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*Original Article*

# Upregulation of Atoh1 correlates with favorable survival in gastrointestinal stromal tumor

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**Abstract:** Atonal homolog 1 (Atoh1) is crucial to the differentiation of many cell types and participates in tumorigenesis and progression. However, the expression of Atoh1 in gastrointestinal stromal tumors (GIST) and its relationship to clinical characteristics of this disease remain poorly understood. In this study, immunohistochemical analysis using tissue microarray (TMA) was employed to evaluate the expression of Atoh1 in GIST and the correlation between Atoh1 expression and clinicopathological features of GIST as well as patient outcome. High Atoh1 cytoplasmic expression was observed in 77.22% of patients with GIST, which was related to the mitotic index ($P$ = 0.010) and AFIP-Miettinen risk classification ($P$ = 0.045). High Atoh1 nuclear expression was seen in 69.49% of cases, which was associated with mitotic index ($P$ = 0.003) and AFIP-Miettinen risk classification ($P$ = 0.001). The Kaplan-Meier method and log-rank test indicated that high Atoh1 cytoplasmic expression, high Atoh1 nuclear expression, small tumor diameter, low mitotic index and TNM stage significantly correlated with improved survival of GIST patients. Overall, the data suggest that Atoh1 high expression correlates with a good prognosis and it may serve as a favorable prognostic factor for GIST. These results also support a role for Atoh1 as a tumor suppressor gene in GIST.

**Keywords:** Atoh1, GIST, tissue microarray, immunohistochemistry, prognosis

## Introduction

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal neoplasms of the gastrointestinal tract with an annual incidence of 10-13 per million people [1, 2]. They are usually detected incidentally during radiologic imaging, endoscopic or surgical procedures [3]. Depending on the size, location and presence of mucosal ulceration, they are mostly diagnosed in the evaluation of patients with abdominal pain, upper gastrointestinal bleeding, dyspepsia, intestinal obstruction, and so forth [4]. GISTs can be located anywhere in the gastrointestinal tract, the most common sites being the stomach (60-70%), followed by small intestine (20-30%), colorectal (5%), and esophagus (< 5%) [5, 6]. GISTs are thought to arise from interstitial cells of Cajal or Cajal-like precursor cells from the muscular plexus in the gut wall [7, 8].

The clinical course of GIST ranges from benign disease and microGIST to highly malignant and inoperable disease [9, 10]. Histologically, they are mesenchymal spindle cells and are immunohistochemically positive for tyrosine kinase c-kit (CD117), platelet-derived growth factor receptor α (PDGFR α) and CD34. These markers have been reported to play important roles in the progression and diagnosis of GISTs [5, 11, 12]. The most specific and sensitive immunohistochemical marker is CD117 and around 95% of GISTs express CD117 [13-15]. Currently, surgery is the treatment of choice for non-metastatic GISTs [16, 17]. Postoperative prognosis correlates with tumor size and mitotic index, and recurrence is associated with a worse outcome [18]. In unresectable or metastatic GISTs, the current first line treatment is imatinib mesylate (Glivec), a receptor tyrosine kinase inhibitor. It has been shown to be effective and well tolerated in clinical trials [19, 20], and adjuvant imatinib treatment prolongs both overall and recurrence-free survival of patients at high risk of recurrence [21]. Therefore, there is an urgent need for novel biomarkers for determining prognosis and to guide molecular targeted therapy.

Atonal homolog 1 (Atoh1), also called the human atonal homolog 1 (Hath1) gene in humans and the mouse atonal homolog 1 (Math1) gene in mice, is a candidate gene that plays a pivotal role in mucous cell metaplasia. Atoh1 is a basic helix-loop-helix (bHLH) transcription factor that is required for intestinal secretory cell differentiation [22]. Recent studies report the loss of Atoh1 expression in human lung cancer [23] and colon cancer [24-26]. The mechanism of development of primary human tumors arising in Atoh1-dependent tissues includes both genetic and epigenetic silencing. Screening for Atoh1 expression, deletion and methylation might be a useful diagnostic tool for early detection and treatment of colon cancer [24]. In GIST, the association between Atoh1 expression and clinical outcome has not been investigated. The potential of Atoh1 as a candidate for molecular targeted therapy of GIST requires further exploration.

