Table 3
Multiple stepwise regression analysis

Independent variables	Nonfrail group Adjusted R^2 value = 0.282 standard regression value	Frail group Adjusted R^2 value = 0.119 standard regression value	Overall Adjusted R^2 value = 0.345 standard regression value
Age (yr)	−0.108*		−0.137**
BMI (kg/m^2)			
Gender (male = 0, female = 1)	0.255**		0.238**
Fear of falling (yes = 1, no = 0)		−0.356**	−0.089*
10-m walking time (s)	−0.202**		−0.172**
Timed up & go test (s)			
Functional reach (cm)			
Five chair stand (s)			−0.147**
Single leg standing (s)	0.306**		0.314**

*$p < 0.05$; **$p < 0.01$.

significant and independent determinants of the average step counts in nonfrail elderly ($R^2 = 0.282$, $p < 0.001$) (Table 3). Stepwise regression analysis also revealed that fear of falling ($\beta = -0.356$, $p < 0.001$) was the only significant and independent determinant of the average step counts in frail elderly ($R^2 = 0.119$, $p < 0.001$) (Table 3).

4. Discussion

In the present study, we showed that the differential factors of physical functions may relate to the daily activities in frail and nonfrail community-dwelling elderly Japanese. Our data implicate that physical daily activities can be maintained in the robust elderly with high physical function, whereas fear of falling plays a more important role for the maintenance of physical daily activities if an older adult becomes functionally impaired and frail. Previous studies also indicated that the low self-efficacy for daily activities reduces physical activity, and psychological well-being is an important predictor for staying physically active.[18,19] Thus, differential approaches should be taken to keep the daily activities depending on their physical fitness in elderly.

The physical functions, age, and gender were associated with daily activities in nonfrail elderly but not in frail elderly. Rantanen et al[20] also reported that the relationship between muscle strength and physical disability in older adults is nonlinear. Moreover, in most of previous reports, the participants were nonfrail older adults.[1–4] Therefore, it has been assumed that there is an association between daily activities and physical functions. In addition, daily activities tended to be greater in women than in men. The reason for greater daily activities in women is often ascribed to activities, such as housework and gardening.[20]

On the other hand, we demonstrated that fear of falling was associated with physical daily activities in frail elderly but not in nonfrail elderly. Fear of falling is shown to be associated with frailty.[21,22] Several studies have indicated that people who are afraid of falling appear to enter a debilitating spiral of loss of confidence, restriction of physical activities, physical frailty, lack of social participation, falls, and loss of independence.[23–28] However, Wolf et al[29] reported that increased core and lower extremity strength with exercise decreases the fear of falling. Moreover, cognitive behavioral therapy has been shown to reduce fear of falling.[30–32]

There were several limitations of this study that warrant mention. First, although we used TUG to define frailty, TUG may not be enough to define frailty. Edmonton frail scale adopts eight other domains, such as cognition, general health status, functional independence, social support, medication use, nutrition, mood, and continence other than TUG.[33] Further study is required to test the levels of these domains in this cohort. Second, participants have used pedometer measurements limited to only 2 weeks. If seasonal changes in activity pattern were taken into consideration, long-term use would be more appropriate. Third, the participant's community was not in the rural area. The present study is the result of being restricted to older adults in the urban area.

This is the first study to demonstrate that differential factors affect daily activities depending on the level of frailty. Future work should determine whether individualized intervention can effectively improve physical activity in both nonfrail and frail elderly.

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Measurements of Stepping Accuracy in a Multitarget Stepping Task as a Potential Indicator of Fall Risk in Elderly Individuals

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Background. Elderly individuals who are at high risk for a fall generally exhibit increased gait variability, a decline in visuomotor control of foot movement, and cognitive impairment, particularly in executive functions. A new walking test, a multitarget stepping task, was developed in the present study to identify elderly individuals with impaired stepping performance on a walkway requiring the involvement of executive functions to find a footfall target.

Methods. Thirty-one high-risk (82.7 ± 6.4 years) and 87 low-risk (80.7 ± 7.9 years) elderly individuals performed the multitarget stepping task on 2 days with a 2-week interval. For the multitarget stepping task, they walked while stepping on squares with an assigned color as a footfall target continuously along the 15 lines while avoiding other colors (distracters). Two types of failure were measured: (a) failure to step precisely on the target (stepping failure) and (b) failure to avoid distracters (avoidance failure). The two groups' performance was compared. A logistic regression analysis was also performed to determine whether the measurements were independently associated with falling.

Results. The high-risk groups showed a significantly higher rate in stepping (64.5 vs 25.3% of participants in the group) and avoidance (54.8 vs 17.2%) failure than the low-risk groups. The test–retest analyses showed good agreement for both measurements. A logistic regression analysis demonstrated that the stepping failure was independently associated with falling (odds ratio = 19.365, 95% confidence interval = 3.28–113.95; $p < .001$).

Conclusions. Measurements of stepping accuracy while performing the multitarget stepping task, particularly precise stepping failure, could contribute to identifying high-risk elderly individuals.

Key Words: Elderly—Fall—MTST.

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ACCIDENTAL falls among elderly individuals are frequently attributed to tripping while walking. Age-related inability to step precisely on the ground (1–7), particularly under challenging conditions (8–11), is likely to cause tripping. Although previous research on stepping behavior in elderly individuals has shown the usefulness of measuring the accuracy of stepping on a ground during gait as a clinical tool to distinguish individuals who are at high risk (HR) for a fall from those who are at low risk (LR), attempts to develop such clinical tests are limited (12). For the current study, we developed a walking test, the multitarget stepping task (MTST). During the test, two types of failure, stepping and avoidance, were measured while the participants walked along a 10-m walkway and stepped on multiple targets. Our objective was to determine whether the indices of stepping accuracy, as well as the time required for performing the task, could be used to identify elderly individuals at risk of falling.

Elderly individuals generally demonstrate increased variability of stride length (1–3). Increased variability in gait has been shown to be associated with a greater risk of falling among elderly participants (1,2). Evidence is increasing that age-related declines in visuomotor control of foot movements are likely to contribute to such stepping variability. When instructed to step precisely on a footfall target, HR elderly individuals showed less accurate and more variable foot placement than younger and LR elderly individuals while stepping on footfall targets (4). Analyses of their gaze behavior showed that the HR elderly individuals looked sooner and longer at imminent stepping targets than younger individuals. This tendency was more pronounced with increased task difficulty (5). The fact that HR elderly individuals looked at targets longer indicates that they require more time to process visual information regarding targets and/or program appropriate motor responses (4–6). Elderly individuals also exhibit impaired visuomotor

control of foot movement in a feed-forward manner. When a target was invisible prior to the onset of stepping on that target, the rate of failure to step onto a footfall target was 42% in older participants but less than 10% in younger participants (6). Overall, these findings suggest that an age-related decline in visuomotor control of foot movement is likely to be one of the causes for impaired stepping performance.

Another factor relating to impaired stepping performance during gait is an age-related decline in the cognitive functions, particularly the executive functions (e.g., attention control, working memory, and problem solving) (12–19). Persad et al. (13) showed that, for elderly participants, difficulties with a complex walking task, including stepping precisely on a ground, have been linked to measures of executive function but were independent of other cognitive functions, such as memory and language.

Alexander et al. (12) developed a Walking Trail Making Test (W-TMT) in which a participant walked at a comfortable pace while stepping on multiple targets in a specific order. Participants were instructed to step on targets so that the number printed beside the stepping target would sequentially increase (W-TMT A) or to step on targets so that the number and letter beside the targets would increase in an alternating manner (ie, 1-A-2-B; W-TMT B). They demonstrated that the difference in the time taken to perform the W-TMT B (ie, cognitively high demand with respect to executive function) from the W-TMT A (ie, cognitively moderate) was extraordinarily high in older participants. With these findings, Alexander et al. concluded that elderly individuals have difficulty in performing accurate stepping movements with increased cognitive demands. A recent study by Persad et al. (14) demonstrated that the time taken to perform the W-TMT B diminished significantly in patients with deficits in executive function. This suggests that performing the W-TMT B involves executive function during gait and, therefore, should be effective to identify HR elderly individuals.

To date, attempts to develop a clinical test to measure the accuracy with which elderly individuals step on the ground have been limited. In the W-TMT, only the time taken to perform the task was measured. Given previous findings on the age-related decline in the visuomotor control of foot movement, measurement of the stepping accuracy itself could be an important contributory factor for identifying HR elderly individuals. Moreover, in the W-TMT, multiple targets and distracters were randomly placed. However, to thoroughly analyze the involvement of age-related decline in the spatiotemporal coordination between eye and foot movement into less stepping accuracy in HR elderly individuals, a more structured arrangement of the targets and distracters would be useful.

Considering these issues, we developed a new clinical test, a MTST, to measure the stepping accuracy in a simplified manner and compare the performance between HR and LR elderly individuals. In the MTST, participants walked along a 10-m walkway on which 15 lines of three colored squares were placed. They were instructed to step on an assigned square (the footfall target) continuously along the 15 lines while avoiding the other squares (distracters). As in the W-TMT (12), performing the MTST involved visual scanning of the targets while simultaneously stepping precisely on the target; participants would, thus, perform the MTST with the involvement of their executive functions. We investigated whether HR elderly individuals had significantly higher rates of stepping failure, that is, stepping on the target and avoiding the distracters, than the LR elderly. The participants also performed other standard clinical tests frequently used in clinical setting to identify elderly individuals at HR of falling, that is, a timed-up-and-go (TUG) test (20), a functional reach (FR) test (21), a one-leg standing (OLS) test (22), and a 10-m walking test (10-m walking) (23). Correlation analyses between each measurement collected from the MTST and each of the other standard clinical tests used were conducted to evaluate their associations. We further examined a logistic regression analysis to clarify which of these measurements, including the measurements taken from the MTST, were independently associated with falling.

