Influence of Photodynamic Therapy on the Expression of Cancer/Testis Antigens in Squamous Cell Carcinoma of the Head and Neck

MARIE-NICOLE THEODORAKI[1], KAI J. LORENZ[2], JULIANE SCHNEIDER[3], JULIA C. THIERAUF[1], GIULIO SPAGNOLI[4], PATRICK J. SCHULER[1], THOMAS K. HOFFMANN[1] and SIMON LABAN[1]

[1]*Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University Medical Center, Ulm, Germany;*
[2]*Department of Oto-Rhino-Laryngology, Hospital of German Armed Forces, Ulm, Germany;*
[3]*Department of Pathology, Hospital of German Armed Forces, Ulm, Germany;*
[4]*Department of Biomedicine, University Hospital, Basel, Switzerland*

Abstract. *Background: Photodynamic therapy (PDT) represents a palliative treatment resulting in induction of inflammatory reactions with importance for the development of an antitumor immunity. Cancer/testis antigens (CTAs) have been associated with poor prognosis in different types of cancer, including head and neck squamous cell carcinoma (HNSCC). Materials and Methods: Tumor tissue samples before and after PDT were evaluated for the expression of four different CTAs by immunohistochemistry. Expression intensity and subcellular expression pattern were assessed. Results: Before PDT, expression of any CTA was detectable in 91%. Comparing the overall expression of CTAs, a decreased expression of all melanoma-associated antigens (MAGEs) post-treatment and a slightly increased expression of New York esophageal squamous cell carcinoma 1 (NY-ESO-1) was visible. The simultaneous cytoplasmic and nuclear expression of pan-MAGE or MAGE-A3/A4 correlated with reduced treatment-failure-free-survival (TFFS). Conclusion: This study investigated the impact of PDT on CTA expression in HNSCC, detecting modified expression patterns after PDT. These changes may have been caused by immunological pressure or epigenetic regulation of CTA expression.*

Abbreviations: HNSCC, head and neck squamous cell carcinoma; PDT, photodynamic therapy; CTA, /testis antigens; FFPE, formalin-fixed, paraffin-embedded.

Correspondence to: Marie-Nicole Theodoraki, MD, Department of Oto-Rhino-Laryngology, Head and Neck Surgery, Ulm University Medical Center, Frauensteige 12, 89070 Ulm, Germany. Tel: +49 73150059523, e-mail: marie-nicole.theodoraki@uniklinik-ulm.de

Key Words: Head and neck squamous cell carcinoma (HNSCC), cancer/testis antigen, MAGE, NY-ESO-1, photodynamic therapy.

Head and neck squamous cell carcinomas (HNSCC) rank among the most common human cancers (1). The prognosis of locoregionally advanced tumors is not satisfying with 40-60% five-year overall survival (OS) (2, 3). Treatment failures are mainly associated with local and regional recurrences (2). If recurrence is non-resectable and re-irradiation cannot be performed, palliative treatment may comprise of systemic chemotherapy (4, 5) or, in selected cases, photodynamic therapy (PDT).

PDT is applicable when the tumor is well-accessible and characterized by a flat growth pattern. Besides the induction of direct tumor-cell damage, PDT triggers a locoregional inflammation, which may lead to immune cell infiltration into the tumor (6). Innate and adaptive immune responses against tumor antigens have also been described (7-9). Abscopal treatment effects have been reported showing regression of distant metastasis after PDT of primary lesions (10). However, the influence of PDT on expression of tumor antigens in human cancers has not been analyzed to date.

Cancer/testis antigens (CTAs) are a class of tumor antigen with highly restricted expression in cancer. Physiological expression is restricted to male germ cells and placenta (11, 12). Over 70 families of CTAs with more than 140 members are listed in a database created by the Ludwig Institute for Cancer Research (http://www.cta.lncc.br/) (13). CTAs have been detected in HNSCC and have been correlated with a poor prognosis and reduced OS (14). Among the most frequently occurring CTAs are melanoma-associated antigens (MAGE)-A1, MAGE-A3, MAGE-A4 and, to a lesser extent, New York esophageal squamous cell carcinoma 1 (NY-ESO-1) with the highest immunogenicity reported for NY-ESO-1 (14-17). CTAs are highly immunogenic and can elicit spontaneous immune responses in CTA-positive cancers (18, 19). Many CTAs have been widely investigated and some of their oncogenic effects are known (*e.g.* MAGE-A, MAGE-

C2, preferentially expressed antigen in melanoma (PRAME)), whereas for others, *e.g.* NY-ESO-1, functions remain unknown (20). Because of the restricted expression on tumor cells, CTAs have the capacity to elicit cancer-specific immune responses.

Two studies focusing on the influence of PDT on the expression of CTAs, both conducted by using murine cell lines and murine tumor models have been published (9, 21). In mice, the induction of a specific immune response against P1A, a naturally occurring murine CTA, was associated with improved treatment results after PDT. Furthermore, PDT was shown to be able to induce MAGE-A4 expression in CTA-negative tumors.

The aim of this study was to assess the impact of PDT on CTA expression in human HNSCC. We hypothesized that PDT may influence CTA repertoire. Therefore, we analyzed CTA expression in paired samples collected before and after PDT by immunohistochemistry.

Materials and Methods

Patients. Eighteen patients with recurrent HNSCC received PDT and were included in the study. Formalin-fixed, paraffin-embedded (FFPE) tissue samples both before and after PDT (11 patients) and after PDT only (7 patients) were evaluated for the expression of CTA MAGE-A1, MAGE-A3/A4, pan-MAGE and NY-ESO-1 protein by immunohistochemistry. Patients received palliative PDT for locally or regionally recurrent superficial HNSCC not amenable to surgery or re-irradiation. Five of these 18 patients were treated twice with PDT, one patient three times. All patients were initially treated according to local head and neck oncology treatment guidelines by surgery and/or (chemo-)radiotherapy. The pathological stage was obtained from the primary pathological report and the stage of distant metastasis was established by the interdisciplinary tumor board report. Clinical follow-up data were available from all patients.

An overview of clinical and pathological data at primary diagnosis is provided in Table I. All patients had given informed consent and the study was approved by the local ethics committee (number: 323/14).

Immunhistochemistry (IHC). Consecutive, freshly cut 4-μm serial sections were used for immunohistochemical analysis and hematoxylin and eosin (HE)-stained reference. All cases were evaluated by two experienced physicians independently.

