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From Cell Biology to Tissue Engineering

GLI1 expression is an important prognostic factor that contributes to the poor prognosis of rhabdomyosarcoma

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Summary. The GLI1 and MDM2 genes are amplified or exhibit copy number gains in rhabdomyosarcoma (RMS). Here, we used immunohistochemistry to determine the relationships between GLI1 and MDM2 protein expression and several clinicopathological variables of RMS. GLI1 and MDM2-positivity rates were 61.36% and 13.64%, respectively. GLI1 expression correlated with presence of the *PAX3-FOXO1* fusion gene (P=0.040) and lymph node metastasis (P=0.034), and a significant association was found between GLI1 expression and overall survival (OS) (P=0.008). However, there was no association between MDM2 expression and any of the clinicopathological parameters or OS. Thus, GLI1 may be a biomarker of poor prognosis in RMS patients, and could itself be a therapeutic target. This contrasts with the apparent lack of clinical importance of MDM2 in RMS pathology, at least in the cohorts we examined.

Key words: Rhabdomyosarcoma, GLI1, MDM2

Introduction

Rhabdomyosarcoma (RMS) is one of the most frequent soft tissue sarcomas in infants and children, and occurs predominantly in the head, neck, trunk, and limbs (Norman et al., 2015). Four RMS subtypes exist based on histopathological features, namely, alveolar RMS (ARMS), embryonal RMS (ERMS), pleomorphic RMS (PRMS), and sclerosing RMS (SRMS) (Rosenberg, 2013). ARMS (approximately 20% of RMS cases), which possesses an aggressive clinical behavior, is associated with a specific t (2;13) translocation (*PAX3-FOXO1*, 55%) or a variant t(1;13) translocation (*PAX7-FOXO1*, 22%) (Linardic, 2008). ERMS (approximately 60% of RMS cases), which generally involves favorable outcomes, is associated with the loss of heterozygosity on chromosome 11 (Gallego Melcon and Sanchez de Toledo Codina, 2007). PRMS and SRMS are rare adult RMS variants and account for less than 20% of all RMS cases. According to the 2013 WHO classification of soft tissue tumors, SRMS has been separated from embryonal RMS, and associated with a favorable outcome and prognosis (Agaram et al., 2014). Although aberrant gene expression is often involved in tumor development and/or progression, the detection of genomic imbalances in RMS is a relatively understudied process. The 12q13-15 region, where both GLI1 and MDM2 genes are located, is frequently found within amplifications in solid tumors, including RMS (Khatib et al., 1993; Reifenberger et al., 1996; Liu et al., 2014a).

GLI1-encoded proteins are important transcription factors in the Hedgehog (Hh) signaling pathway (Li et al., 2012; Choe et al., 2015). A terminal effector in the Hh pathway, GLI1 is a bona fide oncogene, and constitutive overexpression of GLI1 is found in many sarcomas (Forus et al., 1993). Aberrant activation of the Hh signaling pathway stimulates tumorigenesis by regulating cellular proliferation, cellular survival, and epithelial-to-mesenchymal transition (He et al., 2011).

MDM2 is an oncogene that was initially detected in 1987 in spontaneously transformed fibroblasts (Cahilly-

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Snyder et al., 1987). MDM2 protein is present in most normal human tissues, including the liver and lungs. In about 10% of all cancers the MDM2 gene is amplified, whereas the amplification frequency in sarcomas increases to ~33% (Momand et al., 1998). High MDM2 expression levels can accelerate tumorigenesis and increase cancer risk through the negative regulation of the P53 tumor suppressor (Cintra et al., 2013). MDM2 also has P53-independent activities including proliferation, apoptosis, tumor invasion, and metastasis; each of these functions can promote tumor formation (Zhao et al., 2014).

Previous studies have used array comparative genomic hybridization (aCGH) to demonstrate that RMS samples have copy number gains or amplifications in the region where both GLI1 and MDM2 genes are located. Furthermore, we found that they could be classified as proto-oncogenes by functional annotation clustering (Liu et al., 2014a,b). Moreover, we have confirmed that GLI1 mRNA is overexpressed in RMS through quantitative real-time polymerase chain reaction (QRT-PCR) (Liu et al., 2014a,b). Here, we performed a follow-up study to examine GLI1 and MDM2 protein expression in RMS patients. Furthermore, we investigated whether there were correlations between GLI1 or MDM2 protein expression and patient survival or RMS clinicopathological parameters. Ultimately, we aimed to determine whether either of these genes might have utility as a biomarker in RMS.

