Review

Medical application of the monoclonal antibody SKM9-2 against sialylated HEG1, a new precision marker for malignant mesothelioma

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Abstract: Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleural cavity. Pathologically distinguishing MPM from other pleural lesions is often difficult. We searched for marker antigens to facilitate the pathological diagnosis of MPM and found useful markers for the pathological detection of malignant mesothelioma. Among them, the anti-mesothelioma monoclonal antibody SKM9-2, which was isolated as a clone binding to specimens of MPM (but not to specimens of lung adenocarcinoma) by immunohistochemical screening, showed higher specificity and sensitivity than traditional mesothelioma markers. SKM9-2 recognizes both sialylated *O*-glycans and peptide sequences in HEG1, and its glycan modifications are specific to mesothelioma. New effective treatments for MPM are needed because the prognosis of patients with MPM is usually poor. SKM9-2 can be used as a seed for next-generation antibody drugs with strong cytotoxic activities. In this review, we have summarized our research on antibody development for MPM diagnosis and treatment.

Keywords: mesothelioma, diagnosis, antibody, glycopeptide, mucin

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleural cavity caused by persistent asbestos exposure.[1)] Distant metastases of early-stage MPM are uncommon; however, these tumors diffuse widely on the pleura and often recur early after treatment.[2)–4)] MPM is also highly resistant to antitumor drugs and radiation therapy.[5),6)] Therefore, the prognosis of patients with MPM is poor.[7)] The number of MPM cases is approximately 26,000 each year worldwide.[8)]

A specific gene mutation that causes MPM has yet to be identified. Although MPM is often characterized by a loss of function of *BRCA1-associated protein 1* (*BAP1*) and/or homozygous deletion of *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*), these abnormalities are also observed in other tumors and are thus not specific to MPM.[9)–12)] Characteristically, in MPM, there is almost no increase in the levels of specific gene products associated with the malignant transformation of mesothelial cells. It is often difficult to pathologically distinguish MPM from other tumors and/or the non-neoplastic proliferation of mesothelial cells. Indeed, even the two major MPM morphologies, epithelioid-type and sarcomatoid-type, are often indistinguishable from other proliferative lesions without the assistance of further immunohistochemical and molecular genetic analyses. There are no available MPM-specific immunohistochemical markers common to both morphologies.[13),14)] For the pathological diagnosis of MPM, current guidelines require the examination of

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Non-standard abbreviation list: BAP1: BRCA1-associated protein 1; bsTCE: bispecific T cell-engaging antibody fragment; CAR-T: chimeric antigen receptor T cell; CDKN2A: cyclin-dependent kinase inhibitor 2A; CEA: carcinoembryonic antigen; EpCAM: epithelial cell adhesion molecule; GALNT: polypeptide N-acetylgalactosaminyltransferase; HEG1: protein HEG homolog 1; mAb: monoclonal antibody; MPM: malignant pleural mesothelioma; WT-1: Wilms' tumor gene product 1.

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a panel analysis using two or more positive immunohistochemical markers and two or more negative markers.[14] These complicated diagnostic tests for MPM are costly and laborious.

Calretinin, podoplanin, and Wilms' tumor gene product 1 (WT-1) have been used as major positive markers for all malignant mesothelioma containing MPM.[13),14] Although these markers show better specificity and sensitivity than formerly identified markers, such as cytokeratin 5/6 and mesothelin, these cannot necessarily detect all morphological types of mesothelioma, especially the sarcomatoid type.[13] Moreover, the use of calretinin, podoplanin, and WT-1 is limited by their low specificity, because these markers are sometimes present in metastatic carcinoma.[13] To exclude the possibility of metastatic carcinoma, carcinoembryonic antigen (CEA) and epithelial cell adhesion molecule (EpCAM; monoclonal antibody [mAb] clone MOC-31 or Ber-EP4) are used as negative mesothelioma markers in a panel-based analysis.[14] Because the detection of homozygous *CDKN2A/p16* gene deletion by fluorescence *in situ* hybridization and/or the detection of BAP1 function loss also have been examined together,[9)–12] the accuracy of diagnosis is higher than before. However, apart from medical institutions that routinely diagnose mesothelioma, it is a heavy burden for other institutions to perform these tests in all cases of suspected mesothelioma. Therefore, the search for a more precise marker for mesothelioma diagnosis is ongoing. We searched for marker antigens that facilitate the pathological diagnosis of MPM and described two useful markers for the pathological detection of MPM. In this review, we have summarized our research on antibody development for the diagnosis and treatment of MPM.

