FOXM1 expression in rhabdomyosarcoma: a novel prognostic factor and therapeutic target

久田, 正昭

https://doi.org/10.15017/1654706

出版情報：九州大学, 2015, 博士（医学）, 課程博士
バージョン：

FOXM1 expression in Rhabdomyosarcoma: a novel prognostic factor and therapeutic target

Masaaki Kuda[1], Kenichi Kohashi[1], Yuichi Yamada[1], Akira Maekawa[1],Yoshiaki Kinoshita[2], Tetsuya Nakatsura[4], Yukihide Iwamoto[3], Tomoaki Taguchi[2] and Yoshinao Oda[1]

Departments of [1]Anatomic Pathology, [2]Pediatric Surgery, and [3]Orthopedic Surgery, Graduate School of Medical Sciences, Kyushu University

3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan

[4]Division of Cancer Immunotherapy, National Cancer Center Hospital East

6-5-1 Kashiwanoha, Kashiwa, Chiba, 277-8577, Japan

Corresponding author: Yoshinao Oda, MD, PhD

Email. oda@surgpath.med.kyushu-u.ac.jp

Tel. +81-92-642-6061, Fax. +81-92-642-5968,

ABSTRACT

Purpose: The transcription factor Forkhead box M1 (FOXM1) is known to play critical roles in the development and progression of various types of cancer, but the clinical significance of FOXM1 expression in rhabdomyosarcoma (RMS) is unknown. This study aimed to determine the role of FOXM1 in RMS.

Experimental Design: We investigated the expression levels of FOXM1 and vascular endothelial growth factor (VEGF) and angiogenesis in a large series of RMS clinical cases using immunohistochemistry (n=92), and we performed clinicopathologic and prognostic analyses. In vitro studies were conducted to examine the effect of FOXM1 knock-down on VEGF expression, cell proliferation, migration and invasion in embryonal RMS (ERMS) and alveolar RMS (ARMS) cell lines, using small interference RNA (siRNA).

Results: High FOXM1 expression was significantly increased in the cases of ARMS, which has an adverse prognosis compared to ERMS (p=0.0310). The ERMS patients with high FOXM1 expression (n=24) had a significantly shorter survival than those with low FOXM1 expression (n=25; p=0.0310). FOXM1 expression was statistically correlated with VEGF expression in ERMS at the protein level as shown by immunohistochemistry and at the mRNA level by RT-PCR. The in vitro study demonstrated that VEGF mRNA levels were decreased in the FOXM1 siRNA-transfected ERMS and ARMS cells. FOXM1 knock-down resulted in a significant decrease of cell proliferation and migration in all four RMS cell lines and invasion in three of the four cell lines.

Conclusions: Our results indicate that FOXM1 overexpression may be a prognostic factor of RMS and that FOXM1 may be a promising therapeutic target for the inhibition of RMS progression.

Keywords: Rhabdomyosarcoma; FOXM1; VEGF; Prognosis

1. INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue sarcoma in childhood and adolescence. RMS is divided into two major histopathological types: the embryonal and alveolar subtypes [1]. Alveolar RMS (ARMS) and embryonal RMS (ERMS) have distinct morphologic, genetic and biologic alterations [2–4]. ARMS frequently harbors the chromosomal translocation t(2;13)(q35;q14) or t(1;13)(p36;q14), which result in fusion of the paired-box transcription factors PAX3 and PAX7, respectively, to Forkhead box protein O1 (FOXO1) [5, 6]. In ERMS, no specific diagnostic genetic alteration has been identified, but molecular analyses of polymorphic loci have revealed allelic loss in the chromosomal region 11p15 in most cases [7, 8].

RMS is now commonly treated using multimodal therapy, including combination chemotherapy, surgery, and/or radiation therapy. However, despite the increasing cure rates for RMS, children with high-risk RMS tumors including metastatic disease, recurrent tumors, and certain histologies carry a poor prognosis. The identification of new molecular therapeutic targets for RMS is thus required.

Forkhead box protein M1 (FOXM1) belongs to a large family of transcriptional regulators that are characterized by an evolutionarily conserved DNA-binding domain called the Forkhead box or winged helix domain [9–11]. The transcription factor FOXM1 plays a vital role in the regulation of a wide range of biological processes, including cell-cycle progression, cell proliferation, cell differentiation, angiogenesis, apoptosis, DNA damage repair and tissue

homeostasis [12]. Increased levels of FOXM1 expression have been detected in many different types of human cancer [13–22] and sarcomas such as Ewing sarcoma [23, 24], malignant peripheral nerve sheath tumor [25], and osteosarcoma [26, 27]. In a rhabdomyosarcoma cell line, the suppression of FOXM1 was reported to result in the inhibition of cell growth and survival [28].

Many studies have shown that vascular endothelial growth factor (VEGF) plays a vital role in angiogenesis and that it promotes the migration and invasion of human cancer cells [29]. We found previously that VEGF was overexpressed and was associated with prognosis in RMS patients [30]. Some studies have shown that FOXM1 controls an angiogenic switch in malignant tumors by transcriptionally activating VEGF expression through direct binding to Forkhead binding elements of the VEGF promoter [31–33].

In the present study, we investigated FoxM1 and angiogenesis in a large series of RMS clinical cases and conducted a clinicopathologic and prognostic analysis. Using small interference RNA (siRNA), we then examined the effect of FOXM1 knock-down on the VEGF expression in ERMS and ARMS cell lines. We also examined the potential of FOXM1 as a therapeutic target in ERMS and ARMS cell lines.

2. MATERIALS AND METHODS

2.1. Tumor samples

Ninety-two specimens of RMS (ERMS: 49 cases; ARMS: 43 cases) registered in the

Department of Anatomic Pathology, Kyushu University, Japan between 1976 and 2012 were collected from different patients. Frozen tissue samples were available for 21 of the 92 cases (ERMS: 11 cases; ARMS: 10 cases). The diagnosis was done as previously described [30]. We examined PAX3/7-FOXO1 fusion gene transcripts in 35 of the 43 ARMS cases and 12 of the 48 ERMS cases as previously described [34]. We investigated the overall survival period by referring to the medical records, and the survival data were available for 71 cases. We assessed the correlations between clinicopathologic factors (age, sex, histologic subtype, anatomical site and tumor size) and the results of both the immunohistochemical and molecular analyses. This study was approved by the institutional review board at Kyushu University (permission code: 27-78).

2.2. Cell culture and reagents

The human embryonal RMS cell lines RD and RMS-YM were purchased from the Japanese Collection Research Resources Bank (JCRB, Osaka, Japan) and the Riken Bioresource Center (Tsukuba, Japan), respectively. The ARMS cell lines RH30 and RH41 (PAX3-FOXO1-expressing alveolar RMS) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The RD cells were cultured in Eagle's minimal essential medium (E-MEM) with 2× amino acids, vitamins and 10% fetal bovine serum (FBS). The RMS-YM cells were cultured in RPMI1640 medium with 0.1 mM NEAA, 20 mM HEPES and 10% fetal bovine serum. RH30 and RH41 cells were

cultured in RPMI1640 medium with 10% FBS. All of the cells were incubated in a humidified atmosphere of 5% CO2 at 37°C.

