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The paradoxical expression pattern of the epithelial mesenchymal transition-related biomarkers CD44, SLUG, N-cadherin and VSIG1/Glycoprotein A34 in gastrointestinal stromal tumors

Background: The aim of this study was to evaluate the immunohistochemical (IHC) expression of five biomarkers commonly involved in epithelial mesenchymal/mesenchymal epithelial transition (EMT/MET) in gastrointestinal

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stromal tumors (GISTs). **Material and methods:** In 80 consecutive GISTs the IHC examinations were performed using the EMT-related antibodies: E-cadherin, N-cadherin, SLUG, V-set and immunoglobulin domain containing 1 (VSIG1) and CD44. **Results:** The positivity rate was 88.75% for SLUG, 83.75% for VSIG1, 36.25% for CD44 and 10% for N-cadherin. No correlation was noted between the examined markers and clinicopathological parameters. Nuclear positivity for SLUG and VSIG1 was observed in all cases with distant metastasis. The extra-gastrointestinal stromal tumors (e-GISTs) expressed nuclear VSIG1 and SLUG with infrequent positivity for N-cadherin and CD44. The low overall survival was mainly dependent on VSIG1 negativity (p=0.01) and nuclear positivity for SLUG and/or CD44 positivity. **Conclusions:** GIST aggressivity may be induced by nuclear upregulation of SLUG and loss or cytoplasm-to-nuclear translocation of VSIG1. SLUG and VSIG1 may act as activated nuclear transcription factors. The CD44, but not N-cadherin, might also have an independent prognostic value in these tumors. The role of the EMT/MET-related transcription factors in the evolution of GISTs should be elucidated in larger cohorts. This is the first study exploring the IHC pattern of VSIG1 in GISTs.

Core tip: In this paper we proved for the first time in the current literature the possible role of VSIG1 in GISTs in correlation with the expression of the other markers involved in the epithelial mesenchymal/mesenchymal epithelial transition. Based on the obtained results, we hypothesized that the GIST aggressivity may be induced by nuclear upregulation of SLUG and the loss of cytoplasm-to-nuclear translocation of VSIG1.

INTRODUCTION

Despite the existence of several molecular pathways described as being involved in the genesis and evolution of gastrointestinal stromal tumors (GISTs), the invasive and metastatic behavior of GISTs is not completely understood. The aim of this immunohistochemistry (IHC) study was to evaluate the possible role of five of the

biomarkers commonly involved in the epithelial mesenchymal transition/mesenchymal epithelial transition (EMT/MET) and also in maintaining the stem cell capacity of tumor cells in the GIST histogenesis. The reason of this examination is based on some recent studies that proved a negative prognostic role of the EMT/MET-related markers in malignant tumors, including GISTs [1-4].

In carcinomas, the EMT is defined as the loss of the expression of the transmembrane protein E-cadherin and gain in the positivity of tumor cells for mesenchymal markers such as N-cadherin. Another EMT-related biomarker is known as SLUG (SNAIL2), which is a member of the SNAIL family. SLUG is a zinc-finger nuclear transcription protein that can suppress the E-cadherin expression of epithelial cells and favor carcinoma progression [1,2]. There is little known about the clinical significance of E-cadherin, N-cadherin or SLUG in GISTs [3,4]. The first report concerning the clinical significance of SLUG expression in GISTs was published in 2017 [3]. This study is the second.

CD44 is a transmembrane glycoprotein that plays a role in cell-cell adhesion, migration and cell differentiation; during pathological processes, it is involved in tumor cell proliferation, invasion and metastasis [5,6]. CD44 expression is correlated with the phenotype of cancer stem cells but its role in GIST is unclear [7].

V-set and immunoglobulin domain containing 1 (VSIG1), or membrane glycoprotein A34, is a member of the junctional adhesion molecules family expressed in normal gastric mucosa and tumors of the upper, but not lower, gastrointestinal tract. Testicular germ cells and ovarian cancers can also display VSIG1 positivity [2,8,9]. The clinical significance and the function of VSIG1 expression in GISTs or other mesenchymal tumors has not yet been explored in the studies published to date.

MATERIALS AND METHODS

In the present study we retrospectively evaluated the paraffin-embedded specimens provided from 80 consecutive cases of GISTs diagnosed in our clinic from 2003 to 2015. Ethical Committee approval was obtained from the University of Medicine and

Pharmacy of Tirgu-Mures, Romania, and the research was performed according to the Helsinki criteria.

