SPANDIDOS PUBLICATIONS

The expression of hDlg as a biomarker of the outcome in malignant fibrous histiocytomas

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Received July 16, 2009; Accepted October 5, 2009

DOI: 10.3892/or_00000678

Abstract. The human homologue of *Drosophila* disc large tumor suppressor protein (hDlg) is one of the proteins known to act cooperatively in regulating cell polarity and proliferation, suggesting an important connection between epithelial organization and cellular growth control. An abnormal expression of hDlg has been reported in several cancer types. However, the expression of hDlg in soft-tissue sarcomas has not yet been reported. We examined the expression of hDlg immunohistochemically in 46 specimens of malignant fibrous histiocytoma (MFH). The expression of hDlg was negative in 19 specimens, weak in 4, moderate in 16, and strong in 7. The patients with a weak or negative expression of hDlg had a significantly shorter metastasis-free survival rate and disease-free survival rate in comparison with those with a strong or moderate expression in both univariate analysis (p=0.0287 and 0.0237, respectively; log-rank test) and multivariate analysis (p=0.0087 and 0.0126, respectively; Cox proportional hazards regression model). Moreover, the patients with a weak or negative expression of hDlg had a significantly shorter overall survival rate in comparison with those with a strong or moderate expression in a univariate analysis (p=0.0214; log-rank test). This is the first report to demonstrate that a reduced expression of hDlg protein is an independent negative prognostic factor for MFH.

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Key words: hDlg, tumor suppressor gene, malignant fibrous histiocytoma, biomarker, prognosis

Introduction

The *Discs large* (*Dlg*) gene was isolated as a *Drosophila* tumor suppressor gene (1,2). *Drosophila* Dlg and its mammalian homolog 'hDlg' (Sinapse-Associated Protein 97 kDa; SAP97) belong to the membrane-associated guanylate kinase (MAGUK) family of proteins and bear characteristic structural domains including 3 PDZ domains, an SH3 domain, and a guanylate kinase-like (GUK) region (3). All of these domains are involved in protein-protein interactions which allow the MAGUK proteins to act as intracellular scaffolding molecules in the formation of macromolecular signaling complexes (4-7). The PDZ domains are found in a variety of proteins in single or multiple copies (8). The MAGUK PDZ domains bind and mediate the clustering of the membrane receptors and the channel proteins (9). The SH3 domain is a well characterized protein-binding domain that is found in a variety of proteins, and it binds to proline-rich motifs, which may allow hDlg to participate in signaling pathways by forming complexes via the SH3 domains of other proteins (10). Due to sequence divergence, the GUK domain of the MAGUKs shows low or no kinase activity and appears to also act as a protein binding domain (11-14). MAGUKs are known to localize at the regions of cell-to-cell contacts and at the pre-synaptic and post-synaptic density, where they are thought to play an important role in the regulation of cell proliferation and synaptic transmission (8). In addition, hDlg interacts, via its PDZ domain, with the tumor suppressor adenomatous polyposis coli (APC) (15) and with several viral oncoproteins (16,17), thus suggesting that hDlg is involved in cell growth regulation and tumorigenesis. Indeed, the overexpression of hDlg suppresses cell proliferation by blocking cell cycle progression from the G0/G1 phase to the S phase (18).

Epithelial cells display an apico-basal polarity that is required for their correct function. The polarity is mediated by the presence of different cell junctions that depend on the formation of multiprotein networks at the cell membrane (19). Therefore, hDlg is involved in cell growth control, in the

maintenance of cell adhesion and polarity, and in functions that block cell invasion during development (1,2,19). Therefore, the loss of hDlg may explain some of the characteristics of the malignant cells, such as abnormality of the polarity and the high migration ability which is one of the hallmarks of malignant tumors (20,21). Indeed, the abnormality of the expression of hDlg has been reported in several cancers (16,22,24,25). Previous reports have demonstrated the potential importance of hDlg in cancer suppression in the cervix (16,22,23), in gastric cancers (24), and in ductal carcinomas (26). Cavatorta *et al* described that the loss of Dlg may be considered as a late stage marker in the cervical carcinogenic process, although alterations of the level of expression and the intracellular localization of hDlg take place during the different dysplastic stages (22). However, the expression of hDlg in soft-tissue sarcomas has not been reported. In this study, we examined the expression of hDlg in malignant fibrous histiocytoma (MFH).