2.3. Immunohistochemistry

Immunohistochemistry was conducted in 92 tumors as previously described [30, 35]. Double immunohistochemical stain of FOXM1 and VEGF was conducted in 71 RMS (37 ERMS and 34 ARMS) using EnVision Doublestain System (Dako Denmark), according to the vendor's protocol. The following antibodies were used as the primary antibody: anti-FOXM1 (K-19, polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGF (A-20, polyclonal, 1:500; Santa Cruz Biotechnology), anti-CD31 (JC70A, monoclonal, 1:20; DAKO, Glostrup, Denmark), and anti-Ki-67 (MIB-1, monoclonal, 1:100; DAKO).

2.4. Immunohistochemical evaluation

We classified the expression of FOXM1 into five groups according to the percentage of positively staining cells: 0 = absent; 1 = 1%–25%; 2 = 26%–50%; 3 = 51%–75%; 4 = ≥76%. The staining intensity was categorized as follows: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. The proportion and intensity scores were then multiplied to obtain a total score [18]. We then divided the high and low FOXM1 expression by the median value of the total score.

The immunohistochemical score of VEGF expression was classified into five groups and we regarded immunohistochemical scores of 0, 1 and 2 as low expression and scores 3 and 4 as

high expression according to our previous study [36].

The micro-vessel density (MVD) and MIB-1-labeling index (LI) were also estimated as described in our previous studies [35, 36].

2.5. siRNAs and transfection conditions

To achieve the down-regulation of FOXM1, we transfected prevalidated On-Target plus Smart Pool siRNAs-siControle and siFOXM1 (Dharmacon, Brébières, France) into RMS cells (RD, RMS-YM, Rh30, Rh41) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the vendor's reverse transfection protocol.

2.6. Real-time RT-PCR analysis

We isolated total RNA from frozen samples and transfected cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) for VEGF and for FOXM1 was conducted in the same way as in our previous study [30]. The TaqMan assay reagent for FOXM1 is Hs01073586-m1.

2.7. Western blot assay

Whole cell lysates were prepared from transfected cell lines. To confirm the down-regulation of FOXM1, we evaluated the expression of FOXM1 protein by Western blot assay as described

previously [37]. A total of 20 μg protein from each sample was used, and incubated with anti-FOXM1 (1:200 dilution) antibody. Anti-human actin mouse monoclonal antibody (1:5000; Millipore, Bedford, MA) was used as an internal control. Protein levels were standardized by actin, which was assigned an arbitrary level of 10, and the expression signal relative to this was taken as the expression value for each sample.

2.8. VEGF ELISA assay

Transfected cells were seeded into six-well plates, and after 48 h the culture supernatants were collected and the particulates were removed by centrifugation. The numbers of cells in each plate were counted. The protein levels of VEGF were measured using VEGF ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results were normalized by each cell numbers.

2.9. Cell proliferation assay

Transfected cells were seeded in 96-well plates at a concentration of 5000 cells per well in serum-containing growth medium. Viability was assessed every 24 h over a period of 4 days by a WST-8 assay using the Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer's instructions and previous articles [38]. The absorbance at 450 nm was measured by a microplate reader (Model 680 Microplate Reader; Bio-Rad Laboratories, Hercules, CA). All experiments were done in quintuplicate and repeated

three times.

2.10. Cell invasion and migration assay

Cell invasion assays were conducted with transfected cell lines using the 24-well Biocoat Matrigel invasion chamber (BD Biosciences, San Diego, CA) according to the manufacturer's protocol and as described previously [38]. The migration assays were conducted using uncoated Transwell inserts. The transfected cells were seeded into the upper chamber at 1×10^5 per chamber in serum-free media. The outer wells were filled with media containing 10% FBS. The cells were incubated at 37°C with 5% CO_2 for 24 h (invasion assay) or 18 h (migration assay), and then noninvading cells were removed by wiping the chamber surface with a cotton swab. Cells that had migrated through the filter and adhered to its lower surface were fixed and stained with hematoxylin and eosin (H&E). The number of invading or migrating cells on the membrane was counted in five microscopic fields (×400). The results are expressed as the mean number of cells per field. Each assay was conducted in triplicate and repeated three times.

2.11. Statistical analysis

The correlation between two dichotomous variables was estimated by Fisher's exact test. The correlations between immunohistochemical scores and mRNA expression were estimated by the Mann-Whitney U-test. The analyses of overall survival were conducted by the Kaplan-Meier method with the log-rank test. A p-value <0.05 was considered significant. All the data analyses

were performed with JMP statistical software ver. 9.0.2 (SAS Institute, Cary, NC).

3. RESULTS

3.1. Patient characteristics

The clinical and pathological characteristics of the 92 patients with RMS are summarized in Table 1. Sixty patients were children, while 30 patients were adults. The patients were 47 males and 45 females, ranging in age from 1 month to 70 years old. Histopathologically, the 92 specimens included 49 primary ERMS, 41 primary ARMS, and 2 metastatic ARMS. We examined PAX3/PAX7-FOXO1 fusion gene transcripts in 35 of the 43 ARMS cases and 12 of the 49 ERMS cases. PAX3-FOXO1 fusion gene was detected in 20 ARMS cases, and the PAX7-FOXO1 fusion gene was detected in 4 ARMS cases. The PAX3/PAX7-FOXO1 fusion gene transcript was not found in any of the 11 ERMS cases.

Prognostic data were available in 82 cases and the follow-up time ranged from 2 to 255 months (median, 48 months). Therapeutic data was available in 77 patients. Sixty-nine patients were treated with multimodal therapy including combination chemotherapy, and 8 patients were treated with surgery and/or radiation therapy.

3.2. Immunohistochemistry

Tables 2–4 summarize the results of immunostaining. The highlights are described below.

3.2.1. FOXM1 and VEGF expression (immunostaining and mRNA)

VEGF expression was observed mainly in the cytoplasm or endothelium of the tumor cells, whereas FOXM1 expression was recognized in the nucleus and cytoplasm (Fig. 1A–D). When the results of immunohistochemistry and the real-time quantitative RT-PCR were compared, a statistical correlation was identified between the immunohistochemical scores and the mRNA expression levels for FOXM1 (Fig. 1G: p=0.0194). From this result, the reliability of immunohistochemical evaluation of FOXM1 was obtained.

High FOXM1 expression was recognized in 33/43 (74%) specimens of the ARMS group and 24/49 (51%) specimens of the ERMS group. FOXM1 high expression was significantly increased in the specimens of ARMS, which has an adverse prognosis compared with ERMS (Table 2; p=0.0310). Similarly, VEGF high expression was identified in 23/42 (55%) of the ARMS specimens, and 18/46 (38%) of the ERMS specimens. There was no significant difference in VEGF expression between the ARMS and ERMS specimens.

When compared in terms of the existence of fusion gene, high FOXM1 expression was recognized in 19/24 (79.2%) specimens of the PAX3/7-FOXO1 positive ARMS group and 8/11 (63.64%) specimens of the PAX3/7-FOXO1 negative ARMS group. But, there is no significant difference (p=0.3377).

