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Original Article

Expression of MAGE-A3 and/or PRAME tumor antigens in South Korean gastric cancer patients

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Abstract: MAGE-A3 (Melanoma AntiGen-A3) and PRAME (Preferentially expressed Antigen of MElanoma) are two tumor-associated antigens (TAAs) which have been extensively tested to develop antigen-specific cancer immunotherapy. This study aimed at determining the rate of expression of these TAAs in gastric cancer (GC), the association between clinico-pathological factors and TAA mRNA expression as well as investigating TAA expression as potential prognostic marker for patients' overall survival (OS). In addition, the association between the methylation status of the *MAGE-A3* promoter region and its mRNA expression was examined. Archival formalin-fixed paraffin-embedded tissue specimens of resected tumors from 250 GC patients were investigated for TAA mRNA expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and for protein expression of MAGE-A by immunohistochemistry. The methylation status of the *MAGE-A3* promoter region was assessed by methylation-specific PCR. MAGE-A3 mRNA and protein expression was detected in 23.4% and 19.6% of the tumors, respectively, and 23.0% displayed an unmethylated *MAGE-A3* promoter. The overall concordance between the three methods used to investigate expression of MAGE-A (3) was 75.0%. PRAME mRNA expression was found in 20.4% of the tumors, and 33.8% of them expressed at least one of the TAAs. Unlike the tumors in other organs, mRNA expression of either TAA was not associated with any of the clinic-pathological factors examined and neither did it show any potential as a prognostic biomarker for OS. No association was found between OS and the methylation status of the *MAGE-A3* promoter region or with the tumor's protein expression of MAGE-A.

Keywords: Gastric cancer, MAGE-A3 antigen, PRAME antigen, tumor associated antigens, immunohistochemistry, promoter methylation

Introduction

Gastric cancer (GC) is the fourth most common cancer worldwide and the second leading cause of cancer mortality [1, 2]. For the patients with unresectable or reccurent GC, systemic chemotherapy has been shown to improve survival and patient quality of life compared to best supportive care and has become the mainstay of palliative treatment [3], with objective response rates ranging from 20 to 40% and median overall survival (OS) of 8 to 10 months [4].

Among the alternative treatment options being investigated to improve the outcome for GC patients are immune-based therapies. Tumor-specific T lymphocytes have been isolated from GC patients [5-7] and the infiltration of tumors by different types of T cells has been identified as an independent prognostic factor for better OS of GC patients [8]. Conversely, the presence of regulatory T cells which can inhibit anti-tumor T-cell responses has been associated with a negative prognosis in resectable GCs [9-11].

Therapeutic immunization based on tumor-specific or tumor-associated antigens (TAA) provides a possible approach to induce or enhance immunity against GC tumors. These TAAs are defined as antigens expressed on tumor cells that can elicit an immune response in the host, either de novo or by enhancing a pre-existing immune response and making it more robust and durable [12]. In order to induce tumor-specific T cells, peptides derived from TAAs must

be presented to T cells by antigen-presenting cells, which can activate naïve and memory T cells. Many studies have demonstrated the capability of immunogenic peptides derived from TAAs to lyse gastric tumor cells (reviewed in [12]).

Numerous TAAs have been identified and partly characterized, of which a large proportion have been assessed for their potential as targets in antigen-specific cancer immunotherapy. A fundamental preliminary step in assessing the potential of a specific TAA to be used as immunotherapy against a particular type of cancer is to assess the prevalence of the TAA on the tumor cells of the patient population concerned. Only patients with tumors expressing this particular TAA will be able to respond to immunotherapy specifically targeted at this TAA. Tumor expression of a particular TAA may be detected and quantified by different methods. One is by means of quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays on messenger RNA (mRNA) extracted from the tumor cells. Another is by immunohistochemistry (IHC) staining of the protein (antigen) provided that an antigen-specific antibody has been identified and can be used for the staining. Alternatively, gene expression status may be assessed by methylation analysis of the promoter region.

