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ANATOMICAL PATHOLOGY

Grb2-associated binding protein-1 as a biomarker in bone and soft tissue sarcomas

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Summary

The Grb2-associated binding proteins are a family of platform proteins involved in signalling transduction pathways. A major family member, Gab1, modulates the effect of growth factor receptors and intracellular signalling pathways, including the phosphatidylinositol 3-kinase and AKT/mTOR signalling pathways. We have studied the immunohistochemical staining of Gab1 in four separate tissue microarray slides containing Ewing sarcoma, rhabdomyosarcoma, osteosarcoma and synovial sarcoma. The expression of Gab1 was correlated with age, gender, tumour location and clinical stage. Positive staining was identified in 18/32 (56.25%) of Ewing sarcoma, 46/96 (48%) of rhabdomyosarcoma, 18/50 (36%) of synovial sarcoma, and 10/40 (25%) osteosarcoma. Of the 46 positive rhabdomyosarcoma cases, the staining was more prevalent in conventional embryonal (15/27 cases) and pleomorphic (21/30) subtypes than spindle cell (2/15) and alveolar types (8/24). For rhabdomyosarcoma and synovial sarcoma, positive Gab1 staining was significantly more prevalent in tumours of high clinical stage disease (stages III and IV) than low stages (I and II), (p=0.0042 and p=0.0024, respectively). Similarly, Gab1 staining in Ewing sarcoma revealed a significant association with advanced clinical stages (p=0.0189). In conclusion, Gab1 expression in Ewing sarcoma, rhabdomyosarcoma and synovial sarcoma may have prognostic significance and should be further exploited for potential benefit from targeted therapy. These findings are to be confirmed by larger studies with better represented patient populations.

Key words: Gab1; biomarker; immunohistochemistry; Ewing sarcoma; rhabdomyosarcoma; synovial sarcoma; tissue microarray.

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INTRODUCTION

Sarcomas are relatively infrequent malignancies that occur most commonly in the bone and soft tissues in a wide patient age group. Many of these tumours afflict adolescents and young adults and have aggressive behaviours, with rapid metastasis and recurrence. Accurate diagnosis and prognostic classification are particularly important for proper treatment and cure.[1,2]

Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children, exhibits a remarkable heterogeneity with various histological and biological types.[3] The embryonal (ERMS), alveolar (ARMS), spindle cell sclerosing (SRMS) and pleomorphic (PRMS) types are currently recognised. Differentiation of these types is important for gauging prognosis and planning optimal treatment. The ARMS, SRMS and PRMS types are generally associated with specific genetic events and aggressive tumour behaviour, while ERMS comprises a more heterogeneous biological and clinical pattern.[4] Synovial sarcoma is an aggressive neoplasm that predominantly arises in the soft tissues of the extremities. Although it occurs most commonly in young adults and adolescents, it exhibits a wide age range into late adulthood.[5] Ewing sarcoma and osteosarcoma are the most common bone cancers and are also most frequently seen in the second decade of life.[6,7] Modern treatment regimens for these tumours include surgery, chemotherapy and radiotherapy, which have significantly improved the outcome of patients with localised disease. However, metastases are common in up to 25% of patients and are resistant to conventional therapy resulting in higher mortality rates.[7]

The Grb2-associated binding proteins (Gab) are a family of docking proteins that act as platforms for components involved in signalling transduction pathways, resulting in tumour cell survival, proliferation and differentiation.[8] While some growth receptors like the platelet-derived growth factor (PDGF) recruit intracellular signalling pathways directly, other receptors use specific docking proteins to activate intracellular signalling. Gab1 and Gab2 are two of the most commonly expressed docking proteins. As a product of a gene located in chromosomal region 4q31.21, Gab1 acts through protein-protein interaction to modulate the effects of epidermal growth factor receptor (EGFR), insulin growth factor 1 receptor (IGF1R), and fibroblast growth factor receptor (FGFR), and stimulate phosphatidylinositol 3-kinase (PI3K), AKT/mTOR and other signalling cascades.[9–11] These molecules are commonly overexpressed in sarcomas providing continuous signalling for increased cell growth and proliferation.[9–11] Thus, through its interactions with growth factor receptors and signalling pathways, Gab1 plays an important role in sarcoma pathogenesis.[12] In this manuscript, we have studied the protein expression of Gab1 in Ewing sarcoma, rhabdomyosarcoma, synovial sarcoma, and osteosarcoma for possible discriminatory and prognostic roles.

