Clinicopathological significance of the microRNA-146a/WASP-family verprolin-homologous protein-2 axis in gastric cancer

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Key words
Gastric cancer, metastasis, miR-146a, therapy, WASF2

Gastric cancer (GC) is one of the most common malignancies, and cancer invasion and metastasis are the leading causes of cancer-induced death in GC patients. WASP-family verprolin-homologous protein-2 (WASF2), with a role controlling actin polymerization which is critical in the formation of membrane protrusions involved in cell migration and invasion, has been reported to possess cancer-promoting effects in several cancers. However, data of WASF2’s role in GC are relatively few and even contradictory. In this study, we analyzed WASF2 expression in GC tissues and their corresponding adjacent normal tissues. We found that WASF2 was upregulated in GC tissues and high level of WASF2 was associated with lymph node metastasis of GC. Through gain- and loss-of-function studies, WASF2 was shown to significantly increase GC cells migration and invasion, but had no effect on proliferation in vitro. Importantly, WASF2 was also found to enhance GC metastasis in vivo. Our previous research suggested that WASF2 was a direct target of microRNA-146a (miR-146a). Furthermore, we analyzed miR-146a's level in GC tissues and their corresponding adjacent normal tissues. We found that miR-146a was downregulated in GC tissues and low miR-146a level was associated with advanced TNM stage and lymph node metastasis. The level of WASF2 in GC tissues was negatively correlated with miR-146a expression and had inverse clinicopathologic features. The newly identified miR-146a/WASF2 axis may provide a novel therapeutic target for GC.

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Gastric cancer (GC) is one of the most common cancer types and the second leading cause of cancer-induced death. High metastasis potential is one of its characteristics. Most GC patients are diagnosed at advanced stages with distant metastasis. As metastasis accounts for 90% of cancer-related deaths, a better understanding of the metastasis of GC is urgent. It is helpful to identify new diagnostic and prognostic markers and to find novel targeted therapies.

Cell movement is critical for tumor migration, invasion, and metastasis. Recent work has reported that tumor cells can move in two modes: amoeboid mode and mesenchymal mode. Amoeboid movement is characterized by a rounded, blebbing morphology requiring high levels of actomyosin contractility. In contrast, mesenchymal movement is characterized by an elongated cellular morphology, resulting from Ral1/WASP-family verprolin-homologous protein-2 pathway-dependent actin assembly at the leading edge. When cells migrate, they form cellular protrusions called “ruffles” in which the fibrous actin cytoskeleton forms a mesh-like structure. WASF2 is necessary for branching of actin fibers from existing fibers to form a mesh-like structure. It acts as a scaffold for actin nucleation complexes from which a new actin fiber sprouts. It has been confirmed that WASF2 facilitates metastasis of several cancers, and WASF2 was expressed at high levels in breast cancer. Overexpression of WASF2 was seen in node-positive cases as well as in poorly differentiated tumors. A 3-D gel culture system further revealed that the formation of long protrusions, invadopodia, and invasion required WASF2 in MDA-MB-231 cells. Increased expression of WASF2 was also observed to correlate with poor prognosis of hepatocellular carcinoma. In addition, depletion of WASF2 by RNAi significantly suppressed membrane ruffling, cell invasion, and pulmonary metastasis of B16 mouse melanoma cells.