It is widely recognized that important contributors to human carcinogenesis consist of epigenetic alterations including hypermethylation of promoter CpG islands and hypomethylation of the global DNA [13, 14]. Hypermethylation of promoter CpG islands engenders transcriptional silencing of their downstream genes. Many studies have thus reported that tumor suppressor genes are silenced by hypermethylation of their promoter region during carcinogenesis [15]. Conversely, hypomethylation of the promoter region leads to activation of the gene and production of abnormally high levels of protein [13, 14].

Investigation of the methylation status of the promoter region of the genes of interest may therefore be used to assess whether they are silenced or activated. An unmethylated promoter region of a specific gene would then indicate that the gene was expressed.

Two TAAs that have been widely investigated as possible targets for antigen-specific immunotherapy against cancer are melanoma-associated antigen A3 (MAGE-A3) and preferentially expressed antigen of melanoma (PRAME). In addition to the therapeutic target for immunotherapeutics, aberrant expression of PRAME in tumor cells have been found to be correlated with shorter survival in neuroblastoma [16], in ovarian cancer [17] and in breast cancer [18]. The aim of the present study was to assess the prevalence of expression of MAGE-A3 and PRAME in tumors of GC patients, their co-expression and the association between expression of either antigen and clinico-pathological factors or clinical outcomes. MAGE-A3 expression was detected by qRT-PCR of mRNA, by IHC of MAGE-A protein and by assessment of the methylation status of the *MAGE-A3* gene promoter region. PRAME expression was detected by qRT-PCR of mRNA.

To the best of our knowledge, this study is the first assessment of PRAME expression and of co-expression of MAGE-A3 and PRAME in GC.

Materials and methods

Study cohort

Surgically resected formalin-fixed paraffin-embedded (FFPE) tissue specimens were obtained from 250 consecutive GC patients undergoing gastrectomy at the Seoul National University Hospital in South Korea between 1 January and 31 December 2004. Patient and tumor characteristics were recorded from the hospital files and included patient demographics (age, gender), tumor characteristics (histologic type, differentiation grade, depth of invasion, pathological stage (according to Edition 7 of the AJCC Staging Manual), lymph node metastases) and patient survival time. Patient survival data, including dates and causes of death, were obtained from the Korean Central Cancer Registry at the Ministry of Health and Welfare, South Korea.

The study was approved by the Institutional Review Board of Seoul National University Hospital (H-1010-065-336).

Tissue array preparation

All of the specimens were assembled into tissue microarrays. Three representative core tis-

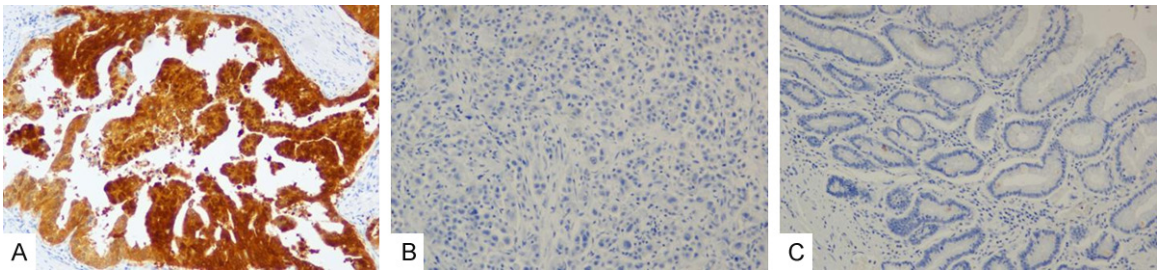

Figure 1. Representative images of MAGE-A positive (A) and MAGE-A-negative (B) tumors and normal gastric cells (C). The MAGE-A3 staining was found in the cytoplasm as well as in the nucleus (original magnification, ×100) (A).

