

Cancer-testis antigens are predominantly expressed in uterine leiomyosarcoma compared with non-uterine leiomyosarcoma

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Received October 11, 2016; Accepted July 27, 2017

DOI: 10.3892/ol.2017.7274

Abstract. Leiomyosarcomas account for ~24% of all adult sarcomas, and develop predominantly either in the uterus [uterine leiomyosarcoma (ULMS)] or in deep soft tissue or the retroperitoneum [non-uterine leiomyosarcoma (NULMS)]. Leiomyosarcomas are relatively chemoresistant tumors, and the prognosis of patients with leiomyosarcomas is poor. Cancer-testis (CT) antigens are considered promising immunotherapeutic targets because of their restricted expression in normal tissue, except in the testis. Little is known about the expression of CT antigens in leiomyosarcomas. In the present study, the protein expression of the CT antigens MAGE family member A (MAGEA)1, MAGEA3, MAGEA4, G antigen 7 (GAGE7) and cancer/testis antigen 1 (NY-ESO-1) in ULMS and NULMS were investigated using immunohistochemistry (IHC), and their expression profiles compared. In ULMS and NULMS, positive expression was observed in 11/32 (31%) and 1/31 (3%; MAGEA1), 15/32 (47%) and 5/31 (16%; MAGEA3), 11/32 (34%) and 3/31 (10%; MAGEA4), 23/32 (72%) and 11/31 (35%; GAGE7) and 3/32 (9%) and 0/31 (0%; NY-ESO-1), respectively. The ULMSs demonstrated significantly higher positive expression of MAGEA1 (P=0.0034), MAGEA3 (P=0.0141), MAGEA4 (P=0.0319) and GAGE7 (P=0.0054) compared with the NULMSs. The ULMSs also had significantly higher IHC scores for MAGEA1 (P=0.0023), MAGEA3 (P=0.0474), MAGEA4 (P=0.011), GAGE7 (P=0.0319) and NY-ESO-1 (P=0.0437). The results of the present study support the potential utility of MAGEA1, MAGEA3, MAGEA4 and GAGE7 in ULMS and GAGE7 in NULMS as immunotherapeutic targets.

Introduction

Soft-tissue sarcomas comprise a rare, complex and heterogeneous group of tumors that account for only 1% of all cancers, and these tumors possess numerous histological subtypes (1). Partially due to the rarity of soft-tissue sarcomas, improvements in their treatment have not been achieved, and novel therapeutic options according to histological subtype are required.

Leiomyosarcomas, defined as malignant tumors with smooth-muscle differentiation, account for 24% of all soft-tissue sarcomas (2). Leiomyosarcomas have the potential to occur in any place in the body, but they develop mainly in the uterus [uterine leiomyosarcoma (ULMS)] or retroperitoneum and deep soft tissue [non-uterine leiomyosarcoma (NULMS)]. ULMSs account for 1-2% of all uterine malignancies (3). Leiomyosarcomas are relatively chemoresistant compared with other sarcoma subtypes, and so their overall prognosis remains poor (4).

Cancer-testis (CT) antigens, which are recognized by specific cytotoxic T lymphocytes (CTLs), are considered promising targets in immunotherapy due to their expression occurring only in tumor tissues of different histological origins and not in normal somatic tissues [except for testis tissue, which has no expression of human leukocyte antigen (HLA) class 1 and is not recognized by CTLs] (5,6). One study noted mRNA expression of CT antigens in several cases of ULMS and NULMS (7), but the available information concerning protein expression of CT antigens in ULMS and NULMS is limited.

In the present study, protein expression of the CT antigens MAGE family member A (MAGEA)1, MAGEA3, MAGEA4, cancer/testis antigen 1 (NY-ESO-1) and G antigen 7 (GAGE7), for which clinical immunotherapy trials are already underway or have been completed, was investigated (8,9). Immunohistochemistry (IHC) was used to validate the potential utility of each of these antigens as a target for immunotherapy in ULMS and NULMS, and comparisons were made between their expression profiles.

Materials and methods

Tissue samples. The paraffin-embedded tissue of 32 ULMS and 31 NULMS cases were obtained from the files of soft-tissue

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Key words: leiomyosarcoma, cancer-testis antigen, MAGE family member A1, cancer/testis antigen 1, G antigen 7

Table I. Antibodies used for immunohistochemical staining.