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MATERIALS AND METHODS

Patients and materials

The Ewing sarcoma tissue microarray slide (TMA) (Moffitt Cancer Center, Tampa, FL, USA) included patients' tumour samples collected from the archives of the Pathology Department over a period of 13 years (1995–2007) with institutional review board approval. Pertinent clinical data including patient age, sex, tumour site and clinical stage were also compiled. Tumour cases sampled in the microarray were from initial diagnostic specimens with confirmed diagnosis of Ewing sarcoma, including 12 cases that had localised disease, eight locally aggressive cases with regional extension and eight others that presented with metastatic disease. The clinical stage was unknown in four cases. Two representative tumour cores (0.6 mm in diameter) of the paraffin-embedded blocks were compiled for the construction of the TMA blocks according to a previously described method.[13]

Rhabdomyosarcoma, synovial sarcoma and osteosarcoma TMA slides were purchased from US Biomax, USA (catalogue numbers SO2082a, SS1001, and OS804c, respectively). Tissue samples were from primary tumour sites, arranged in duplicate paraffin-embedded cores. Each tumour set was accompanied by limited clinical data that included age, gender, tumour location and clinical stage. Patients were categorised as paediatric ($\leq$20 years) or adults (>20 years of age). In rhabdomyosarcoma and osteosarcoma, clinical stages from I to IV, randomly represented in each tumour, were grouped as low (I and II) and high (III and IV) stages. For rhabdomyosarcoma, tumour locations were grouped as head/neck, orbit, parameningeal, trunk, musculoskeletal extremities, bladder/prostate and genitourinary. For Ewing sarcoma, osteosarcoma and synovial sarcoma, tumour locations were grouped as skeletal (bones and joints) or extraskeletal.

Immunohistochemistry

TMA slides were stained with a monoclonal antibody against Gab1 (Santa Cruz Biotechnology, USA), in automated immunohistochemistry protocols. According to the manufacturer, the antibody (H-7) was raised against amino acids 119–316 of the human Gab1 protein. The staining was performed with a Leica instrument (Leica, USA) and was previously validated for routine diagnostic use. After antigen retrieval, deparaffinised tumour slides were sequentially incubated with the primary antibody, a secondary antibody, a polymer conjugate and a colouring reagent that yields a brown colour for positive staining. Separate positive controls were made from medulloblastoma tissue, previously known to be positive for the protein.[14] External negative controls were stained similarly except for omission of primary antibodies.

Measurements and analysis

Staining results were recorded in each core as either positive or negative. Significant staining in or more than 10% of tumour cells was considered positive. The percentage of positive cases in different tumour types was calculated. Absence of staining, or staining in less than 10% of cells, was labelled as negative. Weak staining intensity, that was less than that of the positive control, was considered negative. Only staining of equal or higher intensity than the positive control was recorded. For rhabdomyosarcoma, the prevalence of the staining in different subtypes was also noted.

Fisher's exact Chi-square statistical test of significant differences with a 2×2 contingency table was performed using online software (http://www.socscistatistics.com/tests). The p values were calculated and the results were deemed significant at $p<0.05$. Statistical tests of significant associations were calculated to correlate the positive staining with age, gender, tumour location and clinical stage groups.

RESULTS

Positive staining with Gab1 was recorded as cytoplasmic with no nuclear or membranous immunoreactivity. The results represented in Table 1 reveal positive staining in 18/32 (56.25%) of Ewing sarcoma, 46/96 (48%) of rhabdomyosarcoma, 18/50 (36%) of synovial sarcoma, and 10/40 (25%) osteosarcoma (Fig. 1).

Eighteen Ewing sarcoma cases had positive staining that did not correlate with a specific age group, gender or tumour location (Table 2). Of the 18 cases, 6/8 patients had metastatic disease, 7/8 with regional extension, 4/12 with localised disease and one with unknown stage. More staining was present in advanced tumours (regional extension + metastatic) than localised tumours and the difference was statistically significant (p=0.0189).

