, K. Kobayashi, F. Matsuzuka, K.J.Cl. Kuma, Phosphorylation and inactivation of Tob contributes to the progression of papillary carcinoma of the thyroid, Cancer Lett.

220 (2005) 237–242.
[25] G.T. Park, E.Y. Seo, K.M. Lee, D.Y. Lee, J.M. Yang, Tob is a potential marker gene for the basal layer of the epidermis and is stably expressed in human primary keratinocytes, Br. J. Dermatol. 154 (2010) 411–418.
[26] Y. Yoshida, T. Nakamura, M. Komoda, H. Satoh, T. Suzuki, J.K. Tsuzuku, T. Miyasaka, E.H. Yoshida, H. Umemori, R.K. Kunisaki, Mice lacking a transcriptional corepressor Tob are predisposed to cancer, Genes Dev. 17 (2003) 1201–1206.
[27] C.D.C. Mascarenhas, A.F.D. Cunha, A.F.V. Brugnerotto, S. Gambero, M.H.D. Almeida, M.F. Carazzolle, K.B.B. Pagnano, F.O. Traina, F.F.D. Costa, C.A.D. Souza, Identification of target genes using gene expression profile of granulocytes from patients with chronic myeloid leukemia treated with tyrosine kinase inhibitors, Leuk. Lymphoma 55 (2014) 1861–1869.
[28] J. Kundu, S.M.R. Wahab, J.K. Kundu, Y. Choi, O.C. Erkin, H.S. Lee, G.P. Sang, Y.K. Shin, Tob1 induces apoptosis and inhibits proliferation, migration and invasion of gastric cancer cells by activating Smad4 and inhibiting β-catenin signaling, Int. J. Oncol. 41 (2012) 839–848.
[29] B. Li, Q. Zuo, Y. Zhao, B. Xiao, Y. Zhuang, X. Mao, C. Wu, S. Yang, H. Zeng, Q.J.O. Zou, MicroRNA-25 promotes gastric cancer migration, invasion and proliferation by directly targeting transducer of ERBB2, 1 and correlates with poor survival, Oncogene 34 (2015) 2556–2565.
[30] S.Q. Zhang, K.K. Sun, X.Y. Wu, N. Zhong, H. Zhao, D.C. Li, Clinicopathological significance of cytoplasmic transducer of ErbB2. 1 expression in gastric cancer, Mol. Med. Rep. 12 (2015) 1177–1182.
[31] S. Li, X. Lan, H. Gao, Z. Li, C. Li, W. Wang, S. Song, Y. Wang, C. Li, H. Zhang, Systemic Inflammation Response Index (SIRI), cancer stem cells and survival of localised gastric adenocarcinoma after curative resection, J. Cancer Res. Clin. Oncol. 143 (2017) 2455–2468.
[32] R. Sitarz, M. Skierucha, J. Mielko, G. Offerhaus, R. Maciejewski, W.P. Polkowski, Gastric cancer: epidemiology, prevention, classification, and treatment, Cancer Manag. Res. 10 (2018) 239–248.
[33] S.B. Edge, C.C. Compton, S.B. Edge, C.C. Compton, The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM, Ann. Surg. Oncol. 17 (2010) 1471–1474.
[34] L. Hu, H.Y. Chen, J. Cai, Y. Zhang, C.Y. Qi, H. Gong, Y.X. Zhai, H. Fu, G.Z. Yang, C.F. Gao, Serine threonine tyrosine kinase 1 is a potential prognostic marker in colorectal cancer, BMC Cancer 15 (2015) 246–257.
[35] V.A. Robb, M.A. Gerber, E.K. Hart-Mahon, D.H. Gutmann, Membrane localization of the U2 domain of Protein 4.1B is necessary and sufficient for meningioma growth suppression, Oncogene 24 (2005) 1946–1957.
[36] J. Kawamuratsuzuku, T. Suzuki, Y. Yoshida, T. Yamamoto, Nuclear localization of Tob is important for regulation of its antiproliferative activity, Oncogene 23 (2004) 6630–6638.
[37] R.A. Walker, The erbB/HER type 1 tyrosine kinase receptor family, J. Pathol. 185 (2015) 234–235.
[38] S. Matsuda, J. Kawamuratsuzuku, M. Ohsugi, M. Yoshida, M. Emi, Y. Nakamura, M. Onda, Y. Yoshida, A. Nishiyama, T. Yamamoto, Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity, Oncogene 12 (1996) 705–713.
[39] M. Wobus, R. Rangwala, I. Sheyn, R. Hennigan, B. Coila, E.E. Lower, R.S. Yassin, L.S. Sherman, CD44 associates with EGFR and erbB2 in metastasizing mammary carcinoma cells, Appl. Immunohistochem. Mol. Morphol. Aimm 10 (2002) 34–39.
[40] R. Kuns, J.L. Kissil, I.F. Newsham, T. Jacks, D.H. Gutmann, L.S. Sherman, Protein 4.1B expression is induced in mammary epithelial cells during pregnancy and regulates their proliferation, Oncogene 24 (2005) 6502–6515.
[41] L. Fu, Z. Gao, X. Zhang, H.T. Ying, H.K. Goh, H. Geng, N. Shimizu, J. Tsuchiyama, G.S, Frequent concomitant epigenetic silencing of the stress-responsive tumor suppressor gene CADM1, and its interacting partner DAL-1 in nasal NK/T-cell lymphoma, Int. J. Cancer 124 (2010) 1572–1578.