sue biopsy specimens (diameter 2 mm) were obtained from FFPE GCs (donor blocks) and arranged in triplicate sets of new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Superbiochips Laboratories). Each tissue array block contained up to 60 cores, thus 24 array blocks were prepared during the study.

mRNA antigen expression detected by qRT-PCR arrays

mRNA expression of MAGE-A3 and PRAME was detected and quantified as described in detail previously [19]. In brief, manual dissection of the FFPE tissue specimens was performed by Response Genetics Inc. (RGI, USA) to obtain the minimally required 50 mm^2 of tumor tissue with 50-80% neoplastic cells. Total RNA extraction was performed using the *RNeasy*™ FFPE kit (Qiagen, USA) modified with an additional DNAse digestion step added in order to improve the elimination of genomic DNA. Complementary DNA (cDNA) was synthesized by mixing 15 µL cDNA master mix with 15 µL of RNA and incubating.

MAGE-A3 and *PRAME* genes along with *β-actin* housekeeping gene were amplified using qRT-PCR *TaqMan*® chemistry (ThermoFischer) on the ABI 7900 system (Applied Biosystems) in 384-well plates. 50 ng (100%) cDNA and 0.5 ng (1%) of total RNA extracted from the human melanoma cell line MZ-2-3.0 (referred to as gene expression reference level (GERL) and provided by Ludwig Institute of Cancer Research, Belgium) was included into the RT in parallel as a positive control.

mRNA of a tumor specimen was determined in a semi-quantitative way relative to the GERL. A specimen was declared MAGE-A3-positive if its relative expression of *MAGE-A3* to *β-actin* was ≥ 1% that in the GERL. For *PRAME*, the corresponding threshold was 0.3% of the relative expression of *PRAME* to *β-actin* in the GERL. Probes and primers were used as previously described [19].

Methylation status of MAGE-A3 determined by methylation-specific PCR

For the methylation-specific PCR, 1 µg of genomic DNA extracted by a standard proteinase-K digestion and phenol/chloroform procedure, was denatured with NaOH (final concentration 0.2 M) treated with 3 M sodium-bisulfite (Sigma, USA) and 10 mM hydroquinone (pH 5.0, Sigma) and then incubated at 50°C for 16 hours. After incubation, DNA was purified using a *Wizard* DNA purification kit (Promega, USA) and then treated with NaOH, recovered in ethanol and resuspended in 20 µL of distilled water. After the sodium-bisulfite modification, PCR amplification was performed in a thermal cycler for 1 cycle at 95°C for 5 min, followed by 35 cycles each at 95°C for 30 s, 62°C (unmethylated) or 64°C (methylated) for 30 s, 72°C for 1 min and final extension at 72°C for 10 min. The primer sequences are shown in Table S1 of the Supplementary Material.

MAGE-A protein expression detected by immunohistochemistry

Sections (4 µm) from FFPE blocks were dewaxed in xylene and rehydrated using a graded alcohol series. For the antigen retrieval step, slides were inserted in a rack in diluted retrieval solution (pH 6.0, EDTA) and preheated to 100°C for 6 min and then further heated at 1000 Watt for 5 min. The slides were then

Table 1. Patient and tumor characteristics

Characteristics	Categories	Total N = 250		
		n	%	95% CI
Age	Less than 45 years old	40	16.0	11.7-21.1
	45-54 years old	58	23.2	18.1-28.9
	55-64 years old	59	23.6	18.5-29.4
	65-74 years old	73	29.2	23.6-35.3
	75 years old or more	20	8.0	5.0-12.1
Gender	Female	74	29.6	24.0-35.7
	Male	176	70.4	64.3-76.0
Histology (Laurén)	Intestinal	91	36.4	30.4-42.7
	Diffused	110	44.0	37.8-50.4
	Mixed	47	18.8	14.2-24.2
	Undetermined	2	0.8	0.1-2.9
Histology (Ming)	Infiltrative	230	92.0	87.9-95.0
	Expanding	20	8.0	5.0-12.1
WHO 2000 classification and differentiation grade	Papillary adenocarcinoma	2	0.8	0.1-2.9
	Tubular adenocarcinoma, well differentiated	6	2.4	0.9-5.2
	Tubular adenocarcinoma, moderately differentiated	85	34.0	28.1-40.2
	Tubular adenocarcinoma, poorly differentiated	101	40.4	34.3-46.8
	Mucinous adenocarcinoma	12	4.8	2.5-8.2
	Signet-ring cell carcinoma	38	15.2	11.0-20.3
	Undifferentiated carcinoma	3	1.2	0.2-3.5
	Others*	3	1.2	0.2-3.5
TNM Stage (pooled)	I	44	17.6	13.1-22.9
	II	58	23.2	18.1-28.9
	III	101	40.4	34.3-46.8
	IV	47	18.8	14.2-24.2