Specific expression of intelectin-1 in MPM

Human intelectin-1 is an animal lectin that binds to a bacterial glycan and is only expressed in limited tissue types, including the heart and intestinal tissue.[15),16] Intelectin-1 mRNA is specifically overexpressed in MPM cells,[17] but its protein expression level had not been investigated in human organs. Intelectin-1 protein expression was evaluated in various cancer and non-tumor tissues by immunohistochemical analysis[18] using an anti-intelectin-1 mAb that had been isolated previously.[19] In non-tumor tissues, intelectin-1 is mainly secreted from gastrointestinal goblet cells along with mucus into the intestinal lumen.[18] Clearly, intelectin-1 protein is not expressed in the parenchymal cells of the two organs (cardiac muscles in the heart and adipocytes in the omentum), where high mRNA expression has been reported,[15),20] even though intelectin-1 protein is produced in mesothelial cells that cover these organs.[18] In 88.5% of epithelioid-type MPM, intelectin-1 protein is expressed. It is rarely expressed in lung adenocarcinoma cells (1.1%), which is important to distinguish it from MPM as part of the differential diagnosis. In other cancers, except for mucus-producing adenocarcinomas, intelectin-1 was barely detected, and the positive intelectin-1 ratio in non-mesothelioma tumors was lower than that of other mesothelioma markers (intelectin-1, 2.6%; calretinin, 20.5%; CK5/6, 43.6%; podoplanin, 12.2%; WT-1, 2.6%; mesothelin 27.6%).[18] Thus, intelectin-1 staining may be useful for the differential diagnosis of epithelioid-type MPMs from other cancers.

Intelectin-1 is a secretory protein.[21] The concentration of intelectin-1 in the pleural effusions of patients with MPM was also investigated. Pleural effusion in patients with MPM contains higher intelectin-1 concentrations than those in patients with lung cancer or tuberculosis (MPM, 3047 ng/mL; lung cancer, 323 ng/mL; tuberculosis, 246 ng/mL; each concentration is the mean value).[19] In clinical diagnosis, it is important that the pleural effusion of MPM is distinguished from that of lung cancer or tuberculosis. Thus, intelectin-1 in pleural effusions can also be used as a soluble marker for MPM.

Finding of the novel mesothelioma marker, sialylated HEG1

Pathological analysis of the SKM9-2 antigen in tumors. MPM is a tumor in which the expression of specific oncogene products is rarely detected, making it difficult to pathologically diagnose MPM. Even for major markers (*i.e.*, calretinin and WT-1), it is important to detect their localization in the nucleus rather than their expression. Therefore, it was difficult to identify an excellent marker for MPM by analyzing gene expression profiles.

As tumor markers that are independent of gene expression, there are post-translational modifications in addition to the intercellular localization of target molecules. The change in glycan modification associated with malignant transformation is widely known as a clinical tumor marker, such as CA125 and CA19-9.[22),23] We searched for an mAb that can detect MPM-specific post-translational modifications, particularly glycan modifications, in pathological specimens. Using immunohistochemical

screening to assess binding to MPM specimens but not to lung adenocarcinoma specimens, we succeeded in isolating an mAb clone (SKM9-2) with higher specificity and sensitivity levels than traditional mesothelioma markers and identified sialylated protein HEG homolog 1 (HEG1) as its antigen.[24)]

HEG1 was reported as the encoded product of the heart of the glass gene regulating the concentric zebrafish heart growth.[25)] The mouse *HEG1* gene has been linked to cardiovascular organ development.[26)] Although the physiological function of HEG1 is not well known, HEG1 is observed in the cell–cell junction, and it has functions related to angiogenesis and cell–cell junction signaling.[27),28)] In contrast, the full-length product of the human *HEG1* gene, which we identified as SKM9-2 antigen, is a type I membrane protein and has typical domain structures of mucinous proteins—*i.e.*, it contains numerous Ser/Thr residues, many *N*-glycosylation sites, and EGF domains.[24)] In fact, SKM9-2-recognized HEG1 contained many *O*- and *N*-glycans and was expressed on the apical cell surface, like other mucin membrane proteins.[24)] SKM9-2-recognized HEG1 in mesothelioma cells is sialylated and has mucin-like properties, whereas HEG1 in most other tissues may be in a non-glycosylated form that is not recognized by SKM9-2, which may indicate a different localization than sialylated HEG1. Therefore, the specificity of sialylated HEG1, which we identified as a mesothelioma marker, should be evaluated as an SKM9-2 epitope (SKM9-2 antigen) and not as the HEG1 gene product.