Antibodies for pan-MAGE (clone M3H67) and MAGE-A1 (MA454 clone 1044) were kindly provided by Dr. Gerd Ritter from the New York Branch of the Ludwig Institute for Cancer Research at Memorial Sloan Kettering Cancer Center (22). Stainings with pan-MAGE (stock solution 2.46 mg/ml dilution scale, 1:3324, 0.74 μg/ml), MAGE-A3/A4 (concentration of stock solution unknown; dilution scale, 1:150), MAGE-A1 (stock solution 3.3 mg/ml, dilution scale, 1:150, 22 μg/ml) and NY-ESO-1 (1:100 of stock solution) were carried out with the DakoEnVision+ System-HRP (Dako North America Inc., Carpinteria, CA, USA; for use with mouse primary antibodies) and EDTA buffer at pH 8. Blocking was performed with peroxidase for 5 min and proteinase for 10 min. Primary antibodies were incubated for one hour and the secondary antibody for 30 min. Dako Liquid DAB + Substrate chromogen System was used to visualize the immunostaining (Dako North America Inc.). Testicular tissue was stained as a positive control, whereas tonsil, as well as spleen sections, were used as negative controls.

For the evaluation of immunohistochemical stainings, a well-established scoring system was used (22-25). Nuclear (nuc) and cytoplasmic (cyt) staining intensities (0, negative; 1+, weak; 2+, moderate; 3+, strong), as well as the positive tumor cell fraction in percent, were evaluated separately by three experienced pathologists/physicians. A final score was built from the above parameters: "negative" if the staining intensity was 0 or 1+ in ≤10% of tumor cells; "weakly positive" if the staining intensity was 1+ in >10% and ≤70% of cells or if they displayed a staining intensity of 2+ in ≤30% of tumor cells; "moderately positive" if they showed a staining intensity of 1+ in >70% or 2+ in >30% and ≤70% of cells or 3+ in ≤30% of tumor cells; "strongly positive" if they evidenced a staining intensity of 3+ in >30% of tumor cells. Additionally, an overall score pre- and post-PDT (expression sum-score) was calculated. The product of expression intensity (0-3) and percent of positive tumor cell fraction of the corresponding slides for cytoplasmic expression was added to the same product for nuclear staining with a maximum score of 600 (example: cyt intensity 3+, percent of positive fraction 80 %; nuc intensity 1+, percent of positive fraction 50 %; Result: 3×80 + 1×50=290). Representative examples of different expression intensities, as well as examples of cytoplasmic, nuclear or combined staining patterns, are shown in Figures 1 and 2.

In our prior studies, we showed that clone 57B primarily reacts with MAGE-A3, MAGE-A4 and, to a lesser extent, with MAGE-A6. Therefore, this antibody is referred to as MAGE-A3/A4-specific. The M3H67 clone reacted with MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A8, MAGE-A10, MAGE-B2, MAGE-C2 and, therefore, is referred to as pan-MAGE antibody. MA454 is highly specific for MAGE-A1 (22).

Statistical analysis. For statistical calculations, IBM SPSS Statistics Version 21.0 (http://www-01.ibm.com/support/docview.wss?uid=swg21608060) was used. *p*-Values ≤0.05 were considered significant. Treatment-failure-free survival (TFFS) was defined from the date of PDT until tumor persistence, recurrence or death - whichever occurred first. OS was defined as the time from the date of initial diagnosis until death. Analysis of TFFS and OS was performed using the Kaplan-Meier method. Survival parameters were compared by log rank test. For comparison of the individual CTA expression sum-scores before and after treatment, Wilcoxon test for paired samples and Kendall's coefficient for concordance were used with a significance level of 0.05 and a 95% confidence interval (CI).

Results

Our patient cohort consisted of eleven cases with FFPE samples collected before and after PDT and additional seven patients with specimens obtained after PDT only. In 14 cases, the tumor was located in the pharynx, in three cases in the oral cavity and one case had a laryngeal tumor. All T-stages were included with most patients being >pT2 and pN2 at initial diagnosis. None of the patients had distant metastases. The cohort was heavily pretreated prior to receiving palliative