Methods

Participants

A total of 118 community-dwelling older individuals (mean age, 84.5 ± 6.5years) participated. The exclusion criteria ensured that none of the participants had any indications of the following symptoms: (a) serious visual impairment (cataract, glaucoma, or color blindness), (b) inability to ambulate independently (those requiring the assistance of a walker were excluded), (c) score of less than 7 on the rapid dementia screening test (24), (d) symptomatic cardiovascular disease, (e) neurological and orthopedic disorders, (f) peripheral neuropathy of the lower extremities, or (g) severe arthritis. The participants wore flat-soled footwear while participating in the present study. Written informed consent was obtained from each subject in accordance with the guidelines approved by the Kyoto University Graduate School of Medicine and the Declaration of Human Rights, Helsinki, 1975.

A participant who met the following two criteria was classified as an HR elderly individual: (a) a self-report of the occurrence of at least one fall within the past year and (b) the time required for performing a TUG test was greater than 13.5 seconds (25,26). A fall was defined as any event that led to an unplanned unexpected contact with a supporting surface during walking. Details of the protocols for the TUG test are described later. As a result, 31 HR and 87 LR elderly individuals participated (see Table 1 for participant details). We ensured that there were no significant group

Table 1. Characteristics of Both Groups of Participants

Characteristics	High-Risk Elderly Participants, $n = 31$	Low-Risk Elderly Participants, $n = 87$	p
Age	82.7 ± 6.4	80.7 ± 7.9	.214
Gender distribution (% men)	25.80	26.40	.574
Height (cm)	153.5 ± 10.9	156.8 ± 9.8	.377
Weight (kg)	55.6 ± 12.8	58.1 ± 11.1	.568
Rapid dementia screening test (s)	8.80 ± 1.37	9.21 ± 1.51	.198
Trail Making Test Part A (s)	129.3 ± 43.3	143.6 ± 46.4	.159
Visual acuity score (decimal)	0.65 ± 0.32	0.69 ± 0.33	.550
10 m walking time (s)	21.57 ± 10.65	12.77 ± 3.63	<.001
Number of 10 m walking steps	34.45 ± 13.74	23.02 ± 4.36	<.001
Timed up and go test (s)	20.15 ± 6.26	11.57 ± 3.41	<.001
Functional reach (cm)	14.51 ± 6.31	21.57 ± 7.33	<.001
One-leg standing (s)	2.67 ± 5.57	5.75 ± 7.59	.041

differences in age, gender distribution, height, weight, the score of the rapid dementia screening test, and the visual acuity score (binocular acuity scored on the basis of a Landolt C; Table 1). Furthermore, comparisons of the scores of the TMT-A test between the two groups partly showed did not show any critical group differences in their cognitive impairment (Table 1).

Data Collection and Analyses of the MTST

The MTST was performed on a black elastic mat (10 m long and 1 m wide). There were 45 pieces of a 10 × 10 cm square on the mat (see Figure 1). These squares were arranged into three rows (15 cm between each row) and 15 lines (61 cm between each line). Each square was marked with red, blue, or yellow tape. Each line had one of the three colored squares in a randomized order. One square (blue or yellow) was regarded as a footfall target, whereas the others were distracters. The color of the footfall target was counterbalanced among the participants and announced to each participant prior to initiating walking.

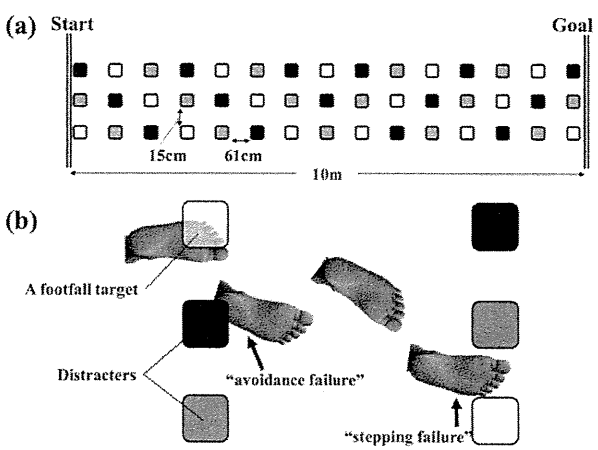

Figure 1. (a) The 10-m walkway used in the multitarget stepping task (MTST). Each square was made of red, blue, or yellow tape. (b) An example of the two types of failure measured in the MTST. A participant intends to step on footfall targets (displayed in white in this figure). The failure to step on the footfall target was regarded as the stepping error. The failure to avoid a distracter was regarded as an avoidance failure. As shown in this figure, avoidance failure was always the result of an accidental step as the participant walked from target to target, but it did not occur as a result of the wrong selection of a target out of the three squares on the line the participants intended to step on.

The participants walked on the mat at a self-selected pace while stepping on the target square placed on each line. The participants were instructed (a) to step on a footfall target with either side of the foot and any part of the sole, (b) to take as many steps as necessary while walking between the lines to comfortably walk toward the next footfall target, and (c) not to step on the distracters. At the beginning of the trial, they stood at the start position with their eyes closed to prevent them from looking at the locations of the targets beforehand. They opened their eyes as soon as they heard an experimenter's command of "Go!" and then started walking toward the first target. The participants performed one main trial. One or two (generally one) practice trials were performed before the main trial until the participants understood the task requirements and were familiar with walking on the mat. To investigate the test–retest reliability of the performance of the MTST, the participants performed the MTST twice (a single main trial each day) with a 2-week interval.

The main dependent measures were two types of failure indicating less accurate stepping performance: a stepping failure (ie, failure to step on the footfall target) and an avoidance failure (ie, failure to avoid distracters). Even a step on the edge of the target was regarded as successful; therefore, the existence of a stepping failure indicated that a participant did not seem to be able to control the placement of the foot on the target. A single experimenter observed the stepping and avoidance failures while walking next to the participant performing the MTST.

The main dependent measures were analyzed statistically from two perspectives. First, the participants who experienced each type of failure at least once were totaled for both the HR and LR groups. For each failure, the numbers, expressed as the frequency of failure occurring in the group (%), were compared statistically between the groups with a chi-square analysis. To investigate the test–retest reliability for the two types of the stepping failure, Kappa coefficients (k values) were calculated. A k value of 0.61–0.80 was

regarded as good agreement (27). Second, each number of failure for each participant was statistically compared between the two groups with a *t* test.

The use of a retrospective fall risk in the present study is less compelling as a risk for falls than the use of prospective falls. To partially address this limitation, we further conducted comparisons of the numbers of each failure for each participant while performing the MTST among three groups, namely, the HR elderly participants who reported more than one fall (the HR multifallers, $n = 11$), those who reported only one fall (the HR single fallers, $n = 20$), and the LR participants ($n = 87$). We expected that the stepping accuracy would be worse for the multifallers than for the single fallers. A one-way analysis of variance was used as a statistical test.

Another dependent measure was the time taken to perform the MTST (a MTST performance time). The time (seccond) required from the verbal command for initiating walking until the participants reached the goal line was measured with a stop watch. A *t* test was performed to statistically compare the time between the groups. To investigate the test–retest reliability for the MTST performance time, the intertrial correlation coefficient = 1.1 between the two measurements with a 2-week interval was calculated.

Data Collection and Analyses of Other Clinical Tests

The other clinical tests that have been used to identify HR elderly adults in many studies (TUG, FR, OLS, and 10 m walking) were measured prior to performing the MTST on the first measurement day. The order of performing these tests was randomized. The participants performed each task for two trials. The score of each task was calculated as an average of the score obtained from the two trials. A *t*-test analysis was examined for each clinical test to statistically compare the scores between the HR and LR groups.

In the TUG, the participants were instructed to stand up from a standard chair with a seat height of 40 cm, walk a distance of 3 m at a comfortable pace, turn, walk back to the chair, and sit down. The time required from the verbal command to begin the task until completion was measured with a stopwatch. A TUG score was defined as the time in seconds for the completion in their second trials. In the FR, the participants initially stood next to a wall while raising one arm at 90° in the sagittal dimension with all fingers extended. The participants then intended to reach forward as far as possible without moving or lifting their feet from an initial upright posture. The distance (cm) between the initial and final fingertip positions of the middle finger was obtained from each of two trials. An FR score was defined as the better performance of their two trials.

In OLS, the participants stood initially with both legs in an upright posture with their eyes were open and their arms positioned to their sides. They were then instructed to stand with only their pivot foot without any assistance. The time the participant could stand on one leg was measured with a stopwatch as an OLS score (second). The participants stopped the OLS if the time exceeded 60 seconds. When a participant could not perform the OLS, his/her OLS score was 0 second. In the 10 m walking, the participants walked alone at their usual speed over a distance of 10 m. The time recorded in the two trials was averaged as the 10 m walking score. The number of steps the participants made during the 10 m walking was also averaged in two trials and used as another score of the 10 m walking.

To quantitatively describe the associations between these clinical tests and the stepping performance in the MTST, correlation analyses were conducted between each of the three measurements in the MTST (ie, the number of stepping and avoidance failures and the MTST performance time) and each of the clinical tests.

Logistic Regression Analysis

A multivariate analysis by means of logistic regression using a stepwise-forward method was performed to investigate which of these measurements (ie, the stepping and avoidance failures; MTST performance time; or scores of TUG, FR, OLS, or 10 m walking) was independently associated with falling. For the independent variables that remained in the final step of the regression model, odds ratios (ORs) with 95% confidence intervals (CIs) were presented.

Results

The frequency of failure occurring in the group (%) and the ORs of the two types of failure that occurred in each group are shown in Table 2. The chi-square test indicated that the HR elderly participants showed significantly higher frequency of the stepping failure than the LR elderly participants ($p < .001$). The HR elderly participants also showed significantly higher frequency of the avoidance failure than the LR elderly participants ($p < .001$). Avoidance failure always occurred as the participants were walking from target to target but not when they intended to step on the target. The investigation of test–retest reliability indicated that the kappa coefficient was .758 for the stepping error and .688 for the avoidance error. Both coefficients showed good agreement between the first- and second-time measurements.