N = Number of patients/samples. n = Number of patients/samples in a given category. % = n/Number of patients/samples with available results × 100. *Others = Other types including squamous cell carcinomas, small cell carcinoma, etc. LL, UL for percentage = Exact 95% Lower and Upper confidence limits. CI = Confidence interval.

transferred to the *Autostainer* 360 (Lab Vision, USA) and the program was run as follows: 1) slide rinse in wash buffer and peroxidase blocking solution, 2) incubation with mouse anti-MAGE A monoclonal antibody 6C1 (Zymed Laboratories Inc., USA) diluted at 1:100 for 60 min, labelled with polymer for 8 min, incubated with DAB (*Envision* kit, DAKO, Denmark) for 10 min and then counterstained in Mayer's hematoxylin. The 6C1 antibody can detect but not distinguish between the proteins MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12.

Three representative tumor cores were obtained from each tumor. MAGE-A was stained in either the cytoplasma or nucleus and often both. The result of the immunostaining experiment was considered positive for MAGE-A if at least one of the three tumor cores had an IHC score ≥ 50 (**Figure 1**) in either the cytoplasma or the nucleus. The IHC score was calculated as follows:

IHC score = ΣIntensity (1, 2 or 3) × Area (0-100%). IHC MAGE-A3 results is shown in Table S2 of the Supplementary Materials.

Statistical methods

This study was exploratory. Thus, the sample size was not calculated on the basis of any prespecified hypotheses and for antigen expression in subgroups determined by patient or tumor characteristics only descriptive statistics are presented. The proportions of FFPE specimens with an antigen expression level above the cut-off value were estimated on the basis of the number of specimens with a valid assay result for the respective antigen excluding specimens with missing or invalid antigen expression results. The percentages of antigen-positive specimens are presented with their exact 95% confidence intervals (CIs). The rates of co-expression of MAGE-A3 and PRAME were estimated on the basis of the number of FFPE specimens with a valid assay result for each antigen.

Kaplan-Meier (KM) curves for OS time were estimated and compared using the log-rank test and unadjusted hazard ratios (HRs) with 95% CIs estimated by means of the Cox proportional hazard regression model.

Table 2. Validity of MAGE-A3 and PRAME expression tests using qRT-PCR assays

Characteristics	Categories	MAGE-A3 (N = 250)			PRAME (N = 250)		
		n	%	95% CI	n	%	95% CI
Validity test	Valid	137	60.4	53.7-66.8	142	62.6	55.9-68.9
	Invalid	52	22.9	17.6-28.9	47	20.7	15.6-26.6
	QNS	38	16.7	12.1-22.2	38	16.7	12.1-22.2
	Missing	23	-	-	23	-	-

N = Number of patients/samples. n = Number of patients/samples in a given category. QNS = Quantity not sufficient. % = n/Number of patients/samples with available results × 100.