MFH is characteristically a tumor of late adult life, with most cases occurring in persons between the age of 50 and 70 years. The tumor occurs most frequently on the lower extremities, especially the thigh, followed by the upper extremity and the retroperitoneum. The vast majority of MFH are high-grade lesions. Despite the recent adequate treatment for MFH, the prognosis is still poor. The 5-year local recurrence-free survival (LRFS), the metastasis-free survival (MFS), and the overall survival rate (OS) were reported as 63-87%, 62-63% and 50-74%, respectively (26-30). Therefore, a biomarker which can predict the high-risk patients is very important because it may be a useful indicator for determining whether adjuvant therapeutic modalities, such as irradiation and chemotherapy, should be performed. The aim of this study was to investigate whether the hDlg protein expression had a prognostic impact on MFH.

Materials and methods

Study population and tissue preparation. The tissue specimens used for this study were obtained from the patients who underwent surgical resection or an open biopsy at the Department of Orthopaedic Surgery, Mie University Graduate School of Medicine after obtaining informed consent according to the institutional review board guidelines. Immediately after surgical removal, the tissue specimens were fixed in 10% buffer formalin solution for 24 h and embedded in paraffin for the histological analysis. The paraffin-embedded tumor tissue specimens, measuring 4 μm in thickness, were placed on silanized slides (Matsunami Japan, Osaka, Japan) and stained with hematoxylin and eosin. The histological sections of all the patients were diagnosed by well-trained pathologists.

In all the cases, immunohistochemical staining was used to diagnose MFHs according to the World Health Organization classification (31). MFH was diagnosed primarily based on the morphological appearance of the results which showed no reactivity for immunostaining, and the fact that a storiform-pleomorphic type was observed in 46 patients.

In this study, we excluded the patients who had distant metastases at initial treatment, or the patients who had recurrent sarcomas after inadequate treatments at the initial hospital.

Table I. Details of patient characteristics.

Characteristics	No. (n=46)
Gender	
Male	22
Female	24
Age (years)	
≤59	14
≥60	32
Location	
Upper extremities	7
Lower extremities	27
Trunk	12
Size (cm)	
<5	10
≥5	36
Depth	
Superficial	10
Deep	36
Primary or recurrence	
Primary	37
Recurrence	9

Clinical and pathologic analysis. The details of the clinicopathologic features are listed in Table I. The study comprised 46 patients (22 males and 24 females). The median age of all the patients was 66 years (range, 42-86 years). The median follow-up for all the patients was 50 months (range, 2-145 months). All the patients underwent a complete tumor resection with a wide margin during the initial surgery at our hospital. The 46 samples consisted of 37 primary lesions and 9 local recurrences.

At the final follow-up, 18 patients were continuously disease-free, 11 patients showed no evidence of disease, 5 patients were alive with disease, 11 patients died of disease, and 1 patient died of an unrelated cause.

Immunohistochemical analysis. The paraffin-embedded tumor tissue specimens measuring 4 μm in thickness were placed on silanized slides (Matsunami Japan) and then deparaffinized in xylol. Antigen retrieval was performed with 0.01 M citrate buffer at 121˚C for 10 min, cooling for 60 min and washing in PBS. The mouse monoclonal anti-SAP97 antibody (clone 2D11, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:50 in the antibody diluent buffer (1% BSA/PBS). Immunohistochemistry was achieved with the Ventana EX system using a DAB universal kit (iview DAB; Ventana Medical Systems, Tucson, AZ, USA), in which the visualization of the bound antibodies was performed using the streptavidin-biotinylated complex method.