The average numbers of each failure occurring in each group are shown in Table 2. The HR elderly participants showed a significantly greater number of stepping and avoidance failures than the LR elderly participants. The average numbers of stepping failures occurring in the HR multifallers, the HR single fallers, and the LR participants were 0.64 ± 1.03, 2.30 ± 2.45, and 0.69 ± 1.53, respectively. The group differences were significant ($p < .01$); the HR single fallers showed significantly greater number of stepping failures than the HR multifallers and LR elderly participants. The average numbers of avoidance failures occurring in the three groups were 1.27 ± 1.73, 1.90 ± 2.55,

Table 2. Group Comparisons of the Stepping and the Avoidance Failure

	High-Risk Elderly Participants, $n = 31$	Low-Risk Elderly Participants, $n = 87$	p
Stepping failure, n (%)	20 (64.5)	22 (25.3)	<.001
Number of stepping failure, times	1.71 ± 2.19	0.69 ± 1.53	<.001
Avoidance failure, number (%)	17 (54.8)	15 (17.2)	<.001
Number of avoidance failure, times	1.68 ± 2.28	0.47 ± 1.28	<.001
Multitarget stepping task performance time (s)	31.58 ± 11.73	21.57 ± 7.64	<.001

and 0.47 ± 1.28, respectively. The significant group differences showed that the HR single fallers had a significantly greater number of stepping failures than the LR elderly participants.

The MTST performance times are also shown in Table 1. The HR elderly participants required significantly more time to perform the MTST than the LR elderly participants ($p < .05$). The investigation of test–retest reliability indicated that the correlation between the first- and second-time measurements was very high (intertrial correlation coefficient = 0.956; 95% CI = 0.92–0.95; $p < .001$).

The average results of the other clinical test are summarized in Table 1. Except for the results of the OLS ($p > .05$), all clinical tests demonstrated that the LR elderly participants had significantly better scores than the HR elderly participants. The correlation between each of the three measurements in the MTST (ie, the number of stepping and avoidance failures and the MTST performance time) and each of the clinical tests is shown in Table 3. The stepping failure was mildly correlated with the time and steps of the 10 m walking and the TUG, OLS, and FR scores, whereas the avoidance failure was mildly correlated with the steps of the 10 m walking and the TUG and OLS scores. The MTST performance time was highly correlated with each of the clinical tests.

The logistic regression analysis indicated that the precise stepping error (OR = 19.365; 95% CI = 3.28–113.95; $p < .001$) and TUG (OR = 1.911; 95% CI = 1.45–2.50; $p = .001$) were the independent variables that remained in the final step of the regression model and, therefore, were considered to be independently associated with falling. The adapted regression model was able to classify 78.0% of cases correctly ($R^2 = .395$, $p < .001$). The specificity was 80.3% and the sensitivity was 75.4%.

Discussion

The aim of the present study was to investigate whether a simplified measurement of stepping accuracy while performing the MTST was able to identify HR elderly individuals. The results demonstrated that 64.5% of the HR elderly participants failed to step precisely on the target at least once. This was a surprisingly high rate of failure when considering our criteria that even a step on the edge of the target was regarded as successful. The HR elderly participants also showed a significantly higher rate of avoidance failure and a slower time for performing the MTST than the LR groups. The test–retest examination showed that these measurements were statistically reliable. Unfortunately, we failed to demonstrate an association between the number of retrospective falls and the number of stepping and avoidance failures; the HR single fallers showed significantly higher frequency of both types of failure. Furthermore, although the logistic regression analysis showed a significantly high OR for the stepping failure (19.365), the very large range of 95% CI indicated that the results need to be interpreted cautiously. Taken collectively, these findings led us to the tentative conclusion that measuring the stepping accuracy while performing the MTST, particularly the stepping error, is potentially an important factor in the identification of HR elderly individuals.

The high rate of stepping failures clearly indicated that HR elderly individuals were unable to step precisely on their intended target, which could result in tripping while walking. The high rate of the avoidance failure also showed that the HR elderly participants were unable to avoid stepping distracters. It is noteworthy that avoidance failure always occurred as a result of an accidental step in the way the participants were walking from target to target but not as a result of the wrong selection of a target from the three

Table 3. The Correlation Variables Between Each of Three Measurements in the Multitarget Stepping Task (MTST) and Each of Clinical Tests

	Number of Stepping Failure	Number of Avoidance Failure	MTST Performance Time
10 m walking time	0.24*	0.15	0.75**
Number of 10 m walking steps	0.21*	0.20*	0.70**
Timed up and go test	0.20*	0.25*	0.70**
One-leg standing	−0.14	−0.19*	−0.35**
Functional reach	−0.21	−0.15	−0.39**
Trail Making Test Part A	0.05	−0.01	0.22
Number of stepping failure		0.345**	0.23*
Number of avoidance failure	0.345**		0.12
MTST performance time	0.23*	0.12	

Note: *$p < .05$. **$p < .01$.

squares in the line that they intended to step on. Avoidance failure, therefore, resulted mainly from incorrect planning of the walking path from target to target and not from the wrong selection of a target from the three squares in a line due to age-related decline in visual acuity and/or visual search. Correlation analyses between each of the three measurements and standard clinical tests showed that the stepping and avoidance failures were correlated only mildly with several tests (Table 3). This was in contrast with the findings that the MTST performance time was highly correlated with all clinical tests. It seems that a decline in stepping accuracy results in an increased fall risk somewhat independently of the balance and gait features assessed by other standard clinical tests.

One possible explanation for the reason that measuring the stepping accuracy, particularly the stepping failure, could predict falls in spite of the multifocal etiology of falls was that these measurements could be associated directly with increased gait variability (1–3) and a decline in the visuomotor control of foot movement (4–6) in HR elderly individuals. This explanation was plausible, given that some clinical tests that have components of measuring the gait variability and visuomotor control of foot movement, such as the Dynamic Gait Index (28) or the Four Square Step Test (29), contributed to identifying HR elderly individuals.

In addition, some factors characterizing the MTST could make a significant contribution to enhance its predictive power. As in the W-TMT (12), to perform the MTST, the participants visually scanned the target while simultaneously attempting to step on it; participants would thus perform the MTST with the involvement of their executive functions. Due to an apparent decline in executive functions (12–15), HR elderly individuals would have difficulty walking in the MTST. Furthermore, the placement of multiple targets on a walkway could test the ability to step quickly in different directions. Because of the difficulty in maintaining a stabilized upright posture after stepping in different directions (29,30), especially with turning behavior (31), the HR elderly individuals may have less accurate stepping performance. To evaluate the validity of these possible explanations, future studies should investigate age-related changes in gaze behavior while performing the MTST; the frequency of turning behavior; and the functional relationship among the gaze, turning behavior, and accuracy of stepping performance.

We failed to demonstrate an association between the number of retrospective falls and the number of stepping and avoidance failures; the HR single fallers showed significantly higher frequency of both types of failure. This was in contrast to the relatively small individual differences in other standard tests (Table 1). We have no clear explanation for the reasons for such large individual differences; future studies should address this issue to reliably predict future falls on the basis of stepping and avoiding failures. Future studies should also address the possibility that the numbers of stepping and avoidance failures are associated with the circumstances under which falls occurred but not with the frequency of falls.

There are several issues that limit the conclusions to be drawn from this study. First, we measured only a single performance of the MTST from each participant; an examination of the within-participant reliability with a different walking path would be necessary in future research. Second, we did not measure participants' executive functions. Whether the difference in executive functions really underlies group differences between HR and LR elderly individuals should be a topic for future research.

In conclusion, the present findings provide general evidence that measuring the accuracy of foot placement while performing the MTST is potentially an effective clinical tool to identify HR elderly individuals. Possible explanations for the reason that measuring the stepping accuracy, especially the stepping failure, could predict falls would be (a) these measurements could be associated directly with increased gait variability and a decline in visuomotor control of foot movement in HR elderly individuals and (b) performing the MTST required the involvement of executive functions to find a footfall target. However, several findings, such as the lack of an association between the number of retrospective falls and the number of stepping and avoidance failures or the very large range in 95% CI observed in the logistic regression analysis, also suggest that the present results need to be interpreted cautiously.

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ORIGINAL ARTICLE

Identification of AFAP1L1 as a prognostic marker for spindle cell sarcomas

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Spindle cell sarcomas consist of tumors with different biological features, of which distant metastasis is the most ominous sign for a poor prognosis. However, metastasis is difficult to predict on the basis of current histopathological analyses. We have identified actin filament-associated protein 1-like 1 (AFAP1L1) as a candidate for a metastasis-predicting marker from the gene expression profiles of 65 spindle cell sarcomas. A multivariate analysis determined that AFAP1L1 was an independent factor for predicting the occurrence of distant metastasis ($P = 0.0001$), which was further confirmed in another set of 41 tumors by a quantitative mRNA expression analysis. Immunohistochemical staining using paraffin-embedded tumor tissues revealed that the metastasis-free rate was significantly better in tumors negative for AFAP1L1 ($P = 0.0093$ by log-rank test). Knocking down the *AFAP1L1* gene in sarcoma cells resulted in inhibition of the cell invasion, and forced expression of AFAP1L1 in immortalized human mesenchymal stem cells induced anchorage-independent growth and increased cell invasiveness with high activity levels of matrix metallopeptidase. Furthermore, tumor growth *in vivo* was accelerated in AFAP1L1-transduced sarcoma cell lines. These results suggest that AFAP1L1 has a role in the progression of spindle cell sarcomas and is a prognostic biomarker.