Table 3. Overall rates of expression of MAGE-A3 and PRAME

Characteristics	Categories	Total (N = 250)		
		n	%	95% CI
MAGE-A3 expression (mRNA) result	Positive	32	23.4	16.6-31.3
	Negative	105	76.6	68.7-83.4
	Missing	113	-	-
MAGE-A3 methylation result	Unmethylated	54	23.0	17.8-28.9
	Methylated	181	77.0	71.1-82.2
	Missing	15	-	-
MAGE-A IHC result	Positive	49	19.6	14.9-25.1
	Negative	201	80.4	74.9-85.1
PRAME expression (mRNA) result	Positive	29	20.4	14.1-28.0
	Negative	113	79.6	72.0-85.9
	Missing	108	-	-

N = Number of patients/samples. n = Number of patients/samples in a given category. % = n/Number of patients/samples with available results × 100. IHC = Immunohistochemistry.

Table 4. Concordance between MAGE-A3 expression tests with the three methods

Categories	N = 136		
	n	%	95% CI
mRNA-negative/Methylated/IHC-negative	87	64.0	55.3-72.0
mRNA-negative/Methylated/IHC-positive	4	2.9	0.8-7.4
mRNA-negative/Unmethylated/IHC-negative	9	6.6	3.1-12.2
mRNA-negative/Unmethylated/IHC-positive	5	3.7	1.2-8.4
mRNA-positive/Methylated/IHC-negative	6	4.4	1.6-9.4
mRNA-positive/Methylated/IHC-positive	5	3.7	1.2-8.4
mRNA-positive/Unmethylated/IHC-negative	5	3.7	1.2-8.4
mRNA-positive/Unmethylated/IHC-positive	15	11.0	6.3-17.5

N = Number of specimens. n = Number of specimens in a given category. % = n/Number of specimens with available results × 100. CI = Confidence interval.

Results

Two-hundred-and-fifty FFPE specimens were tested for antigen expression and *MAGE-A3* promoter methylation status. Patient demographics and tumor characteristics are summarized in **Table 1**.

Percentage of specimens giving valid test results

Each mRNA expression test of an FFPE specimen was done with two replicates. Twenty-three specimens were not tested for mRNA expression (**Table 2**) and for 38 specimens, the amount of tumor cells in the mRNA extracted was insufficient (34/38) or completely absent (4/38).

Valid results were obtained for 60.4% of the specimens tested for mRNA expression of MAGE-A3. The reasons for invalid assay results for MAGE-A3 were test results 'out-of-range' (47/52), inconsistent replicates (3/52) and contamination with genomic DNA (2/52). For mRNA expression of PRAME, valid results were obtained for 62.6% of tested specimens. Invalid test results for PRAME were 'out-of-range'.

All the 250 FFPE specimens gave valid IHC results for MAGE-A protein, whereas *MAGE-A3* methylation status could not be obtained for 15 specimens.

Antigen expression

The three methods for detecting MAGE-A3 expression gave concordant results overall (**Table 3**), with the percentage of specimens with positive results for MAGE-A3 ranging from 19.6% for MAGE-A- IHC to 23.4% for MAGE-A3-mRNA expression. A total of 75% of specimens had fully concordant results across the 3 tests (**Table 4**). Fourteen (10.3%) specimens had MAGE-A3 methylation status results that were

Table 5. Rates of co-expression of MAGE-A3 and PRAME

Characteristics	Categories	N = 136		
		n	%	95% CI
Co-expression MAGE-A3 and PRAME	MAGE-A3-negative/PRAME-negative	90	66.2	57.6-74.1
	MAGE-A3-negative/PRAME-positive	14	10.3	5.7-16.7
	MAGE-A3-positive/PRAME-negative	18	13.2	8.0-20.1
	MAGE-A3-positive/PRAME-positive	14	10.3	5.7-16.7
At least MAGE-A3 or PRAME	MAGE-A3-negative and PRAME-negative	90	66.2	57.6-74.1
	MAGE-A3-positive or PRAME-positive	46	33.8	25.9-42.4

N = Number of patients/samples. n = Number of patients/samples in a given category. % = n/Number of patients/samples with available results × 100.