In terms of GC, it has been reported that increasing the expression of WASF2 is one of the mechanisms that MAC30/transmembrane protein 97 facilitates the proliferation and mobility of GC cells. Similarly, researchers found that downregulation of WASF2 mediated the inhibition of GC cell invasion by overexpression of REIC. These two reports both suggested that WASF2 acted as an oncogene in GC. However, a recent study found that downregulation of WASF2 expression in GC was significantly correlated with lymph node metastasis, and knockdown of WASF2 could increase metastatic potential by promoting growth, invasiveness, and motility. In our previous research, we found that WASF2 was a direct target of microRNA-146a (miR-146a) and involved in the suppression of migration and invasion of GC cells by miR-146a's level in GC tissues and their corresponding adjacent normal tissues. We found that miR-146a was downregulated in GC tissues and low miR-146a level was associated with advanced TNM stage and lymph node metastasis. The level of WASF2 in GC tissues was negatively correlated with miR-146a expression and had inverse clinicopathologic features. The newly identified miR-146a/WASF2 axis may provide a novel therapeutic target for GC.
In this study, we focused on the clinical significance of the miR-146a/WASF2 axis in GC tissues, the function of WASF2 in proliferation, migration, and invasion of GC cells, and its metastasis potential in vivo. We found that WASF2 was upregulated in GC tissues and associated with lymph node metastasis. Upregulation of WASF2 could suppress migration and invasion of GC cells but had no effect on proliferation. Further studies showed that enforced expression of WASF2 in GC cells significantly increased their capacity to develop distal pulmonary metastases in vivo. As a regulator of WASF2, miR-146a expression was negatively associated with WASF2 levels in vivo and its metastasis potential WASF2 in proliferation, migration, and invasion of GC cells, of the miR-146a/WASF2 axis in GC tissues, the function of pulmonary metastases GC cells significantly increased their capacity to develop distal metastasis had lymph node metastasis verified by pathological analysis. The clinicopathologic characteristics of all samples (patient age and gender, tumor size and differentiation, TNM stage, and lymph node metastasis) are shown in Table 1. This study was approved by the Clinical Research Ethics Committee of Fudan University, and written informed consent was obtained from each patient. Human gastric cancer cell lines (MKN-45 and HGC-27) and HEK293T cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37°C in humidified air containing 5% CO2 in RPMI-1640 (MKN-45 and HGC-27) or DMEM (HEK293T) supplemented with 10% FBS.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from GC tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). MicroRNAs were isolated with the miRvana RNA isolation kit (Ambion, Carlsbad, CA, USA), and reverse transcribed with specific RT primers, quantified with a TaqMan probe, and normalized by U6 small nuclear RNA using TaqMan miRNA assays (Applied Bioso, Carlsbad, CA, USA). The relative expression levels were calculated with the 2^(-ΔΔCT) method.

Materials and Methods

Tissue samples and cell lines. Human GC tissues and their corresponding non-tumorous tissues were collected at the time of surgical resection from 60 patients with GC from January 2012 to June 2013 at the Department of General Surgery, Zhongshan Hospital, Fudan University (Shanghai, China). None of the GC patients had received radiotherapy or chemotherapy treatment before biopsy. Patients diagnosed with metastasis had lymph node metastasis verified by pathological analysis. The clinicopathologic characteristics of all samples (patient age and gender, tumor size and differentiation, TNM stage, and lymph node metastasis) are shown in Table 1. This study was approved by the Clinical Research Ethics Committee of Fudan University, and written informed consent was obtained from each patient. Human gastric cancer cell lines (MKN-45 and HGC-27) and HEK293T cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37°C in humidified air containing 5% CO2 in RPMI-1640 (MKN-45 and HGC-27) or DMEM (HEK293T) supplemented with 10% FBS.

Table 1. Expression of WASP-family verprolin-homologous protein-2 (WASF2) detected by immunohistochemistry and clinicopathologic features of gastric cancer patients (n = 60)

| Variable                  | WASF2 expression | P-value |
|---------------------------|------------------|---------|
|                           | Low (n = 24)     | High (n = 36) |
| Sex                       |                  |         |
| Female                    | 12               | 10      | 0.104 |
| Male                      | 12               | 26      |       |
| Age, years                |                  |         |
| ≤60                       | 9                | 11      | 0.590 |
| >60                       | 15               | 25      |       |
| Tumor size, cm            |                  |         |
| ≤5                        | 9                | 19      | 0.297 |
| >5                        | 15               | 17      |       |
| Tumor differentiation     |                  |         |
| Well and moderate         | 12               | 20      | 0.793 |
| Poor                      | 12               | 16      |       |
| TNM stage                 |                  |         |
| I                         | 5                | 3       | 0.089 |
| II                        | 10               | 13      |       |
| III                       | 6                | 19      |       |
| IV                        | 3                | 1       |       |
| Lymph node metastasis     |                  |         |
| Yes                       | 20               | 18      | 0.013*|
| No                        | 4                | 18      |       |