In this study, the expression of hDlg was assessed semi-quantitatively by the following two parameters: the percentage

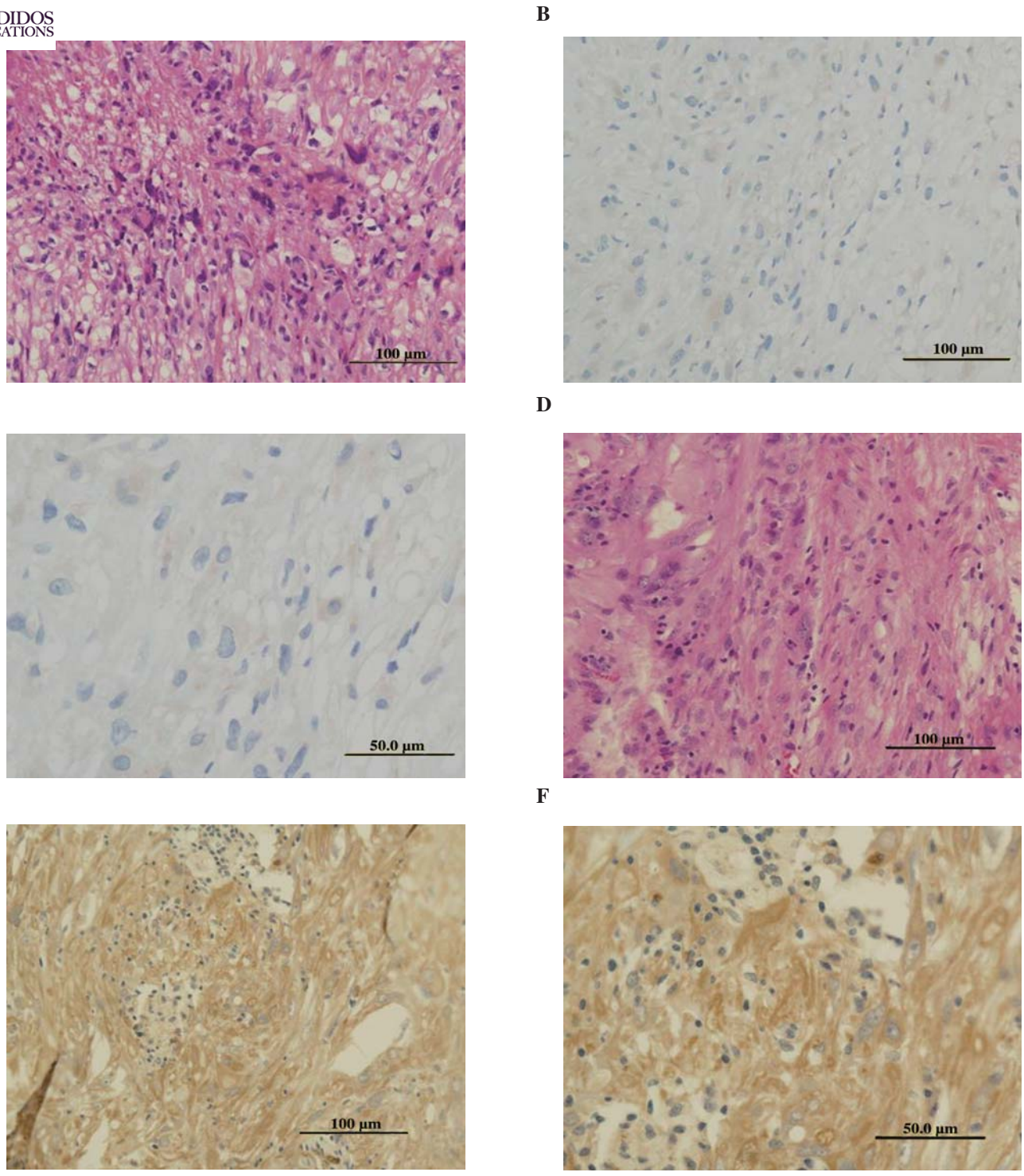

Figure 1. Specimens from a 79-year-old female with malignant fibous histiocytoma [(A) H&E, x200] showing a weak expression of hDlg [(B) and (C) immunostain; (B) x200, (C) x400]. (D-F) Specimens from a 74-year-old male with malignant fibous histiocytoma [(D) H&E, x200] showing a strong expression of hDlg [(E) and (F), immunostain; (E) x200, (F) x400].

of stained tumor cells; and the staining intensity, as referred to the in previous reports (32-34). The percentage of positive cells was rated as follows: 0 point, cases with 10% positive cells (rated as negative, regardless of staining intensity); 2 points, 11-50% positive cells; 3 points, 51-80% positive cells; and 4 points, >80% positive cells. The staining intensity was rated as: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. The points for the percentage of positive cells and the staining intensity were added, and the specimens were categorized into four groups according to their overall score; negative expression, 10% cells stained positive, regardless of intensity, 0-2 points; weak expression, 3 points; moderate expression, 4-5 points; and strong expression, 6-7 points (Fig. 1).