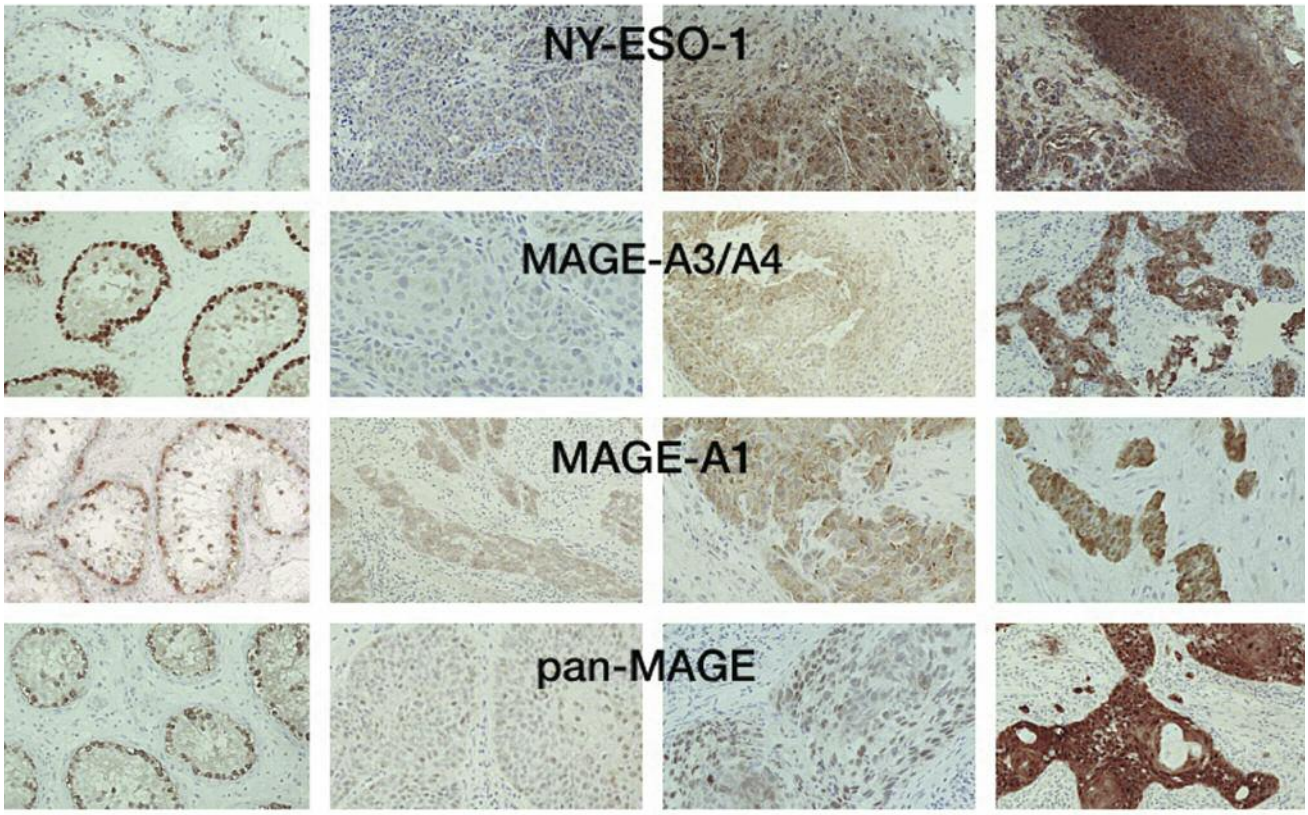

Figure 1. *Expression intensity of the CTA antigens. First column shows the positive controls (testicular tissue), second column presents a weak expression intensity (1+), third column with a moderate (2+) and fourth with the highest intensity (3+). MAGE, Melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1.*

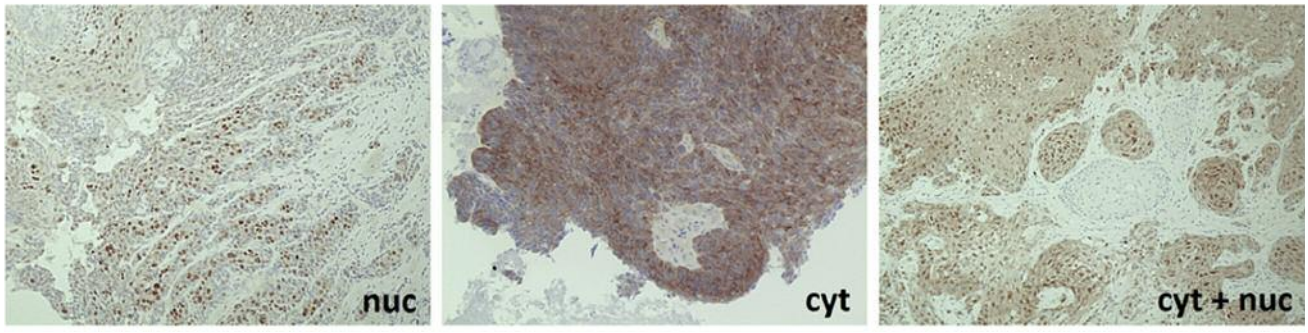

Figure 2. *Intracellular staining patterns. Staining patterns for melanoma-associated antigen (MAGE)- A3/A4 and pan-MAGE. Nuclear (nuc), cytoplasmic (cyt) and combined (cyt+nuc) patterns are shown.*

PDT with a median of three prior treatment lines. The median OS of the entire patient population was 41.6 months and the median TFFS 8.9 months (Table I).

Recurrent HNSCC shows a high expression rate of CTAs. Before PDT, expression of any CTA was found in 10 of 11 patients (91%). Eighty-two % (9/11) were pan-MAGE-positive, 55 % (6/11) were MAGE-A3/A4-positive, 36% (4/11) showed a positivity for MAGE-A1 and 18% (2/11) for NY-ESO-1. Expression rates were higher than expression rates in treatment-naïve patients (22). The comparison of overall expression of CTA (positive or negative) before and after PDT (n=11) showed a decreased expression of all MAGE antigens post treatment and a

Table I. *Clinicopathological characteristics of patients.*

Characteristics	N (%)
Localization (absolute number of patients)	
Oral cavity	3 (16.7)
Hypo-/Oropharynx	14 (77.8)
Larynx	1 (5.5)
Treatment-failure-free-survival (months)	
Median	8.9
Mean	26.9
Overall survival (months)	
Median	41.6
Mean	62.7
Time-to-progression (months)	
Median	7.3
Mean	21.1
Age at diagnosis (Years)	
40-49	1
50-59	8
60-69	8
>70	1
pT category at diagnosis	
pT1	3
pT2	5
pT3	6
pT4	4
pN category at diagnosis	
pN1	4
pN2	10
pN0	4
cM category at diagnosis	
cM0	18
Amount of treatment lines (pre-PDT)	
1	3
2	5
3	6
4	2
≥4	2
Radiotherapy (absolute number of patients)	
No	2
Yes	16
Chemotherapy (absolute number of patients)	
No	5
Yes	13

The TNM classification of malignant tumors has been used (T, size of primary tumor; N, spread to regional lymph nodes; M, distant metastazation); PDT, photodynamic therapy; p, stage defined through pathologic examination.

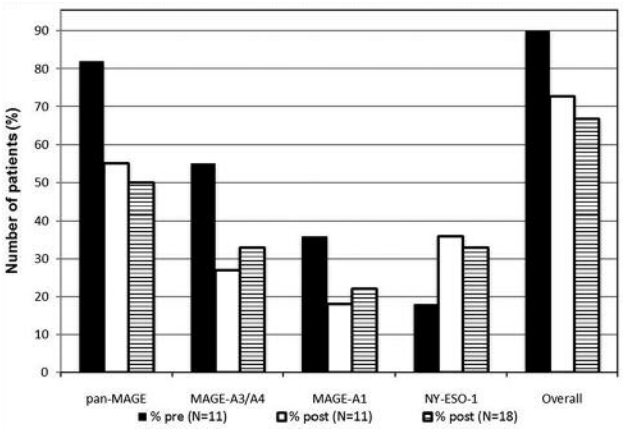

Figure 3. *Overall expression of all CTAs. Overall expression in percent for paired samples pre- and post-photodynamic therapy (PDT) (n=11) or for samples post PDT only (n=18). CTA, Cancer/testis antigen; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1.*

slightly increased expression of NY-ESO-1. After PDT, 55% (6/11) were positive for pan-MAGE, 27% (3/11) for MAGE-A3/A4, 18% (2/11) for MAGE-A1 and 36% (4/11) were positive for NY-ESO-1-specific reagents. In the cohort of the 7 patients with only samples post-treatment available, we found 43% (3/7) being positive for pan-MAGE, 43% (3/7) for MAGE-A3/A4, 29% (2/7) for MAGE-A1 and also 29% (2/7) were positive for NY-ESO-1. In the whole cohort (n=18), including the seven additional post-treatment samples, the results are similar with 50% (9/18) positivity for pan-MAGE, 33% (6/18) for MAGE-A3/A4, 22% (4/18) for MAGE-A1 and 33% (6/18) for NY-ESO-1. The comparison of the expression rate of all CTA combined before and after PDT is consistent with decreased expression rates after PDT (Figure 3).