Among the cases that showed high FOXM1 expression, 18 of the 24 ERMS (75%) and 24 of the 33 ARMS (73%) specimens showed high VEGF expression. There was a significant correlation between FOXM1 and VEGF expression in ERMS (Table 3; p=0.0163).

Within all of the frozen samples of RMS, the expressions of FOXM1 and VEGF mRNA were significantly positively correlated (Fig. 1H, r=4.71, p=0.0128).

To clarify the co-existence of FOXM1 and VEGF in RMS, we conducted double immunohistochemical stain of FOXM1 and VEGF in clinical samples. Co-existence of FOXM1 (DAB; brown) and VEGF (Permanent Red; red) was recognized in ARMS (Fig.1E) and ERMS (Fig.1F). Co-existence of FOXM1 and VEGF was recognized in 25/34 (73.5%) specimens of the ARMS group and 26/37 (70.3%) specimens of ERMS group. There is no significant difference in co-existence between the ERMS and ARMS specimens (p=0.7602). There is also no significant correlation between co-existence and FOXM1 expression in ERMS or ARMS (p=0.0859, p=0.9138, respectively).

When Clinicopathologic variables and the expression of FOXM1 in RMS ware compared, no significant relations were identified between any of the clinicopathological parameters (sex, age, stage and size) and FOXM1 expression in the ERMS or ARMS cases (Table 4).

3.2.2. Microvessel density

We assessed the MVD by immunohistochemical staining of CD31, and the results ranged from 3.00 to 37.25/0.26 mm^2 (12.20±6.55/0.26 mm^2). The MVD in the ERMS specimens was significantly higher than that in the ARMS specimens (Table 2: ERMS, 13.69±6.96; ARMS, 10.58±5.72; p=0.0252). There was a significant correlation between the MVD and the VEGF expression in ERMS (Table 3: p=0.0406), but no significant correlation was identified between

MVD and FOXM1 expression in ERMS or ARMS, or between MVD and VEGF in ARMS (Table 3: p=0.3539, p=0.4447, p=0.4222, respectively).

3.2.3. MIB-1 labeling index

The MIB-1-LI ranged from 0.4 to 75.1 (median, 18.19±14.59), and the median MIB-1-LI values were not significantly different between the ERMS and ARMS subtypes (ERMS median, 18.36±13.88; ARMS median, 18.01±15.46; p=0.9133, Table 2). In addition, no significant correlation was found between the MIB-1-LI and FOXM1 expression in either the ERMS or ARMS subtypes (p=0.5467, p=0.7190, respectively, Table 3).

3.2.4. Prognosis

The ERMS patients with high FOXM1 expression had significantly shorter survivals than those with low FOXM1 expression (p=0.031, Fig. 1I), whereas the FOXM1 expression status in the ARMS cases did not affect the patients' survival (Fig. 1I).

3.3. Effect of FOXM1 knock-down on RMS cells

3.3.1. Effect of FOXM1 knock-down on VEGF expression

In light of the known effects of FOXM1 on angiogenesis [31–33], we examined the level of VEGF in RMS cell lines and assessed the effects of the down-regulation of FOXM1. The effectiveness of FOXM1 siRNA was identified by real-time RT-PCR and Western blotting (Fig.

2A,2B). We observed that the mRNA levels of FOXM1 were significantly decreased in the FOXM1 siRNA-transfected cells compared to the controls in all RMS cell lines ($p \leq 0.001$). VEGF mRNA levels were decreased in the FOXM1 siRNA-transfected cells, and three of the four cell lines revealed significantly decreased VEGF mRNA expression ($p < 0.05$, Fig. 2C). However, no statistically significant difference in VEGF secretions was seen by FOXM1 knock-down (Fig. 2D).

3.3.2. Effect of FOXM1 expression on the proliferation of the RMS cell lines

To clarify whether FOXM1 could be a therapeutic target for rhabdomyosarcoma cells, we examined the effect of FOXM1 knock-down on cell proliferation in RMS cell lines. The effects of FOXM1 knock-down on cell proliferation are shown in Figure 3A. We found that FOXM1 knock-down significantly reduced the cell proliferation in all four RMS cell lines tested ($p < 0.0001$).

3.3.3. Effect of FOXM1 expression on the invasion and migration of the RMS cell lines

In light of the known effects of FOXM1 on tumor cell migration and invasion [39–41], we examined the effect of FOXM1 knock-down on RMS cell migration and invasion. The results demonstrated that FOXM1 knock-down significantly reduced the migration in all the cell lines (Fig. 3B) and invasion in three of the four cell lines (Fig. 3C).

4. DISCUSSION

In recent years, there has been a virtual explosion of evidence indicating the biological significance of FOXM1 (previously known as HFH-11, MPP2, Win, and Trident) in tumor aggressiveness [11]. For example, several studies have evaluated the prognostic significance of FOXM1 in various types of cancer [17, 20, 21, 42, 43]. In gastric cancer, overexpression of FOXM1 was independent prognostic factor for disease-free and overall survival [20]. In advanced non-small cell lung cancer patients, FOXM1 expression is significantly associated with cisplatin-based chemotherapy resistance and poor prognosis [21]. However, the clinical significance of FOXM1 expression in RMS had not been determined prior to the present study, which is the first to clarify the influence of FOXM1 expression on the prognosis of RMS.

In recent investigations of novel potential therapeutic targets of RMS, dysregulation in the RAS pathway, insulin-like growth factor (IGF), hedgehog (Hh), p53, and AKT/mTOR signaling have been shown to be associated with the pathobiology of RMS [44–49]. All of these multiple oncogenic pathways have been reported to crosstalk with a FOXM1 pathway in many different cancers [11]. In colorectal cancer, overexpression of FOXM1 and GLI1 mRNA correlated with Sonic Hh [50]. Coactivation of AKT and Ras signaling lead to activation of mTOR, FOXM1/SKP2 and c-Myc pathways in mouse liver carcinoma model [51]. Thus it is thought that FOXM1 plays important roles in tumor aggressiveness in RMS. In the present study, high FOXM1 expression was significantly increased in ARMS (which has an adverse prognosis) compared with ERMS. The ERMS patients with high FOXM1 expression had significantly

shorter survival compared to those with low FOXM1 expression. Our findings thus suggest that FOXM1 has potential as a prognostic factor in RMS and may be a useful molecular therapeutic target.

In gastric cancer and glioma clinical cases, VEGF has been positively associated with FOXM1 expression [31, 32]. Especially in gastric cancer clinical cases, MVD has been also associated FOXM1 expression [31]. It is evident that FOXM1 down-regulation decreased VEGF expression in various cancer cells [15, 33, 52–54]. In the present study, VEGF expression was positively associated with FOXM1 expression in the immunohistological evaluation of ERMS and the mRNA evaluation of frozen RMS samples. Moreover, the VEGF mRNA levels were decreased in the FOXM1 siRNA-transfected ERMS and ARMS cells, and significantly decreased in three of four cell lines. On the other hand, there was no significant correlation between FOXM1 and MVD expression, but there was a significant correlation between VEGF and MVD expression in ERMS. Therefore, FOXM1 might be indirectly related MVD especially in ERMS. A correlation between FOXM1 expression and angiogenesis in RMS was also suggested, but further extensive study in larger series are needed.