Statistics and analysis. Fisher's exact test and the χ^2 test were used to analyze the associations between the clinical-pathological variables. The local recurrence-free survival

(LRFS) was defined as the time from the initial treatment to the date of the clinically documented local recurrence. The metastasis-free survival (MFS) was defined as the time from the initial treatment to the date of clinically documented distant metastasis. The disease-free survival (DFS) was defined as the time from the initial treatment to the date of clinically documented local recurrence or distant metastasis. The overall survival (OS) was defined as the time from the initial treatment to the date of death attributed to MFH. For a prognostic analysis, the Kaplan-Meier survival analysis and log-rank tests were performed. For a multivariate analysis, a Cox proportional hazards regression model was used to identify the statistically significant differences in the survival and estimate hazard ratios and 95% confidence intervals. Seven prognostic variables including the patient's age, gender, tumor size, tumor depth, primary tumor or recurrent tumor, location (trunk or extremity), and the expression of hDlg were entered into a Cox multivariate analysis model. A p-value <0.05 was considered to be significant. The analysis was performed using the StatView statistical software package (version 5.0; SAS Institute, Cary, NC, USA).

Results

Expression of hDlg and associations between hDlg expression and clinico-pathological variables. The staining of hDlg was observed only in the cytoplasmic area of the sarcoma cells without staining of surrounding myofibroblastic cells. The expression of hDlg was negative in 19 (41.3%) specimens, weak in 4 (8.7%), moderate in 16 (34.8 %), and strong in 7 (15.2%). Fisher's exact test was used to analyze the associations between the hDlg expression and the clinicopathologic variables. The level of hDlg expression was not associated with patient's age, gender, tumor size, and tumor depth (data not shown; the χ^2 test).

Prognostic analysis. We next compared the LRFS, MFS, DFS and OS of the patients showing a high expression of hDlg with that of patients showing a low expression of hDlg. The Kaplan-Meier survival analysis and log-rank tests were performed in all patients (Fig. 2). A univariate analysis demonstrated the level of hDLG expression was significantly associated with MFS (p=0.0047, Table II), DFS (p=0.0062, Table III), and OS (p=0.0214, Table IV). The MFH patients with lower expression of hDlg showed poorer MFS, DFS, and OS compared to those with higher expression of hDlg. However, no significant association was observed between the expression level of hDlg and LRFS (p=0.1098). The trunk MFHs showed poorer MFS compared with the extremity MFHs. There were no significant differences between MFS, DFS or OS, and other prognostic factors including the patient's age, gender, tumor size, and tumor depth in this series (Tables II, III and IV).

A multivariate analysis demonstrated the expression of hDlg to be the only independent prognostic factor for MFS and DFS (p=0.0287 and 0.0237, respectively; Table V). The expression of hDlg was not an independent prognostic factor for OS. There were no significant differences in MDF or OS between initial MFH and recurrent MFH (p=0.5128 and 0.0606, respectively; log-rank test).