Materials and methods

Patients and tissue specimens

A total of 44 formalin-fixed paraffin-embedded RMS samples were selected from the archives of the Department of Pathology of the First Affiliated Hospital, Shihezi University School of Medicine and The First Affiliated Hospital of Xinjiang Medical University, China. All the patients were Chinese. RMS samples were diagnosed and classified according to the Intergroup Rhabdomyosarcoma Study (IRS) staging and grouping system. According to the different clinical stages of patients, treatment strategies (including operation, chemotherapy or radiotherapy) were developed. The tumor samples included 22 ERMS cases, 20 ARMS cases, and 2 PRMS cases. Out of the 20 ARMS patients, 13 had a fusion gene product: using reverse transcription polymerase chain reaction (RT-PCR), the *PAX3-FOXO1* fusion genes were found in 11 patients and the *PAX7-FOXO1* fusion genes in 2 patients. The remaining 7 ARMS patients were fusion gene-negative. By contrast, all 22 ERMS cases and the 2 PRMS cases were negative for the *PAX3-FOXO1*/*PAX7-FOXO1* transcripts. A total of 36 normal muscle tissues were available as controls.

aCGH

Genomic DNA (gDNA) was isolated from tumor samples by QIAamp DNA FFPE tissue kit (Qiagen, Germany). aCGH experiments were performed using standard NimbleGen protocols (NimbleGen Arrays User's Guide: CGH Analysis v5.1). The detailed procedure and conditions followed those used in a previous study (Liu et al., 2014b). Mean log2 ratios of all probes in a chromosome region ≥0.25 were classified as genomic gains, and mean log2 ratios of ≥1.0 were classified as amplification.

Tissue microarray construction

Two representative fields of each tumor sample were selected based on hematoxylin and eosin slides. Tissue microarray (TMA) construction comprised the paraffin blocks that existed in areas corresponding to the selected fields. Each area contained at least 70% tumor cells. The tissue cores were created from paraffin blocks using a tissue arraying instrument (Alphelys, Plaisir, France). Subsequently, the cores were collected using a hollow needle with a diameter of 1.0 mm. Afterward, 3 μm sections of the TMA blocks were cut for subsequent immunohistochemical (IHC) analysis (Sun et al., 2014).

Experimental reagents

The experiments employed rabbit-derived monoclonal antibodies against GLI1 (Abcam, dilution 1:200, Cambridge, MA, USA) and MDM2 (DAKO, dilution 1:100, Carpinteria, CA, USA). The EnVision Detection Kit and DAB chromogenic reagent were purchased from Dako.

IHC staining procedure

The specimen slices were heated in an oven at 67°C for 2 h and then placed into a xylene and ethanol gradient to dewax and rehydrate, respectively. Afterward, the slices were immersed in a citrate buffer (pH 6.0) through high-pressure antigen retrieval for 8 min, and then the slices were left at room temperature for 20 min. Endogenous peroxidase was blocked by 3% H_2O_2-PBS for 10 min. The slides were rinsed three times with PBS for 3 min and then incubated overnight at 4°C with the primary antibodies. After being rewashed with PBS three times for 3 min, the secondary antibodies were reacted for 30 min in an incubator at 37°C. Subsequently, the slides were rinsed again with PBS and then stained with DAB. Neurinoma was used as positive control for GLI1 staining, and liposarcoma was selected as positive control for MDM2 reactivity. For the negative control, the samples were treated with PBS in place of primary antibody.

Evaluation of IHC staining

The expression of GLI1 and MDM2 in tumor tissue and normal muscles was evaluated using a semi-quantitative score. GLI1-positive products were mainly localized in nucleus and cytoplasm, and MDM2-positive

products were mainly localized in the nucleus. The extent of positive staining was scored as follows: 0, ≤5%; 1, 6-25%; 2, 26-50%; 3, 51-100%. The intensity of the specific staining was scored as follows: 0, no staining; 1, buff; 2, yellow; and 3, brown. The extent and intensity scores were multiplied to give the final scores, which ranged from 0 to 9. The staining results were categorized as - (0); + (1-3); ++ (4-6); and +++ (7-9). Two independent pathologists evaluated all the samples.

Statistical analysis

Statistical Package for the Social Sciences (IBM Corp., Armonk, NY, USA) version 17.0 was used to analyze all the statistical data. Statistical significance of the differences between GLI1 and MDM2 protein expression in the RMS samples versus the normal controls was compared using χ^2 or Fisher's exact test. The correlations between GLI1 and MDM2 expression and the clinicopathological factors were determined using the same methods. The correlation between GLI1 and MDM2 expression was determined by using Spearman's rank correlation test. The Kaplan Meier and log-rank methods were adopted to calculate the overall survival (OS) rates, and the OS curves were compared through the log-rank test. A P-value of less than 0.05 was considered statistically significant in all cases.