In the present study, we investigated Atoh1 protein expression in a number of GIST samples by immunohistochemistry using tissue microarray (TMA) sections. Moreover, we assessed the associations between Atoh1 expression and clinicopathological factors to determine its clinicopathological significance in a selected group of GIST patients. Finally, we evaluated the prognostic significance of the Atoh1 protein expression level in GIST.

## Materials and methods

### *Patients and tissue microarray (TMA) analysis*

Formalin-fixed paraffin-embedded GIST specimens (n = 180) from patients who underwent surgery between 2003 and 2010 were obtained from The Nanjing First Hospital affiliated with Nanjing Medical University and The Affiliated Hospital of Nantong University. Clinical data (including sex, age, tumor diameter, gross classification, tumor location, tumor grade, follow-up including 5-year survival and other information) were obtained from the medical records of each patient. The diagnosis of GIST was based on histopathological appearance and positive immunohistochemical staining for c-KIT. None of the patients received adjuvant chemotherapy, radiation therapy or immunotherapy. Survival was calculated from the date of surgery until death or last follow-up. Representative 2.0 mm tissue cores from each patient were used to conduct TMA analysis (Shanghai Outdo Biotech, Shanghai, China). Ethical approval for this study was obtained from The Human Research Ethics Committee of the local hospitals.

### *Immunohistochemical (IHC) analysis*

For IHC analysis, the TMA sections were deparaffinized in 100% xylene and rehydrated in graded ethanol solutions. The sections were then boiled under pressure in citrate buffer (pH 6.0) for 5 min for antigen retrieval. TMA sections were incubated for 1 h with a primary anti-Atoh1 antibody (Biovision, San Francisco, USA) diluted 1:200 in TBS containing 1% bovine serum albumin. After washing, these sections were incubated with anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Atoh1 immunostaining was evaluated independently by two trained pathologists who were unaware of the clinical background of the cases. Positivity of cell staining was recorded as a percentage (0-100%). Staining intensity was graded on a scale of 0 (negative) to 3 (strong). The final Atoh1 staining score was a product of the intensity grading and percentage of positive cells.

The cutoff point for the Atoh1 expression score that was statistically significant in terms of OS (overall survival) was determined using the X-tile software program (Rimm Lab, Yale University, New Haven, CT) as described elsewhere [27]. The degree of staining was quantified using a two-level grading system, and staining scores were defined as follows: for Atoh-1 cytoplasmic, 0-90 was regarded as low expression while 91-300 was regarded as high expression; for Atoh-1 nuclear, 0-15 was regarded as low expression while 16-300 was regarded as high expression.

### *Statistical analysis*

Statistical analyses were carried out using SPSS V.20.0 software (SPSS Inc, Chicago, IL, USA). Relationships between clinicopathological factors and Atoh1 expression were examined with $\chi^2$ tests. For the TMA slides, the following clinical data were assessed: sex, age, tumor diameter, gross classification, tumor location, tumor grade, and other clinicopathological information. Survival curves were estimated using the Kaplan-Meier method. Univariate and multivariate analyses were evaluated using Cox proportional hazards regression models. For all statistical analyses, *P*-values less than 0.05