In this study, there was a significant correlation between the patients' prognosis and their FOXM1 expression and between VEGF and FOXM1 expression in ERMS, but not ARMS. It is known that FOXM1 is transcriptionally upregulated by the Hedgehog-GLI signaling cascade, and the aberrant activation of the receptor tyrosine kinase (RTK)-RAS-RAF signaling cascade leads to ERK-mediated FOXM1 phosphorylation, which results in the transcriptional

upregulation of FOXM1 target genes such as VEGF and MMP2 [55]. The Hedgehog pathway and dysregulation of the RAS signaling pathway are likely relevant to the pathogenesis of ERMS rather than ARMS [56–59]. The results of our study suggest that the overexpression of FOXM1 is directly related to the tumor aggressiveness and various pathogenesis of ERMS.

There is extensive evidence that FOXM1 plays a vital role in cancer cell proliferation and growth following initial tumorigenesis [12]. It is known that FOXM1 knock-down is related to decreased expressions of cell-cycle proteins, cyclin A2, cyclinB1, and Cdc25 phosphatases, and increased expression of the cell-cycle inhibitors p21 and p27 [14, 15, 60]. In the same way, FOXM1 knock-down in various cancer cell lines also results in an inhibition of cell proliferation. We also found that FOXM1 knock-down resulted in a significant inhibition of cell growth in both ERMS and ARMS cell lines. Although there is no significant correlation between the proliferative marker MIB1-LI and FOXM1 expression, FOXM1 was believed to play an important role in cell proliferation in RMS from the results of our proliferation studies.

FOXM1 overexpression leads to a direct upregulation of MMP-2 as well as VEGF [11], whereas FOXM1 regulates MMP-9 expression indirectly via its downstream target JNK1 [40]. Matrix metalloproteinases (MMPs) are crucial in the processes of tumor cell invasion and metastasis, and MMP-2 and MMP-9 are directly linked with angiogenesis and the degradation of the basement membrane collagen leading to metastasis. It is known that the inhibition of FOXM1 results in a decrease of MMP-2 and MMP-9 expression in several types of cancer cells, which leads to a reduction in cancer cell migration and invasion [15, 39, 41, 52]. We also found

that the down-regulation of FOXM1 significantly decreased the migration in all four of the RMS cell lines and invasion in three of the four cell lines. Here, we reached the same conclusion in RMS cells. The results of our cell proliferation, migration and invasion assays provide clear evidence in support of the role of FOXM1 as an oncogene in RMS. We thus suggest that FOXM1 may be a useful molecular therapeutic target in RMS.

In summary, our results showed that FOXM1 overexpression was associated with poor patient survival in ERMS and that FOXM1 overexpression was significantly increased in the cases of ARMS, which are known to have a poorer prognosis compared to ERMS. Our study demonstrated that FOXM1 plays an important role in the proliferation, migration and invasion of both ERMS and ARMS. Moreover, our findings demonstrated that FOXM1 regulated the expression of VEGF in RMS cells. FOXM1 overexpression may therefore be a prognostic factor of RMS, and FOXM1 may serve as a promising therapeutic target for the inhibition of RMS progression.

Acknowledgments

This work was supported by JSPS KAKEN Grant Number 26462708 and Grant Number 25293088. The English used in this article was revised by KN International (http://www.kninter.com/).

Figure Legends

Fig. 1. The immunohistochemical expression of FOXM1 (A,B) and VEGF (C,D) at the primary site of RMS. ARMS (A) and ERMS (B) showing immunoreactivity for FOXM1, the evaluated proportion score 4 (= ≥76%), the intensity score 3 (= strong) and the total score 12. ARMS (C) and ERMS (D) showing diffuse and strong immunoreactivity for VEGF, evaluated as score 4. Original magnification × 200. Double immunohistochemical stain of FOXM1 (DAB: brown, nuclei and cytoplasm) and VEGF (Permanent Red: brown, cytoplasm) (E, F). Co-existence of FOXM1 (brown) and VEGF (red) was recognized in ARMS (E) and ERMS (F). Original magnification × 400. G: Correlation between mRNA expression of FOXM1 and the immunohistochemical expression of FOXM1. The immunohistochemical status was significantly correlated with the corresponding mRNA expression (p=0.0194). H: FOXM1 mRNA vs. VEGF mRNA expression in 21 RMS specimens. There was a positive correlation between the FOXM1 and VEGF mRNA expression levels (r=4.71, p=0.0128). A.U., arbitrary units. I: Survival curve of patients with ERMS or ARMS according to FOXM1 expression. The survival of the ERMS patients with high FOXM1expression was significantly worse than that of the ERMS patients with low FOXM1 expression (p=0.031 by log-rank test).

Fig. 2. FOXM1 expression and the effect of FOXM1 down-regulation on VEGF expression. The efficacy of FOXM1 siRNA for the knock-down of FOXM1 mRNA and protein was confirmed by real-time RT-PCR and Western blotting (A,B). FOXM1 mRNA was significantly decreased

in FOXM1 siRNA-transfected cells (siFOXM1) compared to the siRNA control-transfected cells in all RMS cell lines (siCTR) ($p \leq 0.001$). VEGF mRNA levels were decreased in the FOXM1 siRNA-transfected cells, and the levels three of four cell lines were significantly decreased ($p < 0.05$; C). VEGF secretion was not significantly decreased by FOXM1 knock-down (D).

Fig. 3. Effects of FOXM1 expression on cell proliferation, migration and invasion. A: FOXM1 knock-down caused cell growth inhibition in all four cell lines ($p < 0.0001$). FOXM1 knock-down significantly decreased the migration in all four of the cell lines (B) and invasion in three of the four cell lines (C). siFOXM1; FOXM1 siRNA-transfected cells, siCTR; siRNA control-transfected cells

Fig. 1.