The diagnosis of GISTs was performed according to the modified National Institute of Health consensus classification [10]. The IHC diagnosis was based on the c-KIT/DOG-1/PKCθ panel [11]. The aggressivity was assessed based on the mitotic count associated with the Ki67 index [10].

Tissue microarray (TMA) blocks were constructed for this study. From each case, three representative areas of each GIST tissue (3 mm diameter core) were used as well as the following IHC markers: E-cadherin, N-cadherin, SLUG, VSIG1 and CD44 (Table 1). For each antibody, a cut-off value of 5% was used. The E-cadherin and N-cadherin were quantified in the cell cytoplasm and cytoplasmic and/or membrane CD44 positivity was taken into account (Figure 1). Regarding SLUG and GIST, the cases were considered positive based on nuclear and/or cytoplasmic staining (Figure 2). Two pathologists independently performed the IHC assessment.

Statistical analysis was done with the GraphPad InStat 3 software and two-sided tests with a p-value <0.05 and a 95% confidence interval were considered as statistically significant. Kaplan-Meier curves and log-rank test were used to evaluate the independent prognostic value of the examined biomarkers. The median follow-up was 97 months (range: 12-136 months) and the overall survival (OS) was considered to be the time (in months) from operation to death or last follow-up.

RESULTS

Clinicopathological characteristics

Overall, 80 patients were included in the study, 45 women and 35 men, with a median age of 61.58 ± 11.84 years (range from 19 to 80 years). The most common location of GISTs was the stomach (n=35), followed by the small intestine (n=25), colorectum (n=6) and extra-gastrointestinal area (n=14) with a median tumor size of 6.47 ± 1.34 cm (range: 0.4-21 cm). The spindle cell morphology predominated (n=64), followed by epithelioid (n=2) and mixed architecture (n=14). There were no lymph node metastases observed in

the examined cases. Distant metastases (n=11) were localized in the peritoneum (n=6) and the liver (n=5) (Table 2).

Immunohistochemical features

E-Cadherin positivity was not noted in the examined cases. Most of the cases (n=71; 88.75%) showed SLUG positivity and VSIG1 positivity was seen in 67 of the 80 cases (83.75%), CD44 and N-cadherin showed positivity in 29 out of 80 (36.25%) and 8 out of 80 cases (10%) respectively.

Not one of the four positive markers (SLUG, CD44, N-cadherin and VSIG1) was statistically correlated with the clinicopathological factors, which included gender, age, tumor size, mitotic rate, tumor location, histological type, intratumoral necrosis, risk degree, Ki67 proliferation index, local invasion, presence or absence of distant metastasis. Most of the extra-gastrointestinal stromal tumors (e-GISTs) displayed SLUG and VSIG1 expression without N-cadherin and CD44 positivity (Table 2).

All of the cases with distant metastasis showed the immunophenotype SLUG nuclear positivity, VSIG1 nuclear positivity, N-cadherin± and CD44±. All 13 cases, which were negative for VSIG1, displayed nuclear SLUG positivity and were negative for N-cadherin. They were included in the cases with a high mitotic rate, high Ki67 index and the high-risk group.

The nine SLUG negative cases that displayed positivity for VSIG1 (predominantly in the cytoplasm) but not for N-cadherin, did not present necrosis and were included in the cases with a low mitotic rate, Ki67 negative and low-risk group.

All of the six c-KIT negative cases expressed SLUG positivity and were negative for N-cadherin. These cases were also positive or negative for CD44 or VSIG1. The expression of SLUG was not correlated with N-Cadherin expression (p=0.58). A reverse correlation was seen between PKCθ and N-cadherin (p=0.029) and also between N-cadherin and VSIG1 (p=0.021). The VSIG1 expression was directly correlated with the PKCθ pattern (p=0.012) (Table 3).

Clinical outcome

The patients with VSIG1-negative GISTs showed a shorter OS than those with tumors that display VSIG1 positivity (p=0.01). A univariate Cox regression analysis showed that OS also decreased with CD44 positivity (p=0.06) and slightly decreased in patients with SLUG or N-cadherin positive GISTs (Figure 3). The VSIG1 expression was the most significant independent prognostic factor.