Individual expression profiles before and after PDT are displayed in Table II. Only in two cases the overall expression (positive or negative) remained constant. In three cases, a new NY-ESO-1 expression was noted after PDT (# 1, 2 and 4), whereas, in cases 3 and 4, a new MAGE-A1 expression was found after therapy. Three cases were negative for post-PDT CTA expression, despite a positive pre-PDT expression (# 7-9). Interestingly, in both cases, #10 and #11, the pan-MAGE expression before and after therapy was positive. However, the MAGE-A3/A4 expression varied. As antigens MAGE-A3/A4 are also included in the antigen-group recognized by the pan-MAGE antibody, the exclusive pan-MAGE expression appears to be due to expression of other antigens of the MAGE-family. We then compared expression profiles of the different antigens (positive *vs.* negative) before and after PDT. Pearson's Chi^2 analysis revealed a significant correlation between MAGE-A3/A4 expression and pan-MAGE expression after treatment (p=0.01). The same result was observed for MAGE-A1 and pan-MAGE (p=0.022), as well as between NY-ESO-1 and MAGE-A1 (p=0.048), after PDT.

To assess the expression intensity of the different antigens before and after therapy we used an expression sum-score. Wilcoxon signed-rank test for related samples

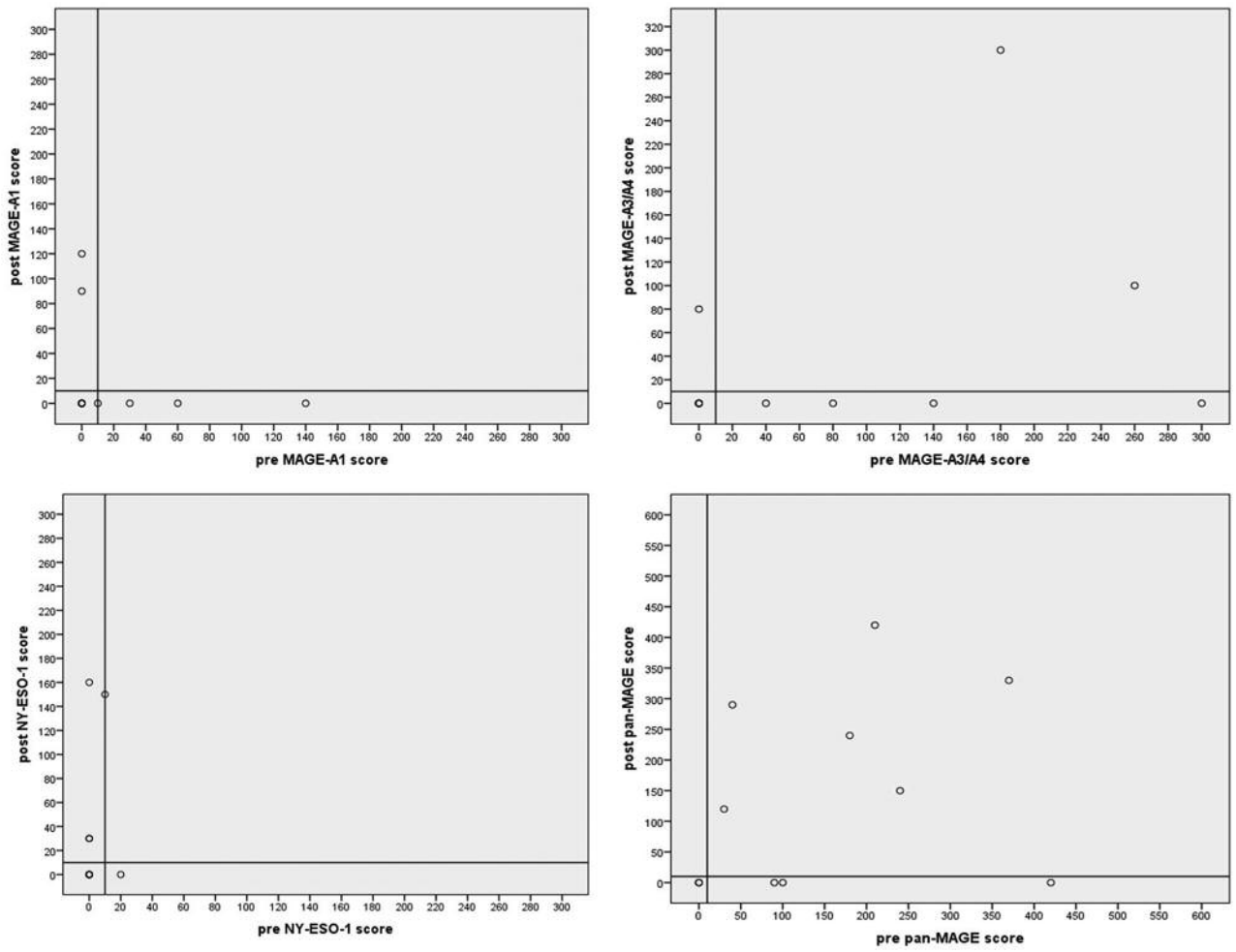

Figure 4. *Expression sum-scores of all cancer/testis antigens (CTAs). The horizontal and vertical lines demonstrate the 10% mark separating positive and negative tumors. The upper left area shows patients with pre-PDT-negative and post-PDT-positive results, in the upper right area are double-positive results. The lower left area contains patients with a double negative expression and the lower right area with pre-PDT-positive and post-PDT-negative expression of the analyzed CTA. MAGE, Melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1.*

was used to compare expression intensities before and after PDT. However, the median of differences was not significantly different for MAGE-A1 (p=0.753), MAGE-A3/A4 (0.204), pan-MAGE (p=0.858) and NY-ESO-1, although a trend to significance (0.078) was noticed in the latter case. The highest Kendall's coefficient for concordance was found for pan-MAGE (0.739). For all other antigens, the concordance coefficient was lower (MAGE-A1: 0.414; MAGE-A3/A4: 0.257; NY-ESO-1: 0.180). A graphic illustration of expression sum-scores before and after PDT is displayed in Figure 4. Interestingly, not a single case of cancer positive for MAGE-A1 expression before PDT was still positive after therapy. A similar trend was seen in MAGE-A3/A4 and NY-ESO-1. In contrast, a nearly linear correlation was found in the pan-MAGE expression-intensity before and after PDT.