Antigen	Clone (catalog no.)	Host species	Dilution	Retrieval	Source
MAGEA1	MA454 (sc 20033)	Mouse	1:1,000	pH9 Pressure Boiler	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
MAGEA3	1H1 (ab140678)	Mouse	1:500	pH9 Microwave	OriGene Technologies, Inc., Rockville, MD, USA
MAGEA4	CPTC- MAGEA4-1 (ab2138142)	Mouse	1:500	pH6 Microwave	DSHB, University of Iowa, Iowa City, IA, USA
GAGE7	Polyclonal (PA5-26760)	Rabbit	1:500	pH9 Microwave	Thermo Fisher Scientific, Inc., Waltham, MA, USA
NY-ESO-1	E978 (sc-53869)	Mouse	1:100	pH6 Pressure Boiler	Santa Cruz Biotechnology, Inc., Dallas, TX, USA

MAGEA, MAGE family member A; GAGE7, (G antigen 7; NY-ESO-1, cancer/testis antigen 1; MA454, anti-MAGE1 antibody; 1H1, anti-EBV antibody; CPTC-MAGEA4-1, melanoma-associated antibody; E978, NY-ESO-1 monoclonal antibody).

tumors registered at the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University (Fukuoka, Japan) between April 1988 and December 2014. Each tumor was re-classified according to the World Health Organization classification, by bone and soft-tissue tumor pathologists and gynecological pathologists (10,11). The tissue samples had been obtained from open biopsy specimens or surgically resected tumors. All the 31 NULMS tissues were located at the extremities (24 was located at lower extremities and 7 was at the upper extremities), and no retroperitoneal cases were included. Among the 31 NULMS cases, 11 males and 20 females were included. The mean age of the NULMS patients was 60 years (range, 32-78 years) and the mean age of the ULMS patients was 48 years (range, 29-63 years). In the 32 ULMS cases, 15 patients were receiving chemotherapy and the remaining 17 tissue samples were obtained prior to the patients receiving chemotherapy, including biopsy specimens. In the 31 NULMS cases, 12 patients were receiving chemotherapy and the remaining 19 tissue samples were obtained prior to the patients receiving chemotherapy, including biopsy specimens. The present study was approved by the Ethics Committee of Kyushu University (Fukuoka, Japan) and conducted according to the Ethical Guidelines for Epidemiological Research enacted by the Japanese government.

IHC and evaluation. IHC was conducted as previously described (12). Antigen retrieval, the primary antibodies and dilutions used are summarized in Table I. The slides were incubated with the primary antibodies overnight at 4°C. The DAKO™ REAL™ Envision detection system was employed for detection, which contained horseradish peroxidase-conjugated antibodies (undiluted; catalog no. K5007; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), and incubated with slides for 90 min at room temperature. Slides were examined using Olympus light BX 43 microscope (Olympus Corporation, Tokyo, Japan). The IHC results were assessed by three investigators who were blinded to the clinical data of the patients. A consensus assessment was adopted as the IHC result.

Table II. Immunohistochemistry results in ULMS and NULMS.

Antigen	Positive cases/Total n (positive %)		P-value
	ULMS (%)	NULMS (%)	
MAGEA1	10/32 (31)	1/31 (3)	0.0034 ^a
MAGEA3	15/32 (47)	5/31 (16)	0.0141 ^a
MAGEA4	11/32 (34)	3/31 (10)	0.0319 ^a
GAGE7	23/32 (72)	11/31 (35)	0.0054 ^a
NY-ESO-1	3/32 (9)	0/31 (0)	0.2381

^aP<0.05, as assessed using Fisher's exact (test, MAGEA, MAGE family member A; GAGE7, G antigen 7; NY-ESO-1, cancer/testis antigen 1; ULMS, uterine leiomyosarcoma; NULMS, non-uterine leiomyosarcoma).

The percentage of immunoreactive cells and staining intensity were assessed in the most representative areas. The proportion of immunoreactive cells was scored as 0 to 4 points as follows: 0, <5%; 1, 5-<25%; 2, 25-<50%; 3, 50-<75%; 4, ≥75%, and the intensity was scored as 0 to 3 points as follows: 0, negative; 1, weak staining; 2, moderate staining; 3, strong staining. The total score (proportion score + intensity score) was obtained, and the cases with a total score ≥3 were judged as positive. A total score ≥5 was defined as high expression as previously described (12).