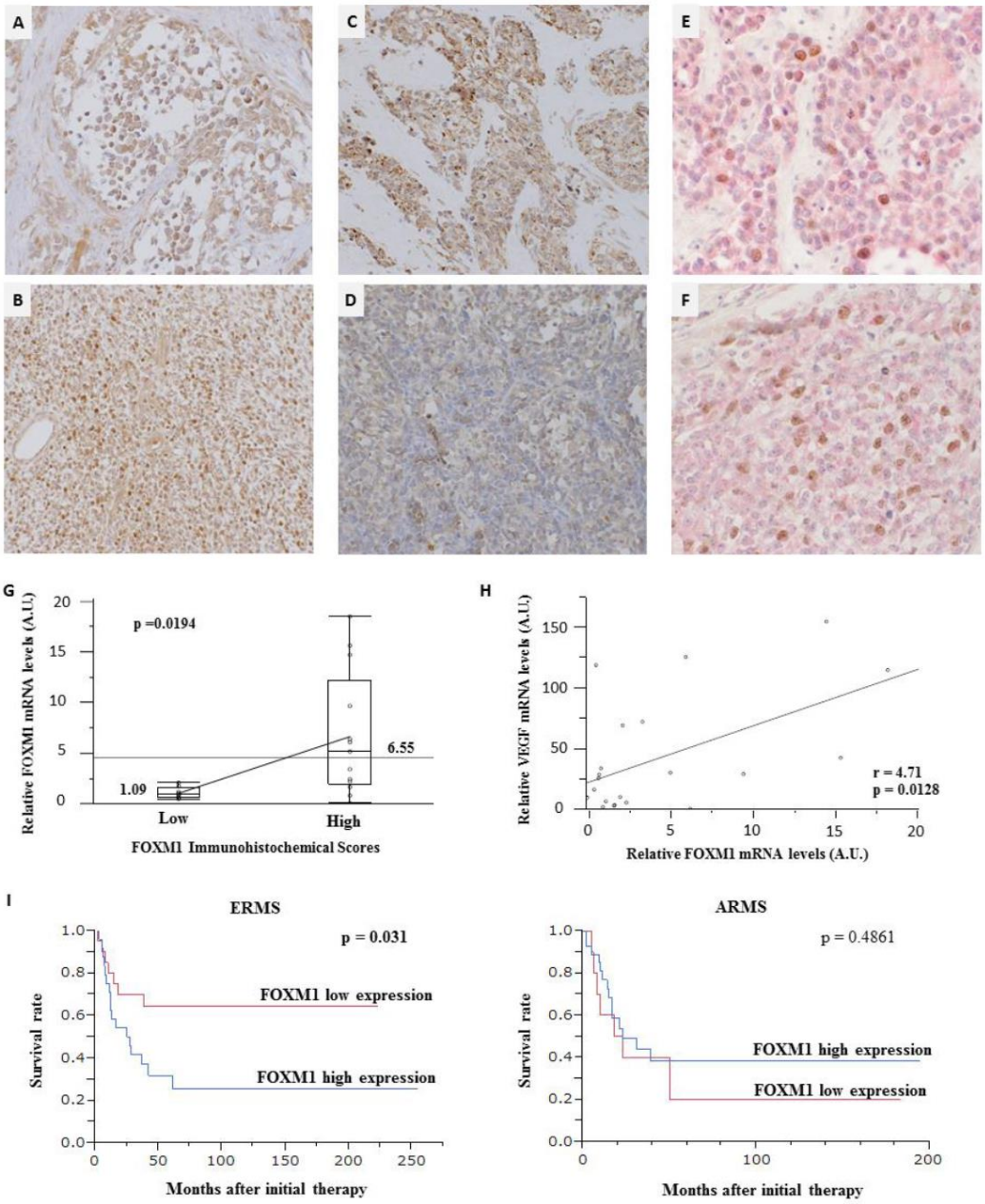

A
C
E
B
D
F
G
Relative FOXM1 mRNA levels (A.U.)
p =0.0194
6.55
1.09
Low
High
FOXM1 Immunohistochemical Scores
H
Relative VEGF mRNA levels (A.U.)
r = 4.71
p = 0.0128
Relative FOXM1 mRNA levels (A.U.)
I
ERMS
p = 0.031
FOXM1 low expression
FOXM1 high expression
Survival rate
Months after initial therapy
ARMS
p = 0.4861
FOXM1 high expression
FOXM1 low expression
Survival rate
Months after initial therapy

Fig. 2.

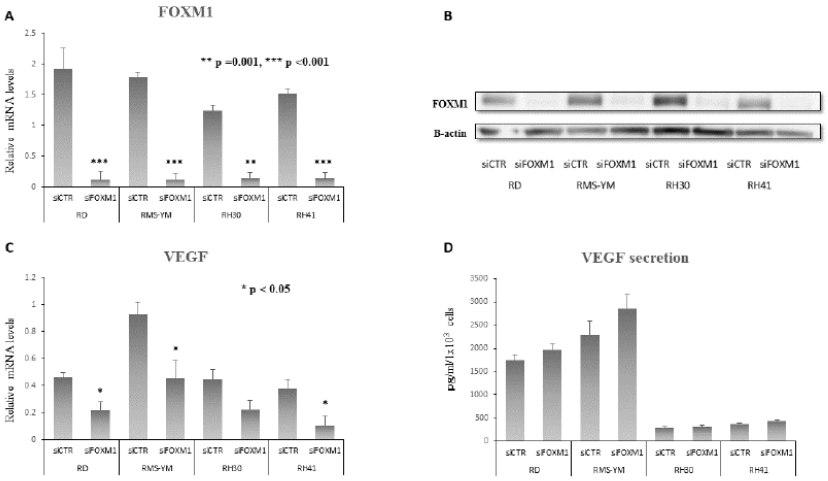
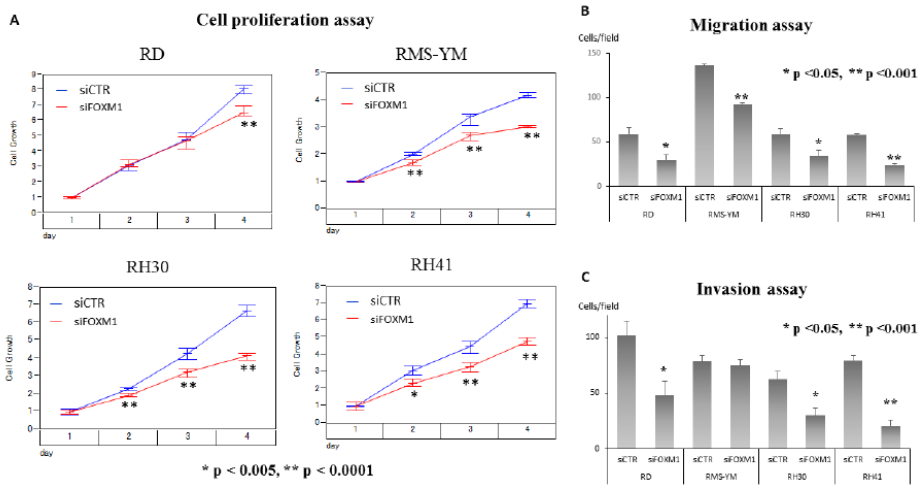

A
FOXM1
** p =0.001, *** p <0.001
Relative mRNA levels
siCTR siFOXM1 siCTR siFOXM1 siCTR siFOXM1 siCTR siFOXM1
RD RMS-YM RH30 RH41
B
FOXM1
B-actin
siCTR siFOXM1 siCTR siFOXM1 siCTR siFOXM1 siCTR siFOXM1
RD RMS-YM RH30 RH41
C
VEGF
* p < 0.05
Relative mRNA levels
D
VEGF secretion
pg/ml/1x10^5 cells

Fig. 3.