Table 6. Subgroup analyses of MAGE-A3 or PRAME expression (mRNA) according to age, gender, tumor histology and tumor stage

Variable	Categories	MAGE-A3 expression				PRAME expression			
		N	n	%	95% CI	N	n	%	95% CI
Age	26-44	23	1	4.3	0.1-21.9	24	2	8.3	1.0-27.0
	45-54	34	4	11.8	3.3-27.5	35	7	20.0	8.4-36.9
	55-64	25	6	24.0	9.4-45.1	28	4	14.3	4.0-32.7
	65-74	45	17	37.8	23.8-53.5	45	12	26.7	14.6-41.9
	≥ 75	10	4	40.0	12.2-73.8	10	4	40.0	12.2-73.8
Gender	Female	35	5	14.3	4.8-30.3	39	5	12.8	4.3-27.4
	Male	102	27	26.5	18.2-36.1	103	24	23.3	15.5-32.7
Histology (Lauren)	Intestinal	55	16	29.1	17.6-42.9	55	16	29.1	17.6-42.9
	Diffused	64	7	10.9	4.5-21.2	67	8	11.9	5.3-22.2
	Mixed	18	9	50.0	26.0-74.0	20	5	25.0	8.7-49.1
	Undetermined	0	0	-	-	0	0	-	-
Histology (Ming)	Infiltrative	124	29	23.4	16.3-31.8	128	24	18.8	12.4-26.6
	Expanding	13	3	23.1	5.0-53.8	14	5	35.7	12.8-64.9
Histology (WHO)	Papillary AC	2	0	0.0	0.0-84.2	1	0	0.0	0.0-97.5
	Tubular AC, well diff.	5	1	20.0	0.5-71.6	5	1	20.0	0.5-71.6
	Tubular AC, moderately diff.	48	17	35.4	22.2-50.5	48	16	33.3	20.4-48.4
	Tubular AC, poorly diff.	53	9	17.0	8.1-29.8	56	8	14.3	6.4-26.2
	Mucinous AC	5	1	20.0	0.5-71.6	6	1	16.7	0.4-64.1
	Signet-ring cell carcinoma	23	3	13.0	2.8-33.6	24	1	4.2	0.1-21.1
	Undifferentiated carcinoma	0	0	-	-	0	0	-	-
	Others	1	1	100	2.5-100	2	2	100	15.8-100
Stage	I	26	3	11.5	2.4-30.2	25	5	20.0	6.8-40.7
	II	35	13	37.1	21.5-55.1	36	8	22.2	10.1-39.2
	III	50	8	16.0	7.2-29.1	54	11	20.4	10.6-33.5
	IV	26	8	30.8	14.3-51.8	27	5	18.5	6.3-38.1

N = Number of FFPE specimens in this category giving a valid test result for the antigen concerned. n = Number of specimens in a given category with a valid test result positive for the antigen concerned. AC = Adenocarcinoma. diff. = Differentiated. % = (n/N) × 100. CI = Confidence interval.

discordant with the results for mRNA detection and IHC (nine with mRNA-negative/unmethylation/IHC-negative and five with mRNA-positive/methylation/IHC-positive).

Of the 142 specimens with valid PRAME assay results, 29 (20.4%) were PRAME-positive (**Table 3**). Of the 136 FFPE specimens with valid mRNA expression assay results for both antigens, 14