**Table 1.** Association of ATOH-1 expression with clinical characteristics of GIST

| Groups | N | Cytoplasm staining of ATOH-1 | | | | Nucleus staining of ATOH-1 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Low or no expression (%) | High expression (%) | Pearson $x^2$ | *P* value | Low or no expression (%) | High expression (%) | Pearson $x^2$ | *P* value |
| Total | 180 | 41 (22.78) | 139 (77.22) | | | 54 (30.51) | 123 (69.49) | | |
| Gender | | | | | | | | | |
| Male | 72 | 21 (29.17) | 51 (70.83) | 3.206 | 0.073 | 25 (35.21) | 46 (64.79) | 1.024 | 0.312 |
| Female | 92 | 16 (17.39) | 76 (82.61) | | | 25 (27.78) | 65 (72.22) | | |
| Unknown | 16 | 4 | 12 | | | 4 | 12 | | |
| Age | | | | | | | | | |
| ≤ 60 years | 96 | 24 (25.00) | 72 (75.00) | 0.954 | 0.329 | 30 (31.58) | 65 (68.42) | 0.000 | 0.982 |
| > 60 years | 65 | 12 (18.46) | 53 (81.54) | | | 20 (31.75) | 43 (68.25) | | |
| Unknown | 19 | 5 | 14 | | | 4 | 15 | | |
| Tumor size | | | | | | | | | |
| < 5 cm | 38 | 5 (13.16) | 33 (86.84) | 2.969 | 0.227 | 13 (35.14) | 24 (64.86) | 0.863 | 0.650 |
| 5-10 cm | 77 | 19 (24.68) | 58 (75.32) | | | 22 (28.57) | 55 (71.43) | | |
| > 10 cm | 38 | 11 (28.95) | 27 (71.05) | | | 13 (36.11) | 23 (63.89) | | |
| Unknown | 27 | 6 | 21 | | | 6 | 21 | | |
| Mitotic index (per 50 HPFs) | | | | | | | | | |
| 0-5 | 68 | 14 (20.59) | 54 (79.41) | 9.230 | 0.010* | 14 (20.59) | 54 (79.41) | 11.555 | 0.003* |
| 6-10 | 46 | 7 (15.22) | 39 (84.78) | | | 15 (34.09) | 29 (65.91) | | |
| > 10 | 32 | 14 (43.75) | 18 (56.25) | | | 17 (54.84) | 14 (45.16) | | |
| Unknown | 34 | 6 | 28 | | | 8 | 26 | | |
| Gross classification | | | | | | | | | |
| Single nodule | 13 | 5 (38.46) | 8 (61.54) | 0.160 | 0.689 | 1 (7.69) | 12 (92.31) | 2.934 | 0.087 |
| Multiple nodules | 22 | 7 (31.82) | 15 (68.18) | | | 7 (33.33) | 14 (66.67) | | |
| Unknown | 145 | 29 | 116 | | | 46 | 97 | | |
| Tumor location | | | | | | | | | |
| Stomach | 79 | 17 (21.52) | 62 (78.48) | 0.334 | 0.846 | 23 (29.49) | 55 (70.51) | 0.267 | 0.875 |
| Intestine | 55 | 13 (23.64) | 42 (76.36) | | | 17 (32.08) | 36 (67.92) | | |
| Others | 26 | 7 (26.92) | 19 (73.08) | | | 9 (34.62) | 17 (65.38) | | |
| Unknown | 20 | 4 | 16 | | | 5 | 15 | | |
| AFIP-Miettinen risk classification | | | | | | | | | |
| Very low-low risk | 30 | 4 (13.33) | 26 (86.67) | 4.028 | 0.045* | 2 (6.67) | 28 (93.33) | 10.298 | 0.001* |
| Moderate-high risk | 44 | 15 (34.09) | 29 (65.91) | | | 17 (40.48) | 25 (59.52) | | |
| Unknown | 106 | 22 | 84 | 6.019 | 0.111 | 35 | 70 | | |
| Grade | | | | | | | | | |
| I | 47 | 10 (21.28) | 37 (78.72) | | | 11 (23.40) | 36 (76.60) | 6.388 | 0.094 |
| II | 56 | 10 (17.86) | 46 (82.14) | | | 16 (29.09) | 39 (70.91) | | |
| III | 27 | 8 (29.63) | 19 (70.37) | | | 13 (50.00) | 13 (50.00) | | |
| IV | 15 | 7 (46.67) | 8 (53.33) | | | 6 (42.86) | 8 (57.14) | | |
| unknown | 35 | 6 | 29 | | | 8 | 27 | | |

*$P < 0.05$; HPFs: high-power fields.

were regarded as statistically significant. All statistical tests were two-sided.

