Expression and Clinicopathological Significance of CD9 in Gastrointestinal Stromal Tumor

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INTRODUCTION

Knowledge on the etiology, pathogenesis, diagnosis and treatment of gastrointestinal stromal tumors (GIST) have reached maturity in recent years. GIST prognosis has been also improved significantly. However, the reason that only certain parts of GIST patients relapse or metastasize after operation in the same site remains unclear. Moreover, non-gastric stromal tumor (GST), intestinal and extra-gastrointestinal stromal tumor (EGIST) relapses or metastasizes more easily than GST, even with the same diameter and mitotic counts, which is a phenomenon that should be explored (1). Although metastasis mainly spreads through blood and implantation metastasis in GIST, the mechanism of metastasis remains unclear, and current studies on the mechanism of metastasis in GIST are limited.

CD9 protein that belongs to the transmembrane 4 superfamily (TM4SF) is a transmembrane glycoprotein. This protein is present in various tumors, which indicates that CD9 expression is inversely correlated with recurrence and/or metastasis, and can be utilized as a prognostic marker (2, 3). This study investigates CD9 expression in GIST and analyzes its correlation with clinicopathological significance, risk classification and prognosis.

MATERIALS AND METHODS

Patients and tissue samples

Surgical samples were selected from West China Hospital, Sichuan University of China between January 2002 and June 2010. The following inclusion criteria were used: pathologically confirmed negative surgical margins (R0) after resection, immunohistochemically confirmed CD117-positive tumor; no pre-operative imatinib mesylate (IM) treatment; no severe systemic disease or combined tumor. The exclusion criteria were as follows: less than 18 yr of age; pregnant or breastfeeding patients; with severe systemic disease or combined tumor; and poor compliance.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm paraffin sections. The sections were soaked through water and then incubated in 3% H₂O₂ methanol solution for 10 min. Soaked in 0.01 M/L EDTA (pH = 9.0) and repaired in a separated water environment for 10 min through pressure heating. Then, blocked with goat serum for 20 min at room temperature. Rabbit monoclonal CD9 antibody (purchased from Abcam Ltd., Cambridge, UK) was applied overnight with a dilution of 1:300 at 4°C
and incubated for 30 min with biotinylated goat anti-mouse immunoglobulin G. The sections were detected with a secondary antibody for 30 min at room temperature and visualized through incubation by using DAB (1:50 dilution) for 5 min to 10 min. The sections were then counterstained, dehydrated and mounted.

The stained specimens were reviewed by two pathologists who had no knowledge of the clinical status of the patients. The sections were scored semi-quantitatively based on previously described method (4), which considered cell staining intensity and percentage. Intensities were classified as 0 (no staining), 1 (weak staining), 2 (distinct staining), and 3 (very strong staining). The cell staining percentage was classified as 0 (< 10%), 1 (10% to 25%), 2 (26% to 50%), 3 (51% to 75%) and 4 (> 75%). The total score for each specimen was calculated by using the following equation: total score = score of intensity multiplied by score of percentage. Specimens with a total score > 3 were classified as CD9-positive (+), whereas specimens with total score ≤ 3 were classified as CD9-negative (−).

Data and statistical analysis
The last follow-up was conducted on December 31, 2012. Recurrence-free survival (RFS) is the date of operation to the date of recurrence and/or distant metastasis, which is the endpoint for GIST patients. Patients who survived without recurrence and/or metastasis were censored on the date of the last follow-up. The data were evaluated by using SPSS version 18.0. Measurement data were expressed as mean ± standard deviation. Categorical data from different groups were compared by using the chi-square test or Fisher’s exact test. Cumulative RFS was estimated by using the Kaplan–Meier method with a one-sided log-rank test. Hazard regression model and 95% confidence interval (CI) were described based on the Cox proportion HR model. \( P < 0.05 \) was considered statistically significant.

Ethics statement
The study protocol was approved by the institutional review board of West China Hospital, Sichuan University, China (No. 2013-64), and an informed consent was obtained from every of the patients.

