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Cancer testis antigen expression in primary and recurrent vulvar cancer: Association with prognostic factors

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ABSTRACT

Cancer testis tumour associated antigens (C/T-TAAs) were investigated in several gynaecologic and non-gynaecologic neoplasms as possible prognostic markers and targets for immunotherapy. The objective of the present study was to evaluate C/T-TAA expression patterns and prognostic significance in patients affected by vulvar cancer.

Melanoma antigen E (MAGE)-A1, MAGE-A4 and NY-ESO-1 expression was determined by immunohistochemistry in paraffin-embedded tissue specimens from 45 primary and 14 recurrent vulvar carcinomas treated with surgery.

MAGE-A1, MAGE-A4 and NY-ESO-1 were expressed in 25 (42%), 38 (64%) and 40 (68%) of the 59 samples, respectively. MAGE-A4 was significantly more frequently expressed in tumours with lymph node metastases ($p < 0.002$) and in recurrent tumours ($p < 0.02$). NY-ESO-1 was more highly expressed by moderately or poorly differentiated tumours ($p < 0.01$).

This study demonstrates that vulvar cancer frequently expresses C/T-TAAs. Antigen expression correlates with the presence of lymph node metastases and poor tumour differentiation.

1. Introduction

Vulvar cancer is a relatively rare disease. In developed countries, it is the fourth gynaecologic malignancy after endometrial, ovarian and cervical cancer. In the USA, 3700 new cases are diagnosed and 900 deaths occur annually.[1] Epidermoid carcinoma accounts for 86% of these patients.[2] Most patients are elderly, with a peak of incidence in the eighth decade.[2] Nearly 35% of the patients are diagnosed at FIGO stage III or IV with a 5-year overall survival of 43% and 13%, respectively.[2]

The standard treatment for resectable vulvar cancer includes radical surgery and postoperative radiotherapy in selected cases.[3] Considering the age group primarily affected by this disease, clinical and pathological prognostic factors are constantly being explored in order to minimise unnecessary treatments. Furthermore, new molecules and biological mechanisms are being investigated to propose target therapies that can increase survival with acceptable morbidity.

Tumour associated antigens (TAAs) encoded by the cancer/testis (C/T) gene family are a group of proteins expressed by

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the trophoblast, germ line and cancer cells.[4,5] Most of these genes are localised on the X-chromosome, often as multigene families.[6] The biological function of C/T-TAAs both in normal and in neoplastic tissues remains poorly understood. Some authors believe that these genes are involved in the regulation of the cell cycle.[6] During the last decade, 44 C/T gene families such as melanoma antigen E (MAGE)-A, NY-ESO, LAGE, GAGE and SSX have been identified and their expression is being studied in numerous cancers.[6] As far as cancerogenesis is concerned, scattered information has been gained. It has been demonstrated that MAGE-A genes are necessary for differentiation[7] and provide tumour cells with high resistance to TNFα-mediated cytotoxicity.[8] Furthermore, overexpression of C/T-TAA genes is associated with resistance to frequently used chemotherapy agents.[9]

Some authors have attempted to correlate the expression of C/T-TAAs with the prognosis of patients affected by different tumours such as ovarian[10] and non-small cell lung cancers,[11] while the previous work by our group[12] demonstrated a correlation between C/T-TAAs and commonly recognised prognostic factors in patients affected by cervical cancer.

The peculiar characteristics of C/T-TAAs, being expressed primarily by gonadal and neoplastic tissues,[5] and the capacity of this group of antigens to elicit a spontaneous humoral and cellular immune response,[13] make these antigens particularly attractive targets for immunotherapy in gynaecologic patients.

The objective of the present analysis was to determine the prevalence of MAGE-A1, MAGE-A4 and NY-ESO-1 expression in vulvar cancer patients in order to identify possible targets for immunotherapy and to correlate their expression with commonly recognised prognostic factors.

2. Material and methods

2.1. Patients' characteristics

The ethical committee of the Policlinico Umberto I Hospital, University of Rome 'Sapienza' granted IRB approval for this study. Data including age, tumour extension, treatment protocol, histology, lymph node metastases and surgical outcome were obtained from clinical charts and pathological records. Disease extension of primary tumours is reported both as UICC TNM classification and FIGO staging system,[2] whereas description of recurrent disease is reported as pathological tumour size. Radical surgery was defined as histologically documented free resection margins.

Follow-up was closed on March 2007 and carried out within the Gynaecology Department of the involved institutions starting from the time of pathologic diagnosis.