Based on the above-mentioned aspects, we presume that the loss of VSIG1 is an independent predictor of low OS whereas nuclear positivity for VSIG1 might indicate risk for distant metastasis. The cytoplasmic expression of a GIST is not an indicator of high risk. SLUG positivity indicates an increased risk of metastatic behavior whereas the loss of SLUG positivity is associated with longer OS. Double nuclear positivity for SLUG and VSIG1 indicates aggressive behavior especially for e-GISTs. The GISTs might be classified as tumors with high (SLUG nuclear positivity, VSIG1 negative or nuclear positivity, N-cadherin± and CD44±) or low risk for MET-induced aggressivity (SLUG negative, VSIG1 negative or cytoplasmic positivity, N-cadherin± and CD44±).

DISCUSSION

The EMT/MET-related biomarkers examined in the present study may have induced aggressivity as result of their role as nuclear transcription factors. It is important to note that CD44 is also known as a stemness-related biomarker.

About 20-50% of GISTs can display SLUG expression [3,12-15]. Due to the cut-off value of 5% used here, compared to the 20% used in other studies, the positivity rate was found to be higher (88.75%) in our study [3]. Although a possible link between the KIT signaling pathway and the SLUG transcription factor has been proven in experimental studies, it was not proven in our material [3]. SLUG is also proposed to have stemness properties [3] but we did not find it to correlate with CD44. In GISTs, SLUG positivity is considered to be an indicator of a high cell proliferation rate but not for cancer progression [3,12,13] especially in extra-gastric tumors [12-14].

In line with the literature, we confirm the role of SLUG in GISTs aggressivity, especially for e-GISTs. SLUG acts as a nuclear transcription factor, being more frequently

expressed by large GISTs with pleomorphic nuclei and high mitotic index [3], and as an indicator of risk for systemic metastases and/or local invasions [3,15].

In the present material, double nuclear positivity for SLUG and VSIG1 has been identified in the metastatic cases and the loss of VSIG1 is associated with a lower OS. Although no data regarding the role of VSIG1 in GIST have been published, its nuclear positivity indicates its possible role as a nuclear transcription factor. In normal gastric epithelium, VSIG1 plays the role of the junctional adhesion molecule that can be lost in carcinomas and as an indicator for a worse clinical outcome [8,9]. In mesenchymal tumors such as GISTs, its loss may indicate a lower survival rate whereas membrane/cytoplasm to nuclear transcription may stimulate tumor cells' proliferation and their migration in the blood vessels. As VSIG1 is considered to be a novel target for antibody-based cancer immunotherapy [8], this therapy may benefit patients with VSIG1 positive metastatic GISTs. We found a direct correlation between VSIG1 and the expression of PKCθ and a reverse correlation with N-cadherin expression.

The potential role of N-cadherin in increasing the metastatic potential of GISTs was previously proposed [16] but not confirmed [4].

The cell-cell adhesion molecule E-Cadherin and AE1/AE3 keratin might be expressed by one third of GISTs [12,13] as an indicator of low invasion properties and low risk for recurrence [17,18]. In leiomyosarcomas the increased expression of E-Cadherin and decreased SLUG expression was associated with decreased cell proliferation, invasion, and migration [19]. In this study lower levels of aggressive behavior were shown by SLUG negative GISTs.

The CD44 stemness marker was expressed in one quarter of the cases but its positivity can be shown by more than 70% of the GISTs [20,21]. The role of CD44 in GISTs' tumor progression and metastasis has been analyzed in a few studies, however the results are controversial. CD44 positivity might be an indicator of better prognosis [20]. The high-risk group GISTs displayed a significant loss of CD44 expression [21]. Being universally expressed in GISTs, CD44 and CD133 may represent a linkage rather than cancer stem cell markers [22,23]. We did not prove a statistical correlation between CD44 and SLUG

despite its well-defined role in GISTs. A slightly lower OS was proven for CD44 positive cases compared with CD44 negative ones.

CONCLUSIONS

We hypothesized that the EMT/MET of GISTs involves the upregulation of the nuclear transcription factors SLUG and VSIG1. The main shortfall of this paper is the small number of examined cases. The role of the adhesion molecule N-cadherin and stemness factor CD44 in GISTs should be further explored in studies which include a higher number of GISTs. The possible predictive role of VSIG1 expression for immunotherapy and the prognostic significance of its subcellular localization also deserve further exploration.