## Results

### *Clinical features of GIST*

The average age of the 180 patients was 57.78 years (range, 16-96 years). There were 38 cases with a tumor diameter < 5 cm, 77 with a tumor diameter between 5-10 cm, and 38 with tumor diameter ≥ 10 cm. The location of GIST included the stomach (79 cases), intestine (55 cases), esophagus (7 cases), and other organs (19 cases). The mitotic index per 50 high-power fields (HPFs) was < 5 in 68 GISTs, between 6 and 10 in 46 GISTs, and > 10 in 32 GISTs. All cases were stratified according to AFIP-Miettinen

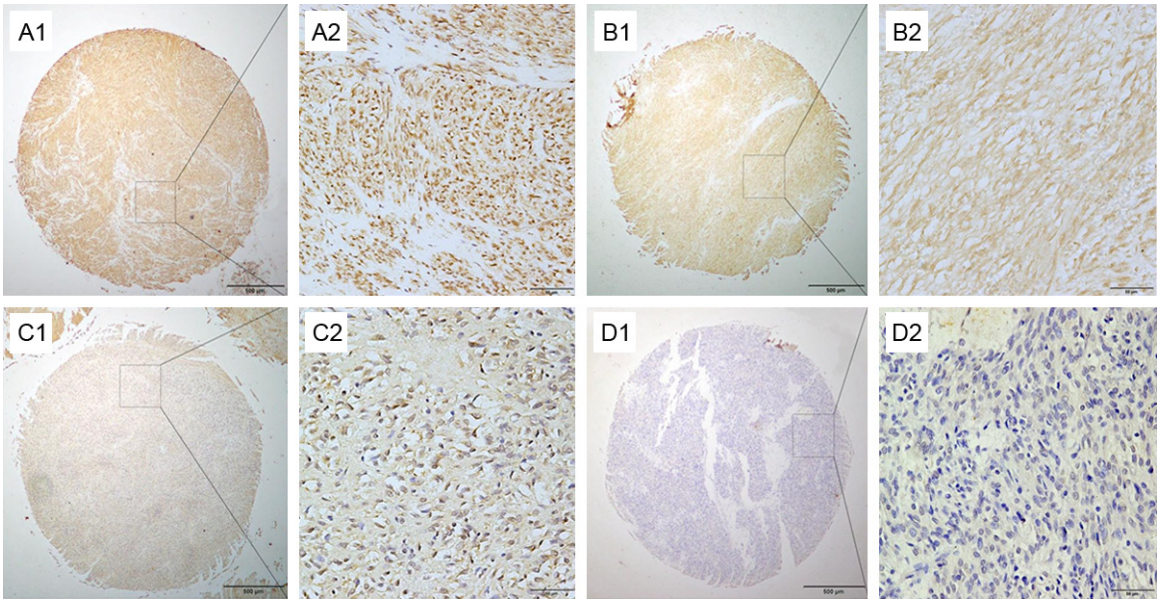


**Figure 1.** Representative immunohistochemistry (IHC) images showing expression of Atoh1 in tissue microarray sections of GIST. (A1) and (A2) Positive staining in the cytoplasm and nucleus. (B1) and (B2) high Atoh1 cytoplasmic expression. (C1) and (C2) High Atoh1 nuclear expression. (D1) and (D2) A negative IHC reaction. Original magnification was ×40 for (A1-D1); and ×400 for (A2-D2).

risk classification (very low to low risk, 30 cases; moderate to high risk, 44 cases). Detailed clinicopathological data are shown in **Table 1**.

*Expression of Atoh1 in GIST by IHC analysis*

We performed IHC analysis to examine Atoh1 expression in GIST. Positive staining was localized mainly in the cytoplasm and nucleus of GIST cells. High Atoh1 cytoplasmic expression was detected in 139 (77.22%) of 180 cases of GIST. High Atoh1 nuclear expression was seen in 123 (69.49%) of 177 cases of GIST. Typical IHC staining patterns for Atoh1 in GIST are shown in **Figure 1**.