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Keywords: AFAP1L1; spindle cell sarcoma; metastasis; prognostic marker

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Introduction

Sarcomas are non-epithelial malignant tumors that develop in mesenchymal tissue and classified into two groups on the basis of cellular morphology. Small round cell sarcomas, also known as blue tumors from their color on hematoxylin and eosin staining, include alveolar rhabdomyosarcomas and Ewing's sarcomas that develop in soft tissue and bone, respectively. Both have tumor-specific chromosomal translocations creating tumor-specific fusion genes (Helman and Meltzer, 2003; Toguchida and Nakayama, 2009). Spindle cell sarcomas, named for their flattened, elongated and fibroblastic morphology, far outnumber the first group. Malignant fibrous histiocytomas (MFHs) and ostesarcomas, the prototypes of this second group, develop in soft tissue and bone, respectively. They are generally radio- and chemoresistant, although high-dose, multidrug combination chemotherapy is effective for osteosarcomas. Except for some tumors, such as synovial sarcomas, which are characterized as having *SYT–SSX* fusion genes (Clark *et al.*, 1994), most spindle cell sarcomas lack tumor-specific genetic alterations (Toguchida and Nakayama, 2009). Mutations of tumor suppressor genes such as the *RB* and *p53* genes are found in many cases but not all (Toguchida *et al.*, 1992; Wadayama *et al.*, 1994). The histopathological complexity of soft tissue sarcomas is an issue. For example, MFH used to be considered the most prevalent soft tissue sarcoma but now the pathological concept is controversial (Fletcher *et al.*, 2001). To overcome this complexity, a number of gene expression profiling studies have been performed to classify spindle cell sarcomas and identify prognostic markers (Nielsen *et al.*, 2002; Lee *et al.*, 2004; Francis *et al.*, 2007; Nakayama *et al.*, 2007). Our group has also identified such markers using a custom-made cDNA microarray consisting of 23 040 genes in various types of malignant tumors (Nagayama *et al.*, 2002). As for synovial sarcomas, we have identified fibroblast growth factor (Ishibe *et al.*, 2005), Wnt-FZD10 (frizzled homolog 10) (Nagayama *et al.*, 2005) and retinoic acid

signals as molecular targets in synovial sarcomas (Ishibe *et al.*, 2008). For other types of tumors, however, the identification of tumor-specific markers is made difficult by small numbers of cases. Therefore, we have taken a different approach using the microarray data, trying to identify genes associated with aggressive phenotypes irrespective of the pathological diagnosis. Using distant metastasis as a discriminating factor, we have identified actin filament-associated protein 1-like 1 (AFAP1L1) as a candidate prognostic marker for spindle cell sarcomas. Here, we report that AFAP1L1 has a significant role in the progression of spindle cell sarcomas and is a prognostic marker as well as a potential target for molecular therapy.

Results

Identification of AFAP1L1 as a metastasis-related gene in spindle cell sarcomas

Genome-wide gene expression profiles of 65 soft tissue spindle cell sarcomas (STSs) were analyzed with a cDNA microarray system consisting of 23 040 genes, and mesenchymal stem cells (MSCs) were used as a reference (Nagayama *et al.*, 2002). The expression of each gene in tumor samples was demonstrated as the ratio of the signal intensity in tumor samples and MSCs (Nagayama *et al.*, 2002). Tumor samples were classified as positive for the gene if the ratio was more than 1.0, and negative if the ratio was 1.0 or less. Distant metastases developed in 29 cases, the remaining 36 patients being metastasis-free until the last follow-up (minimum of 61 months). The fraction of cases positive for each gene was calculated among tumors with and without metastasis, and statistical analyses were performed to identify genes associated with distant metastasis. This procedure identified the *AFAP1L1* gene, for which 21 of 29 cases with metastasis (Figure 1a) and 11 of 36 cases without metastasis (Figure 1b) were positive, and the difference was statistically significant ($P=0.0011$, Fisher's exact test). When the 65 cases were divided into AFAP1L1 (+) and AFAP1L1 (−) groups on the basis of the value relative to that of MSC, the metastasis-free fraction of AFAP1L1 (+) cases was lower than that of AFAP1L1 (−) cases (Figure 1c). AFAP1L1 is a member of the AFAP family along with AFAP-110 and AFAP1L2. AFAP-110 was identified as one of several major substrates of the viral oncogenic protein tyrosine kinase v-Src (Kanner *et al.*, 1990; Flynn *et al.*, 1993) and has been shown to function as an actin filament crosslinking protein with a fundamental role in the actin cytoskeleton's arrangement (Baisden *et al.*, 2001a). Moreover, it has been demonstrated that AFAP-110 is overexpressed in breast and prostate cancers and contributes to tumorigenic growth by regulating focal contact sites (Dorfleutner *et al.*, 2007; Zhang *et al.*, 2007). No previous reports concerning the function or involvement in cancer of AFAP1L1 have been published.

Although the overall amino-acid sequence similarity between AFAP-110 and AFAP1L1 is as much as 44%,

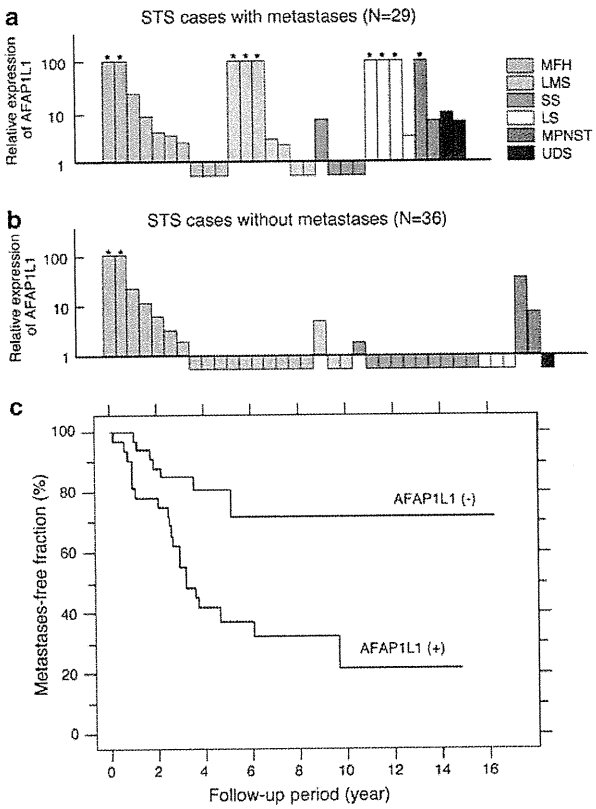

Figure 1 Identification of the *AFAP1L1* gene as a metastasis-associated gene of STSs. Expression of the *AFAP1L1* gene in STSs with metastasis ($N=29$) (**a**), and without metastasis ($N=36$) (**b**). The expression level of the *AFAP1L1* gene in each tumor is demonstrated relative to that in human bone marrow stem cells (hBMSCs). For convenience, the highest value is set as 100. Samples with a relative value of more than 100 are indicated by asterisks, and those with a relative value of less than 1.0, by rectangles. LMS, leiomyosarcoma; LS, liposarcoma; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; SS, synovial sarcoma; UDS, undifferentiated sarcoma. (**c**) Metastasis-free fraction of the initial set of STSs. A total of 65 STSs were divided by the expression level of the *AFAP1L1* gene relative to that in hBMSCs: the relative value in AFAP1L1 (+) tumors was more than 1.0 and the relative value in AFAP1L1 (−) tumors was equal to or less than 1.0. The metastasis-fee fraction of each group was demonstrated by a Kaplan–Meier curve.

AFAP1L1 shared most of the predicated domain structures with AFAP-110, such as two pleckstrin homology domains, two Src homology 2 domains (SH2), and a putative leucine zipper domain (Figure 2a) (Baisden *et al.*, 2001a). A polyclonal antibody was raised against its 79 N-terminal amino acids, which showed no homology with AFAP-110 (Figure 2a). Western blotting using this antibody showed a band with a molecular size of 90–100 kD in cell lines expressing the *AFAP1L1* gene (Figure 2b). When a set of sarcomas and human MSCs (hMSCs) were analyzed by quantitative PCR (qPCR), clear differences in the expression patterns between AFAP-110 and AFAP1L1 were found (Figure 2c). The expression of AFAP1L1 was much higher in sarcoma