Results

12q15 (MDM2) amplified in RMS detected by aCGH

To the best of our knowledge, very few aCGH studies have been performed on RMS samples, and all of these studies used cancer cell lines. In the present study, aCGH was performed using high-quality DNA obtained from 20 FFPE samples in order to identify the potential drivers of RMS pathogenesis. By aCGH, we identified 2 cases of MDM2 amplification and 5 cases of MDM2 copy number gain from a total of 20 RMS samples (Fig. 1).

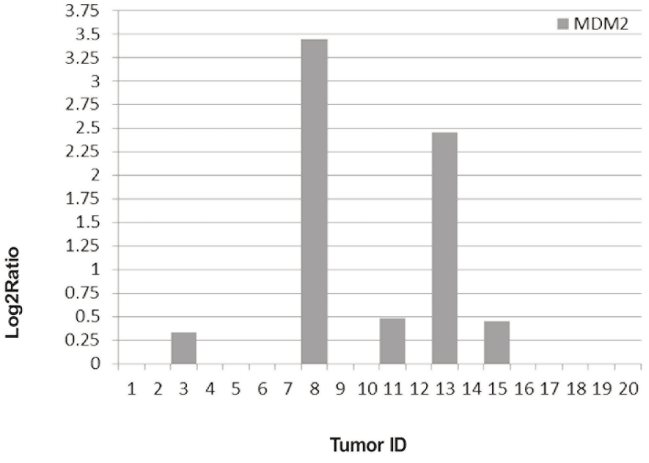

Fig. 1. RMS tumors with copy number gains at the MDM2 locus. Bars represent log2 values for MDM2 from 20 RMS samples.

GLI1 and MDM2 expression in RMS samples

While previous studies have reported GLI1 amplifications and copy number gains in RMS by using aCGH or QRT-PCR (Liu et al., 2014a), there are no comprehensive reports of GLI1 protein expression in this disease. The frequency of GLI1 positivity in the 44 RMS samples was 61.36%; this was significantly higher than that of the normal muscle samples (χ^2=33.345, P<0.001). The GLI1 (+) expression rate was 27.27% (12/44); the GLI1 (++) expression rate was 25% (11/44); and the GLI1 (+++) expression rate was 9.09% (4/44). Twelve cases from the aCGH samples harbored GLI1 gene gains, 9 of which also had detectable levels of GLI1 protein.

Since a recent aCGH study reported MDM2 amplification or copy number gain in RMS, we decided to examine MDM2 protein expression in our samples. This revealed an MDM2 positivity rate of 13.64% among the 44 RMS samples, which was significantly higher than the rate in normal muscle samples (χ^2=5.307, P=0.021). The MDM2 (+) expression rate was 11.36% (5/44), and the MDM2 (++) expression rate was 2.28% (1/44). Five cases among the aCGH samples harbored MDM2 copy number gains, and three of these exhibited simultaneous MDM2 protein expression. Table 1 summarizes the IHC staining results, and Fig. 2 depicts the corresponding images.

Correlation of the clinicopathological parameters with GLI1 and MDM2 expression

Combined with the above results, Table 2 shows the relationship between GLI1 and MDM2 expression and the associated clinicopathological factors. GLI1 expression was related to fusion gene expression (χ^2=4.208, P=0.040) and lymph node metastasis

Table 1. GLI1 and MDM2 expression in rhabdomyosarcoma patients and normal muscle tissue.

Tissue Type	n	GLI1		MDM2	
		Negative (%)	Positive (%)	Negative (%)	Positive (%)
RMS	44	17(38.64)	27(61.36)	38(86.36)	6(13.64)
ARMS	20	6(30)	14(70)	18(90.00)	2(10.00)
ERMS	22	11(50)	11(50)	18(81.82)	4(18.18)
PRMS	2	0(0.00)	2(100.00)	2(100.00)	0(0.00)
Normal muscle tissue	36	36(100.00)	0(0.00)	36(100.00)	0(0.00)

RMS, rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; PRMS, pleomorphic rhabdomyosarcoma.