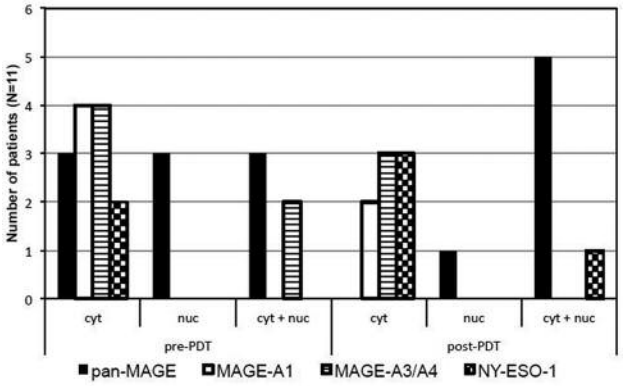

Figure 5. *Expression pattern (nuc, nuclear; cyt, cytoplasmic; cyt+nuc, cytoplasmic+nuclear) of all cancer/testis antigens (CTAs) pre- and post-photodynamic therapy (PDT); n=11. MAGE, Melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1.*

Table II. *CTA expression. Individualized overall cancer/testis antigen (CTA) expression before and after photodynamic therapy (PDT).*

	pre-PDT					post-PDT				
Patient	MAGE-A1	MAGE-A3/A4	pan-MAGE	NY-ESO-1	Any CTA	MAGE-A1	MAGE-A3/A4	pan-MAGE	NY-ESO-1	Any CTA
1	–	–	–	–	–	–	–	–	+	+
2	+	+	+	–	+	–	–	–	+	+
3	–	–	+	+	+	+	–	+	+	+
4	–	–	+	–	+	+	–	+	+	+
5	–	+	+	–	+	–	+	+	–	+
6	–	+	+	–	+	–	+	+	–	+
7	+	–	–	–	+	–	–	–	–	–
8	+	+	+	–	+	–	–	–	–	–
9	+	+	+	+	+	–	–	–	–	–
10	–	–	+	–	+	–	+	+	–	+
11	–	+	+	–	+	–	–	+	–	+
12			n.a.			+	+	+	–	+
13			n.a.			–	–	–	–	–
14			n.a.			–	–	–	–	–
15			n.a.			–	+	+	–	+
16			n.a.			–	–	–	–	–
17			n.a.			+	+	+	+	+
18			n.a.			–	–	–	+	+
x/n	4/11	6/11	9/11	2/11	10/11	4/18	6/18	9/18	6/18	12/18
	36.4%	54.5%	81.8%	18.2%	90.9%	22.2%	33.3%	50.0%	33.3%	66.7%

(+)=Positive staining; (–)=negative staining; n.a.: not available. MAGE: Melanoma-associated antigen; NY-ESO-1: New York esophageal squamous cell carcinoma 1.

Changes in the intracellular expression pattern of the CTAs are visible post-PDT. Taking a closer look at the subcellular expression pattern of the different CTA, we found pan-MAGE, MAGE-A3/A4 and NY-ESO-1 to be predominantly expressed in the cytoplasm and less frequently in the nucleus after PDT. In contrast, MAGE-A1 was exclusively localized in the cytoplasm. A purely nuclear expression was only found for pan-MAGE after PDT (Figure 5). In most cases, a nuclear staining was accompanied by an intermediate or strong cytoplasmic staining.

Comparing the expression pattern of CTAs before and after PDT, we noted changes in the expression of almost all antigens (Figure 5). Before PDT, the expression pattern of pan-MAGE was equally divided between purely cytoplasmic, purely nuclear and combined expression. Post-PDT, purely cytoplasmic expression was never observed. Instead, MAGE A3/A4 was expressed in the cytoplasm or in both nuclei and cytoplasms combined before PDT, whereas post-PDT only a cytoplasmic expression was detectable. An additional simultaneous (combined) cytoplasmic and nuclear expression was detected for NY-ESO-1 post-PDT compared to a purely cytoplasmic expression before therapy. Only the expression of MAGE-A1 was constantly observed exclusively in the cytoplasmic area.

The subcellular expression pattern of pan-MAGE and MAGE-A3/A4 affects the TFFS of HNSCC patients. We found that CTAs expression did not correlate with common clinicopathological criteria, such as age, sex, TNM-status or OS, as determined by Kaplan-Meier analysis. However, the presence of a combined cytoplasmic and nuclear pan-MAGE expression correlated significantly with reduced TFFS (p=0.009). Similar results were noticed for MAGE-A3/A4 in the pairwise comparison of 'cyt+nuc' to negative expression (p=0.008), but not in the overall comparison (p=0.086) (Figure 6).

Discussion

To the best of our knowledge, this is the first study on the impact of PDT on CTA expression in human HNSCC. CTAs have been in the focus of oncologic research in the past decades in different types of malignancies (26-29). CTAs are a group of self-antigens. However, their expression in normal tissue is restricted to fetal and adult germ cells (11, 12) and they appear to play a role in placental development (30). Although CTAs are self-antigens, they can be immunogenic if expressed in cancer tissue. Normal tissue expression is usually not accessible to the immune system due to the physiological blood-testis-barrier (31) and absence of HLA class I determinants on the surface of germ cells (32). Hence, CTAs present an interesting target for specific immunotherapy (33).