*Association between Atoh1 expression and clinicopathological parameters*

The relationship between high Atoh1 expression and clinicopathological features of 180 cases of GIST is shown in **Table 1**. High Atoh1 cytoplasmic expression was related to mitotic index ($P = 0.010$) and AFIP-Miettinen risk classification ($P = 0.045$). High Atoh1 nuclear exprression was related to mitotic index ($P = 0.003$) and AFIP-Miettinen risk classification ($P = 0.001$). In contrast, no statistically significant correlation was found between Atoh1 expression and other clinical parameters, including sex, age, gross classification, and tumor location.

*Survival analysis*

Several known predictive factors of poor outcome in GIST were assessed to confirm that our cohort of patients were representative of those with GIST (**Table 2**). As expected, Atoh1 protein cytoplasmic overexpression ($P < 0.001$) and nuclear overexpression ($P = 0.008$) were significantly associated with 5-year survival by Cox regression univariate analysis. In addition, other prognostic factors such as tumor diameter ($P = 0.002$), mitotic index ($P < 0.001$), tumor grade ($P < 0.001$) were also statistically significant. All these factors were included in the multivariable analysis. Low Atoh1cytoplasmic expression ($P = 0.034$) and high mitotic index ($P = 0.002$) were identified as independent predictive factors for poor outcome. Kaplan-Meier survival cu-rves demonstrated that patients with high Atoh1 cytoplasmic expression and low mitotic index had a significantly longer survival time (**Figure 2**).

## Discussion

Although rare, GISTs are the most common mesenchymal tumor of the gastrointestinal

**Table 2.** Univariate and multivariable analysis of prognostic factors for 5-year survival in GIST

| | Univariate analysis | | | | Multivariate analysis | | | |
|---|---|---|---|---|---|---|---|---|
| | HR | *P* > \|z\| | 95% CI | | HR | *P* > \|z\| | 95% CI | |
| ATOH-1 cytoplasm expression | .202 | < 0.001* | .095 | .428 | .370 | 0.034* | .147 | .929 |
| High vs. low | | | | | | | | |
| ATOH-1 nucleus expression | .358 | 0.008* | .167 | .768 | | | | |
| High vs. low | | | | | | | | |
| Gender | .529 | 0.093 | .252 | 1.111 | | | | |
| Male vs. Female | | | | | | | | |
| Age (years) | .860 | 0.686 | .414 | 1.786 | | | | |
| ≤ 60 vs. > 60 | | | | | | | | |
| Tumor diameter (cm) | 2.468 | 0.002* | 1.389 | 4.382 | | | | |
| < 5 vs. 5-10 vs. > 10 | | | | | | | | |
| Mitotic index (per 50 HPFs) | 3.727 | < 0.001* | 2.240 | 6.201 | 19.451 | 0.002* | 2.844 | 133.036 |
| 0-5 vs. 6-10 vs. > 10 | | | | | | | | |
| Gross classification | 1.414 | 0.581 | .413 | 4.839 | | | | |
| Single vs. multiple | | | | | | | | |
| Tumor position | 1.244 | 0.379 | .765 | 2.023 | | | | |
| Stomach vs. intestine vs. other | | | | | | | | |
| AFIP-Miettinen risk classification | 2.451 | 0.068 | .936 | 6.417 | | | | |
| Very low-low risk vs. Moderate-high risk | | | | | | | | |
| Tumor grade | 2.306 | < 0.001* | 1.598 | 3.326 | | | | |
| Stage I-II vs. Stage III-IV | | | | | | | | |

*$P < 0.05$; HPFs: high-power fields.