In our first study,[24)] the SKM9-2 antigen positivity rate was 98% (89/91 cases) in epithelioid-type MPM, 90% (19/21) in biphasic MPM, and 64% (9/14) in sarcomatoid-type MPM. Naso *et al.* reported a positive rate of 94% (65/69) in epithelioid-type MPM and 44% (14/32) in sarcomatoid-type MPM.[29)] Hiroshima *et al.* showed that the positive rate was 97% (122/126, total of tissue microarray and whole section results) in epithelioid-type malignant mesothelioma, 94% (64/68) in biphasic malignant mesothelioma, and 81% (22/27) in sarcomatoid-type malignant mesothelioma.[30)] This report by Hiroshima *et al.* contained not only MPM but also malignant peritoneal mesotheliomas (23 epithelioid, 3 biphasic, 1 sarcomatoid), malignant mesothelioma of tunica vaginalis (1 epithelioid), and one well-differentiated papillary mesothelioma. We also observed that SKM9-2-stained rare cell types, comprising malignant peritoneal mesothelioma (3/3), one malignant pericardial mesothelioma, one malignant mesothelioma of the tunica vaginalis, and one recurrent well-differentiated papillary mesothelioma with invasive foci in the peritoneum.[24)] Thus, SKM9-2 recognizes not only MPM but also various malignant mesotheliomas. Table 1 summarizes the positivity rates of the mesothelioma markers in these three studies. SKM9-2 antigen (sialylated HEG1) was better than conventional markers (calretinin, WT-1, and podoplanin) for detecting all major histological types of malignant mesothelioma (epithelioid-type/biphasic/sarcomatoid-types): SKM9-2, 97%/93%/62%; calretinin, 92%/70%/19%; WT-1, 81%/48%/24%; podoplanin, 84%, 52%, and 45%. The SKM9-2 antigen was particularly detected at a higher positive rate in sarcomatoid-type MPM than in the other markers. These results suggested that the SKM9-2 antigen is a sensitive histopathological marker for various mesothelioma types, including MPM.

In our study, SKM9-2 antigen expression was negative in major non-mesothelial tumors that should be distinguished from MPM, with a specificity of 99%.[24)] In subsequent histopathological studies by Naso *et al.*[29)] and Hiroshima *et al.*,[30)] the SKM9-2 antigen was focally and weakly expressed in some lung squamous cell carcinomas or some ovarian carcinomas (Table 2). Weak SKM9-2 antigen expression was also detected at a high rate in angiosarcomas and leiomyosarcomas in the analysis by Hiroshima *et al.*[30)] SKM9-2 antigen was observed as weak cytoplasmic staining in almost all positive cases of non-mesothelial carcinomas, except for ovarian carcinoma (Table 2). This difference in staining localization may be useful in distinguishing epithelioid-type MPM from metastatic carcinoma of the pleura.

SKM9-2 antigen is not expressed in many non-mesothelial tumors and normal tissues, including the liver.[24)] However, the expression of HEG1, whose recognition by SKM9-2 has not been investigated, is associated with hepatocellular carcinoma prognosis.[31)] Because SKM9-2 antigen cannot be detected with anti-HEG1 antibodies other than SKM9-2 or mRNA analysis, further studies are needed to determine the positive rate of SKM9-2 antigen in hepatocellular carcinoma.