RESULTS

Characteristics of the patients
A total of 74 patients included 50 males and 24 females, with a mean age of 52.9 ± 12.2 (from 29 to 84) yr. The pathological types included 38 GST, 23 intestinal GIST, and 13 EGIST. Based on The NCCN guideline for risk classification of GIST (5), 10 tumors were low risk, 11 were intermediate risk, and 53 were high risk.

CD9 expression in GIST and the relationship between CD9 expression and clinicopathological features of GIST
Fig. 1A shows that the immunohistochemical positive reaction product of CD9 was mainly localized in the cytoplasm and/or cell membrane. About 59.5% (44/74) of the 74 GIST specimens were classified as CD9-positive. By contrast, 40.5% (30/74) were classified as CD9-negative (Fig. 1B).

Table 1 summarizes the immunoreactivities in GIST. No significant association was observed between CD9 expression and age \( (P = 0.333) \), sex \( (P = 0.712) \), and location \( (P = 0.769) \) when CD9 expression was compared with various clinical features. However, a highly significant association was found between

![Fig. 1. CD9 immunohistochemistry in GIST. (A) CD9-positive expression in low-risk GIST (× 400), (B) CD9-negative expression in high-risk GIST (× 400).](http://dx.doi.org/10.3346/jkms.2013.28.10.1443)
CD9 expression and tumor diameter (P = 0.028), mitotic count (P = 0.035), and risk classification (P = 0.018).

Relationship between CD9 expression and RFS
The 74 patients were followed up regularly through telephone and outpatient visits. The average follow-up time was 49 months (6 to 132 months). 9 patients recurred, 16 patients had a distant metastasis, 5 patients recurred and merging with a distant metastasis. After excluding seven patients who received IM treatment post-operation and three who underwent a follow-up time of < three years post-operation, sixty-four patients left, who underwent at least 36 months of follow-up without post-operative IM adjuvant treatment. In the 64 patients, the three-year RFS rate was 78.4% (29/37) in 37 patients with CD9-positive expression compared with 33.3% (9/27) in 27 patients with CD9-negative expression (P = 0.001). The Cox proportion hazards regression (HR, 0.352; 95% CI: 0.153 to 0.813; P = 0.015) showed that CD9 expression is an independent prognostic factor of RFS. The cumulative RFS curve of 64 patients in relation to CD9 expression showed that the CD-positive group has a significantly better cumulative RFS rate compared with the group with CD9-negative expression (P = 0.015) (Fig. 2).

The relationship of CD9 expression in GST with clinicopathological features, risk classification and RFS
Table 2 shows the lack of significant association between CD9 expression and age (P = 0.578), sex (P = 0.542), and mitotic count (P = 0.645) in the 38 GSTs. However, a highly significant association was observed between CD9 expression and tumor diameter (P = 0.031) and risk classification (P = 0.023).

Two patients who received IM treatment post-operation and three patients who underwent a follow-up time of < three years post-operation were excluded. The three-year RFS rate of the
remaining 33 patients was 88.9% (16/18) in 18 patients with CD9-positive expression compared with 33.3% (5/15) in 15 patients with CD9-negative expression \( (P = 0.001) \). The Cox proportion hazards regression \( (HR, 0.104; 95\% CI, 0.021 \text{ to } 0.528; P = 0.006) \) showed that CD9 expression was also an independent prognostic factor of RFS for GST. The cumulative RFS curve showed that the RFS of patients with CD9-positive expression group was significantly better than that of the CD9-negative expression group \( (P = 0.006) \) (Fig. 3).

**The relationship of CD9 expression in non-GST with clinicopathological features, risk classification and RFS**

No significant relationship was observed between CD9 expression and clinicopathological features, risk classification, and operative three-year RFS \( (P > 0.05) \) of the 36 non-GST patients. The Cox proportion hazards regression \( (HR, 0.710; 95\% CI, 0.214 \text{ to } 2.353; P = 0.575) \) showed that CD9 expression was not an independent prognostic factor.

**DISCUSSION**

Despite complete resection, nearly half of the primary GIST recurred or had a metastasis, and showed poor outcome (6). Jouen et al. found that non-GIST relapsed or metastasized more easily than GST even with the same diameter and mitotic count. However, the reason behind this phenomenon remains unclear because the mechanism is yet to be classified.