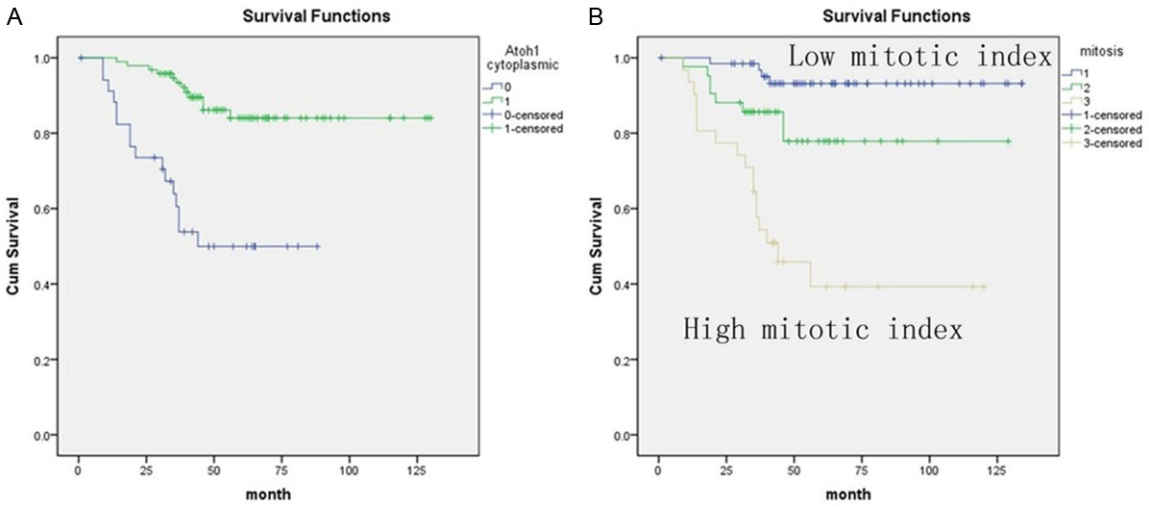


**Figure 2.** Analysis of survival of GIST patients by Kaplan-Meier method. A. Survival curves based on Atoh1 cytoplasmic expression. Atoh1 cytoplasmic = 1 is the high expression group (green line); Atoh1 cytoplasmic = 0 is the low and no expression group (blue line). B. Overall survival in cases with a low mitotic index was significantly longer than in cases with a high mitotic index.

tract [28]. GISTs are characterized by the presence of mutations in receptor tyrosine kinases; activating mutations are present in KIT and PDGFRA in approximately 80 and 10% of GISTs, respectively [1]. In GISTs, adjuvant therapy with imatinib has lead to dramatic improvements in long-term survival and delayed the development of metastasis [21, 29]. Approximately

60% of patients with GIST are cured by surgery alone, and imatinib therapy may benefit only a limited number of individuals [30]. Therefore, assessment of the postoperative risk of metastasis is important. In addition, there is an urgent need for novel biomarkers that relates to the mechanism of disease for determining prognosis and to guide therapy.

Recently, some studies reported the loss of ATOH1 expression in human colorectal cancer (CRC) [25, 26]. CRC is a common cancer with high mortality (36%) and represents 11% of all cancer deaths annually [31]. A growing number of studies have suggested that loss of ATOH1 strongly enhances the formation and progression of tumors. In turn, gain of ATOH1 strongly inhibits tumor cell growth in vitro in human cell lines [24, 26]. At present, the actual function of Atoh1 in GIST remains unclear. Therefore, we attempted to examine the relationship between Atoh1 expression and various clinicopathological parameters in GIST.

In the present investigation, Atoh1 protein expression in GIST tissues was evaluated using IHC, and results showed that 77.22% of cases exhibited high Atoh1 cytoplasmic expression while 69.49% of cases exhibited high Atoh1 nuclear expression. Furthermore, we found that strong Atoh1 expression in GIST correlated significantly with mitotic index and AFIP-Miettinen risk classification.

Our data clearly showed that high cytoplasmic and nuclear expression of Atoh1 was associated with significantly improved survival. Our results are in agreement with studies that reported that loss of Atoh1 expression in human lung cancer [23] and colon cancer [24-26]. Multivariate analysis indicated that mitotic index could be considered an independent factor for poor prognosis in GIST.

To our knowledge, this is the first report of the differential expression of Atoh1 in GIST, and indicates that Atoh1 may constitute a novel prognostic marker for GIST. Our findings demonstrated a high expression of Atoh1 in GIST specimens, and that the high expression was associated with a good prognosis. Further experiments are necessary to determine whether Atoh1 acts as a tumor suppressor in GIST. Studies elucidating the signaling pathways and potential mechanisms of Atoh1 in GIST are needed.

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## Disclosure of conflict of interest

None.

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