2.2. Primary tumours

Paraffin-embedded samples from 45 patients affected by primary squamous vulvar cancer FIGO stages I–IVa treated with primary radical surgery were retrieved from the archives of the Department of Surgical Pathology of the Universities of Rome 'Sapienza' and 'Campus Bio-Medico'.

Surgical procedures were carried out with the triple incision technique.[14] Patients with lesions involving only the vulva were subjected to radical vulvectomy or wide local excision[15] in the attempt to obtain at least a 1 cm free resection margin.[16] In case of lower urethral involvement a partial distal urethrectomy was performed. Superficial and deep groin lymph nodes were always removed.[17] Ipsilateral groin lymphadenectomy was reserved only to patients with Ib lateral tumours. Pelvic lymphadenectomy was carried out in the presence of frankly metastatic groin nodes or enlarged pelvic lymph nodes at preoperative imaging. Patients with multiple lymph node metastases were treated with adjuvant chemotherapy[18] or radiotherapy.[19]

2.3. Recurrent tumours

Paraffin-embedded samples from 14 patients affected by squamous cell vulvar cancer, with local recurrence that was treated with radical surgery, were retrieved from the archives of the above-mentioned institutions. Recurrence was judged as a lesion occurring at least 6 months after primary surgical treatment.

2.4. Immunohistochemistry

Serial formalin-fixed, paraffin-embedded cervical tumour samples were deparaffinized in xylene, followed by absolute

Table 1 – Patients and tumour characteristics of 45 primary and 14 recurrent squamous cell vulvar neoplasms

Characteristics		(%)
Primary cancer (45 patients)		
Median age in years (range)	71 (41–85)	
T stage		
1A	3	(7)
1B	8	(18)
2	31	(69)
3	3	(7)
Median depth of invasion in mm (25–75°)	7 (6–9)	
Grade		
1	10	(22)
2	19	(42)
3	16	(35)
Positive nodal status[a]	13/42	(31)
FIGO stage		
IA	3	(7)
IB	4	(9)
II	22	(49)
III	14	(31)
IVa	2	(4)
Recurrent cancer (14 patients)		
Median age in years (range)	70 (52–82)	
FIGO stage at diagnosis		
IB	5	(36)
II	4	(29)
III	5	(36)
Median lesion size (25–75°)	53 (36–69)	
Median depth of invasion in mm (25–75°)	6 (4–10)	
Grade		
1	3	(21)
2	8	(57)
3	3	(21)

a 42 Patients subjected to lymphadenectomy.

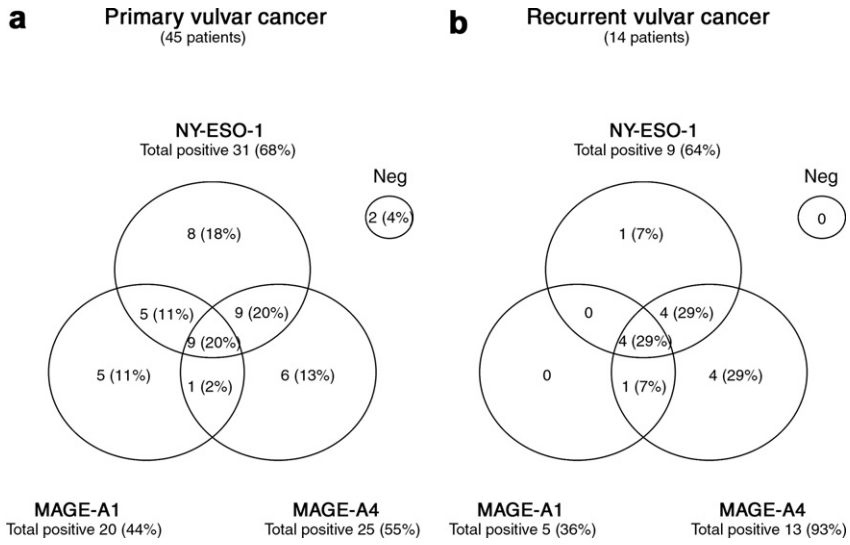

Fig. 1 – Co-expression rates of MAGE-A1, MAGE-A4 and NY-ESO-1 in primary (a) and recurrent (b) vulvar tumours.