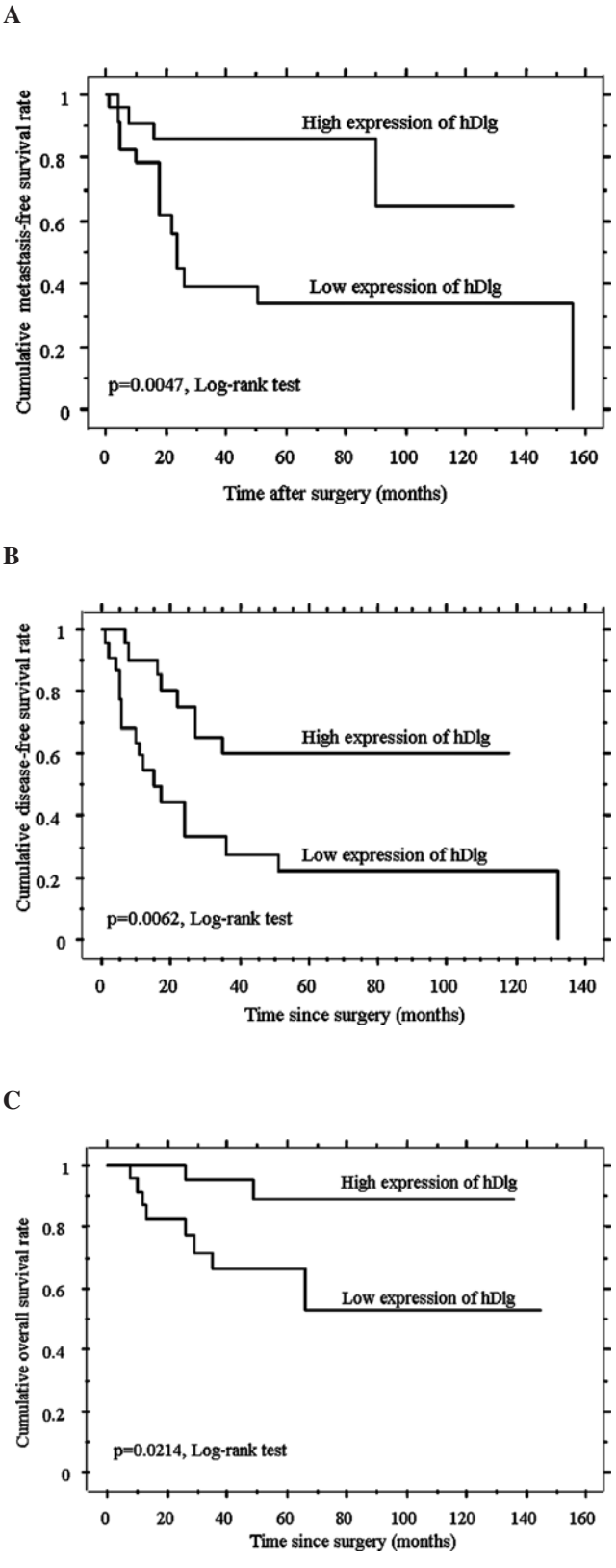

Figure 2. (A) Cumulative metastasis-free survival (MFS) of patients with a moderate or strong expression of hDlg, compared to patients with a negative or weak expression in MFHs. (B) Cumulative disease-free survival (DFS) of patients with a moderate or strong expression of hDlg, compared to patients with a negative or weak expression in MFHs. (C) The cumulative overall survival (OS) of patients with a moderate or strong expression of hDlg, compared to patients with a negative or weak expression in MFHs. Kaplan-Meier curves confirmed that the metastasis-free survival rate, disease-free survival rate, and overall survival rates for patients with moderate or strong expression of hDlg were significantly reduced.

Table II. Univariate metastasis-free survival analysis in 46 patients with MFH.

Factors	No.	5-Year metastasis-free survival rate (%)	P-value[a]
Patient's age (years)			
<60	14	69.6	0.4332
≥60	32	57.0	
Gender			
Male	22	62.0	0.7230
Female	24	59.7	
Size (cm)			
<5	10	61.7	0.7298
≥5	36	57.1	
Depth			
Superficial	10	39.4	0.2276
Deep	36	66.2	
Location			
Trunk	12	28.1	0.0035
Extremity	34	73.2	
hDLG1[b,c]			
Low	23	33.5	0.0047
High	23	86.1	

[a]Log-rank test. [b]hDLG1 human homologue of *Drosophila* discs large tumor suppressor protein. [c]Low means negative/weak, and high means moderate/strong.

Table III. Univariate disease-free survival analysis in 46 patients with MFH.

Factors	No.	5-Year disease-free survival rate (%)	P-value[a]
Patient's age (years)			
<60	14	59.8	0.1735
≥60	32	34.4	
Gender			
Male	22	48.7	0.3701
Female	24	36.7	
Size (cm)			
<5	10	24.0	0.3436
≥5	36	47.1	
Depth			
Superficial	10	40.5	0.9312
Deep	36	42.4	
Location			
Trunk	12	33.3	0.092
Extremity	34	45.4	
hDLG1[b,c]			
Low	23	25.9	0.0062
High	23	59.7	

[a]Log-rank test. [b]hDLG1 human homologue of *Drosophila* discs large tumor suppressor protein. [c]Low means negative/weak, and high means moderate/strong.