(χ^2=4.476, P=0.034), but was unrelated to the other clinicopathological parameters. When the fusion gene status of RMS was taken into account, a significantly higher level of GLI1 was detected in fusion gene-positive ARMS (11/13) compared with fusion gene-negative ARMS, ERMS, and PRMS (16/31) in both cohorts (χ^2=4.208, P=0.040). This was also revealed by a comparison between fusion gene-positive ARMS (11/13) and ERMS (11/22) in both cohorts (χ^2=4.194, P=0.041). In contrast, there was no significant difference in the levels of GLI1 protein between fusion gene-positive (11/13) and fusion gene-negative (3/7) ARMS in both cohorts (χ^2= 3.778, P=0.052). No differential protein expression of GLI1 was observed when ERMS (11/22) was compared with fusion gene-negative ARMS (3/7) (χ^2= 0.109, P=0.742). Our data showed that, of the 10 RMS patients with lymph node metastasis, 7 were of the ARMS subtype and 3 were of the ERMS subtype. GLI1 was expressed in 6/7 of the ARMS samples and 3/3 of the ERMS samples.

Although the expression of MDM2 was clearly higher in RMS patients than in the control group, MDM2 levels were not associated with any of the clinicopathological parameters. The Hh signal pathway and MDM2 expression have been implicated in medulloblastoma pathology (Malek et al., 2011) and pancreatic cancer (Sheng et al., 2014). However, when we further investigated the relationship between GLI1 and MDM2 expression, a significant correlation in RMS was not found (r=0.079, P=0.581).

Relationship between GLI1 and MDM2 expression and OS

Based on the above data, we then evaluated whether expression of either GLI1 or MDM2 protein was associated with a particular prognosis. A total of 37 cases with more than 60 months of clinical follow-up information were used in our study, as shown in Table 3. The collective OS time of all the patients ranged from 1.5-117 months, but the collective median overall survival time was only 25 months.

A significant association was found between GLI1 expression and OS (χ^2=7.104, P=0.008); patients with detectable GLI1 expression had a less favorable outcome compared to GLI1-negative patients (Fig. 3). The median survival time of the patients with GLI1 expression was 19 months (range: 1.5-57 months) shorter than those without GLI1 expression with 38 months (range: 5-117 months). There was no association between MDM2 expression and OS (χ^2=0.140, P=0.708).

Since ARMS and ERMS patients have different prognoses, we carried out survival analysis separately for each group. GLI1 protein expression and OS were not associated in the ARMS group (χ^2=0.179, P=0.672). Indeed, among fusion gene-positive patients, there was no relationship between GLI1 protein expression and OS (χ^2=0.164, P=0.686). However, GLI1 expression did have a significant impact on OS in ERMS (χ^2=6.934, P=0.008), where the patients with GLI1 expression