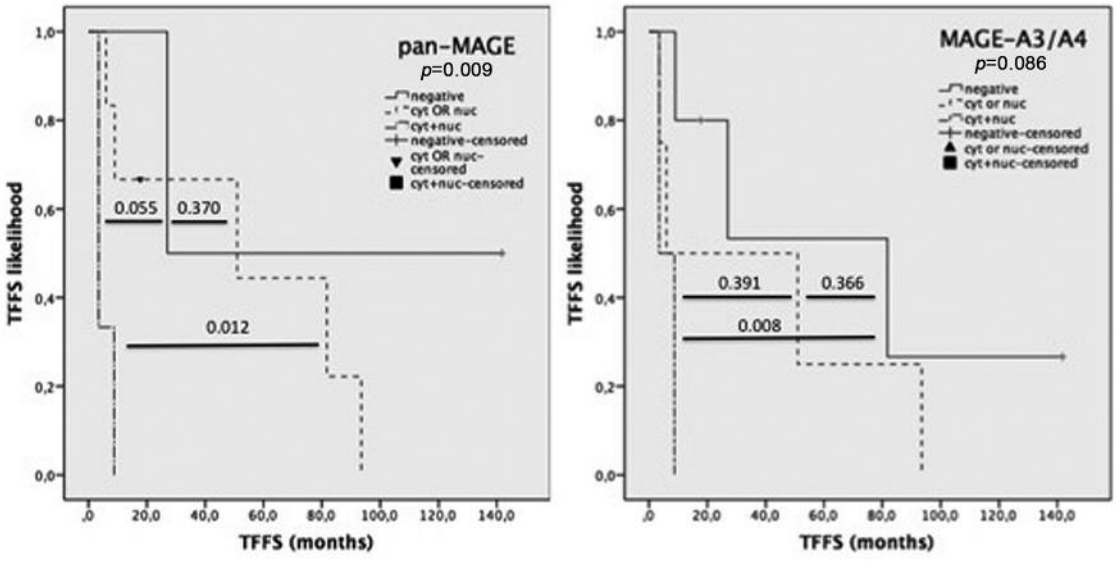

Figure 6. *Treatment-failure-free survival (TFFS). Categorized by pan-MAGE and MAGE-A3/4 expression pattern respectively. Kaplan-Meyer curves of TFFS for the cohort of n=11 comparing patients with simultaneous cytoplasmic and nuclear (cyt+nuc) to only cytoplasmic (cyt) or only nuclear (nuc) expression and negative patients. The overall p-values are given. Simultaneous nuclear and cytoplasmic expression of pan-MAGE is a significant marker or poor TFFS. Simultaneous nuclear and cytoplasmic expression of MAGE-A3/A4 is significantly associated with reduced survival compared to negative patients. MAGE, Melanoma-associated antigen.*

The present patient cohort was heavily pre-treated before receiving PDT in a palliative setting for local or regional recurrence as indicated by the median number of 3 prior treatment lines. Although the available patient number was limited, the analysis of biopsy pairs before and after PDT in eleven patients is a strength of the study. We did not find a correlation of CTA expression with clinicopathological parameters, which is in line with previously published data in HNSCC (17, 22), although one group described increased expression rates of CTA in advanced T-categories (15).

Compared to our previous results on CTA expression in previously untreated HNSCC (22), the expression rates found here were approximately two-fold higher for all CTAs evaluated. Ninety percent of the pre-PDT specimens were positive for at least one of the analyzed antigens.

CTA expression has been associated with a poor outcome in different tumor types (22, 34, 35). On the other hand, improved survival due to cancer antigen-specific immune responses has been reported as well (36-38). In our cohort, the expression of pan-MAGE and MAGE-A3/A4 was associated with significantly reduced TFFS. In particular, the simultaneous cytoplasmic and nuclear expression before PDT had a negative impact on TFFS. These findings are in line with our previously published results in treatment-naïve, surgically-treated patients with HNSCC (22). Interestingly, after PDT the expression pattern changed in all CTAs except for MAGE-A1. Overall, it appears that simultaneous nuclear and cytoplasmic expression decreases post-PDT, whereas the combined expression of NY-ESO-1 increases compared to the expression pre-PDT.

Physiologically, CTAs appear to be involved in fetal and placental development consistent with similarities suggested between gametogenesis and cancer (39, 40). In cancer, they appear to play a role by enhancing tumor proliferation and preventing apoptosis, thus supporting the malignant phenotype (17, 41-43). This could be an explanation why CTA expression rates, as an indicator of cancer aggressiveness, were higher in our palliative treatment setting than in treatment-naïve patients. Overall, CTA expression appears to be a rather dynamic process. After PDT, the expression frequency of all MAGE-antigens was reduced in contrast to a slightly increased NY-ESO-1 expression. In contrast to our findings, Koltun *et al*. showed in a murine squamous cell carcinoma cell line that the procedure of PDT itself could induce the expression of MAGE-A4 (21).

One possible mechanism underlying the modified expression of CTAs detected after PDT could be represented by epigenetic modulation. It is well-known that CTA expression is silenced in somatic cells by gene methylation and may be reactivated in neoplastic cells by demethylation. DNA-methylation and histone post-translational modifications represent the best described epigenetic factors for CTA regulation (44). Demyanenko *et al*. showed that PDT can influence the

expression of proteins involved in epigenetic regulations and histone modifications in the mouse cerebral cortex (45).

Alternatively, reduced MAGE antigen expression could be explained by immunological cancer "editing" resulting in the elimination of MAGE-positive cancer cells. It has been shown previously that cancer therapy, such as radio- and chemotherapy, may lead to an auto-vaccination through the induction of cell death and inflammation, thus activating an immune response (38, 46). Mroz *et al*. detected the induction of specific immune responses against the mouse-specific CTA P1A on murine mastocytoma cells with inhibition of tumor growth and increased survival (9). Thus, the loss of CTA expression could also mirror the elimination of CTA-positive cells by the immune system or an escape mechanism from such an immune response (immune editing) (46). Due to the retrospective nature of this study, no patient material to test this hypothesis was available.

One limitation of this study is represented by the relatively small size of the patient cohort and the possibly associated selection bias. However, PDT is not a frequently used treatment option but, rather, only amenable in selected cases. Therefore, we focused on intra-individual comparison before and after PDT. Another possible bias lies in the heterogeneity of CTA expression in cancer tissue and in the possibility of having sampled non-representative parts of the tumor. By evaluating large, representative biopsies on whole slides, we reduced this potential bias. Finally, due to the retrospective nature of the study, tissue collection time points were not standardized after PDT. The different time intervals between PDT and the sampling of biopsy specimen might have an influence on antigen-expression. In a currently ongoing prospective study, we collect tumor material, patient serum and peripheral blood mononuclear cells (PBMC) before and after PDT at defined time points to test the hypothesis that PDT induces immune responses to CTA.

Conclusion

The expression of CTAs in HNSCC change dynamically during oncological treatment with higher expression rates in heavily pretreated patients. We examined the effects of PDT on HNSCC and found changes in CTA expression patterns after PDT. These changes may result from immunological pressure or may be caused by epigenetic regulation of the CTA expression through PDT. These hypotheses are currently being investigated in a prospective cohort with standardized intervals of tumor biopsy sampling and corresponding serum and PBMC samples.

Acknowledgements

The Authors thank Prof. Gerd Ritter for providing the MAGE-A1 antibody (MA454, clone 1044) and critical reading of the manuscript.

References

1 Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. CA Cancer J Clin *65(1)*: 5-29, 2015.

2 Forastiere AA, Zhang Q, Weber RS, Maor MH, Goepfert H, Pajak TF, Morrison W, Glisson B, Trotti A, Ridge JA, Thorstad W, Wagner H, Ensley JF and Cooper JS: Long-term results of rtog 91-11: A comparison of three nonsurgical treatment strategies to preserve the larynx in patients with locally advanced larynx cancer. J Clin Oncol *31(7)*: 845-852, 2013.