Pleural effusion is often a primary sign of MPM. Therefore, cytological examination of pleural effusion is important for identifying MPM. However, the cytological features of MPM cells in the effusion tended to overlap with those of other carcinoma

Table 1. Positive rate of mesothelial cell markers in mesotheliomas

MPM marker MPM type	Tsuji *et al.*[24]	Naso *et al.*[29]	Hiroshima *et al.*[30]	Total positive number/cases
Sialylated HEG1 (SKM9-2 antigen)				
Epithelioid	89/91	65/69	122/126	276/286 (97%)
Biphasic	19/21	ND	64/68	83/89 (93%)
Sarcomatoid	9/14	14/32	22/27	45/73 (62%)
Calretinin				
Epithelioid	62/71	65/69	105/113	232/253 (92%)
Biphasic	13/14	ND	35/55	48/69 (70%)
Sarcomatoid	1/8	7/32	0/2	8/42 (19%)
WT-1				
Epithelioid	64/71	62/69	75/108	201/248 (81%)
Biphasic	12/14	ND	20/53	32/67 (48%)
Sarcomatoid	6/8	4/32	0/2	10/42 (24%)
Podoplanin				
Epithelioid	63/71	66/69	83/112	212/252 (84%)
Biphasic	11/14	ND	24/53	35/67 (52%)
Sarcomatoid	3/8	16/32	0/2	19/42 (45%)

ND, no data.

Table 2. Positive rate of SKM9-2 antigen in non-mesothelial tumors

Cancer	Tsuji *et al.*[24]			Naso *et al.*[29]			Hiroshima *et al.*[30]			Total positive number/cases
	m	c	N	m	c	N	m	c	N	
Lung carcinoma										
Adenocarcinoma	0	0	78	0	3	73	0	0	36	3/187 (2%)
SCC	0	0	20	0	16	60	0	5	23	21/103 (20%)
Ovarian carcinoma	0	0	10	3	0	17	6	0	9	9/36 (25%)
Gastric carcinoma	0	0	10				0	0	6	0/16 (0%)
Colon carcinoma	0	0	10				0	0	7	0/17 (0%)
Breast carcinoma	0	0	10				0	0	7	0/17 (0%)
Urothelial carcinoma	1	0	10				0	0	6	1/16 (6%)
Angiosarcoma	0	0	10				3	3	6	6/16 (38%)
Leiomyosarcoma	0	1	10				0	6	6	7/16 (44%)

SCC, squamous cell carcinoma; m, membranous staining number; c, cytoplasmic staining number; N, total number.

cells or reactive mesothelial cells. Hiroshima *et al.* observed the expression of SKM9-2 antigen in mesothelioma cells, other cancer cells, and reactive mesothelial cells in the effusions.[32] Mesothelioma cells expressed the SKM9-2 antigen at a rate of 100% (41/41 cases), but lung cancer cells and other cell lines, except ovarian carcinoma, had no SKM9-2 antigen (0%, 0/34). Although some ovarian carcinoma cells, especially serous carcinoma, expressed the SKM9-2 antigen (29%, 6/21), these staining intensities were weak or moderate, and the staining extension was focal. Reactive non-neoplastic mesothelial cells showed strong membranous staining at a high rate (77%, 20/26). The authors recommend cell block analysis using SKM9-2 antigen, claudin 4, BAP1, and methylthioadenosine phosphorylase for

cases with effusions of unknown origin as follows: immunohistochemistry with SKM9-2 antigen and claudin 4 to validate the mesothelial origin and immunohistochemistry with BAP1 and methylthioadenosine phosphorylase to confirm malignancy.[32]

Identification of sialylated HEG1 as an SKM9-2 antigen. Because the SKM9-2 antigen was detected as a large band (~400 kDa) on western blotting in a sialylation-dependent manner, it was expected to be a large mucinous glycoprotein. The antigen was purified by precipitation in acidic conditions to concentrate sialylated mucin, size-exclusion chromatography, and lectin-affinity chromatography. The purified antigen was identified by mass spectrometry as HEG1, which has not been investigated as a protein molecule.[24] HEG1 does not belong to the mucin gene family because it does not have typical tandem repeat structures. However, human HEG1 includes several repeated homologous amino acid sequences and poly Ser sequences, caused by the duplication of DNA sequences. Because HEG1 has a type I membrane protein containing EGF domains and many sialylated *O*- and *N*-glycans, HEG1 may be a gene related to mucinous glycoproteins.