Statistical analysis. Fisher's exact test and the Mann-Whitney U-test were used as appropriate to evaluate the associations between two variables. A two-sided P-value of <0.05 was considered to indicate a significant difference. The data analyses were performed using the JMP statistical software package (v.12.2.0; SAS Institute, Inc., Cary, NC, USA).

Table III. Results of immunohistochemistry, determining high and low expression levels.

Antigen	Uterine leiomyosarcoma		Non-uterine leiomyosarcoma	
	Low, N (%)	High, N (%)	Low, N (%)	High, N (%)
MAGEA1	8 (25)	2 (6)	1 (3)	0 (0)
MAGEA3	14 (31)	1 (3)	3 (10)	2 (6)
MAGEA4	7 (22)	4 (13)	2 (6)	1 (3)
GAGE7	19 (59)	4 (13)	9 (29)	2 (6)
NY-ESO-1	2 (6)	1 (3)	0 (0)	0 (0)

MAGEA, MAGE family member A; GAGE7, G antigen 7; NY-ESO-1, cancer/testis antigen 1.

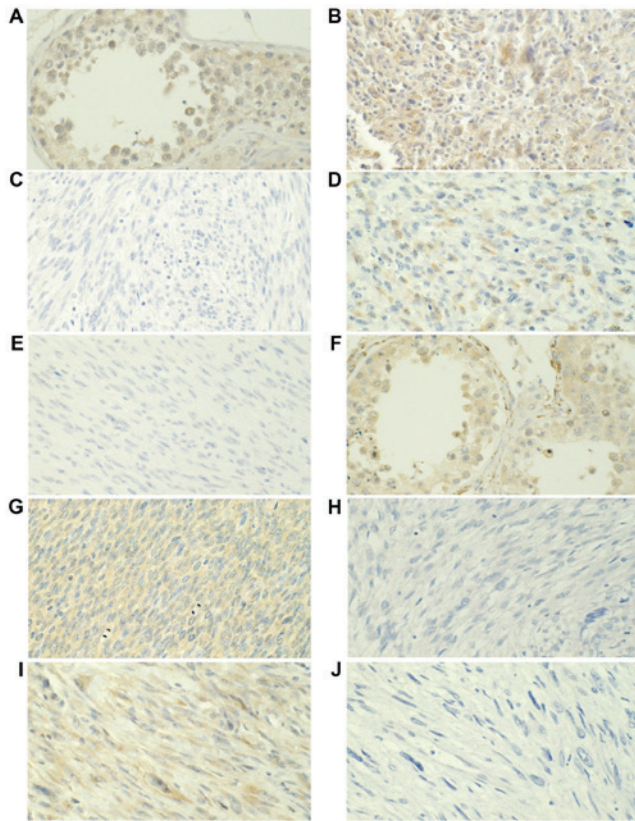


Figure 1. MAGEA1 and MAGEA3 protein expression was assessed using immunohistochemistry. MAGEA1 expression in (A) the testis, (B) positive and (C) negative staining in ULMS, and (D) positive and (E) negative staining in NULMS. MAGEA3 expression in (F) the testis, (G) positive and (H) negative staining in ULMS, and (I) positive and (J) negative staining in NULMS. Magnification, x400. MAGEA, MAGE family member A; ULMS, uterine leiomyosarcoma; NULMS, non-uterine leiomyosarcoma.

Results

IHC results. The IHC results are presented in Tables II and III and Figs. 1-3. MAGEA4, GAGE7 and NY-ESO-1 were localized in the cytoplasm and nuclei, whereas MAGEA1 and MAGEA3 were localized mainly in the cytoplasm. In the