Table 1 Gab1 staining results in different tumours and subtypes

Tumour	Number/total (%)
Ewing sarcoma	18/32 (56.25%)
Rhabdomyosarcoma	46/96 (48.0%)
Embryonal	15/27 (55.6%)
Pleomorphic	21/30 (70.0%)
Alveolar	8/24 (33.3%)
Spindle cell	2/15 (13.3%)
Osteosarcoma	10/40 (25.0%)
Synovial sarcoma	18/50 (36.0%)

A total of 96 rhabdomyosarcoma cases were present in the array. Patients' ages ranged from 1 to 91 years (median age 36) with 20 patients in the paediatric age group. The M/F ratio was approximately 1.8:1. Gab1 staining was present in 46 cases (48%) with no predilection to a specific age group. Of the paediatric patients, Gab1 expression was present in 7/20. Of the 46 total positive cases, more staining was noted in ERMS and PRMS: 2/15 SRMS, 21/30 PRMS, 15/27 ERMS and 8/24 ARMS (Fig. 2). Gab1 staining was more prevalent in ERMS and PRMS than SRMS and the difference was statistically significant (p=0.0097 and p=0.0004, respectively). Gab1 staining was also significantly more prevalent in PRMS than ARMS (p=0.0129, respectively). There was no significant difference in the staining of ERMS versus ARMS (p=0.1602). Positive staining was more prevalent in high clinical stage patients (44/81) compared to low stage patients (2/15) and the difference was statistically significant (p=0.0042). However, the different clinical stages were not well-represented as there was no patient in stage I and all stage IV patients were negative (0/3). Similarly, all SRMS cases were stage II, two of which were positive (2/15). Thus, the difference in the staining may be due to tumour histology, i.e., SRMS cases commonly being non-expressers. All of the PRMS and ERMS were stage III (with positive rates of 21/30 and 15/27, respectively). ARMS included 21 in stage III, of which eight were positive, and three in stage IV that were negative (total positive rate of 8/24). With respect to tumour locations, the staining was more prevalent in the head and neck than other locations. However, there was no significant difference in the staining of favourable sites (head and neck, orbit, bile duct and genitourinary, 41/26 or 53.8%) versus unfavourable sites (all other sites, 3/10 or 30%) (p=0.2742).

A total of 50 synovial sarcoma cases were included in the array; patients were 14–72 years old (median 36 years), and had a male/female ratio of 5:2. Only two patients were in the paediatric group and both tumours had positive staining. Positive cases were mostly from patients in stages III and IV (13/21, Table 2) with a significant difference in the staining between high (III/IV) and low (I/II) stage groups (p=0.0024). All tumours were all located in the extremities and no visceral locations were present.

The osteosarcoma TMA contained 40 cases from patients ranging in age from 11 to 64 years (median 28 years) with a male/female ratio of 2.8:1 and a paediatric/adult ratio of

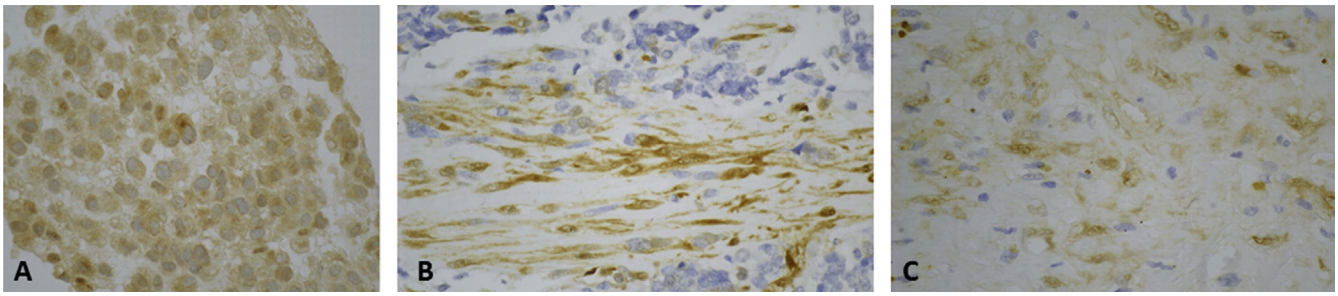

Fig. 1 Examples of positive staining patterns in different tumours: (A) Ewing sarcoma, (B) synovial sarcoma, and (C) osteosarcoma.