A
Cell proliferation assay
RD
RMS-YM
RH30
RH41
siCTR
siFOXM1
Cell Growth
day
* p < 0.005, ** p < 0.0001
B
Migration assay
Cells/field
* p <0.05, ** p <0.001
C
Invasion assay
Cells/field
* p <0.05, ** p <0.001

1
2

Table 1. Clinicopathological characteristics of the 92 patients with primary rhabdomyosarcoma (RMS)

Parameter	No. of cases
Age (yr)	
≤15	60
>15	30
Unknown	2
Sex	
Male	47
Female	45
Histology (primary)	
Embryonal	48
Alveolar	44
Stage (at diagnosis)	
1	16
2	6
3	43
4	12
Unknown	15
Location of primary tumor	
Favorable	16
Unfavorable	61
Unknown	15
Tumor size (cm)	
≤5	26
>5	50
Unknown	16

1

Table 2. Immunostaining results

	RMS	ERMS	ARMS	p-value
FOXM1	(n=92)	(n=48)	(n=44)	
High expression		24 (50%)	33 (75%)	0.0128*
Low expression		24 (50%)	11 (25%)	
VEGF	(n=89)	(n=46)	(n=43)	
High expression		17 (37%)	24 (55.8%)	0.0737
Low expression		29 (67%)	19 (44.2%)	
MVD	(n=85)	(n=44)	(n=41)	
Median±s.d.	12.20±6.55	13.75±7.03	10.59±5.65	0.0227*
High expression		27 (61.4%)	15 (36.6%)	0.0217*
Low expression		17 (38.6%)	26 (63.4%)	
MIB-1-LI	(n=86)	(n=43)	(n=43)	
Median±s.d.	18.19±14.59	18.35±14.04	18.02±15.28	0.2189
High expression		22 (51.2%)	16 (37.2%)	0.1919
Low expression		21 (48.8%)	27 (62.8%)	

MVD, microvessel density; MIB-1-LI, MIB-1 labeling index; RMS, rhabdomyosarcoma; ERMS, embryonal RMS; ARMS, alveolar RMS.

*p<0.05

2
3

Table 3. Correlations between FOXM1 expression and VEGF expression, MVD or MIB-1 LI, and between VEGF expression and MVD in ERMS and ARMS

		ERMS			ARMS		
	FOXM1	Low	High	p	Low	High	p
VEGF							
Low		13	6		3	9	
High		8	18	0.0163*	7	24	1.0000
MVD							
Low		10	7		7	19	
High		11	16	0.3539	2	13	0.4447
MIB-1 LI							
Low		9	12		7	20	
High		12	10	0.5467	3	13	0.7190
		ERMS			ARMS		
	VEGF	Low	High	P	Low	High	p
MVD							
Low		14	14		12	5	
High		3	17	0.0406*	14	10	0.4222

Abbreviations are explained at Table 2..

* p<0.05.

4

1

Table 4. Correlation between FOXM1 expression and clinicopathological parameters in ERMS and ARMS

		ERMS			ARMS		
	FOXM1	Low	High	*P*	Low	High	*P*
Sex							
Male		14	10		4	19	
Female		10	14	*0.3868*	7	14	*0.3028*
Age							
< 15		17	20		6	17	
≧15		5	4	*0.7178*	5	16	*1.000*
Stage							
1,2		8	6		3	5	
3,4		12	16	*0.5154*	7	20	*0.6614*
Size							
<5cm		7	5		5	9	
≧5cm		13	17	*0.4994*	3	17	*0.2278*

Abbreviations: RMS, rhabdomyosarcoma; ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma.

2

3

References

1 Parham DM, FG. B: Embryonal rhabdomyosarcoma. Alveolar rhabdomyosarcoma.; in Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds): World health organization classification of tumours who classification of tumours of soft tissue and bone. Lyon, IARC Press, 2014, pp 127-132.

2 Kelly KM, Womer RB, Sorensen PH, Xiong QB, Barr FG: Common and variant gene fusions predict distinct clinical phenotypes in rhabdomyosarcoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 1997;15:1831-1836.

3 Parham DM: Pathologic classification of rhabdomyosarcomas and correlations with molecular studies. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 2001;14:506-514.

4 Lae M, Ahn EH, Mercado GE, Chuai S, Edgar M, Pawel BR, Olshen A, Barr FG, Ladanyi M: Global gene expression profiling of pax-fkhr fusion-positive alveolar and pax-fkhr fusion-negative embryonal rhabdomyosarcomas. The Journal of pathology 2007;212:143-151.

5 Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW: Fusion of pax3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer research 1993;53:5108-5112.

6 Davis RJ, D'Cruz CM, Lovell MA, Biegel JA, Barr FG: Fusion of pax7

1 to fkhr by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma.
2 Cancer research 1994;54:2869-2872.
3 7 Scrable HJ, Witte DP, Lampkin BC, Cavenee WK: Chromosomal
4 localization of the human rhabdomyosarcoma locus by mitotic recombination
5 mapping. Nature 1987;329:645-647.
6 8 Visser M, Sijmons C, Bras J, Arceci RJ, Godfried M, Valentijn LJ,
7 Voute PA, Baas F: Allelotype of pediatric rhabdomyosarcoma. Oncogene
8 1997;15:1309-1314.
9 9 Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A,
10 Clevers H, Medema RH: Foxm1 is required for execution of the mitotic programme
11 and chromosome stability. Nature cell biology 2005;7:126-136.
12 10 Laoukili J, Stahl M, Medema RH: Foxm1: At the crossroads of ageing
13 and cancer. Biochimica et biophysica acta 2007;1775:92-102.
14 11 Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH: Forkhead
15 box m1 transcription factor: A novel target for cancer therapy. Cancer treatment
16 reviews 2010;36:151-156.
17 12 Koo CY, Muir KW, Lam EW: Foxm1: From cancer initiation to
18 progression and treatment. Biochimica et biophysica acta 2012;1819:28-37.
19 13 Teh MT, Wong ST, Neill GW, Ghali LR, Philpott MP, Quinn AG: Foxm1
20 is a downstream target of gli1 in basal cell carcinomas. Cancer research

2002;62:4773-4780.

14 Kim IM, Ackerson T, Ramakrishna S, Tretiakova M, Wang IC, Kalin TV, Major ML, Gusarova GA, Yoder HM, Costa RH, Kalinichenko VV: The forkhead box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. Cancer research 2006;66:2153-2161.

15 Ahmad A, Wang Z, Kong D, Ali S, Li Y, Banerjee S, Ali R, Sarkar FH: Foxm1 down-regulation leads to inhibition of proliferation, migration and invasion of breast cancer cells through the modulation of extra-cellular matrix degrading factors. Breast cancer research and treatment 2010;122:337-346.

16 Nakamura S, Hirano I, Okinaka K, Takemura T, Yokota D, Ono T, Shigeno K, Shibata K, Fujisawa S, Ohnishi K: The foxm1 transcriptional factor promotes the proliferation of leukemia cells through modulation of cell cycle progression in acute myeloid leukemia. Carcinogenesis 2010;31:2012-2021.

17 Priller M, Poschl J, Abrao L, von Bueren AO, Cho YJ, Rutkowski S, Kretzschmar HA, Schuller U: Expression of foxm1 is required for the proliferation of medulloblastoma cells and indicates worse survival of patients. Clinical cancer research : an official journal of the American Association for Cancer Research 2011;17:6791-6801.

18 Chu XY, Zhu ZM, Chen LB, Wang JH, Su QS, Yang JR, Lin Y, Xue LJ, Liu XB, Mo XB: Foxm1 expression correlates with tumor invasion and a poor

prognosis of colorectal cancer. Acta histochemica 2012;114:755-762.