HEG1 mRNA is detected in various organs such as the heart and lungs; however, SKM9-2 does not bind to most tissues in these organs.[24] As SKM9-2 recognition requires not only HEG1 peptide sequences but also sialylated glycans, the high specificity of SKM9-2 to mesothelioma may be attributed to mesothelioma-specific glycan modifications on HEG1.[24] The SKM9-2 epitope was determined by the binding of SKM9-2 to truncated HEG1 and candidate epitope-fused glycosylphosphatidylinositol-anchor proteins.[33] The peptide epitope of SKM9-2 is located near the center of the HEG1 molecule ^{893}-SKSPSLVSLPT-^{903}. Alanine substitutions for Ser^{893}, Lys^{894}, Pro^{896}, Ser^{897}, Val^{899}, or Ser^{900} resulted in the loss of recognition by SKM9-2. The attached glycan and modified residues were analyzed using mass spectrometry and lectin binding analyses. The epitope contains a non-glycosylated Ser^{897} residue and two disialylated core 1 *O*-linked glycan (disialyl T)-modified serine residues Ser^{893} and Ser^{900}. Surface plasmon resonance analysis showed that SKM9-2 associates with a glycosylated epitope containing two T-antigens without sialic acid. However, the antibody rapidly dissociates from the non-sialylated glycopeptide. SKM9-2 cannot bind non-glycosylated peptides.[33] These results suggested that antigen recognition by SKM9-2 requires peptide sequences and two site-specific modifications with sialylated glycans.

Disialyl T, the glycan required for SKM9-2 recognition, is not the type of glycan that is exclusively expressed in mesothelioma. Disialyl T is found in various normal and tumor cells. Regarding the mesothelioma-specific disialyl T modification recognized by SKM9-2, the position of the glycan-modified residue is more important than the unique structure of disialyl T. At present, it is unclear what regulates the site-specific modification of disialyl T on HEG1. The characteristic expression of polypeptide N-acetylgalactosaminyltransferase (GALNT) in MPM cell lines has not been found yet. Site-specific modification of disialyl T in the SKM9-2 epitope may be controlled by GALNT isoforms and/or a collaboration of several GALNTs that function as follow-up enzymes.

The biological effects of glycosylation in the SKM9-2 epitope region are unclear. HEG1 has functions related to angiogenesis and cell–cell junction signaling.[27),28] The extracellular domain of HEG1 is classified into two regions separated by the SKM9-2 epitope: an *N*-terminal glycosylated domain containing several large *N*-glycans and many *O*-glycans; and three highly conserved EGF domains in the juxtamembrane.[24),33] Glycosylation of the SKM9-2 epitope region may interfere with enzymatic cleavage of that region, inhibiting the release of the *N*-terminal glycosylated domain. The persistence of the glycosylated domain may keep the EGF domain away from its interacting molecules by intercalating into cell–cell junctions. If the conserved EGF domains are responsible for cell–cell junction signaling, the signals that maintain cell–cell junctions may be inhibited, and thus, disruption of the cell–cell junction can induce cell migration and/or cell detachment from the pleura. Further studies are required to clarify the biological function of HEG1 and the effects of glycosylation on the SKM9-2 epitope region.

Further clinical applications of SKM9-2. Mesothelioma is resistant to most drug treatments.[5),6] Combination treatment with pemetrexed and cisplatin or carboplatin has been the mainstay first-line chemotherapy treatment for a long time.[34] Adjuvant or neoadjuvant chemotherapy with this combination, surgery (pleurectomy or extrapleural pneumonectomy), and adjuvant radiation therapy has been used as the trimodal approach to mesothelioma, resulting in a median survival of 20–29 months.[35] However, the trimodal approach is limited to the resectable stages of mesothelioma.