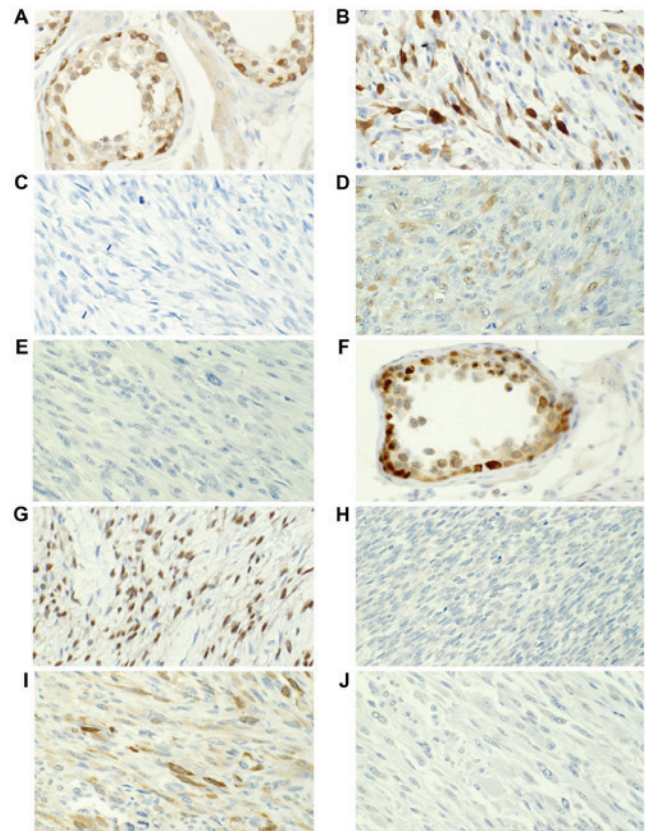


Figure 2. MAGEA4 and GAGE7 protein expression was analyzed using immunohistochemistry. MAGEA4 expression in (A) the testis, (B) positive and (C) negative staining in ULMS, and (D) positive and (E) negative staining in NULMS. GAGE7 expression in (F) the testis, (G) positive and (H) negative staining in ULMS, and (I) positive and (J) negative staining in NULMS. Magnification, x400. MAGEA, MAGE family member A; ULMS, uterine leiomyosarcoma; NULMS, non-uterine leiomyosarcoma; GAGE7, G antigen 7.

ULMS and NULMS samples, positive staining was observed in 10 of 32 (31%) and 1 of 31 (3%) for MAGEA1, 15 of 32 (47%) and 5 of 31 (16%) for MAGEA3, 11 of 32 (34%) and 3 of 31 (10%) for MAGEA4, 23 of 32 (72%) and 11 of 31 (35%) for GAGE7, and 3 of 32 (9%) and 0 of 31 (0%) for NY-ESO-1, respectively (Table II). The positive staining rates for MAGEA1 ($P=0.0034$), MAGEA3 ($P=0.0141$), MAGEA4 ($P=0.0319$) and GAGE7 ($P=0.0054$) were significantly higher in the ULMSs compared with the NULMSs. The ULMSs tended to have a higher positive staining rate for NY-ESO-1 compared with the NULMSs, but the difference was not significant. No significant difference was observed in the expression of any of the CT antigens between male and female patients (data not presented).

In addition, the immunohistochemical scores of the ULMS cases were significantly higher for MAGEA1 ($P=0.0023$), MAGEA3 ($P=0.0474$), MAGEA4 ($P=0.011$), GAGE7 ($P=0.0319$) and NY-ESO-1 ($P=0.0437$) compared with the NULMS (Fig. 4). In the ULMS and NULMS samples, high expression was observed in 2 of 32 (6%) and 0 of 31 (0%) samples for MAGEA1, 1 of 32 (3%) and 2 of 31 (6%) for MAGEA3, 4 of 32 (13%) and 1 of 31 (3%) for MAGEA4, 4 of 32 (13%) and 2 of 31 (6%) for GAGE7, and 1 of 32 (3%) and 0 of 31 (0%) for NY-ESO-1, respectively. No significant