Table 2 Gab1 staining patterns in relation to different clinical parameters

Clinical characteristics	Positive staining (total number of cases)			
	Ewing sarcoma	Rhabdomyosarcoma	Synovial sarcoma	Osteosarcoma
Age	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$
Paediatric	5 (8)	7 (20)	2 (2)	3 (17)
Adult	13 (24)	39 (76)	16 (48)	7 (23)
Location	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$
Musculoskeletal	9 (20)	21 (43)	18 (50)	10 (40)
Extraskeletal	5 (10)	25 (53)	0	0
Clinical stage	**$p=0.0189$**	**$p=0.0042$**	**$p=0.0024$**	$p>0.05$
Low (localised or stages I/II)	4 (12)	2 (15)	5 (29)	10 (39)
High (advanced or stages III/IV)	13 (16)	44 (81)	13 (21)	0 (1)

Bold p values are statistically significant.

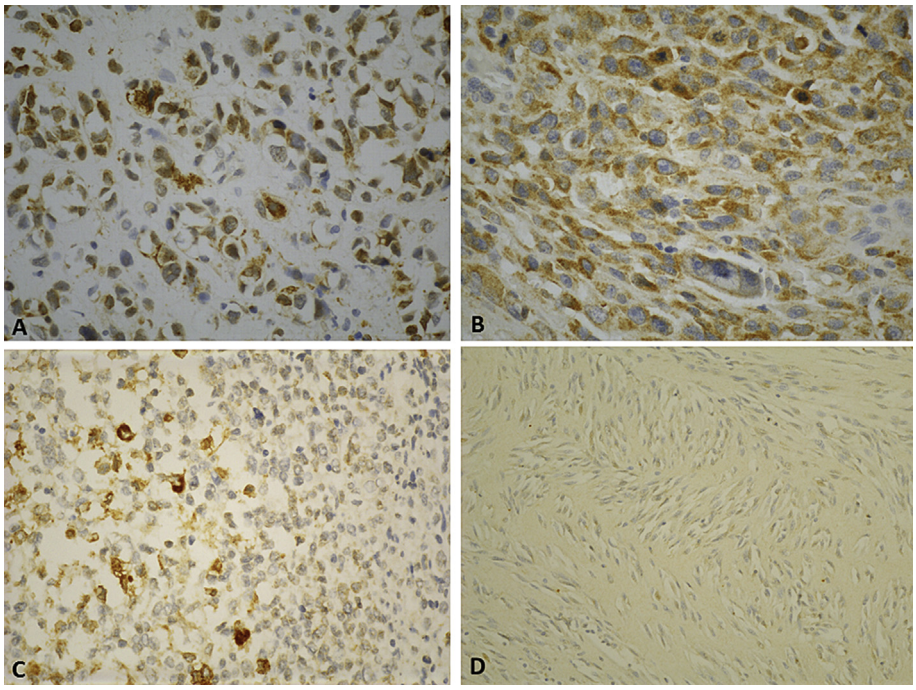

Fig. 2 Examples of different staining patterns in different rhabdomyosarcoma (RMS) tumours. (A) Diffuse staining in embryonal and (B) pleomorphic RMS, (C) focal staining in alveolar RMS, and (D) weak/negative staining in spindle cell RMS.

17:23. Ten cases revealed positive staining, three of which were paediatric <20 years, four <30 years, and six >30 years of age. There was no significant difference in the staining pattern between paediatric versus older patients (p=0.4709). All of the positively stained tumours were in the low clinical stage group (stages I and II; 10/39) and no staining was seen in the one patient in the high stage (i.e., metastatic) group (0/1). Tumours were all located in skeletal locations (including four axial locations) and no extraskeletal locations were present (Table 2). Of the positive cases, seven were in the femur (7/26), one in the tibia (1/5), one in the fibula (1/1) and one in the jaw (1/2).