Recently, the efficacy of immune checkpoint inhibitors has been confirmed in the CheckMate 743 trial,[36] including primarily unresectable mesothelioma patients, and some trials.[37] Antitumor effects of immune checkpoint inhibitors are mediated by cytotoxic lymphocytes. Thus, specific cellular immunotherapies using cytotoxic T lymphocytes and antibody fragments, such as chimeric antigen receptor T cell (CAR-T) therapy, may have even greater therapeutic effects. Mesothelin-targeted CAR-T therapy has been investigated in several phase I clinical trials.[37] Combination immunotherapy with CAR-T cells and immune checkpoint inhibitors showed promising effects in a population of 18 patients with MPM; 8 patients achieved stable disease for $\geq$6 months and 2 exhibited complete metabolic response on positron emission tomography scans. The median overall survival was 23.9 months, and the 1-year overall survival was 83%.[38] Improved mesothelin-targeted CAR-T cells that are more cytotoxic to mesothelioma have also been developed to elicit a powerful therapeutic effect.[39] In contrast, bispecific T cell-engaging antibody fragment (bsTCE), such as clinically approved BiTE, has also been developed for cancer therapy as another cellular immunotherapy using cytotoxic T lymphocytes and antibody fragments.[40] In immunotherapies using cytotoxic T lymphocytes, the high specificity of antibody fragments to the target tumor is a principal factor in improving the therapeutic effect. Because SKM9-2 does not bind to most organs,[24] CAR-T or bsTCE using SKM9-2 is expected to be effective in treating mesothelioma.

The CheckMate 743 trial showed that the clinical benefits of nivolumab plus ipilimumab were more significant in non-epithelioid-type MPM than in epithelioid-type MPM.[36] The reason for this difference is not yet clear; non-epithelioid-type MPM may have more neoantigens than epithelioid-type MPM, and it may be relevant that the protein expression profile of epithelioid-type MPM often resembles that of normal reactive mesothelial cells. Because cytotoxic T lymphocytes can attack epithelioid-type MPM using SKM9-2 fragments, such as CAR or bsTCE, combination immunotherapy with these SKM9-2 fragments and immune checkpoint inhibitors can expand target subtypes and be clinically beneficial. Because the epithelioid type is the predominant MPM subtype, expanding the target of treatment using immune checkpoint inhibitors would be important in mesothelioma therapy.

The next generation of antibody drugs, such as CAR-T and bsTCE, has strong cytotoxic activity; thus, it is important to certify tumor specificity, namely, whether the medication has no effect on non-tumorous tissues. However, in the case of glycan-modified antigens, such as the SKM9-2 epitope, it is difficult to show that the antibody does not bind to healthy tissues because the target expression in tissues cannot be confirmed by comprehensive mRNA-based expression analysis. For example, sialylated HEG1, which contains the SKM9-2 epitope, is poorly expressed in non-tumorous tissues as indicated by immunohistochemistry. However, because this section is only a part of the whole, it cannot be proven that there is no sialylated epitope in the "other regions" of the organ in which HEG1 mRNA expression is observed. Additionally, SKM9-2 does not recognize HEG1 in rodents used in nonclinical studies. Thus, it is difficult to guarantee organ safety in traditional nonclinical studies. To solve this problem, investigating the tissue distribution of radioisotope-labeled antibodies may be required in subhuman primates. Few academic institutions can perform isotope imaging and animal experiments using radioactive labels. Moreover, it is difficult for academic institutions to conduct isotope experiments on primates. Such pharmacokinetic verification issues may be a major obstacle to the research and development of antibody drugs against disease-specific glycan-related antigens, which are attracting attention as new targets owing to the depletion of targets for antibody drugs.[41]

Recently, preclinical studies of the next generation of radioimmunotherapy using antibodies against mesothelin or podoplanin on mesothelioma cells were reported.[42–44] In particular, antibodies labeled with α-particle emitting radioisotopes, such as thorium-227 or actinium-225, showed strong anti-mesothelioma activities.[43),44] Such α-particle emitters have a greater linear energy transfer than β-emitters and deposit more energy into target cells, which can induce stronger DNA damage to the cells.[45] SKM9-2 labeled with an α-particle emitter may be promising as a labeled-antibody drug against mesothelioma more than antibody-drug conjugates, because mesothelioma has a strong drug resistance. Furthermore, creating a development environment for radioisotope-labeled antibody drugs may help to solve the above pharmacokinetic validation issues. In the next generation of drug development using SKM9-2, we are proceeding with research and development to solve various these problems individually.

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Additional information

Competing interests. The authors declare that they are holders of patents (JP6859498, EP3418304, and ZL201780011527.6) that cover the antibody against HEG1 and its usage. These patents were filed by the Kanagawa Prefectural Hospital Organization and The University of Tokyo. These patents were licensed in part by LSI Medience Co. or Nihon Medi-Physics Co., Ltd.

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Profile

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Profile

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