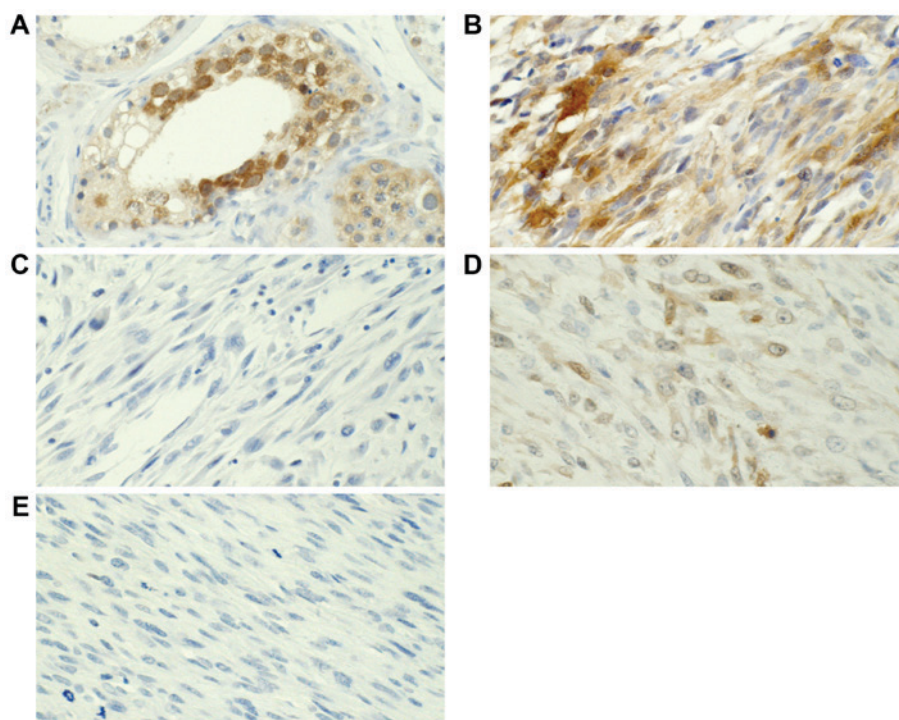


Figure 3. NY-ESO-1 expression was analyzed using immunohistochemistry. (A) NY-ESO-1 expression in the testis. (B) NY-ESO-1-positive and (C) -negative staining in ULMS. (D) NY-ESO-1--positive and (E) -negative staining in NULMS. Magnification, x400. NY-ESO, cancer/testis antigen 1; ULMS, uterine leiomyosarcoma; NULMS, non-uterine leiomyosarcoma.

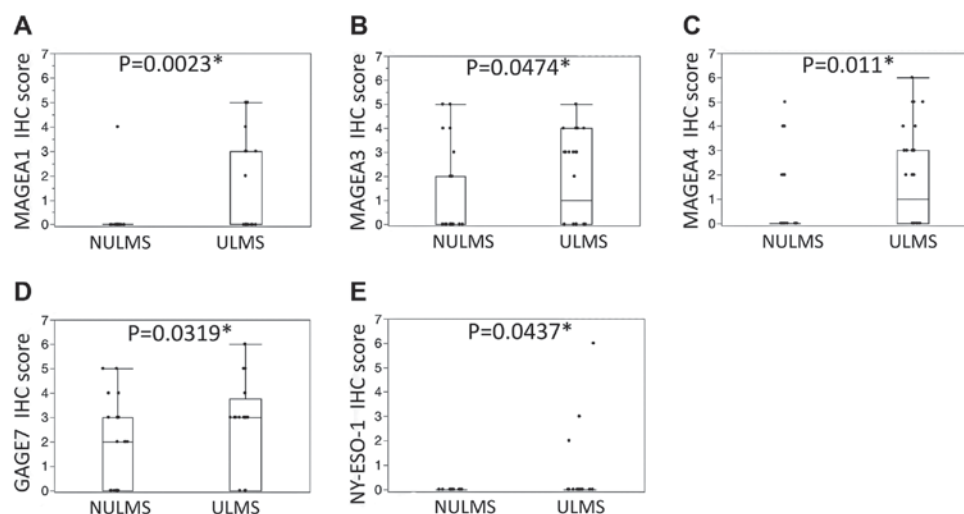


Figure 4. Immunohistochemical scores in NULMS and ULMS. The ULMSs demonstrated significantly higher scores for (A) MAGEA1, (B) MAGEA3, (C) MAGEA4, (D) GAGE7 and (E) NY-ESO-1 compared with the NULMS cases. ULMS, uterine leiomyosarcoma; NULMS, non-uterine leiomyosarcoma; MAGEA, MAGE family member A; GAGE7, G antigen 7; NY-ESO, cancer/testis antigen 1.

association was observed between the expression of any of the CT antigens (data not presented).