ethanol, 95% ethanol and distilled water. Before immunostaining procedures, sections were incubated for 5 min in citrate buffer (pH 6.0) in a pressure cooker at 112 °C to enhance the immunoreactivity of samples. Endogenous peroxidase activity was quenched by treatment with 3% H_2O_2. After blocking of the unspecific sites, the sections were incubated at room temperature for 1 h with mouse monoclonal (MoAb) α-NY-ESO-1 antibody (D8.38 clone) (1:5 dilution) or MoAb α-MAGE-A1 (77B clone) or MoAb α-MAGE-A4 (57B clone) (1:10 dilution). The 57B antibody recognises highly homologous MAGE-A3, MAGE-A6 and MAGE-A12 molecules, but predominantly MAGE-A4 in paraffin-embedded materials.[20–22] A biotin-labelled secondary antibody was used to detect the primary antibody, followed by peroxidase-labelled streptavidin. The reaction was developed by adding 3-amino-9-ethylcarbazole (AEC) (ScyTek, Utah, USA) and the tissue sections were counterstained with haematoxylin (ScyTek). Staining was graded according to the number of positive tumour cells as follows: focal or ⩽5%, negative; >5–20%, weak; >20–50%, moderate; >50%, strong. Negative control slides were incubated with buffer instead of primary antibody. Two independent investigators blinded to the patient clinical informations evaluated all specimens.

2.5. *Statistical analysis*

Statistical analyses were performed with the Fisher's exact test or χ^2 test as appropriate. Patients were censored if lost to follow-up or if alive at the end of the study period. Survival curves were plotted by means of Kaplan–Meier method and compared by using the Log rank test. A p value (two-tailed) lower than 0.05 was considered significant.

3. Results

3.1. *Patients' characteristics*

Characteristics of patients affected by primary and recurrent tumours are reported in Table 1. Briefly, most patients were affected by FIGO stage II or III moderately or poorly differentiated disease. Approximately a third of the patients had lymph node metastases. Mean tumour size was 33 mm. Mean tumour infiltration was 7 mm.

Patients affected by recurrent disease had significantly larger lesions ($p < 0.0001$). FIGO stage at primary diagnosis was equally distributed between stages I and III. Mean tumour maximal diameter and depth of invasion were 57 mm and 8 mm, respectively. Most patients had moderately or poorly differentiated tumours.

3.2. *MAGE-A1, MAGE-A4 and NY-ESO-1 expression*

MAGE-A1, MAGE-A4 and NY-ESO-1 specific positivities were detected in 25 (42%), 38 (64%) and 40 (68%) of the 59 samples, respectively. Only two samples (3%) expressed none of the three C/T-TAAs investigated. Thirteen (22%) samples expressed all three antigens. Of interest was the high proportion of vulvar cancer sample that expressed MAGE-A4 and NY-ESO-1. Co-expression rates for primary and recurrent tumours are reported in Fig. 1.

Table 2 – C/T-TAA expression rates in primary and recurrent squamous cell vulvar neoplasms

	MAGE-A1	MAGE-A4	NY-ESO-1
	Patients (%)	Patients (%)	Patients (%)
Primary			
Negative	25 (55)	20 (44)	14 (31)
Weakly (5–20%)	6 (13)	5 (11)	6 (13)
Moderate (>20–50%)	11 (24)	4 (9)	11 (24)
Strongly (>50%)	3 (7)	16 (35)	14 (31)
Recurrent			
Negative	9 (64)	1 (7)	5 (36)
Weakly (5–20%)	0	0	2 (14)
Moderate (>20–50%)	3 (21)	1 (7)	3 (21)
Strongly (>50%)	2 (14)	12 (86)	4 (29)

In the 59 samples examined, MAGE-A1 was strongly, moderately and weakly expressed by 5, 14 and 6 samples, respectively. MAGE-A4 was strongly, moderately and weakly expressed by 28, 5 and 5 samples, respectively. NY-ESO-1 was strongly, moderately and weakly expressed by 18, 14 and 8 samples, respectively. Data regarding expression intensity for primary and recurrent tumours are reported in Table 2.

Examples of antigen distribution within the tumour can be visualised in Fig. 2. MAGE-A4 and MAGE-A1 showed a strong nuclear and cytoplasmatic expression (Fig. 2a and b), whereas NY-ESO-1 was mostly weakly expressed and was identified only in the cytoplasm (Fig. 2c). Only neoplastic tissue displayed positive staining.