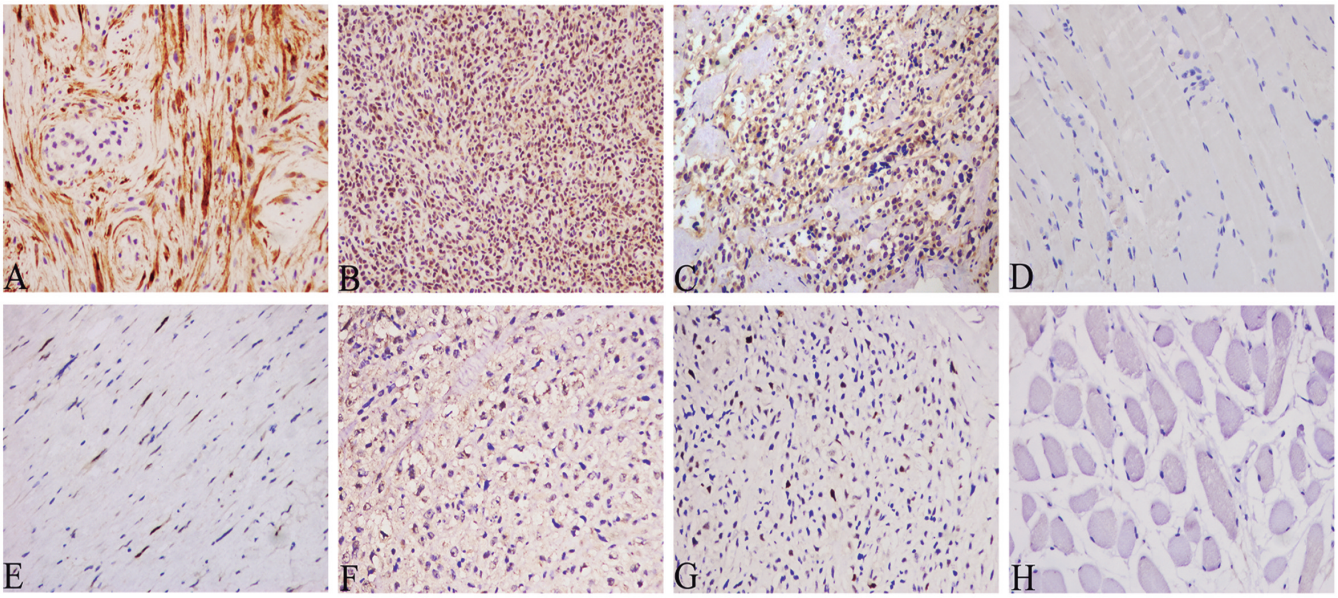

Fig. 2. GLI1 and MDM2 protein expression in the RMS samples. **A.** As control, GLI1-positive staining in the nucleus and cytoplasm of a neurinoma. **B.** GLI1 positive staining in the nucleus and cytoplasm of ERMS neoplastic cells. **C.** ARMS neoplastic cells showing nuclear immunoreactivity for GLI1. **D.** GLI1 was not found in muscle cells. **E.** A liposarcoma control showing nuclear immunoreactivity for MDM2. **F.** ERMS neoplastic cells showing nuclear immunoreactivity for MDM2. **G.** ARMS neoplastic cells showing nuclear immunoreactivity for MDM2. **H.** MDM2 was not found in muscle cells. × 200.

experienced worse outcomes and a greater risk of death after surgery than those without GLI1 expression. With the same trend, the patients with GLI1 expression in ERMS had shorter median survival time (18 months, range: 6-57 months). The opposite was found in GLI1-negative patients (median survival time 60 months, range: 13-117 months).

Discussion

RMS is the most common soft tissue sarcoma in children. Despite recent improvements to our understanding of the molecular pathogenesis of RMS, patient outcomes remain poor. This has prompted the search for biomarkers of theranostic value in the disease. Previous studies used aCGH to show that the gain and amplification frequencies of the 12q13.3-14.1 region where *GLI1* is located are 60% and 30%, respectively (Liu et al., 2014a), whereas the gain and amplification frequencies of the 12q15 region that contains MDM2 are 25% and 10%, respectively. In this study, immunohistochemistry was used to confirm GLI1 and MDM2 protein expression in RMS.

GLI1 is an important component of the Hh signaling pathway, which contributes to the growth and development of various tumors, such as those involving the prostate, lung, brain, and digestive tract (Feng et al., 2007). The Hedgehog (Hh) protein family is a group of secreted signaling molecules. Ptch is a receptor for Hh ligands, while Smo is the membrane-bound receptor that transduces Hh ligand signals. In the absence of Hh, Smo is inhibited by Ptch. Once Hh ligands bind to Ptch, Smo is derepressed and transduces the Hh signals; this cascade converges on the GLI1, GLI2, and GLI3 transcription factors (Fig. 4). GLI2 and GLI3 have both transcriptional activation and repression properties. By contrast, GLI1 is exclusively a transactivator and is also transcriptional target of the Hh pathway itself (Kim et

Table 2. Basic clinical characteristics of patients with GLI1 and MDM2 expression.

Variables	Cases	GL11		χ^2 value	P value	MDM2		χ^2 value	P value
		negative	positive			negative	positive		
Gender				0.096	0.757			3.088	0,079
Male	22	8	14			21	1		
Female	22	9	13			17	5		
Age (yrs)				1.800	0.180			0.554	0.457
≤5	13	7	6			12	1		
>5	31	10	21			26	5		
Ethnicity				0.029	0.865			0.412	0.521
Han	24	9	15			20	4		
Other minorities[1]	20	8	12			18	2		
Tumor diameter				0.170	0.680			0.132	0.717
≤5 cm	25	9	16			22	3		
>5 cm	19	8	11			16	3		
Histologic type				1.739	0.187			0.573	0.449
ARMS	20	6	14			18	2		
ERMS	22	11	11			18	4		
Fusion Gene				4.208	*0.040			0.554	0.457
PAX3/7-FKHR+	13	2	11			12	1		
PAX3/7-FKHR-[2]	31	15	16			26	5		
Location				4.099	0.251			1.677	0.642
head and neck	19	7	12			17	2		
extremities and trunk	11	3	8			10	1		
genitourinary tract	7	5	2			6	1		
thoracic cavity	7	2	5			5	2		
TNM Stage				3.725	0.054			0.014	0.905
I and II	23	12	11			20	3		
III and IV	21	5	16			18	3		
Lymph node metastasis				4.476	*0.034			0.145	0.703
No	34	16	18			29	5		
Yes	10	1	9			9	1		
Distant metastasis				1.884	0.170			0.554	0.457
No	31	14	17			26	5		
Yes	13	3	10			12	1		

*: significant difference; [1]: including Uygur (n=17), Kazak (n=2) and Hui (n=1); [2]: including the fusion gene-negative ARMS (n=7), ERMS (n=22) and PRMS (n=2).

al., 2009).