*Statistically significant.
chambers coated without or with Matrigel (BD Pharmingen, San Jose, CA, USA). Gastric cancer cells were suspended in serum-free medium at $3 \times 10^5$/mL (migration) or $1 \times 10^5$/mL (invasion), and 0.1 mL suspension was added in the top chamber and incubated for 48 h. The bottom chamber was filled with medium containing 10% FBS as chemoattractant. Cells that migrated to the underside of the membrane were stained with Giemsa (Sigma Chemical Company, Saint Louis, MO, USA) and counted. All experiments were performed in triplicate.

**In vivo metastasis assays.** For in vivo pulmonary metastasis assays, $1 \times 10^6$ MKN-45 cells infected with either the shWASF2 lentivirus or the control lentivirus were suspended in 200 mL PBS and then injected into nude mice (5-week-old BALB/c-nu/nu, $n = 7$ per group) through the lateral tail vein. Five weeks after injection, mice were killed and the lungs were extracted. Visible lung metastases were measured and counted with a fluorescence microscope. Then the lungs were paraffin-embedded, sectioned, and stained with HE. All research involving animals complied with protocols approved by the Committee on Animal Care of Zhongshan Hospital, Fudan University.

**Statistical analysis.** Data were shown as mean ± SEM and analyzed using Student’s t-test or the two-tailed Mann–Whitney U-test. All statistical analyses were carried out with spss 16.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

### Results

**WASP-family verprolin-homologous protein-2 frequently upregulated in GC and associated with lymph node metastasis.** In order to explore the expression and significance of WASF2 in GC, we first evaluated the protein level of WASF2 in 60 cases of GC and their matched adjacent non-cancerous tissues by immunohistochemical staining. The results showed that WASF2 was significantly upregulated in GC tissues when compared with their matched adjacent non-cancerous tissues ($P < 0.01$; Fig. 1a,b). Strong staining (score 2 or 3) was observed in 36 of 60 GC tissues (60%) but only in 17 of 60 cases from non-cancerous gastric tissues (29%) ($P < 0.001$; Fig. 1c). The correlation between WASF2 expression and clinico-pathological features of GC patients was analyzed (Table 1), which showed that WASF2 levels were remarkably higher in GC patients with lymph node metastasis than in those without ($P = 0.013$).

**WASP-family verprolin-homologous protein-2 promotes migration and invasion of GC In Vitro.** Our previous study found that WASF2 level was the highest in MKN-45 among four GC cell lines (MKN-45, SGC-7901, MGC-803, and HGC-27) and the lowest in HGC-27. So, in an attempt to understand the function of WASF2 in GC cells, we established HGC-27 cell lines stably expressing WASF2 by lentivirus, and knocked down…

![Fig. 1. WASP-family verprolin-homologous protein-2 (WASF2) is upregulated in human gastric cancer (GC). (a) Representative staining intensities of WASF2 in GC tissues. (a) GC case 1, score 0; (b) GC case 2, score 1; (c) GC case 3, score 2; (d) GC case 4, score 3. (b) Two cases of WASF2 immunohistochemical staining in GC tissues and matched non-cancerous tissues (NT). Case 1, adenocarcinoma; case 2, mucinous carcinoma. (c) Statistical analysis of WASF2 expression according to immunohistochemical staining scores.](https://www.wileyonlinelibrary.com/journal/cas)
WASF2 in MKN-45 cell lines. Successful overexpression and underexpression of WASF2 was confirmed by Western blot analysis (Fig. 2a). Mobility of MKN-45 cells in wound healing assays was significantly decreased with WASF2 knockdown (Fig. 2b); however, overexpression of WASF2 increased wound healing in HGC-27 cells (Fig. 2c). Similarly, we found that knockdown of WASF2 suppressed the migration and invasion of GC cells in Matrigel chamber assays, whereas upregulation of WASF2 resulted in a dramatic increase in GC cell migration and invasion (Fig. 2d,e). However, WASF2 had no effect on the proliferation of GC cells (Figs. S1,S2, Doc. S1).