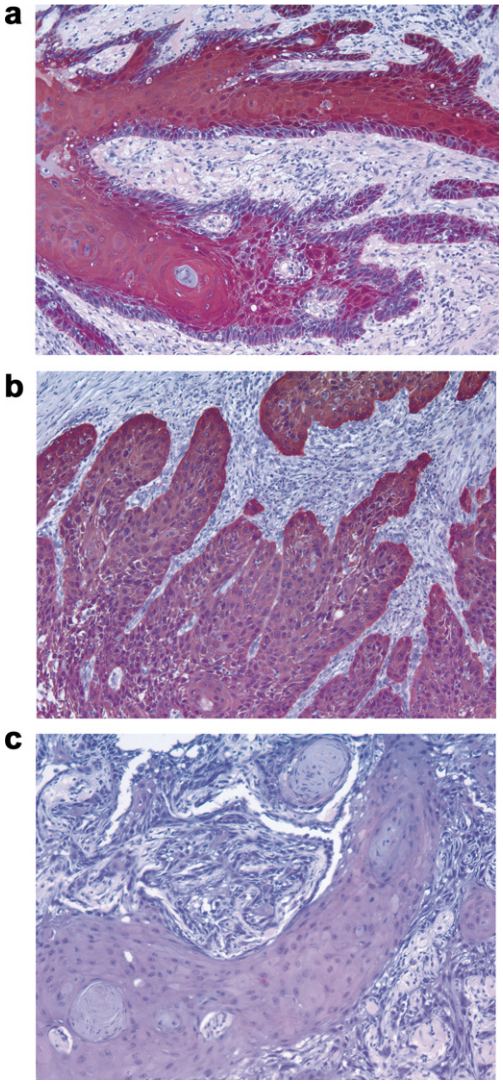

Fig. 2 – (a) Nuclear and cytoplasmic expression of MAGE-A4 in vulvar cancer tissue. Paraffin-embedded tissue was stained with MoAb 57B. Original magnification 10×. (b) Nuclear and cytoplasmic expression of MAGE-A1 in vulvar cancer tissue. Paraffin-embedded tissue was stained with MoAb 77B. Original magnification 10×. (c) Cytoplasmic expression of NY-ESO-1 in cervical cancer tissue. Paraffin-embedded tissue was stained with MoAb D8.38. Original magnification 20×.

The comparison between antigen expression in primary and recurrent tumours showed no significant difference in expression rates for MAGE-A1 and NY-ESO-1. However, MAGE-A4 was significantly more highly expressed in recurrent tumours ($p < 0.02$) (Fig. 3).

3.3. *Clinicopathologic parameters and MAGE-A1, MAGE-A4 and NY-ESO-1 expression*

Correlations between C/T-TAA expression and established prognostic factors are shown in Table 3. MAGE-A1 expression was not significantly correlated to commonly identified prognostic factors. In primary neoplasms, MAGE-A4 was more frequently expressed in tumours from patients with lymph node metastases ($p < 0.002$). NY-ESO-1 instead was more highly expressed by moderately or poorly differentiated tumours both in primary ($p < 0.01$) and recurrent (9/11 versus 0/3; $p < 0.05$) neoplasms.

3.4. *Survival analyses in patients affected by primary tumours*

Although overall and progression free survival was not statistically correlated to the expression of any single C/T-TAA, patients expressing MAGE-A1, MAGE-A4 or NY-ESO-1 all suffered from a more severe prognosis (Fig. 4).

4. Discussion

The objective of the present study was to evaluate the expression of C/T-TAAs in squamous vulvar cancer and to identify possible correlations with commonly recognised clinical and pathological prognostic factors.

This study demonstrated that vulvar cancer frequently expressed C/T-TAAs. In particular, more than 95% of vulvar cancer expressed at least one C/T-TAA and, furthermore, the same neoplasm frequently expressed multiple C/T-TAAs. In these patients, NY-ESO-1 and MAGE-A4 appeared to be expressed more frequently than MAGE-A1. When positive staining was detected, MAGE-A4 staining usually appeared to be of a higher intensity as compared to MAGE-A1 and NY-ESO-1. These data were consistent with previous reports by our group and by some authors in other squamous malignancies such as cervical,[12,23,24] bladder[25] and head and neck cancers.[26]

Important correlations between C/T-TAA expression and commonly recognised prognostic factors emerged. In this series, a significant association between MAGE-A4 and lymph node metastases was identified; in fact, only a single patient with positive lymph nodes was MAGE-A4 negative. A similar behaviour has been reported by Hansel and colleagues, that identified members of the MAGE gene family in subsets of gastrointestinal neoplasms associated with a high incidence of lymph node metastases.[27] NY-ESO-1 appeared to be primarily expressed by moderately or poorly differentiated tumours as previously reported in breast[28] and cervical cancer.[12] In contrast, MAGE-A1 expression did not appear to be related to any of the prognostic factors investigated.