3 Vermorken JB, Remenar E, van Herpen C, Gorlia T, Mesia R, Degardin M, Stewart JS, Jelic S, Betka J, Preiss JH, van den Weyngaert D, Awada A, Cupissol D, Kienzer HR, Rey A, Desaunois I, Bernier J and Lefebvre JL: Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. N Engl J Med *357(17)*: 1695-1704, 2007.

4 Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, De Raucourt D, Bokemeyer C, Schueler A, Amellal N and Hitt R: Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med *359(11)*: 1116-1127, 2008.

5 Vermorken JB and Specenier P: Optimal treatment for recurrent/metastatic head and neck cancer. Ann Oncol *21(Suppl 7)*: vii252-261, 2010.

6 Castano AP, Mroz P and Hamblin MR: Photodynamic therapy and anti-tumour immunity. Nat Rev Cancer *6(7)*: 535-545, 2006.

7 Nowis D, Makowski M, Stoklosa T, Legat M, Issat T and Golab J: Direct tumor damage mechanisms of photodynamic therapy. Acta Biochim Pol *52(2)*: 339-352, 2005.

8 Brackett CM and Gollnick SO: Photodynamic therapy enhancement of anti-tumor immunity. Photochem Photobiol Sci *10(5)*: 649-652, 2011.

9 Mroz P, Vatansever F, Muchowicz A and Hamblin MR: Photodynamic therapy of murine mastocytoma induces specific immune responses against the cancer/testis antigen p1a. Cancer Res *73(21)*: 6462-6470, 2013.

10 Thong PS, Ong KW, Goh NS, Kho KW, Manivasager V, Bhuvaneswari R, Olivo M and Soo KC: Photodynamic-therapy-activated immune response against distant untreated tumours in recurrent angiosarcoma. Lancet Oncol *8(10)*: 950-952, 2007.

11 De Plaen E, Arden K, Traversari C, Gaforio JJ, Szikora JP, De Smet C, Brasseur F, van der Bruggen P, Lethe B, Lurquin C *et al*: Structure, chromosomal localization, and expression of 12 genes of the mage family. Immunogenetics *40(5)*: 360-369, 1994.

12 Zendman AJ, Ruiter DJ and Van Muijen GN: Cancer/testis-associated genes: Identification, expression profile, and putative function. J Cell Physiol *194(3)*: 272-288, 2003.

13 Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, Chen YT, Chua R, Gurung S, Gnjatic S, Jungbluth AA, Caballero OL, Bairoch A, Kiesler E, White SL, Simpson AJ, Old LJ, Camargo AA and Vasconcelos AT: Ctdatabase: A knowledge-base of high-throughput and curated data on cancer-testis antigens. Nucleic Acids Res *37(Database issue)*: D816-819, 2009.

14 Cuffel C, Rivals JP, Zaugg Y, Salvi S, Seelentag W, Speiser DE, Lienard D, Monnier P, Romero P, Bron L and Rimoldi D: Pattern and clinical significance of cancer-testis gene expression in head and neck squamous cell carcinoma. Int J Cancer *128(11)*: 2625-2634, 2011.