19 Huang C, Qiu Z, Wang L, Peng Z, Jia Z, Logsdon CD, Le X, Wei D, Huang S, Xie K: A novel foxm1-caveolin signaling pathway promotes pancreatic cancer invasion and metastasis. Cancer research 2012;72:655-665.

20 Okada K, Fujiwara Y, Takahashi T, Nakamura Y, Takiguchi S, Nakajima K, Miyata H, Yamasaki M, Kurokawa Y, Mori M, Doki Y: Overexpression of forkhead box m1 transcription factor (foxm1) is a potential prognostic marker and enhances chemoresistance for docetaxel in gastric cancer. Annals of surgical oncology 2013;20:1035-1043.

21 Wang Y, Wen L, Zhao SH, Ai ZH, Guo JZ, Liu WC: Foxm1 expression is significantly associated with cisplatin-based chemotherapy resistance and poor prognosis in advanced non-small cell lung cancer patients. Lung cancer 2013;79:173-179.

22 Li X, Qi W, Yao R, Tang D, Liang J: Overexpressed transcription factor foxm1 is a potential diagnostic and adverse prognostic factor in postoperational gastric cancer patients. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico 2014;16:307-314.

23 Christensen L, Joo J, Lee S, Wai D, Triche TJ, May WA: Foxm1 is an oncogenic mediator in ewing sarcoma. PloS one 2013;8:e54556.

24 Sengupta A, Rahman M, Mateo-Lozano S, Tirado OM, Notario V: The dual inhibitory effect of thiostrepton on foxm1 and ews/fli1 provides a novel therapeutic option for ewing's sarcoma. International journal of oncology 2013;43:803-812.

25 Yu J, Deshmukh H, Payton JE, Dunham C, Scheithauer BW, Tihan T, Prayson RA, Guha A, Bridge JA, Ferner RE, Lindberg GM, Gutmann RJ, Emnett RJ, Salavaggione L, Gutmann DH, Nagarajan R, Watson MA, Perry A: Array-based comparative genomic hybridization identifies cdk4 and foxm1 alterations as independent predictors of survival in malignant peripheral nerve sheath tumor. Clinical cancer research : an official journal of the American Association for Cancer Research 2011;17:1924-1934.

26 Wang IC, Chen YJ, Hughes D, Petrovic V, Major ML, Park HJ, Tan Y, Ackerson T, Costa RH: Forkhead box m1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the scf (skp2-cks1) ubiquitin ligase. Molecular and cellular biology 2005;25:10875-10894.

27 Grant GD, Brooks L, 3rd, Zhang X, Mahoney JM, Martyanov V, Wood TA, Sherlock G, Cheng C, Whitfield ML: Identification of cell cycle-regulated genes periodically expressed in u2os cells and their regulation by foxm1 and e2f transcription factors. Molecular biology of the cell 2013;24:3634-3650.

28 Wan X, Yeung C, Kim SY, Dolan JG, Ngo VN, Burkett S, Khan J,

1 Staudt LM, Helman LJ: Identification of foxm1/bub1b signaling pathway as a
2 required component for growth and survival of rhabdomyosarcoma. Cancer
3 research 2012;72:5889-5899.
4 29 Ellis LM, Hicklin DJ: Vegf-targeted therapy: Mechanisms of
5 anti-tumour activity. Nature reviews Cancer 2008;8:579-591.
6 30 Miyoshi K, Kohashi K, Fushimi F, Yamamoto H, Kishimoto J, Taguchi
7 T, Iwamoto Y, Oda Y: Close correlation between cxcr4 and vegf expression and
8 frequent cxcr7 expression in rhabdomyosarcoma. Human pathology
9 2014;45:1900-1909.
10 31 Zhang Y, Zhang N, Dai B, Liu M, Sawaya R, Xie K, Huang S: Foxm1b
11 transcriptionally regulates vascular endothelial growth factor expression and
12 promotes the angiogenesis and growth of glioma cells. Cancer research
13 2008;68:8733-8742.
14 32 Li Q, Zhang N, Jia Z, Le X, Dai B, Wei D, Huang S, Tan D, Xie K:
15 Critical role and regulation of transcription factor foxm1 in human gastric cancer
16 angiogenesis and progression. Cancer research 2009;69:3501-3509.
17 33 Karadedou CT, Gomes AR, Chen J, Petkovic M, Ho KK, Zwolinska AK,
18 Feltes A, Wong SY, Chan KY, Cheung YN, Tsang JW, Brosens JJ, Khoo US, Lam
19 EW: Foxo3a represses vegf expression through foxm1-dependent and -independent
20 mechanisms in breast cancer. Oncogene 2012;31:1845-1858.

1 34 Jin L, Majerus J, Oliveira A, Inwards CY, Nascimento AG, Burgart LJ,
2 Lloyd RV: Detection of fusion gene transcripts in fresh-frozen and formalin-fixed
3 paraffin-embedded tissue sections of soft-tissue sarcomas after laser capture
4 microdissection and rt-pcr. Diagnostic molecular pathology : the American journal
5 of surgical pathology, part B 2003;12:224-230.
6 35 Oda Y, Tateishi N, Matono H, Matsuura S, Yamamaoto H, Tamiya S,
7 Yokoyama R, Matsuda S, Iwamoto Y, Tsuneyoshi M: Chemokine receptor cxcr4
8 expression is correlated with vegf expression and poor survival in soft-tissue
9 sarcoma. International journal of cancer Journal international du cancer
10 2009;124:1852-1859.
11 36 Oda Y, Yamamoto H, Tamiya S, Matsuda S, Tanaka K, Yokoyama R,
12 Iwamoto Y, Tsuneyoshi M: Cxcr4 and vegf expression in the primary site and the
13 metastatic site of human osteosarcoma: Analysis within a group of patients, all of
14 whom developed lung metastasis. Modern pathology : an official journal of the
15 United States and Canadian Academy of Pathology, Inc 2006;19:738-745.
16 37 Kohashi K, Oda Y, Yamamoto H, Tamiya S, Matono H, Iwamoto Y,
17 Taguchi T, Tsuneyoshi M: Reduced expression of smarcb1/ini1 protein in synovial
18 sarcoma. Modern pathology : an official journal of the United States and Canadian
19 Academy of Pathology, Inc 2010;23:981-990.
20 38 Endo M, Yamamoto H, Setsu N, Kohashi K, Takahashi Y, Ishii T, Iida

1 K, Matsumoto Y, Hakozaki M, Aoki M, Iwasaki H, Dobashi Y, Nishiyama K,
2 Iwamoto Y, Oda Y: Prognostic significance of akt/mtor and mapk pathways and
3 antitumor effect of mtor inhibitor in nf1-related and sporadic malignant peripheral
4 nerve sheath tumors. Clinical cancer research : an official journal of the American
5 Association for Cancer Research 2013;19:450-461.
6 39 Dai B, Kang SH, Gong W, Liu M, Aldape KD, Sawaya R, Huang S:
7 Aberrant foxm1b expression increases matrix metalloproteinase-2 transcription
8 and enhances the invasion of glioma cells. Oncogene 2007;26:6212-6219.
9 40 Wang IC, Meliton L, Tretiakova M, Costa RH, Kalinichenko VV, Kalin
10 TV: Transgenic expression of the forkhead box m1 transcription factor induces
11 formation of lung tumors. Oncogene 2008;27:4137-4149.
12 41 Uddin S, Ahmed M, Hussain A, Abubaker J, Al-Sanea N, AbdulJabbar
13 A, Ashari LH, Alhomoud S, Al-Dayel F, Jehan Z, Bavi P, Siraj AK, Al-Kuraya KS:
14 Genome-wide expression analysis of middle eastern colorectal cancer reveals foxm1
15 as a novel target for cancer therapy. The American journal of pathology
16 2011;178:537-547.
17 42 Sun H, Teng M, Liu J, Jin D, Wu J, Yan D, Fan J, Qin X, Tang H,
18 Peng Z: Foxm1 expression predicts the prognosis in hepatocellular carcinoma
19 patients after orthotopic liver transplantation combined with the milan criteria.
20 Cancer letters 2011;306:214-222.