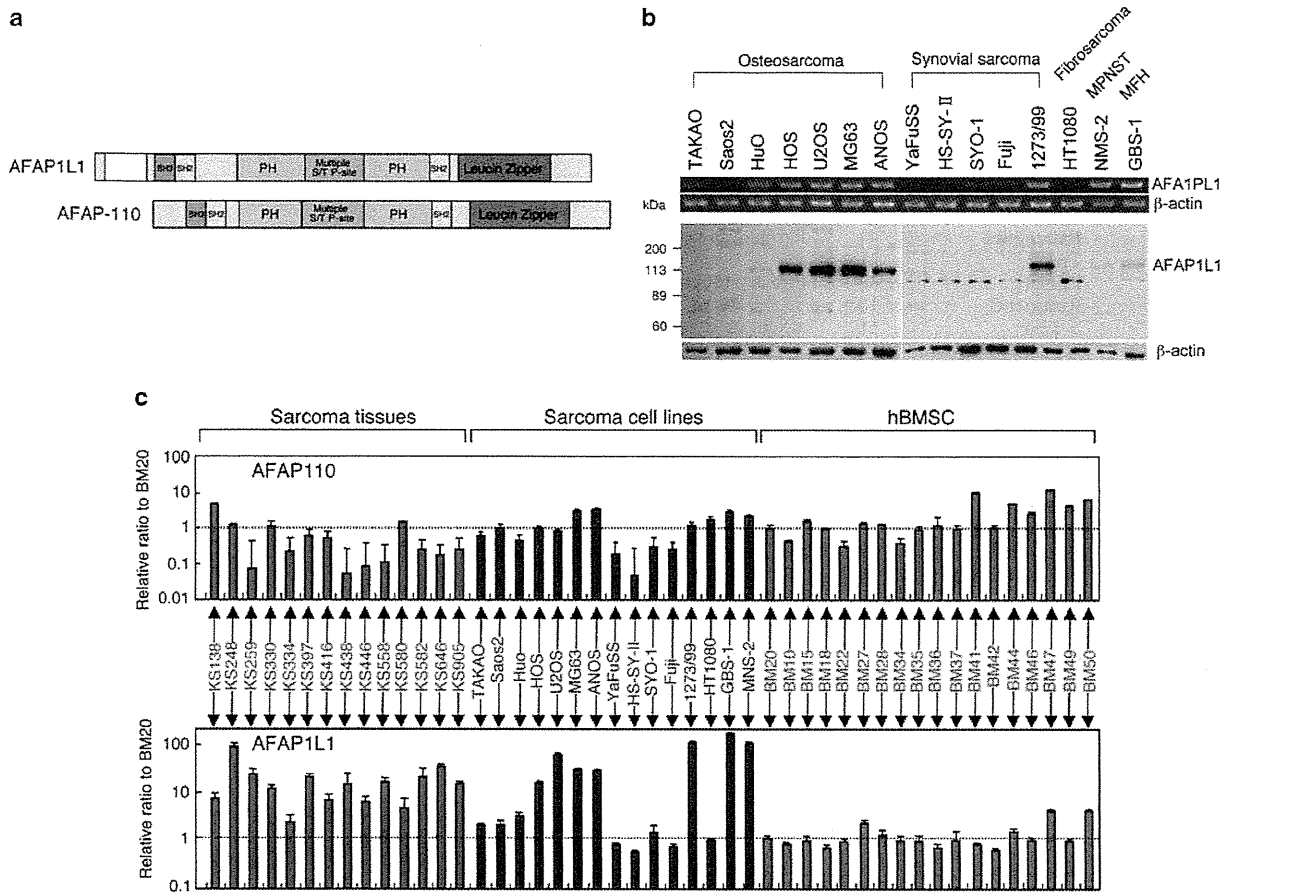

Figure 2 Comparison of AFAP-110 and AFAP1L1. (**a**) Predicted domain features of AFAP1L1 and AFAP-110. An oligopeptide derived from the hatched region was used as an antigen to raise a polyclonal antibody for APAP1L1.Validation of the anti-AFAP1L1 antibody. LZ, leucine zipper domain; PH, pleckstrin homology domain; SH2, Src homolog 2 motif. (**b**) Expression of AFAP1L1 in sarcoma cell lines. mRNA expression was analyzed by reverse transcription–PCR and protein expression was analyzed by western blotting using anti-AFAP1L1 antibody. β-Actin was used as a control. (**c**) Comparison of AFAP-110 and AFAP1L1 expression in cell lines and tissue samples. mRNA expression of AFAP-110 and AFAP1L1 in sarcoma tissues, sarcoma cell lines and human bone marrow stem cells (hBMSCs) was analyzed by qPCR. The expression level of each gene is demonstrated as a value relative to that in hBMSCs (BM20).

tissues and cell lines than in normal hMSCs ($P<0.0001$, Man–Whitney U test), whereas the expression of AFAP-110 was slightly higher in MSCs than in sarcoma tissues and cell lines ($P=0.0122$, Man–Whitney U test) (Figure 2c). On the basis of these results, we focused on the *AFAP1L1* gene.

Confirmation of the significance of AFAP1L1 in multivariate analyses

To confirm the significance of AFAP1L1 expression in the clinical behavior of STSs, the 65 cases were divided into two or more groups on the basis of clinicopathological factors, such as gender, age, location, size, depth, previous treatment history, surgery, and chemotherapy, pathological stage and pathological diagnosis, in addition to the expression of AFAP1L1 (Table 1). These factors were known to contribute to the prognoses in sarcoma patients (Ottaiano *et al.*, 2005). When the occurrence of distant metastases was used as an endpoint, AFAP1L1 expression was confirmed as a significant factor along with age and FNCLCC grade in univariate analyses (Table 1). These three factors were found to contribute independently to the prognosis in multivariate analyses (Table 1).

Association of AFAP1L1 expression with metastasis was confirmed in the second set of tumors

To confirm the significance of *AFAP1L1* gene expression, a second set of tumors consisting of 41 STSs was analyzed (Supplementary Table 1). Their clinical characteristics were almost equivalent to those of the tumors used in the initial analyses except for the pathological classification, due to a recent refinement of the diagnostic criteria for MFH (Fletcher *et al.*, 2002). STSs with no definitive features, which might be diagnosed as MFHs, were classified as 'undifferentiated sarcomas' in

Table 1 Cox's proportional hazards model analysis of factors relating to distant metastases in patients with STS

Variables	*Classes*	*No. of cases*	*Comparison*	*Hazard ratio*	*95% CI*	*Unfavorable/ Favorable*		P-*value*
Univariate analysis								
Gender	Female	39	Female vs male	0.872	0.416–1.828			0.7173
	Male	26						
Age	>60	31	≧60 vs <60	2.411	1.116–5.208	≧60	60>	0.0251
	<60	34						
Location	Extremities	44	Extremities vs trunk	0.759	0.350–1.645			0.4840
	Trunk	21						
History	Primary	49	Primary vs recurrence	1.212	0.517–2.843			0.6578
	Recurrence	16						
Size	>5 cm	51	≧5 cm vs <5 cm	1.787	0.620–5.154			0.2826
	<5 cm	14						
Depth	Superficial	24	Superficial vs deep	1.185	0.550–2.552			0.6640
	Deep	41						
FNCLCC	Grade 1	9	Grade 3 vs others	2.652	1.259–5.587	Grade 3	Grade 1 or 2	0.0103
	Grade 2	28						
	Grade 3	28						
Surgery	Wide	33	Wide vs others	0.954	0.460–1.981			0.9004
	Marginal	29						
	Intralesional	3						
Chemotherapy	Performed	29	Performed vs not performed	0.639	0.298–1.367			0.2482
	Not performed	36						
Diagnoses	MFH	27	MFH vs others	0.709	0.329–1.527			0.3795
	Synovial sarcoma	14						
	Leiomyosarcoma	10						
	Liposarcoma	7						
	MPNST	4						
	UDS	3						
AFAP1L1	Positive	32	Positive vs negative	3.611	1.592–8.195	Positive	Negative	0.0021
	Negative	33						
Multivariate analysis								
Age				2.908	1.069–7.907	≧60	60>	0.0365
FNCLCC				3.607	1.409–9.231	Stage 3	Stage 1 or 2	0.0075
AFAP1L1				4.001	1.656–9.665	Positive	Negative	0.0021

Abbreviations: AFAP1L1, actin filament-associated protein 1-like 1; CI, confidence interval; FNCLCC, Fédération Nationale des Centres de Lutte Contre le Cancer; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; STS, soft tissue spindle cell sarcoma; UDS, undifferetiated sarcoma.

most cases. During the follow-up period (4–210 months for all patients; 29–210 months for living patients), 18 developed distant metastases and 23 were free from metastasis until the last follow-up, and the qPCR analysis was used to evaluate the expression of the *AFAP1L1* gene in tumors of each group. In all, 48 of 65 samples in the first set of tumors were available for the qPCR analysis, which showed that the expression level of the *AFAP1L1* gene was significantly higher in tumors with metastasis ($N=29$) than without ($N=19$) ($P=0.0347$, Man–Whitney U test) (Figure 3a). A similar difference was found in the second set of tumors: The expression level of the *AFAP1L1* gene was significantly higher in tumors with metastasis ($N=23$) than without metastasis ($N=19$) ($P=0.0093$, Man–Whitney U test). Therefore, the association of *AFAP1L1* gene expression with metastatic activity was confirmed in the second set of STSs.

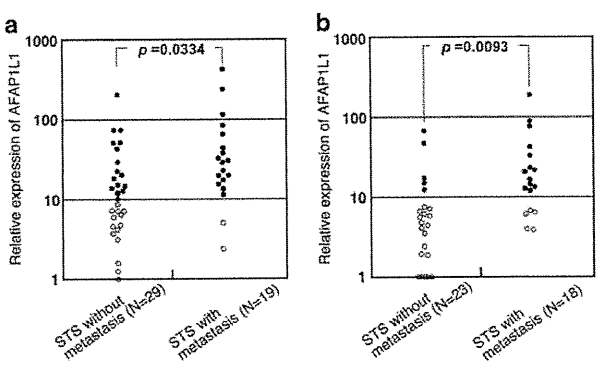

Figure 3 qPCR analyses of AFAP1L1 expression in STS. The expression level of the *AFAP1L1* gene in the first (**a**) and second (**b**) sets of STSs is demonstrated relative to that in human bone marrow stem cells.

Immunohistochemical analysis of AFAP1L1 protein in sarcomas

The expression of the AFAP1L1 protein was analyzed in paraffin-embedded specimens of 36 STSs from the second set of tumors, in which AFAP1L1 had already been analyzed by qPCR. On the basis of staining intensity of positive cells, tumors were classified into four groups; negative (−, 15 cases), weakly positive (+, 6 cases), moderately positive (++, 10 cases), and