Discussion

In this study, we investigated whether hDlg protein expression has a prognostic impact on MFH. A univariate analysis demonstrated that the MFH patients with the lower expression of hDlg showed poorer MFS, DFS, and OS compared to those with higher expression of hDlg. A multivariate analysis demonstrated the expression of hDlg was the only independent prognostic factor for MFS and DFS. Our results clearly indicate, for the first time, that reduced expression of hDlg was associated with a more aggressive behavior of tumor cells in MFH.

MFH is the most common soft-tissue sarcoma (STS) diagnosed in adult patients, constituting as much as 34% of all STS (28). MFH often shows an aggressive clinical course. Despite adequate treatment, the prognosis remains poor. The 5-year LRFS, MFS and OS were reported to be 63-87%, 62-63% and 50-74%, respectively (26-30). Biomarkers which can predict the high-risk patients are very important, because such biomarkers may be a useful indicator for determining whether adjuvant therapeutic modalities such as irradiation and chemotherapy should be performed. In MFH, the tumor size, tumor location, microscopic tumor necrosis and histologic grade have been shown to be important prognostic factors (27,30,35,36). Some of these factors are incorporated into staging systems that form the basis for decision making regarding adjuvant therapy and follow-up. However, these prognostic factors do not always reliably predict the patient's outcome because of the presence of histologic heterogeneity which reflects the biologic behavior of MFH. Therefore, objective prognostic factors which more closely reflect the histological grading are required.

hDlg is a cell-junction localized protein that is a regulator of cell polarity and proliferation (8,37). In epithelial cells, hDlg colocalizes with E-cadherin at sites of cell-cell interaction, where it is thought to have both structural and signaling roles in association with the cytoskeleton (38,39). Reuver *et al* described that hDlg can be found in an E-cadherine/catenin adhesion complex and this interaction seems to be mediated by the attachment of hDlg to the cortical cytoskeleton (38). Cavatora *et al* (22) analyzed the intracellular and tissue distribution pattern of hDlg in normal cervical epithelium and cervical cancer with immunochemistry. Thereafter, they described that hDlg was localized in the basal, parabasal and intermediate layers, but absent in the upper layer which

Table IV. Univariate overall survival analysis in 46 patients with MFH.

Factors	No.	5-Year overall survival rate (%)	P-value[a]
Patient's age (years)			
<60	14	90.0	0.0544
≥60	32	67.8	
Gender			
Male	22	64.8	0.3446
Female	24	85.2	
Size (cm)			
<5	10	77.1	0.8472
≥5	36	74.5	
Depth			
Superficial	10	74.1	0.1330
Deep	36	75.6	
Location			
Trunk	12	59.3	0.421
Extremity	34	75.8	
hDLG1[b,c]			
Low	23	66.1	0.0214
High	23	84.2	

[a]Log-rank test. [b]hDLG1 human homologue of *Drosophila* discs large tumor suppressor protein. [c]Low means negative/weak, and high means moderate/strong.

consisted of differentiated cells in the normal cervical epithelium. This distribution pattern was similar to that of the adherences junction protein E-cadherin (40). In contrast, in the low-grade squamous intraepithelial lesion, the expression of hDlg was observed in the cytoplasmic area of the tumor cell with more intense staining, compared to normal tissue. In high-grade squamous intraepithelial lesion, cytoplasmic staining of hDlg was observed throughout the full thickness of the epithelium, which was composed of basal-type undifferentiated cells. Moreover, in the invasive cervical cancer, expression of Dlg was extremely weak or even absent. Lin *et al* (41) also evaluated the hDlg expression in cervical cancer and reported that in invasive cervical cancer, decreased hDlg expression was observed.