1 43 Xia L, Huang W, Tian D, Zhu H, Zhang Y, Hu H, Fan D, Nie Y, Wu K:
2 Upregulated foxm1 expression induced by hepatitis b virus x protein promotes
3 tumor metastasis and indicates poor prognosis in hepatitis b virus-related
4 hepatocellular carcinoma. Journal of hepatology 2012;57:600-612.
5 44 Cao L, Yu Y, Darko I, Currier D, Mayeenuddin LH, Wan X, Khanna C,
6 Helman LJ: Addiction to elevated insulin-like growth factor i receptor and initial
7 modulation of the akt pathway define the responsiveness of rhabdomyosarcoma to
8 the targeting antibody. Cancer research 2008;68:8039-8048.
9 45 Xu J, Timares L, Heilpern C, Weng Z, Li C, Xu H, Pressey JG, Elmets
10 CA, Kopelovich L, Athar M: Targeting wild-type and mutant p53 with small
11 molecule cp-31398 blocks the growth of rhabdomyosarcoma by inducing reactive
12 oxygen species-dependent apoptosis. Cancer research 2010;70:6566-6576.
13 46 Kawabata N, Ijiri K, Ishidou Y, Yamamoto T, Nagao H, Nagano S,
14 Maeda S, Komiya S, Setoguchi T: Pharmacological inhibition of the hedgehog
15 pathway prevents human rhabdomyosarcoma cell growth. International journal of
16 oncology 2011;39:899-906.
17 47 Renshaw J, Taylor KR, Bishop R, Valenti M, De Haven Brandon A,
18 Gowan S, Eccles SA, Ruddle RR, Johnson LD, Raynaud FI, Selfe JL, Thway K,
19 Pietsch T, Pearson AD, Shipley J: Dual blockade of the pi3k/akt/mtor (azd8055) and
20 ras/mek/erk (azd6244) pathways synergistically inhibits rhabdomyosarcoma cell

growth in vitro and in vivo. Clinical cancer research : an official journal of the American Association for Cancer Research 2013;19:5940-5951.

48 van Gaal JC, Roeffen MH, Flucke UE, van der Laak JA, van der Heijden G, de Bont ES, Suurmeijer AJ, Versleijen-Jonkers YM, van der Graaf WT: Simultaneous targeting of insulin-like growth factor-1 receptor and anaplastic lymphoma kinase in embryonal and alveolar rhabdomyosarcoma: A rational choice. European journal of cancer 2013;49:3462-3470.

49 Srivastava RK, Kaylani SZ, Edrees N, Li C, Talwelkar SS, Xu J, Palle K, Pressey JG, Athar M: Gli inhibitor gant-61 diminishes embryonal and alveolar rhabdomyosarcoma growth by inhibiting shh/akt-mtor axis. Oncotarget 2014;5:12151-12165.

50 Ho C, Wang C, Mattu S, Destefanis G, Ladu S, Delogu S, Armbruster J, Fan L, Lee SA, Jiang L, Dombrowski F, Evert M, Chen X, Calvisi DF: Akt (v-akt murine thymoma viral oncogene homolog 1) and n-ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes rapid carcinogenesis by way of mtor (mammalian target of rapamycin complex 1), foxm1 (forkhead box m1)/skp2, and c-myc pathways. Hepatology 2012;55:833-845.

51 Douard R, Moutereau S, Pernet P, Chimingqi M, Allory Y, Manivet P, Conti M, Vaubourdolle M, Cugnenc PH, Loric S: Sonic hedgehog-dependent proliferation in a series of patients with colorectal cancer. Surgery 2006;139:665-670.

52 Wang Z, Banerjee S, Kong D, Li Y, Sarkar FH: Down-regulation of forkhead box m1 transcription factor leads to the inhibition of invasion and angiogenesis of pancreatic cancer cells. Cancer research 2007;67:8293-8300.

53 Jiang L, Wang P, Chen L, Chen H: Down-regulation of foxm1 by thiostrepton or small interfering rna inhibits proliferation, transformation ability and angiogenesis, and induces apoptosis of nasopharyngeal carcinoma cells. International journal of clinical and experimental pathology 2014;7:5450-5460.

54 Wen N, Wang Y, Wen L, Zhao SH, Ai ZH, Wang Y, Wu B, Lu HX, Yang H, Liu WC, Li Y: Overexpression of foxm1 predicts poor prognosis and promotes cancer cell proliferation, migration and invasion in epithelial ovarian cancer. Journal of translational medicine 2014;12:134.

55 Katoh M, Igarashi M, Fukuda H, Nakagama H, Katoh M: Cancer genetics and genomics of human fox family genes. Cancer letters 2013;328:198-206.

56 Martinelli S, McDowell HP, Vigne SD, Kokai G, Uccini S, Tartaglia M, Dominici C: Ras signaling dysregulation in human embryonal rhabdomyosarcoma. Genes, chromosomes & cancer 2009;48:975-982.

57 Zibat A, Missiaglia E, Rosenberger A, Pritchard-Jones K, Shipley J, Hahn H, Fulda S: Activation of the hedgehog pathway confers a poor prognosis in embryonal and fusion gene-negative alveolar rhabdomyosarcoma. Oncogene 2010;29:6323-6330.

58 Pressey JG, Anderson JR, Crossman DK, Lynch JC, Barr FG: Hedgehog pathway activity in pediatric embryonal rhabdomyosarcoma and undifferentiated sarcoma: A report from the children's oncology group. Pediatric blood & cancer 2011;57:930-938.

59 Shukla N, Ameur N, Yilmaz I, Nafa K, Lau CY, Marchetti A, Borsu L, Barr FG, Ladanyi M: Oncogene mutation profiling of pediatric solid tumors reveals significant subsets of embryonal rhabdomyosarcoma and neuroblastoma with mutated genes in growth signaling pathways. Clinical cancer research : an official journal of the American Association for Cancer Research 2012;18:748-757.

60 Chan DW, Yu SY, Chiu PM, Yao KM, Liu VW, Cheung AN, Ngan HY: Over-expression of foxm1 transcription factor is associated with cervical cancer progression and pathogenesis. The Journal of pathology 2008;215:245-252.