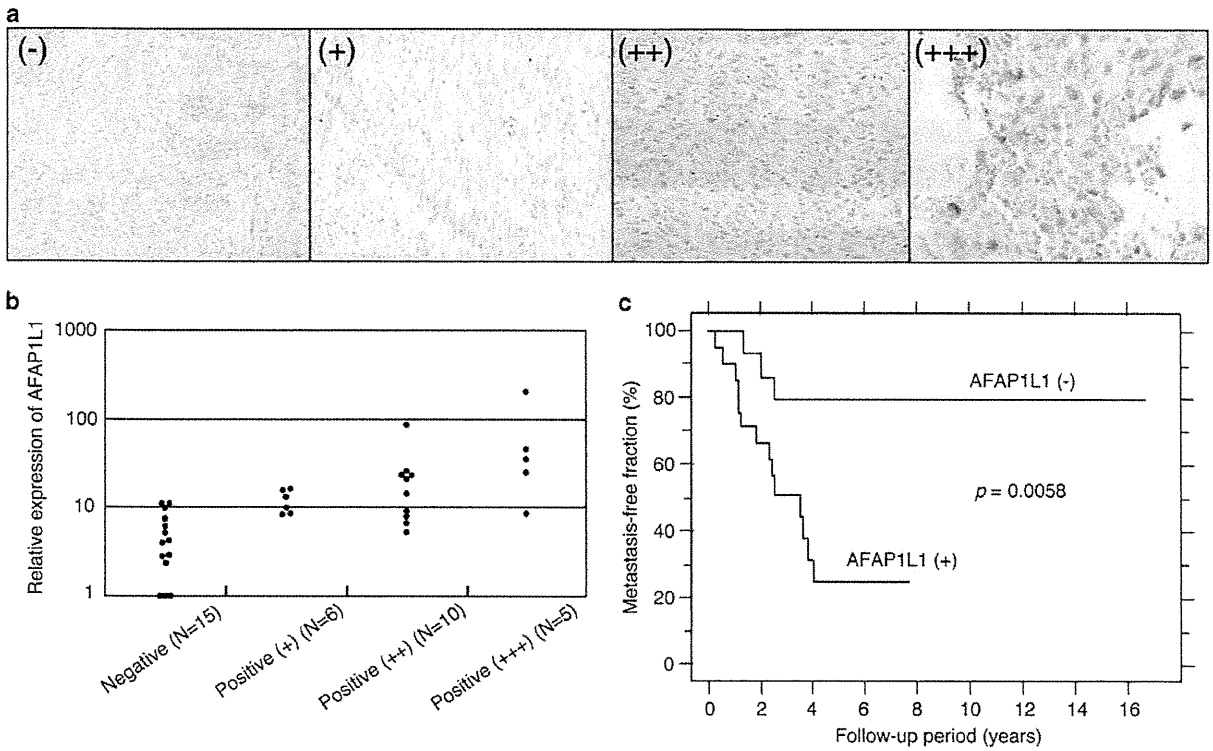

Figure 4 Expression of the AFAP1L1 protein in tumor tissues. (**a**) Expression of the AFAP1L1 protein in tumor tissues. Representative cases with negative (−), weak (+), moderate (++) or strong (+++) staining of AFAP1L1 are shown. (**b**) Relationship between the mRNA expression and immunostaining of AFAP1L1 in tumors. The mRNA level of the *AFAP1L1* gene in tumors used in the immunohistochemical analyses is demonstrated relative to that in human bone marrow stem cells. (**c**) Metastasis-free fraction of tumors with positive and negative staining of the AFAP1L1 protein. In all, 36 tumors were divided into AFAP1L1-negative (−; 15 cases) and -positive (+, ++, or +++; 21 cases) groups, and the metastasis-fee fraction of each group was demonstrated by a Kaplan–Meier curve.

strongly positive (+++, 5 cases) (Figure 4a). The staining intensity of each tumor was consistent with the result of qPCR (Figure 4b). In positive cases, AFAP1L1 was detected predominantly in the cytoplasm of tumor cells. When tumors were simply divided into AFAP1L1-negative (−, 15 cases) and positive cases (+, ++ and +++, 21 cases), the stepwise regression model showed that the metastasis-free fraction of AFAP1L1-negative cases was significantly higher than that of positive cases (Figure 4c). There was no significant difference between tumors positive and negative for the staining in terms of patient's age, tumor size and tumor depth. As for FNCLCC grade, however, the number of high-grade tumor (grade 3) in positive cases (12/21 cases) was significantly higher than that in negative cases (3/15 cases) ($P = 0.0407$, Fisher's exact test). Therefore, the association of AFAP1L1 expression with metastasis was confirmed by both mRNA and protein analyses, suggesting AFAP1L1 to be a prognostic marker of spindle cell sarcomas.

Inhibition of AFAP1L1 expression reduced the invasiveness

We next generated AFAP1L1-knocked down cells to investigate the function of AFAP1L1 using an RNA interference system mediated by a lentivirus. U2OS and 1273/99 cells were used in these experiments because they had abundant AFAP1L1 expression among sarcoma cell lines tested (Figure 5a). Two non-targeting sequences for mammalian genes (control RNAi-1 and -2) and two AFAP1L1-targeting sequences (AFAP1L1 RNAi-1 and -2) were employed for further experiments. The transduction efficiency was 90–95% in U2OS cells and 85–90% in 1273/99 cells, as determined by counting EmGFP-positive cells (data not shown), and the knock-down of AFAP1L1 was confirmed effective by western blotting (Figure 5a). As for proliferative ability, no differences were found between the AFAP1L1-knock-down cells and control cells in the U2OS (Figure 5b) and 1273/99 cell lines (data not shown). However, matrigel invasion assays revealed that knocking down AFAP1L1 resulted in reduced cell invasiveness (Figure 5c).

Induction of AFAP1L1 gene expression conferred invasiveness

The cells of origin for sarcomas remain unclear but one possible candidate is the MSC (Matushansky *et al.*, 2007), so we chose immortalized human MSCs (ihMSCs) as a recipient for AFAP1L1 transduction. ihMSCs were established in our laboratory and shown to be fully transformed when the activated H-*ras* gene had been

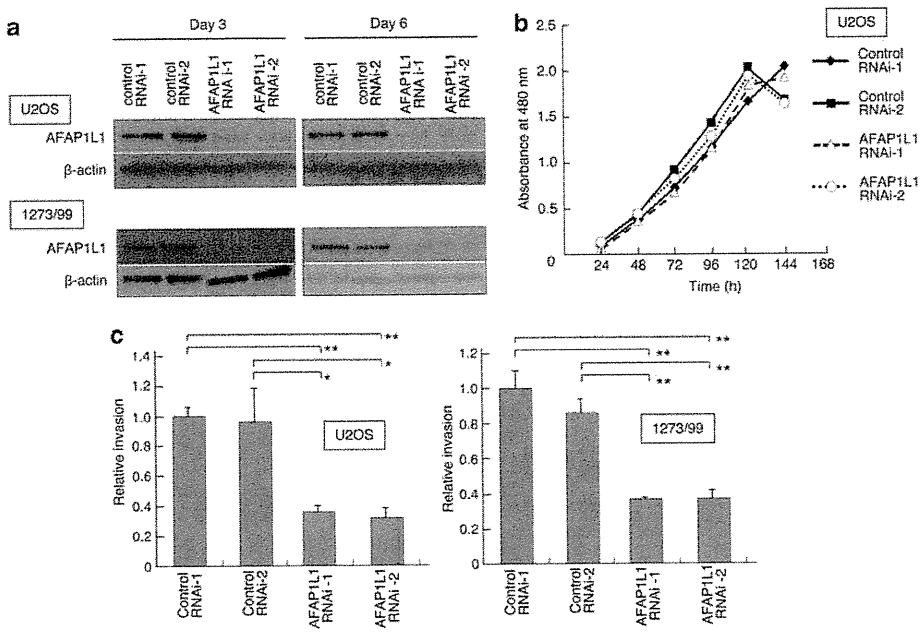

Figure 5 Downregulation of AFAP1L1 expression decreased invasiveness of sarcoma cells. (**a**) Western blotting of U2OS and 1273/99 cells transduced with microRNAs. Either non-targeting (control RNAi-1 or -2) or AFAP1L1-targeting (AFAP1L1 RNAi-1 or -2) microRNA was introduced and the expression of AFAP1L1 was analyzed 3 and 6 days later. (**b**) Growth curves of U2OS cells transduced with mircoRNAs. Cell viability was evaluated by WST-8 assay. (**c**) Invasive ability of U2OS and 1273/99 cells transduced with mircoRNAis. Matrix invasiveness was calculated as described in materials and methods, and demonstrated as fold change relative to control RNAi-1 cells. $^{**}P<0.01$; $^{*}P<0.05$.

introduced (Shima *et al.*, 2007). pLenti6/AFAP1L1 was transduced into ihMSCs, and several clones stably expressing AFAP1L1 were established (ihMSC/AFAP1L1) and used for further experiments (Figure 6a). The growth of AFAP1L1-transduced clones showed no significant change compared with that of the parental ihMSCs or ihMSC/LacZ cells (data not shown), whereas invasiveness and anchorage-independent growth were exaggerated in all four ihMSC/AFAP1L1 clones (Figures 6b and c).

Tumor invasiveness correlates with the activity of matrix metalloproteinases (MMPs), such as gelatinases, MMP-2 and MMP-9 (Egeblad and Werb, 2002; Overall and Kleifeld, 2006). To determine whether the increased invasive ability of the ihMSC/AFAP1L1 clones was related to increased excretion of MMPs, gelatin zymography was performed. The ihMSC/AFAP1L1 clones showed significantly increased activity of MMP-9 but not MMP-2 compared with control cells (Figure 6d). The increased excretion of MMP-9 in two ihMSC/AFAP1L1 clones was also confirmed by enzyme-linked immunosorbent assay (Figure 6e). These results suggest that AFAP1L1 endows ihMSCs with invasiveness, at least partly, by regulating MMP-9's excretion. In clinical samples, however, the expression level of MMP-9 was not clearly associated with that of AFAP1L1 ($R^2 = 0.248$) (Supplementary Figure S1).

Acceleration of tumor growth by AFAP1L1 expression in vivo

The inoculation of ihMSC/AFAP1L1 clones subcutaneously into immunodeficient mice produced no tumors (data not shown), indicating that the overexpression of AFAP1L1 in immortal cells was not enough for full transformation. To investigate whether AFAP1L1 modifies the phenotype of sarcoma cells without endogenous AFAP1L1 expression *in vivo*, stably expressing cell lines were generated using Saos2 cells as a recipient (Saos2/AFAP1L1) (Figure 7a). The growth of Saos2/AFAP1L1 clones *in vitro* showed no significant change compared with that of parental Saos2 or Saos2/LacZ control cells, but the invasive activity of Saos2/AFAP1L1 clones increased, which was consistent with that of ihMSC/AFAP1L1 clones (data not shown). These Saos2 clones were subcutaneously inoculated into the back of non-obese diabetic/severe combined immunodeficient mice to evaluate tumorigenesis and metastasis. Tumor growth was accelerated in Saos2/AFAP1L1 clones, although the extent of the increase seemed not to completely match the level of AFAP1L1 expression (Figures 7b and c). There was no metastasis by any Saos2/AFAP1L1 clones or control cells, suggesting that the expression of AFAP1L1 was not enough to produce distant metastasis in this animal model.