Overall survival data did not highlight any significant correlation with MAGE-A1, MAGE-A4 and NY-ESO-1 expression.

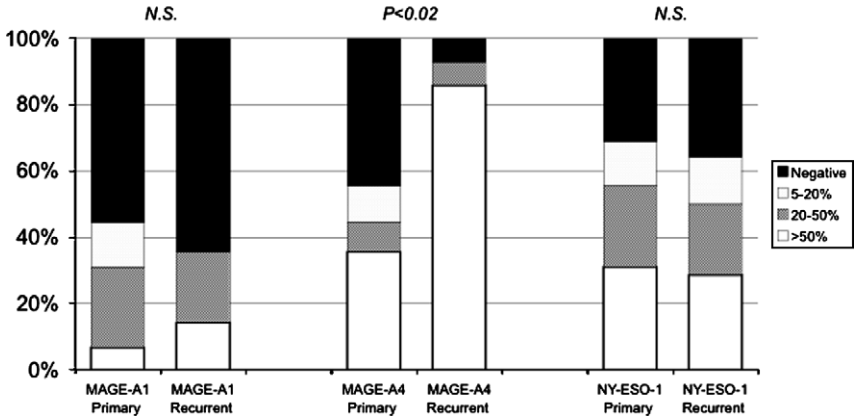

Fig. 3 – Comparison between C/T-TAA expression in primary and recurrent vulvar tumours. Each histogram represents the percentage of negative, weak (5–20%), moderate (20–50%) and strong (>50%) antigen expression (in black, dotted, diagonal lines and white, respectively).

Table 3 – Correlation between C/T-TAA expression rates and commonly recognised prognostic factors

Characteristics	Primary vulvar cancer					
	MAGE-A1		MAGE-A4		NY-ESO-1	
	+/tot	*p*	+/tot	*p*	+/tot	*p*
Age						
<71 years	7/22	NS	12/22	NS	15/22	NS
>71 years	10/23		13/23		16/23	
Tumour size						
T1	6/11	NS	7/11	NS	9/11	NS
T2–T3	14/34		18/34		22/34	
Tumour differentiation						
Well	6/10	NS	6/10	NS	3/10	<0.01
Moderate-poor	14/35		19/35		28/35	
Nodes[a]						
N0	13/29	NS	11/29	<0.002	22/29	NS
N1	5/13		12/13		7/13	

a 42 Patients subjected to groin lymphadenectomy.

The analysis on the single antigens separately showed a non-significant more severe prognosis in patients with positive staining. It should be noted that survival was not a primary objective of the study and population size and follow-up had a power, admittedly below what would be adopted in studies investigating survival.

An interesting and important observation is that MAGE-A4 was significantly more highly expressed by recurrent tumours. This was revealed by the fact that all but one patient affected by disease recurrence expressed MAGE-A4, additionally high intensity staining could be observed in most patients. A possible hypothesis could be that MAGE-A4 is associated with a negative prognosis. Future experimental models and prospective clinical trials carried out on larger populations will be required in order to confirm and precisely address this issue.

Since the recognition that C/T-TAAs were expressed with high frequency in Melanomas,[29] great attention has been paid to their possible role as targets for immunotherapy. Recently, van Baren and colleagues[30] reported the results obtained in 30 patients affected by advanced metastatic melanoma vaccinated with ALVAC virus encoding MAGE antigens. A partial response was observed in one patient, and two additional patients showed disease stabilisation lasting more than 6 months. The relatively low response rate reported by this and several previous immunotherapy clinical trials has been partially ascribed to the fact that patients usually enrolled have large tumour burden and/or have received several lines of systemic therapies and therefore are unlikely to generate an adequate immune response.[31] This hypothesis is supported by the results reported by a double-blind, randomised, placebo-controlled trial in patients affected by non-small cell lung cancer in which vaccination with MAGE-A3 was carried out after complete surgical resection.[32] The preliminary data, although not significant, indicated a trend towards an improvement in disease free interval (33%; HR 0.67). Obviously, definitive conclusions will only be available at the completion of the study.