Discussion

As previously discussed, CT antigens are considered to be a promising target for immunotherapy due to their restricted expression in normal tissues (except in the testes, which do not express HLA molecules). MAGEA1 expression was detected in myxoid liposarcoma (11.0%) (13), medulloblastoma (4.0%) (14),

head and neck squamous cell carcinoma (13.0%) (15), seminoma (16.6%) (16) and esophageal squamous cell carcinoma (38.8%) (17) as demonstrated by IHC. MAGEA3 expression was detected in gastric carcinoma (45.0%) (18), head and neck squamous cell carcinoma (45.0%) (19), invasive intrahepatic cholangiocarcinoma (47.0%) (20), and prostate cancer (85.8%) (21) as demonstrated by IHC. MAGEA4 expression was detected in uterine endometrioid adenocarcinomas (12.0%), uterine papillary serous carcinomas (63.0%), uterine carcinosarcoma (91.0%) (22), urinary squamous cell carcinoma (45.5%) (23),

cervical squamous cell carcinoma (33.0%) (24), breast cancer (74.0%) (25) and oral squamous cell carcinoma (56.5%) (26). NY-ESO-1 expression was detected in invasive ductal carcinoma of the breast (11.2%) (27), metastatic malignant melanoma (32.0%) (28), head and neck squamous cell carcinoma (4.3%) (29) and non-small cell lung cancer (25.0%) (30). GAGE7 expression was detected in spermatocytic seminoma (67.0%), seminomas (4.0%) (31) and advanced breast carcinoma (43.9%) (32).

Clinical trials of immunotherapeutic targets for CT antigens have been actively performed. NY-ESO-1, MAGEA3 and MAGEA4 are of particular interest to researchers and clinicians, and developments in immunotherapeutic techniques including peptide vaccines, dendritic cell vaccines and T cell receptor gene transduced T lymphocytes for these CT antigens are currently underway (33-35).

However, little is known about the expression of CT antigens in sarcomas, and even less is known about their expression in leiomyosarcoma. Previously, NY-ESO-1 expression in various sarcomas had been analyzed using IHC and revealed that leiomyosarcomas demonstrated negligible NY-ESO-1 protein expression (36,37). Another study surveyed the mRNA expression of CT antigens in several ULMS and NULMS cases and reported that ULMS demonstrated relatively higher mRNA expression of CT antigens compared with NULMS (38). The results of the present study coincide with these results. However, there appear to be no other studies that evaluate the protein expression of CT antigens other than NY-ESO-1 in ULMS or NULMS. The present investigation therefore appears to be the first to determine the protein expression of CT antigens in ULMS and NULMS. The results of the present study revealed that the expression of CT antigens was dominant in ULMS compared with NULMS. Additionally, considering the rate of positivity and high expression, GAGE7 and MAGEA4 may be potential targets for immunotherapy in ULMS cases.

Although the functions of CT antigens remain unclear, it has been reported that MAGEA1 may act as a transcriptional repressor of genes required for differentiation (39). MAGEA4 was demonstrated to promote apoptosis in non-small cell lung cancers (40), and additionally was revealed to induce growth by inhibiting apoptosis in normal oral keratinocytes (41). The functions of NY-ESO-1, MAGEA3 and GAGE7 are poorly understood. The expressions of these CT antigens were reported to be regulated by hypomethylation of the promoter region (42,43). Further studies on the functions and epigenetic regulation of these CT antigens in ULMS and NULMS are needed to elucidate the reason why expression of CT antigens is increased in ULMS compared with NULMS.

The oncofetal protein U3 small nucleolar ribonucleoprotein (IMP3) has been revealed to be expressed in ULMS (7 of 15, 47%) and NULMS (36 of 67, 54%) (44). Oncofetal protein may potentially be a promising target for immunotherapy because it is highly expressed in fetal tissue and malignant tumors but rarely observed in adult benign tissues (45,46). Similarly to the results of the IMP3 study, the results of the present study support the potential utility of MAGEA1, MAGEA3, MAGEA4 and GAGE7 as targets for immunotherapy for ULMS. GAGE7 may also be a potential immunotherapeutic target for NULMS.

One limitation of the present study was that sample-size of the study was too small to be conclusive. Another limitation

was that only IHC expression was studied, thus further studies including western blotting or reverse transcription-quantitative polymerase chain reaction may be beneficial.

In conclusion, the analysis in the present study indicated that the CT antigens MAGEA1, MAGEA3, MAGEA4 and GAGE7 are frequently expressed in ULMS, and the expressions of these proteins were higher in ULMS compared with NULMS. GAGE7 and MAGEA4 have potential use as immunotherapeutic targets in ULMS.

Acknowledgements

The authors would like to thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University for the technical support. The present study was supported by JSPS KAKEN (grant no. 25293088).

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