IM has significantly improved the prognosis of advanced or high-risk GIST patients (7). However, secondary resistance caused by gene amplification or secondary gene mutation caused by prolonged IM treatment has become the main factor that affects prognosis (8). An in-depth study of the mechanism of recurrence and/or metastasis may screen patients with high risk of recurrence or metastasis post-operation and provide a basis for implementing individualized treatment to improve prognosis.

Tumor recurrence and/or metastasis is a complex process accompanied with multiple genes and regulated by the interaction of a number of genes, adhesion molecules, enzymes, and protein. Epigenetic changes occur in small GISTs, such as 14q, 15q, and 22q, which result in malignant transformation and metastasis (9-11). Okamoto et al. (12) found that the methylation level of tumor suppressor genes, such as RASSF1A, P1, CDH1, and MGMT4, is correlated with tumor malignancy. Thus, this study examined whether some proteins plays important roles in the mechanism of recurrence and/or metastasis in GIST.

CD9, which belongs to TM4SF, can inhibit proliferation and metastasis by inhibiting the activation of the Wnt signaling pathway (13), the degradation of transforming growth factor \( \alpha \) (TGF-\( \alpha \)) (14), and the secretion of metalloproteinas (15). CD9 downregulation is a poor prognostic marker in various cancers and is correlated with tumor invasion and metastasis in gastric cancer (16), lung cancer (17), and bladder cancer (18). Setoguchi et al. (19) found that CD9 in metastatic liver GIST is downregulated by micro-array and PCR. The present study employed immunohistochemistry analysis in detecting CD9 expression to investigate its correlation with clinicopathological features, risk classification, and prognosis.

In our study, forty-four (59.5%) cases are CD9 positive. Positive reaction product was mainly localized in the cytoplasm and/or cell membrane, similar to other reports (20, 21). The positive rates of CD9 were higher in cases with \( \leq 5 \) cm diameter, \( \leq 10/50 \) HPF mitotic count, and low-/intermediate-risk group than in cases with \( > 5 \) cm diameter, \( > 10/50 \) HPF mitotic counts, and high-risk group \( (P < 0.05) \). These results suggest that reduced CD9 expression plays an important role in tumor progression and malignant behaviors. Comparing the RFS of CD9-positive with CD9-negative group, the universal analysis showed that the postoperative three-year RFS rate of the CD9-positive group is higher than that of the CD9-negative group (78.4% vs 33.3%, \( P < 0.001 \)). Multivariate analysis \( (HR, 0.352; 95\% CI, 0.153 \text{ to } 0.813; P = 0.015) \) showed that CD9 expression is an independent predictor of RFS. It indicates CD9 is an important role in the invasion process and metastasis in GIST, the loss of CD9 increases the risk of recurrence or metastasis. Thus, CD9 may be a favorable predictor of tumor progression or aggressive behavior in GIST.
ed that the postoperative five-year RFS rate of the CD9-positive group is higher than that of the CD9-negative group in GST. However, a statistically significant relationship was not found between these two groups in intestinal GST. This finding is attributed to the different functions and signal transduction pathways in different organs (22). In our study, the subgroup analysis of GST in this study showed that the positive rate of CD9 expression of the \( \leq 5 \) cm group is higher than that of the \( > 5 \) cm group \( (P = 0.031) \). The CD9-positive rate declined when risk increased \( (P = 0.023) \). The postoperative three-year rate of the CD9-positive group was higher than that of the CD9-negative group \( (88.9\% \text{ vs } 33.3\%, P = 0.001) \). However, no significant difference was found between CD9 expression and clinicopathological features risk classification and RFS in non-GST. This finding is consistent with the findings of Setoguchi. Thus, unlike non-GST, it indicates that CD9 expression may be organ specific, CD9 plays an more important role in the progression and/or metastasis of GST.

In conclusion, CD9 expression is closely related to the diameter, risk classification, and RFS of GST, especially in GST. CD9 expression is downregulated during the malignant transformation process or metastasis in GST. This mechanism of CD9 could be a potential prognostic marker in GST and may guide individualized treatment for patients.

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DISCLOSURE

The authors have no conflicts of interest to disclose.

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