Discussion

Tumor type-specific molecular markers have been searched for in a variety of malignant tumors, in order to predict biological phenotype and/or serve as a target

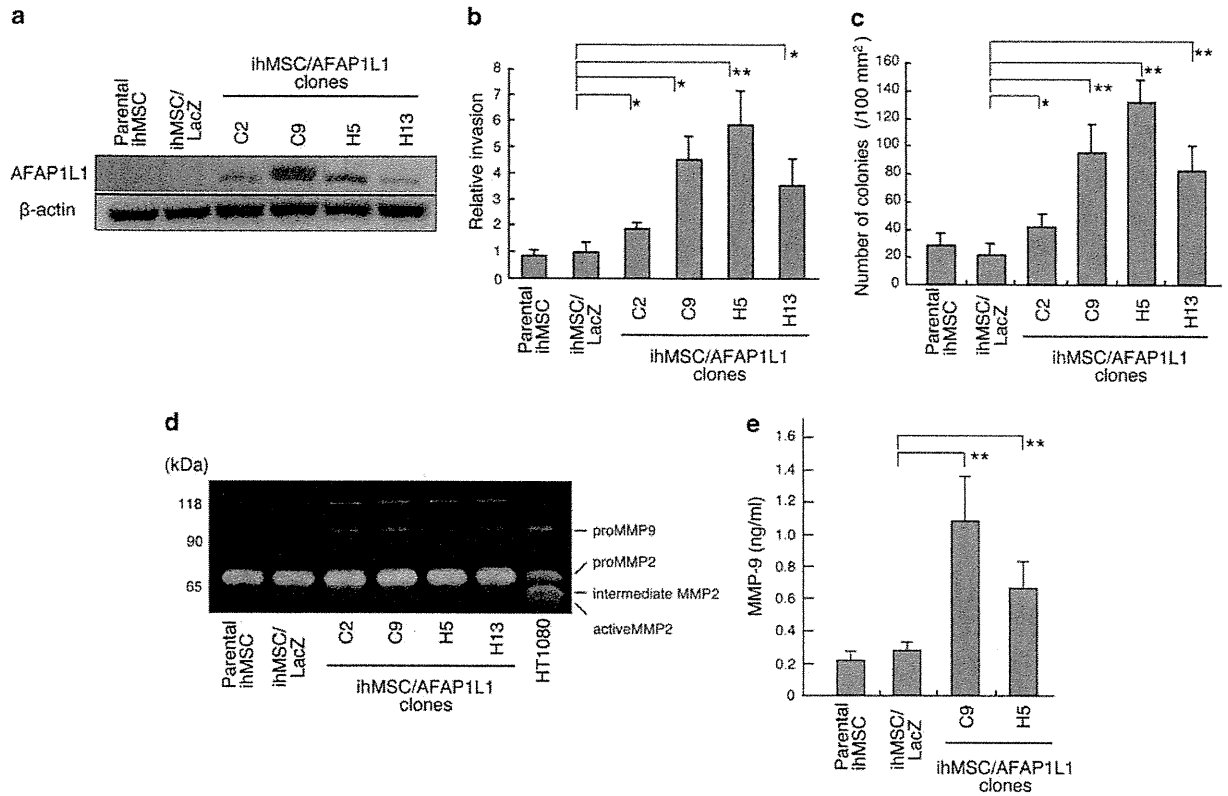

Figure 6 Upregulation of AFAP1L1 expression increased invasiveness and anchorage-independent growth of ihMSCs. (**a**) Western blotting of ihMSC clones stably expressing AFAP1L1. ihMSCs transduced with a LacZ-expressing lentivirus were used as a control. (**b**) Invasive ability of ihMSC clones transduced with AFAP1L1. Matrix invasiveness was calculated as described in materials and methods and demonstrated as fold change relative to LacZ-transduced cells. (**c**) Anchorage-independent growth of ihMSC clones transduced with AFAP1L1. (**d** and **e**) Production of MMP-9 in ihMSC clones transduced with AFAP1L1. Gelatin zymography (**d**) and enzyme-linked immunosorbent assay (**e**) demonstrated increased secretion of MMP-9 in ihMSC clones transduced with AFAP1L1. $^{**}P<0.01$; $^{*}P<0.05$.

for therapy. In the case of spindle cell sarcomas, however, the diversity and rarity of tumors have hampered such efforts. The importance of this study lies in having identified AFAP1L1 as a metastatic and a prognostic marker of spindle cell sarcomas, irrespective of pathological diagnosis. However, the significance of this gene in the development of distant metastasis should be carefully evaluated because our strategy focused on the contribution of single genes. Recently, two studies using large numbers of samples have been published relating to prognostic and therapeutic markers for soft tissue sarcomas. Barretina *et al.* (2010) performed an intensive analysis of 722 protein-coding and miRNA genes using a combination of DNA sequencing and a single-nucleotide polymorphism array and identified several tumor subtype-specific genetic alterations, some of which could be molecular targets for therapy. Chibon *et al.* (2010) identified a set of 67 genes on the basis of genomic and expression profiling and established a complexity index in sarcomas, which can predict the prognosis of patients. Among 67 genes, most were related to mitosis and chromosome management, and the *AFAP1L1* gene was not included.

Clinical results clearly demonstrated the association of AFAP1L1 with metastatic behavior of sarcomas in our cohorts, but the molecular mechanisms underlying this association are not yet clear. The results of knockdown and forced-expression experiments using sarcoma cell lines suggest that AFAP1L1 is involved in the process of invasion. Increased invasion of the matrix gel was confirmed in AFAP1L1-introduced ihMSC clones in association with an increase in excretion of MMP-9, a common feature of highly invasive malignant cells. We searched the ONCOMINE cancer array database (http://www.oncomine.org) and found a moderate correlation ($R^2=0.6964$) between AFAP1L1 and MMP-9 in glioblastomas (Sun *et al.*, 2006). We have no clear explanation of why we failed to find a clear association between the expression level of AFAP1L1 and that of MMP-9 in clinical samples (Supplementary Figure S1). This may be due to the multifactorial control of MMP-9 expression (St-Pierre *et al.*, 2004), and factors other than MMP-9 may be involved in the role of AFAP1L1 in tumor cell invasion. In this respect, it is intriguing that although the introduction of AFAP1L1 expression caused no change in growth profiles *in vitro*,

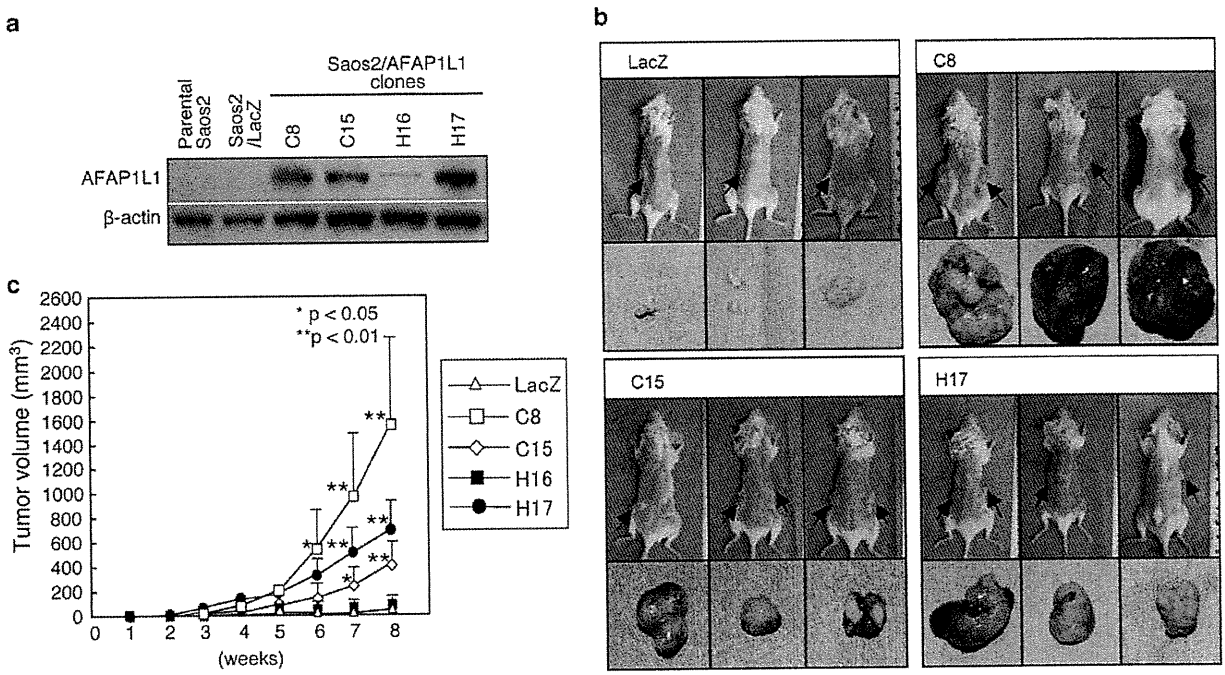

Figure 7 Acceleration of tumor growth by AFAP1L1 *in vivo*. (**a**) Western blotting of Saos2 clones stably expressing AFAP1L1. Saos2 cells transduced with a LacZ-expressing lentivirus were used as a control. (**b**) Macroscopic finding of tumors that developed in mice injected with AFAP1L1- or LacZ-transduced Saos2 clones. Tumors are indicated by arrows. (**c**) Growth curve of tumors shown in (**b**). $^{**}P<0.01$; $^{*}P<0.05$. Statistical examination was carried out by analysis of variance. Six mice were used for each cell clone.

the formation of tumor masses was accelerated *in vivo*. This suggested a role for AFAP1L1 at the interface between tumor cells and environments. Although the *AFAP1L1* gene was identified as a metastasis-associated gene from clinical data, we failed to find an association of metastasis with the expression of AFAP1L1 in experiments *in vivo*. The current experimental system using the subcutaneous inoculation of osteosarcomas cells may not be appropriate for evaluating the function of AFAP1L1.