In the present study, in MFHs with relatively benign clinical course, the intensive staining of hDlg was observed in the cytoplasmic area of the tumor cells. In MFHs with a relatively poorer clinical outcome, weak or no staining was observed. Our observations of hDlg redistribution may be rationalized by the fact that both alterations of intracellular localization of hDlg and reduced expression of hDlg may play an important role in the biological behavior of the malignant cells, such as loss of polarity, high migration ability and invasive characteristics. The cytoplasmic accumulation of hDlg in patients with relatively good outcome may be causing the breakdown of cellular contacts between adjoining tumor cells, which do not have a critical impact on the prognosis. Moreover, the loss of hDlg may be considered a late stage marker in the carcinogenic process. Several mechanisms of the decreased expression of hDlg in patients with poor prognosis can be considered. The first possibility is that the MFHs without expression of hDlg may have a genetic point mutation or deletion in the h*Dlg* gene locus, although there are no reports to date which indicate such a genetic aberration of the h*Dlg* gene. Ishidate *et al* reported that the overexpression of hDlg suppresses cell proliferation by blocking cell cycle progression from the G0/G1 to S phase (18). hDlg also binds to the cytoplasmic domain of tumor necrosis factor α converting enzyme (TACE), which is the metalloprotease-disintegrin resoponsible for the ectodomain shedding of several proteins, including tumor necrosis factor α (TNFα) (23). hDlg also binds via its PDZ domains to the carboxy-terminus of the adenomatous polyposis coli (APC)

Table V. Multivariate analysis of the metastasis-free survival and of the disease-free survival 46 patients with MFH.

	The metastasis-free survival			The disease-free survival		
Factors	Relative risk	95% Confidence interval	P-value[a]	Relative risk	95% Confidence interval	P-value[a]
Patient's age (years) (<60 vs. ≥60)	0.405	0.096-1.717	0.2200	0.401	0.112-1.428	0.1584
Gender (male vs. female)	1.013	0.359-2.854	0.9812	1.665	0.681-4.071	0.2635
Size (cm) (<5 vs. ≥5)	2.057	0.563-7.515	0.2750	1.795	0.635-5.075	0.2699
Depth (superficial vs. deep)	0.861	0.210-3.530	0.8347	2.286	0.542-9.644	0.2602
Primary vs. recurrent tumor	0.596	0.158-2.252	0.4452	1.006	0.337-3.003	0.9908
Location (trunk vs. extremity)	0.227	0.060-0.853	0.0281	0.227	0.077-0.994	0.0489
hDLG1[b,c] (low vs. high)	5.092	1.153-13.416	0.0287	02.978	1.157-7.666	0.0237

[a]Cox proportional hazards regression model. [b]hDLG1 human homologue of *Drosophila* discs large tumor suppressor protein. [c]Low means negative/weak, and high means moderate/strong.

pressor and forms an APC-hDlg complex, which is for APC-mediated growth suppression (15,18,37). Therefore, the functional abrogation of hDlg due to genetic aberration of h*Dlg* gene may lead to uncontrollable cell proliferation and cell invasion. The second possibility is that viral infection may play a definitive role in development of MFH in some patients similar to patients with cervical cancer. It is now firmly established that high-risk human papilloma virus (HPV) plays a causal role in the development of cervical cancer. The E6 protein of high-risk HPV interacts with the PDZ domains of hDlg. Their E6 oncoproteins target hDlg for ubiquitin-mediated proteolysis and degradation of hDlg by the proteosome pathway (16,22,23,43). Similar to HPV E6, both HTLV-1 Tax and Ad9 E4 ORF1 also bind Dlg through a consensus PDZ-binding motif located at their C terminus (16). Suzuki *et al* reported that Tax abrogates the cell cycle regulatory function of hDlg possibly through APC, leading to deregulation of growth of HTLV-1-infected T-cells (17). In MFH, viral protein such as HPV E6, HTLV-1 Tax and Ad9 E4 ORF1 may play a definitive role in carcinogenesis. The third possibility is that reduced expression of hDlg is generated only as a result of the disruption of the normal cell architecture due to an aberration of the other key molecules which control cell polarity and cell growth. Further investigations are therefore warranted.

In conclusion, immunohistologically, the expression of hDlg protein was negative in 19 (41.3%) specimens, weak in 4 (8.7%), moderate in 16 (34.8 %) and strong in 7 (15.2%). The patients with negative or weak expression of hDlg showed a significantly poorer MFS and DFS in comparison to those with a moderate or strong expression based on the findings of both univariate and multivariate analyses. These results suggest that the reduced expression of hDlg is an independent prognostic marker for the MFH patients. Further investigations are necessary to clarify the mechanisms of a reduced expression of hDlg in MFHs with an aggressive clinical course.

Acknowledgements

The authors thank Miss Chiyuki Ueno and Mrs. Katsura Chiba for their assistance in preparing the clinical records.

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