**Knockdown of WASF2 suppresses distal pulmonary metastases of GC in vivo.** Given that WASF2 promoted the migration and invasion of GC cells in vitro, we further determined whether WASF2 could affect tumor metastasis in vivo. MKN-45 cells with lentivirus-mediated WASF2 knockdown or with empty vector as control were injected into nude mice through the lateral tail vein. Five weeks later, the mice were killed and the lungs were removed. As shown in Figure 3(a), fewer metastatic nodes were observed on the surface of lungs in the WASF2 knockdown group when compared to the control. Staining with HE further confirmed that both the number and size of the lung micrometastatic nodes were significantly lower in the WASF2 underexpression group ($P = 0.018$; Fig. 3a,b).

**MicroRNA-146a level inversely correlated with WASF2 protein expression in GC and has an opposite relationship with clinicopathologic features.** In our published data, we found that miR-146a is a tumor suppressor in GC cells through binding.

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**Fig. 2.** WASP-family verprolin-homologous protein-2 (WASF2) promotes the migration and invasion of gastric cancer cells in vitro. (a) Western blot was used to detect WASF2 levels in HGC-27 cells after infection with WASF2 expression lentivirus, and in MKN-45 cells with shWASF2 lentivirus. (b, c) Cell migration was assessed by scratch assay. (b) Wound healing rate in MKN-45 cells transfected with shWASF2 lentivirus was significantly decreased. (c) Wound healing rate in HGC-27 cells transfected with WASF2 expression lentivirus was obviously increased. (d) Transwell migration assays of MKN-45 cells infected by shWASF2 lentivirus and HGC-27 cells by WASF2 expression lentivirus. LEFT, representative images; right, quantification of five randomly selected fields. Values shown are expressed as mean ± SD. (e) Transwell Matrigel invasion assays of MKN-45 cells infected by shWASF2 lentivirus and HGC-27 cells by WASF2 expression lentivirus. MOCK, cells infected by empty WASF2 expression lentivirus; NC, cells infected by empty shWASF2 lentivirus.
the 3’-UTR of WASF2.\(^\text{(15)}\) We wanted to determine whether miR-146a levels are negatively associated with WASF2 expression in GC tissues and to further clarify the role of the miR-146a/WASF2 axis in GC. To confirm miR-146a levels in GC patients, quantitative real-time PCR analysis was carried out in 60 coupled samples of GC. The results showed that miR-146a was significantly downregulated in GC tissues when compared with the corresponding non-tumorous samples (median, 0.120 vs. 0.728, \(P < 0.001\); Fig. 4a). Next, the 60 clinical cases were divided into two groups based on miR-146a expression in cancer tissues: low miR-146a expression (\(n = 30\)) and high miR-146a expression (\(n = 30\)). The correlation between miR-146a level and clinicopathologic features of GC are summarized in Table 2. In the correlation analysis between miR-146a and clinicopathological features, it suggested that the level of miR-146a had a negative correlation with TNM stage and lymph node metastasis status (\(P = 0.018\) and \(P < 0.001\), respectively; Table 2). The GC specimens were further stratified based on TNM stage; the results showed that miR-146a levels were gradually decreased with stage progression, especially between stage II and III (\(P = 0.009\); Fig. 4b). Similarly, when stratified based on the status of lymph node metastasis, the expression of miR-146a was significantly downregulated in GC with lymph node metastasis when compared with those
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Table 2. Expression of microRNA-146a (miR-146a) detected by real-time PCR and clinicopathologic features of gastric cancer patients (n = 60)