In conclusion, this study demonstrates that vulvar cancers frequently express C/T-TAAs. Antigens' specific staining correlated to lymph node metastases and tumour differentiation. Furthermore we identified MAGE-A4 expression in most

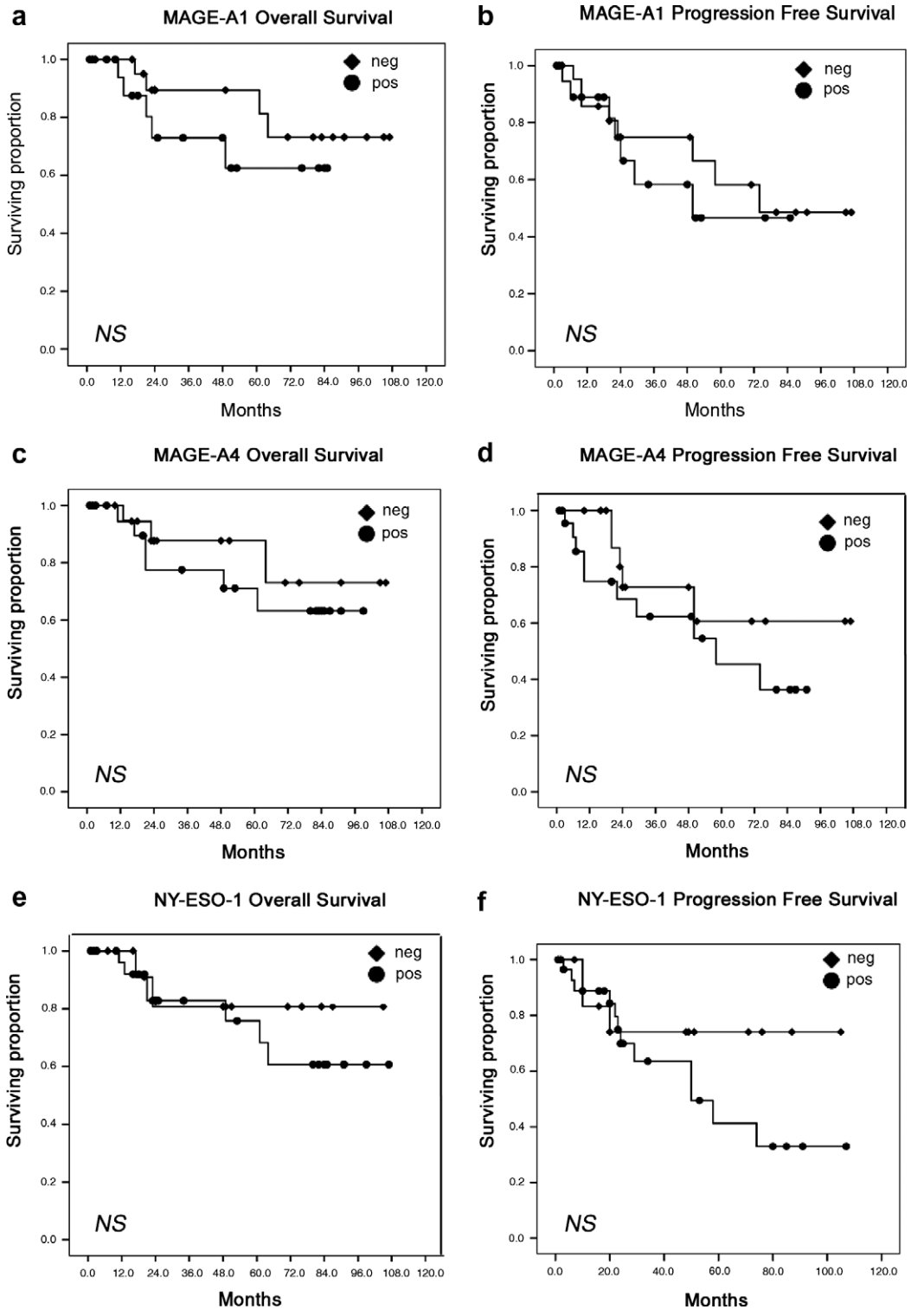

Fig. 4 – Estimated overall survival (OS) (a) and disease free survival (DFS) (b) in MAGE-A1 positive and MAGE-A1 negative patients. Estimated overall survival (OS) (c) and disease free survival (DFS) (d) in MAGE-A4 positive and MAGE-A4 negative patients. Estimated overall survival (OS) (e) and disease free survival (DFS) (f) in NY-ESO-1 positive and NY-ESO-1 negative patients.

recurrent tumours. Thus, this antigen, like other important component of C/T-TAAs, might represent an attractive therapeutic target. Prospective studies with the objective of defining the prognostic significance and the immunogenicity *in vivo* of these antigens are currently ongoing and will help define their role as immunological targets.

Conflict of interest statement

All authors declared there are no conflicts of interest related to this work.

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