15 Figueiredo DL, Mamede RC, Spagnoli GC, Silva WA, Jr., Zago M, Neder L, Jungbluth AA and Saggioro FP: High expression of cancer testis antigens mage-a, mage-c1/ct7, mage-c2/ct10, ny-eso-1, and gage in advanced squamous cell carcinoma of the larynx. Head Neck *33(5)*: 702-707, 2011.
16 Scanlan MJ, Gure AO, Jungbluth AA, Old LJ and Chen YT: Cancer/testis antigens: An expanding family of targets for cancer immunotherapy. Immunol Rev *188*: 22-32, 2002.
17 Atanackovic D, Blum I, Cao Y, Wenzel S, Bartels K, Faltz C, Hossfeld DK, Hegewisch-Becker S, Bokemeyer C and Leuwer R: Expression of cancer-testis antigens as possible targets for antigen-specific immunotherapy in head and neck squamous cell carcinoma. Cancer Biol Ther *5(9)*: 1218-1225, 2006.
18 Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, Nicolay HJ, Sigalotti L and Maio M: The biology of cancer testis antigens: Putative function, regulation and therapeutic potential. Mol Oncol *5(2)*: 164-182, 2011.
19 van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A and Boon T: A gene encoding an antigen recognized by cytolytic t lymphocytes on a human melanoma. Science *254(5038)*: 1643-1647, 1991.
20 Gjerstorff MF, Andersen MH and Ditzel HJ: Oncogenic cancer/testis antigens: Prime candidates for immunotherapy. Oncotarget *6(18)*: 15772-15787, 2015.
21 Koltun M, Banas M, Majewski P, Krzykawska-Serda M and Kwitniewski M: Cancer testis antigens: Searching for novel mechanisms of photodynamic therapy-mediated antitumor immune responses. Photodiagnosis Photodyn Ther *11(4)*: 546-548, 2014.
22 Laban S, Atanackovic D, Luetkens T, Knecht R, Busch CJ, Freytag M, Spagnoli G, Ritter G, Hoffmann TK, Knuth A, Sauter G, Wilczak W, Blessmann M, Borgmann K, Muenscher A and Clauditz TS: Simultaneous cytoplasmic and nuclear protein expression of melanoma antigen-a family and ny-eso-1 cancer-testis antigens represents an independent marker for poor survival in head and neck cancer. Int J Cancer *135(5)*: 1142-1152, 2014.
23 Clauditz TS, Wang CJ, Gontarewicz A, Blessmann M, Tennstedt P, Borgmann K, Tribius S, Sauter G, Dalchow C, Knecht R, Munscher A and Wilczak W: Expression of insulin-like growth factor ii mrna-binding protein 3 in squamous cell carcinomas of the head and neck. J Oral Pathol Med *42(2)*: 125-132, 2013.
24 Minner S, Kraetzig F, Tachezy M, Kilic E, Graefen M, Wilczak W, Bokemeyer C, Huland H, Sauter G and Schlomm T: Low activated leukocyte cell adhesion molecule expression is associated with advanced tumor stage and early prostate-specific antigen relapse in prostate cancer. Hum Pathol *42(12)*: 1946-1952, 2011.
25 Simon R, Mirlacher M and Sauter G: Immunohistochemical analysis of tissue microarrays. Methods Mol Biol *664*: 113-126, 2010.
26 Wang M, Li J, Wang L, Chen X, Zhang Z, Yue D, Ping Y, Shi X, Huang L, Zhang T, Yang L, Zhao Y, Ma X, Li D, Fan Z, Zhao L, Tang Z, Zhai W, Zhang B and Zhang Y: Combined cancer testis antigens enhanced prediction accuracy for prognosis of patients with hepatocellular carcinoma. Int J Clin Exp Pathol *8(4)*: 3513-3528, 2015.
27 Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, Badros AZ, Garfall A, Weiss B, Finklestein J, Kulikovskaya I, Sinha SK, Kronsberg S, Gupta M, Bond S, Melchiori L, Brewer JE, Bennett AD, Gerry AB, Pumphrey NJ, Williams D, Tayton-Martin HK, Ribeiro L, Holdich T, Yanovich S, Hardy N, Yared J, Kerr N, Philip S, Westphal S, Siegel DL, Levine BL, Jakobsen BK, Kalos M and June CH: Ny-eso-1-specific tcr-engineered t cells mediate sustained antigen-specific antitumor effects in myeloma. Nat Med *21(8)*: 914-921, 2015.
28 Sahin U, Tureci O, Chen YT, Seitz G, Villena-Heinsen C, Old LJ and Pfreundschuh M: Expression of multiple cancer/testis (ct) antigens in breast cancer and melanoma: Basis for polyvalent ct vaccine strategies. Int J Cancer *78(3)*: 387-389, 1998.
29 Sigalotti L, Coral S, Altomonte M, Natali L, Gaudino G, Cacciotti P, Libener R, Colizzi F, Vianale G, Martini F, Tognon M, Jungbluth A, Cebon J, Maraskovsky E, Mutti L and Maio M: Cancer testis antigens expression in mesothelioma: Role of DNA methylation and bioimmunotherapeutic implications. Br J Cancer *86(6)*: 979-982, 2002.
30 Jungbluth AA, Silva WA Jr., Iversen K, Frosina D, Zaidi B, Coplan K, Eastlake-Wade SK, Castelli SB, Spagnoli GC, Old LJ and Vogel M: Expression of cancer-testis (ct) antigens in placenta. Cancer Immun *7*: 15, 2007.
31 Bart J, Groen HJ, van der Graaf WT, Hollema H, Hendrikse NH, Vaalburg W, Sleijfer DT and de Vries EG: An oncological view on the blood-testis barrier. Lancet Oncol *3(6)*: 357-363, 2002.
32 Fiszer D and Kurpisz M: Major histocompatibility complex expression on human, male germ cells: A review. Am J Reprod Immunol *40(3)*: 172-176, 1998.
33 Kalejs M and Erenpreisa J: Cancer/testis antigens and gametogenesis: A review and "brain-storming" session. Cancer Cell Int *5(1)*: 4, 2005.
34 Zhang W, Barger CJ, Link PA, Mhawech-Fauceglia P, Miller A, Akers SN, Odunsi K and Karpf AR: DNA hypomethylation-mediated activation of cancer/testis antigen 45 (ct45) genes is associated with disease progression and reduced survival in epithelial ovarian cancer. Epigenetics *10(8)*: 736-748, 2015.
35 Ogata K, Aihara R, Mochiki E, Ogawa A, Yanai M, Toyomasu Y, Ando H, Ohno T, Asao T and Kuwano H: Clinical significance of melanoma antigen-encoding gene-1 (mage-1) expression and its correlation with poor prognosis in differentiated advanced gastric cancer. Ann Surg Oncol *18(4)*: 1195-1203, 2011.
36 Lee HJ, Kim JY, Song IH, Park IA, Yu JH and Gong G: Expression of ny-eso-1 in triple-negative breast cancer is associated with tumor-infiltrating lymphocytes and a good prognosis. Oncology *89(6)*: 337-344, 2015.
37 Ademuyiwa FO, Bshara W, Attwood K, Morrison C, Edge SB, Karpf AR, James SA, Ambrosone CB, O'Connor TL, Levine EG, Miliotto A, Ritter E, Ritter G, Gnjatic S and Odunsi K: Ny-eso-1 cancer testis antigen demonstrates high immunogenicity in triple negative breast cancer. PLoS One *7(6)*: e38783, 2012.
38 Karbach J, Gnjatic S, Biskamp M, Atmaca A, Weidmann E, Brandt K, Wahle C, Bernhard H, Knuth A and Jager E: Long-term complete remission following radiosurgery and immunotherapy in a melanoma patient with brain metastasis: Immunologic correlates. Cancer Immunol Res *2(5)*: 404-409, 2014.
39 Old LJ: Cancer/testis (ct) antigens – a new link between gametogenesis and cancer. Cancer Immun 1(1, 2001.
40 Simpson AJ, Caballero OL, Jungbluth A, Chen YT and Old LJ: Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer *5(8)*: 615-625, 2005.

41 Nardiello T, Jungbluth AA, Mei A, Diliberto M, Huang X, Dabrowski A, Andrade VC, Wasserstrum R, Ely S, Niesvizky R, Pearse R, Coleman M, Jayabalan DS, Bhardwaj N, Old LJ, Chen-Kiang S and Cho HJ: Mage-a inhibits apoptosis in proliferating myeloma cells through repression of bax and maintenance of survivin. Clin Cancer Res *17(13)*: 4309-4319, 2011.

42 Por E, Byun HJ, Lee EJ, Lim JH, Jung SY, Park I, Kim YM, Jeoung DI and Lee H: The cancer/testis antigen cage with oncogenic potential stimulates cell proliferation by up-regulating cyclins d1 and e in an ap-1- and e2f-dependent manner. J Biol Chem *285(19)*: 14475-14485, 2010.

43 Cappell KM, Sinnott R, Taus P, Maxfield K, Scarbrough M and Whitehurst AW: Multiple cancer testis antigens function to support tumor cell mitotic fidelity. Mol Cell Biol *32(20)*: 4131-4140, 2012.

44 Karpf AR and Jones DA: Reactivating the expression of methylation silenced genes in human cancer. Oncogene *21(35)*: 5496-5503, 2002.

45 Demyanenko SV, Uzdensky AB, Sharifulina SA, Lapteva TO and Polyakova LP: Pdt-induced epigenetic changes in the mouse cerebral cortex: A protein microarray study. Biochim Biophys Acta *1840(1)*: 262-270, 2014.

46 von Boehmer L, Mattle M, Bode P, Landshammer A, Schafer C, Nuber N, Ritter G, Old L, Moch H, Schafer N, Jager E, Knuth A and van den Broek M: Ny-eso-1-specific immunological pressure and escape in a patient with metastatic melanoma. Cancer Immun *13*: 12, 2013.

Received May 9, 2016
Revised June 8, 2016
Accepted June 9, 2016