DISCUSSION

This study highlights the expression of Gab in a subset of high grade bone and soft tissue sarcomas, reflecting the role of this protein in their sarcomagenesis. Gab1 is a multi-substrate binding protein that possesses an N-terminal pleckstrin homology domain, a Met-binding domain that mediates interactions with the Met receptor and other receptor tyrosine kinases, and a large C-terminal portion containing multiple tyrosine residues. Gab1 is capable of recruiting several SH2 domain-containing signalling molecules including Grb2, Shp2, PLCγ, Crk1, PI3K, and Nck and thus is an important central signalling modulator of several growth factors.[15] The interactions with these proteins highlight the complex spatial and temporal regulatory mechanisms involved in signalling pathways. Gab1 mutations promote tumourigenesis and cancer progression.[16,17] Expression of Gab1 has also been identified in many cancers, including medulloblastoma, epithelial ovarian carcinomas, hepatocellular carcinoma, and intrahepatic cholangiocarcinoma.[14,18–20] Inhibition of Gab1 suppresses proliferation and metastasis of breast cancer, hepatocellular carcinoma and colorectal carcinoma.[21–23]

Few published studies have addressed the expression of Gab1 in sarcomas. Overexpression of Gab1 was noted in a subset of Ewing sarcoma tumours in this study in association with advanced disease, thus highlighting a role in its sarcomagenesis. Through its function as a docking protein and its close association with IGF1R, EGFR, FGFR, and c-Met, Gab1 can activate downstream PI3K signalling and other transduction pathways. Thus, it can serve as a cross-link of signalling pathways resulting in persistent tumour growth. In support of this notion, feedback activation of Gab1 has been detected following MAPK inhibition in BRAF mutant melanoma cells.[24] In Ewing sarcoma, independent Gab1 activation can bypass IGF1R pathway and hence explain resistance to IGF1R targeted therapy. Thus, combination targeted therapy including Gab1 inhibitors may be worth pursuing as a treatment strategy for IGF1R inhibition-resistant tumours.

Rhabdomyosarcoma also activates FGFR and IGFR which require Gab1 protein for signalling.[25] An early study has identified Grb2 docking proteins making a complex with SHC domains.[26] In clinical tissue samples, our study revealed Gab1 expression in a subset of RMS, predominantly in the ERMS and PRMS subtypes. Its low prevalence in SRMS and ARMS types may help differentiate these types from other EMRS patterns and hence may be diagnostically useful. Conventional ERMS may show focal spindling of tumour cells or round cells and thus may be confused with SRMS or ARMS, especially in small-size specimens. In this setting, positive Gab1 immunostaining favours ERMS. Although our patient cohort is not well distributed with respect to clinical stages, the study shows significant prevalence of staining in high stage tumours.

In synovial sarcoma, both hepatocyte growth factor (HGF) and its receptor (c-Met) are overexpressed.[27] Co-expression of HGF and c-MET in clinical samples correlated with a poor prognosis in synovial sarcoma patients. At least in cell lines, activation of HGF pathway is induced by the adaptor protein Crk, which induces phosphorylation of Gab1 in response to c-Met stimulation resulting in sustained proliferation and migration of human synovial sarcoma.[28] These findings are substantiated by our own result of Gab1 overexpression in synovial sarcoma tumour tissues. Furthermore, Gab1 expression was mainly seen in high clinical stage tumours (stages III and IV), indicating that it may correlate with aggressive biological behaviour and hence can be used as a marker of tumour progression.[27] In non-small lung cancer cell lines, the novel drug erlotinib inhibited tumour growth through down-regulation of EGFR and its downstream signalling mediators including Gab1.[29] Thus, inhibition of Gab1 by erlotinib potentially may be useful in targeted therapy of other tumours expressing Gab1.

Osteosarcoma revealed Gab1 staining in 25% of cases. Although no previous studies have directly addressed the role of Gab1 in this tumour, osteosarcoma is known to express EGFR and activates PI3K/Akt pathways, both of which require docking proteins for signalling.[30,31] Inhibition of PI3K and downstream pathways in preclinical trials have already paved the way for potential therapeutic opportunities in osteosarcoma and other sarcomas with many clinical trials underway.[32,33] Since Gab1 is a part of this signalling cascade, Gab1 inhibition becomes an attractive alternative method of suppressing tumour growth, particularly in synergistic combination with other inhibitors.[34]

In conclusion, Gab1 shows heterogeneous expression in the studied bone and soft tissue sarcomas. Its expression in Ewing sarcoma, rhabdomyosarcoma and synovial sarcoma may have a possible link to prognosis. This study is limited by small sample size and unequal representation of clinical stage groups. However, the findings are encouraging for subsequent confirmatory studies with larger case numbers and/or other genetic/proteomic studies on better represented patient populations.

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Conflicts of interest: The authors state that there are no conflicts of interest to disclose.

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