Recently, Yamanaka et al. studied the expression of Hh signal transcription factors in pediatric tumor cells. The authors investigated the expression of GLI1 in various pediatric tumor cell lines via QRT-PCR, and found that the expression of GLI1 mRNA was remarkably increased in RMS (RMS-YM, RD, RH30) cell lines (Yamanaka et al., 2010). Furthermore a study of ERMS (the more typical RMS subtype) revealed that expression of GLI1, -2, and -3 was higher in the tumor samples than in normal human fetal skeletal muscle cells (Tostar et al., 2010). Importantly, treatment of all the ERMS cells with GANT61, which can block the transcriptional activity of GLI1/GLI2, reduced growth/proliferation and induced apoptosis. In vivo, GANT61 treatment reduced ERMS cell growth. Knockdown of GLI1 and GLI3 mRNA (but not that of GLI2) with siRNA leads to a reduction in proliferation. Together with the knowledge that GANT61 does not inhibit GLI3, these data strongly suggest that GLI1 is a potential therapeutic target in ERMS cells. Consistent with this idea, GLI1 expression is reportedly high in cells derived from both RMS subtypes, and GANT61 is equally effective in suppressing the growth of both ARMS and ERMS xenografts (Srivastava et al., 2014).

GLI1 expression in the ARMS subtype appears to be

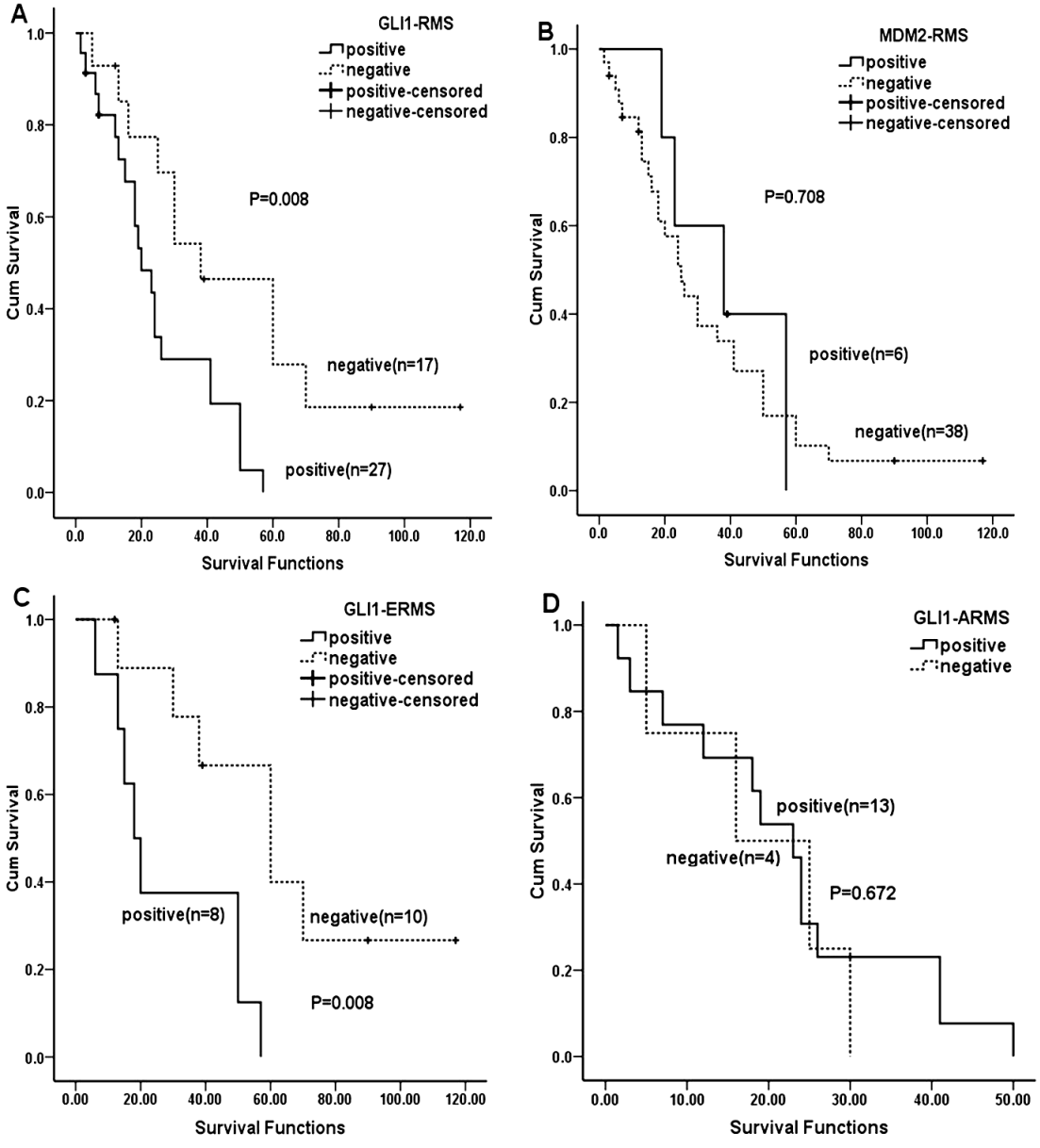

Fig. 3. Kaplan Meier OS curves for GLI1 and MDM2-negative and -positive patients. **A.** GLI1-positive RMS patients have a significantly shorter survival period after surgery than their GLI1-negative counterparts (χ^2=7.104, P=0.008). **B.** There was no association between MDM2 expression and OS (χ^2=0.140, P=0.708). **C.** GLI1 protein expression significantly affects OS in ERMS (χ^2=6.934, P=0.008): GLI1-positive patients experienced worse outcomes and a greater risk of death after surgery than their GLI1-negative counterparts. **D.** There was no correlation between GLI1 protein expression and OS in the ARMS patients (χ^2=0.179, P=0.672).