| Variables             | miR-146a expression | P-value |
|-----------------------|---------------------|---------|
|                       | Low (n = 30)        |         |
|                       | High (n = 30)       |         |
| Sex                   |                     |         |
| Male                  | 12                  | 10      | 0.789   |
|                       | 18                  | 20      |         |
| Age, years            |                     |         |
| ≤50                   | 8                   | 12      | 0.412   |
| >60                   | 22                  | 18      |         |
| Tumor size, cm        |                     |         |
| ≤5                    | 12                  | 16      | 0.438   |
| >5                    | 18                  | 14      |         |
| Tumor differentiation |                     |         |
| Well and moderate     | 18                  | 14      | 0.438   |
| Poor                  | 12                  | 16      |         |
| TNM stage             |                     |         |
| I                     | 2                   | 6       | 0.018*  |
| II                    | 8                   | 15      |         |
| III                   | 16                  | 9       |         |
| IV                    | 4                   | 0       |         |
| Lymph node metastasis |                     |         |
| Yes                   | 26                  | 12      | <0.001* |
| No                    | 4                   | 18      |         |

*Statistically significant.

without lymph node metastasis (P < 0.0001; Fig. 4c). Further attempts to find the correlation between WASF2 expression and miR-146a showed that high expression of WASF2 was more likely to be seen in GC tissues with low levels of miR-146a. In contrast, low expression of WASF2 was significantly correlated with high levels of miR-146a in GC tissues (P = 0.003; Fig. 4d).

Discussion

In the present study, we found that the level of WASF2 was commonly higher in GC tissues when compared with corresponding adjacent normal tissues. Higher WASF2 levels were significantly associated with lymph node metastasis of GC. Furthermore, WASF2 overexpression by lentivirus was found to promote migration and invasion in GC cells in vitro and knockdown of WASF2 suppress GC metastasis in vivo, but have no effect on proliferation. Conversely, downregulation of WASF2 inhibited migration and invasion of GC cells. These results indicate that WASF2 might be a novel oncogene regulating metastasis of GC.

WASP-family verprolin-homologous protein-2 is a member of the Wiskott–Aldrich syndrome protein (WASP) family that consists of the WASP (WASP and N-WASP) and WASF (WASF1, WASF2, and WASF3) subfamilies. Through the actin-related protein 2/3 complex, WASF2 induces rapid actin polymerization, resulting in the formation of lamellipodia and the initiation of amoeboid movement. Atypical WASF2 expression and activity were found in several types of human cancer including breast cancer, lung cancer, and colorectal cancer. Functional assays showed that WASF2 promoted cancer invasion and metastasis and correlated with poor prognosis.

Consistent with our study, Xu et al. suggested that increasing the expression of WASF2 is one of the mechanisms that MAC30/transmembrane protein 97 facilitates the proliferation and mobility of GC cells. Similarly, Xu et al. also found that downregulation of WASF2 mediated the inhibition of GC cells invasion by REIC overexpression. Interestingly, in research by Jia et al., they reached the opposite conclusion, that WASF2 was downregulated in GC and knockdown of WASF2 could increase metastatic potential by promoting the growth, invasiveness, motility, and adhesiveness of GC cells. How do we reconcile the above opposing conclusions? It has been discovered that WASF2 can act as a tumor suppressor in benign tumors (stabilizing cell–cell adhesion) and, at the same time, as a driver of epithelial–mesenchymal transition in migrating cancer cells (inducing actin meshwork formation at the cellular leading edge). The quantitative regulation of WASF isoform levels may underlie the dual function of WASF2 in invasion. The WASF isoforms share components of the WASF complex and there is dynamic equilibrium among them. In the earlier stages of cancer progression, cancer cells inhibit WASF2 (possibly through WASF3 overproduction, which is verified as an invasion promoter in several cancers) to acquire dissociating abilities. Later, some cancer cells may restore WASF2 activity (possibly through re-repression of WASF3 or through overexpression of Abi) to increase cancer cell motility, which leads to metastatic progression. As most patients with GC are diagnosed at advanced stages, results show that WASF2 is overexpressed in GC tissues compared with corresponding adjacent normal tissue.