AFAP1L1 is a paralogue of AFAP-110, which is an SH2/SH3-binding partner for Src (Flynn *et al.*, 1993; Guappone and Flynn, 1997; Qian *et al.*, 1998). AFAP-110 contains several protein-binding motifs at its amino terminus and functions as an adapter protein for actin filaments (Qian *et al.*, 2000; Baisden *et al.*, 2001b; Qian *et al.*, 2004). It is also required to control protein kinase Cα-mediated activation of c-Src and the subsequent formation of podosomes (Gatesman *et al.*, 2004). AFAP1L2, also known as XB130, is another paralogue of AFAP-110. AFAP1L2 associates with Src as well (Xu *et al.*, 2007) and is predominantly expressed in the thyroid (Lodyga *et al.*, 2009). AFAP1L2 cooperates with RET/PTC (rearranged in transformation/papillary thyroid carcinomas), a thyroid-specific tyrosine kinase, to increase phosphorylation of AKT, suggesting a role in thyroid cancer (Lodyga *et al.*, 2009). In addition, although AFAP1L2 has structural similarities to chicken AFAP-110 with which it was identified in a search of databases, it does not associate with actin filaments, suggesting a different role from that of AFAP-110 (Lodyga *et al.*, 2009). We have performed a series of experiments to examine the association of AFAP1L1 with actin filaments but found no definite evidence of one (data not shown). Although we speculate that AFAP1L1 acts as an adapter protein on the basis of its structural similarity to AFAP-110, AFAP1L1 functions in sarcoma cells via mechanisms distinct from those of AFAP-110, which may confer aggressive biological features.

Materials and methods

Clinical samples

Tumor samples were obtained at resection surgery in Kyoto University Hospital and preserved as described previously (Nagayama *et al.*, 2002). At least 90% of the viable cells in each specimen were identified as tumor cells. Primary hMSCs (hMSCs) were obtained and cultured by a method reported previously (Shibata *et al.*, 2007). All samples were approved for analysis by the ethics committee of the Faculty of Medicine, Kyoto University.

Cell lines

HS-SY-II was kindly provided by H Sonobe (Kochi University, Japan), SYO-1 by A Kawai (Okayama University, Japan), Fuji by S Tanaka (Hokkaido University, Japan), 1273/99 by O Larsson (Karolinska Institute, Sweden), NMS-2 (malignant peripheral nerve sheath tumor) by A Ogose (Niigata University, Japan) and GBS1 (MFH) by H Kanda (The Cancer Institute of the Japanese Foundation for Cancer

Research, Japan). ANOS, YaFuSS and an immortalized hMSC (ihMSC) line were established in our laboratory as described previously (Aoyama *et al.*, 2004; Ishibe *et al.*, 2005; Shima *et al.*, 2007). Other cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) or Japanese Collection of Research Bioresources (JCRB) (Ibaraki, Japan). Cells were maintained in RPMI 1640 medium or Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37 °C under 5% CO_2.

Production of anti-AFAP1L1 polyclonal antibody
The polyclonal antibody for AFAP1L1 was raised by immunizing rabbits with glutathione *S*-transferase-fused polypeptides corresponding to codon 35–113 of the human *AFAP1L1* gene and purified with standard protocols using affinity columns.

Immunohistochemical and immunocytochemical analyses
Immunohistochemical experiments using paraffin-embedded specimens of soft tissue sarcomas were performed as described previously (Kohno *et al.*, 2006). The anti-AFAP1L1 antibody was used at a concentration of 1 μg/ml. For immunocytochemistry, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and blocked with 1% bovine serum albumin in PBS. Slides were incubated with the anti-AFAP1L1 antibody or an anti-Flag M2 antibody (Sigma-Aldrich) overnight and then with a corresponding Alexa Fluor-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). When indicated, rhodamine–phalloidin (Invitrogen) was used to stain actin fibers. Nuclei were stained with 4,6-diamidino-2-phenylindole. Cells were viewed with an IX81 (OLYMPUS, Tokyo, Japan) and photographed. The scoring was performed by two researchers without information on the clinical data for each sample.

Transient AFAP1L1 expression vectors
The coding region of the *AFAP1L1* gene was cloned into the pCAGGS vector (pCAG/AFAP1L1WT) tagged at the N-terminus with 3 × Flag. Transfection of these vectors was carried out using the Amaxa electroporation system (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instructions.

Lentiviral production
The BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen) was used to knock down the *AFAP1L1* gene. Two oligonucleotides targeting AFAP1L1 (Hmi456004 and 456007, designated AFAP1L1 RNAi-1 and -2) and two control microRNAs that have no homology with mammalian gene sequences (designated control RNAi-1 and -2) were designed by and purchased from Invitrogen. They were annealed and ligated into the pcDNA6.2-GW/EmGFP-miR, in which microRNA expression was driven by a cytomegalovirus promoter with simultaneous expression of EmGFP. The cassette was subsequently cloned into pLenti6/V5-DEST, and cells were infected with supernatant containing microRNA lentiviruses (4.2×10^6 TU/ml) using the ViraPower Lentiviral Expression System (Invitrogen). These microRNA-transduced cells were prepared without drug selection or single-cell cloning. To generate cells stably expressing AFAP1L1, its gene was cloned into pLenti6/V5-DEST by a PCR-based method (pLenti6/AFAP1L1). As a control, a β-galactosidase gene-expressing vector (pLenti6/LacZ) was used. Cells were infected with these lentiviruses and selected with blasticidin (Invitrogen) for two weeks. Several clones of pLenti6/AFAP1L1-transduced cells were isolated by limiting dilution. pLenti6/LacZ-transduced control cells were used without cloning.

Western blot analyses
Western blot analyses were performed as described previously (Kohno *et al.*, 2006). Membranes were incubated overnight with the anti-AFAP1L1 (1:1000), anti-Flag M2 (1:4000; Sigma-Aldrich) or anti-β-actin (1:4000; Sigma-Aldrich) antibody.

Matrigel invasion assay
Cell suspensions (2.5×10^4) in 0.5 ml of DMEM without fetal bovine serum were placed in the upper chambers of 8 μm control cell culture inserts (BD Biosciences, Franklin Lakes, NJ, USA) or BioCoat matrigel invasion chambers (BD Biosciences), and 0.5 ml of DMEM containing 5% fetal bovine serum was placed in each lower chamber. After incubation for 22 h at 37 °C under 5% CO_2, cells on the upper surface of the membrane were mechanically removed. The membranes were fixed, and stained with 1% Toluidine blue. Cells were counted in five randomly chosen fields under a magnification of × 100. Cell invasiveness was calculated by dividing the number of cells invading through the matrigel membrane by the number invading the control insert.

Cell growth assay
Cells were seeded on 96-well plates at a density of 1000 per well in quadriplicate. The next day, cell viability was assessed by WST-8 using a Cell Counting Kit (DOJINDO, Kumamoto, Japan) every 24 h, according to the manufacturer's instructions.

Colony formation in soft agar
Cells (1×10^4) were suspended in DMEM containing 0.35% agarose and layered on a solidified 0.7% agarose layer in 60-mm tissue culture plates and cultured at 37 °C under 5% CO_2. After 4 weeks of incubation, p-iodonitrotetrazolium violet (Sigma-Aldrich) was added to count viable colonies.

Gelatin zymography
Gelatinolytic activity of the supernatant was analyzed as described elsewhere. Briefly, cells (4×10^5) were incubated with DMEM containing 10% fetal bovine serum for 24 h and then the medium was replaced with 0.5 ml of OptiMEM1 (Invitrogen) containing 0.1% bovine serum albumin. After 16 h of incubation, the conditioned medium was analyzed on a 10% Tris–glycine gel containing 0.1% gelatin. The gel was treated with renaturating buffer for 30 min and with developing buffer for 12 h at 37 °C. Bands of gelatinolytic activity were visualized after staining the gels with 0.1% coomassie brilliant blue R-250 (Thermo Fisher Scientific Inc.) and then destaining. The digested bands were scanned by ChemiDocXRS (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Enzyme-linked immunosorbent assay
The expression of MMP-9 was quantified using a commercially available enzyme-linked immunosorbent assay system (Amersham matrix metalloproteinase-9 human biotrak ELISA system, GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

Animal experiments
All experiments with animals were approved by the Animal Research Committee (Graduate School of Medicine, Kyoto University) and conducted according to the Guidelines for Animal Experiments of Kyoto University. Cells (5×10^6)

suspended in 100 μl of PBS were injected subcutaneously into the hind flank region of female non-obese diabetic/Shi-*scid* Jic (non-obese diabetic/severe combined immunodeficient) mice at 5 weeks of age (Clea Japan, Tokyo, Japan). Tumor volumes were calculated using the formula: (length × width × height × 3.14)/6.

Reverse transcription and real-time qPCR

RNA extraction and reverse transcription were performed as described previously (Kohno *et al.*, 2006). Real-time qPCR analyses were performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Taqman probes for AFAP1L1 (5′-GGCCCTTCCTCTGGGA CCCGGC-3′) and AFAP-110 (Taqman Gene Expression Assays Hs00222181_m1) were purchased from Applied Biosystems. 18S rRNA was also purchased from Applied Biosystems and used as an endogenous reference. Information on primers is available upon request.

Statistical analyses

Statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA). Univariate and multivariate analyses were performed using Cox's proportional hazards model. The statistical significance of Kaplan–Meier curves was assessed by log-rank (Mantel–Cox) test. For comparisons of two individual data points, a two-sided Student's *t*-test was applied to assess statistical significance. An analysis of variance with *post hoc* testing was used for comparisons of more than three.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)