significantly higher than in the ERMS type (Oue et al., 2010). Since the ARMS subtype is typically characterized by presence of a fusion gene, we inferred that the level of GLI1 expression might correlate with *PAX3/7-FOXO1* status. In support of this, the level of GLI1 expression was significantly higher (P=0.040) in the fusion gene-positive ARMS (11/13) than in the fusion gene-negative ARMS, ERMS, and PRMS (16/31). This was also the case following a comparison between fusion gene-positive ARMS (11/13) and ERMS (11/22) in both cohorts (P=0.041). In contrast to our results, Zibat and colleagues considered that Hh signaling was significantly higher in ERMS and in the fusion gene-negative ARMS than in the fusion gene-positive ARMS in RMS patients (Zibat et al., 2010). We suggest that more samples should be analyzed in order to clarify the relationship between GLI1 and fusion gene expression. Moreover, the interferenced GLI1 expression in the ARMS cells with fusion gene expression can be considered to detect the *PAX3/7- FOXO1* expression.

Although there are several reports on the role of GLI1 in RMS, we believe this is the most comprehensive in terms of examining correlations between clinical data and GLI1 expression. The level of GLI1 protein was significantly correlated with lymph node metastasis (P=0.034). ARMS manifests with an aggressive clinical behavior, whereas ERMS is generally associated with a favorable outcome. Our data show that the 10 RMS patients with lymph node metastasis were composed of 7 ARMS and 3 ERMS samples; GLI1 was expressed in 6 of 7 ARMS samples and 3 of 3 ERMS samples. Despite this apparent correlation, the small sample size makes it difficult to determine whether GLI1 was a driver of lymph node metastasis in these patient groups.

The RMS patients with GLI1 expression experienced worse outcomes and a greater risk of death after surgery than GLI1-negative patients. During the follow-up period, all fusion gene-positive patients and ARMS patients eventually died. Statistical analysis showed that there was no association between GLI1 expression and OS. However, in the situation of ERMS, the GLI1-negative patients had a longer survival time and improved survival rates. Together with other reports on the GLI family in RMS, our current data indicate that GLI1 may be a novel candidate for molecular targeting in this disease.

MDM2, which is an E3 ubiquitin ligase, negatively regulates the levels of the tumor suppressor protein, P53,

Table 3. The follow-up results of 44 cases of patients with rhabdomyosarcoma postoperative.