Our previously published data showed that WASF2 was modulated by miR-146a as a direct target, and mainly mediated miR-146a’s suppression of the migration and invasion of GC cells. In this study, we directly focused on the comparative analysis of miR-146a levels in GC tissues and their corresponding adjacent normal tissues. It was revealed that miR-146a was frequently downregulated in human GC, and that downregulated miR-146a was significantly associated with advanced clinical stage and lymph node metastasis. The inverse correlation between the level of miR-146a and WASF2 in GC tissues further proved that WASF2 was a target of miR-146a.

MicroRNA-146a was initially discovered during a systematic study aiming to identify miRNAs that play potential roles in the innate immune response to microbial infection. In addition to acting as a modulator of the innate immune response, miR-146a also plays a role in the modulation of adaptive immunity. It is well known that tumor escapes from immune recognition, and tumor-mediated suppression of anti-tumor immunity plays a part in the development and progression of neoplasms. Consistent with this concept, many recent studies have suggested a role for miR-146a in the initiation and maintenance of neoplastic processes. MicroRNA-146a exerts an antitumor effect in breast cancer, pancreatic cancer, and GC by targeting the 3′-UTR of EGFRI mRNA. Other studies showed that the miR-146a/NOTCH1 and miR-146a/C-X-C chemokine receptor type 4 axes play an important role in glioblastomas and leukemia, respectively. Together with our results, Kogo and coworkers examined miR-146a levels in 90 GC samples and found that miR-146a levels in cancer tissues were significantly lower than those in corresponding non-cancerous tissues. Lower levels of miR-146a were associated with lymph node metastasis and venous invasion, depending on higher levels of interleukin 1 receptor associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) as its targets. The link between
miR-146a and GC is also related to the possible effects of this miRNA on the inflammatory response of Helicobacter pylori, one of the most important causes of GC. Helicobacter pylori induces miR-146a expression in a nuclear factor-kB-dependent way. In turn, miR-146a downregulates the expression of target genes, including IRAK1, TRAF6, IL-8, and, through this mechanism, modulates the inflammatory response. Above all, miR-146a plays roles in multiple physiopathologic processes, mainly through an immune-associated mechanism. In this study, the miR-146a/WASF2 axis we first illustrated may expand the mechanism through which miR-146a plays in GC, and the miR-146a/WASF2 axis may have therapeutic potential to suppress GC metastasis.

As cancer is a complex process involving multiple factors and steps, the efficacy of anticancer agents designed to target single therapeutic proteins is often suboptimal. MicroRNAs are single-stranded small non-coding RNAs that can regulate gene expression by hindering translation or degrading target mRNA post-transcriptionally. MicroRNA-based therapeutics may be attractive alternatives to protein-based cancer therapy as single onco-miRNAs always downregulate multiple anti-oncogenes, while tumor suppressor miRNAs downregulate several onco-genes. Considering miR-146a suppresses gastric metastasis by targeting EGFR, IRAK1, TRAF6, and WASF2 (as shown in this study), it suggests that miR-146a may be an attractive therapeutic target for GC therapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information can be found online in the supporting information tab for this article:

Fig. S1. Effect of WASP-family verprolin-homologous protein-2 (WASF2) on proliferation curves of gastric cancer cells. (a) MKN-45 cells were stably transfected with pLKO.1-TRC or pLKO.1-shWASF2. (b) HGC-27 cells were transfected with pCDH or pCDH-WASF2. Cells were seeded

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in 96-well plates and cell growth was monitored every 24 h with Cell Counting Kit-8. Results are presented as mean ± SD of four replicates.

**Fig. S2.** Effect of WASP-family verprolin-homologous protein-2 (WASF2) on anchorage-independent growth of gastric cancer cells. (a) MKN-45 cells were stably transfected with pLKO.1-TRC or pLKO.1-shWASF2. (b) HGC-27 cells were transfected with pCDH or pCDH-WASF2. Cells were seeded in 6-well plates for 2 weeks and the colony numbers were counted. Colony formation rates were scored and presented as mean ± SD of four replicates.

**Doc. S1.** Supplementary methods.