Number	Histologic type	GLI1	MDM2	Survival state	Survival time (Month)
1	ARMS	++	-	Death	50
2	ARMS	+	-	Death	41
3	ARMS	+	-	Death	24
4	ARMS	+	-	Loss	None
5	ARMS	++	+	Death	19
6	ARMS	+	-	Death	24
7	ARMS	+	-	Death	1.5
8	ARMS	-	-	Death	25
9	ARMS	±	-	Loss	None
10	ARMS	-	-	Loss	None
11	ARMS	-	-	Death	30
12	ARMS	+	-	Death	3
13	ARMS	+	-	Death	18
14	ARMS	+++	-	Death	12
15	ARMS	-	-	Death	5
16	ARMS	+	-	Death	41
17	ARMS	+	-	Death	7
18	ARMS	+	+	Death	23
19	ARMS	+	-	Death	26
20	ARMS	-	-	Death	16
21	ERMS	-	-	Death	60
22	ERMS	±	-	Death	70
23	ERMS	+	-	Death	13
24	ERMS	-	-	Survival	12
25	ERMS	-	-	Death	60
26	ERMS	-	-	Survival	117
27	ERMS	++	-	Death	50
28	ERMS	-	-	Survival	90
29	ERMS	+	-	Death	15
30	ERMS	+	-	Loss	None
31	ERMS	-	-	Death	13
32	ERMS	++	-	Death	20
33	ERMS	+	-	Death	18
34	ERMS	+	++	Death	57
35	ERMS	+	-	Death	50
36	ERMS	++	+	Loss	None
37	ERMS	+	-	Loss	None
38	ERMS	-	+	Death	38
39	ERMS	-	-	Loss	None
40	ERMS	-	+	Survival	39
41	ERMS	-	-	Death	30
42	ERMS	+++	-	Death	6
43	PRMA	+++	-	Survival	7
44	PRMS	+	-	Survival	3

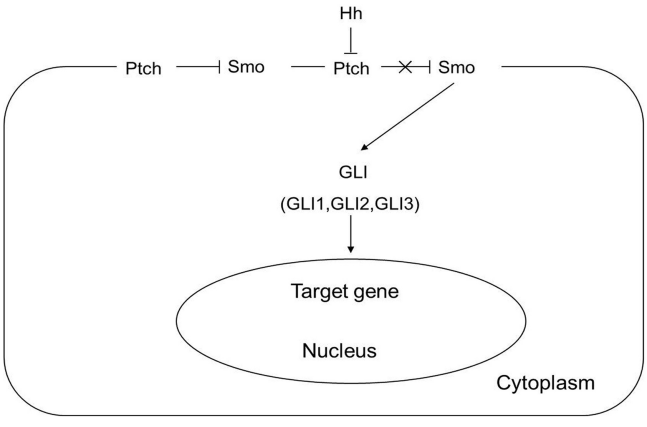

Fig. 4. Hedgehog (Hh) signaling transduction pathways. In the absence of Hh, Smo is inhibited by Ptch, whereas the binding of Hh to Ptch derepresses Smo and activates zinc finger DNA-binding proteins GLI1, GLI2, and GLI3. The GLI proteins mediate Hh signaling by translocating from the cytoplasm to the nucleus where they act as transcription factors to upregulate target genes.

through ubiquitin-dependent degradation (Honda et al., 1997). Although MDM2 is a bona fide oncogene involved in several human malignancies, all published reports indicate a relatively low prevalence of MDM2 alteration in RMS (Taylor et al., 2000; Ragazzini et al., 2004; Stock et al., 2009; Ognjanovic et al., 2012), ranging from 10% to 20% (Taylor et al., 2000). MDM2 expression frequency in the present study was only 13.6% (6/44), and was unrelated to all the clinicopathological parameters. We also found no association between MDM2 expression and OS. Together, these findings suggest that MDM2 expression may not be a primary genetic alteration in RMS. The interplay between the Hh signaling pathway and MDM2 play a clear role in medulloblastoma and pancreatic cancer. However, it appears that the carcinogenic potential of GLI1 may not be affected by MDM2 in RMS (r=0.079, P=0.581).

Our systematic study has shown that the expression of GLI1 and MDM2 is higher in tumors of RMS patients than in the corresponding normal tissue. The frequency of GLI1 protein expression was significantly higher than that of MDM2. GLI1 expression was related to the presence of a fusion gene and to lymph node metastasis, whereas MDM2 expression was not associated with any of the tested clinicopathological parameters. GLI1 expression was an important factor associated with patient survival, and patients with GLI1 expression were at a higher risk of death after surgery. In summary, GLI1 contributes to a poor prognosis in RMS patients, and its targeting may be a valid therapeutic strategy.

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