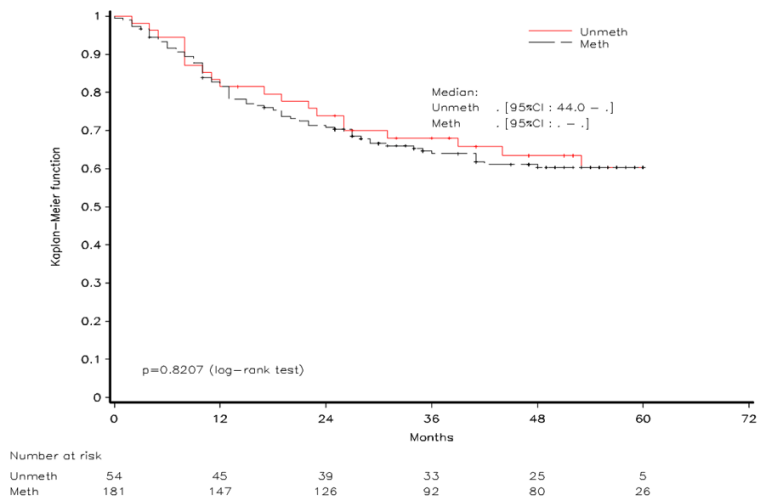

Figure 2. Kaplan-Meier curves for OS by MAGE-A3 methylation status of the specimen. HR for MAGE-A3 unmethylated: 0.94 (95% CI: 0.57-1.56; P = 0.82) (Wald test from Cox regression model).

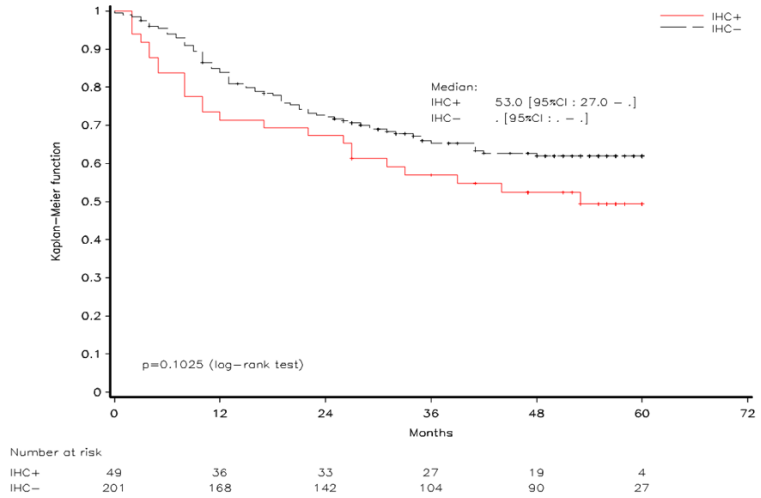

Figure 3. Kaplan-Meier curves for OS by MAGE-A protein expression of the specimen. HR for MAGE-A3-positive: 1.46 (95% CI: 0.92-2.32; P = 0.11) (Wald test from Cox regression model).

(10.3%) expressed both while 46 (33.8%) expressed at least one of them (**Table 5**).

The exploratory analyses of the mRNA expression of the TAAs in subgroups determined by patient and tumor characteristics showed similar results for MAGE-A3 and PRAME (**Table 6**). TAA mRNA expression was independent of tumor stage and there was no obvious association between tumor TAA mRNA expression and any of the other clinico-pathological factors investigated.

Overall survival by MAGE-A3 methylation status, protein expression of MAGE-A and mRNA expression of the antigens

Kaplan-Meier curves for OS according to the tumor's MAGE-A3 methylation status (**Figure 2**) overlap almost completely. The Kaplan-Meier curves for OS according to the specimens' expression of MAGE-A3 protein are more separated (**Figure 3**) but are not statistically significantly different (P = 0.11). Likewise, there were no indications of an association between OS and mRNA expression of the antigens, neither for MAGE-A3 nor for PRAME (**Figures 4** and **5**).

Discussion

This is the first study of PRAME expression in GC, while mRNA MAGE-A3 expression has been reported in several previous studies, with rates of MAGE-A3-positive GC tumors varying between 30% and 41.6% [20-23]. None of these studies reported statistically significant associations between clinico-pathological tumor characteristics and mRNA expression of MAGE-A3, in line with findings in the current study.

Another study investigated MAGE-A expression in GC cell lines as well as primary gastric tumor specimens using both IHC and analysis of *MAGE-A3* promoter methylation status for detection. Of 1097 cancer tissues, 15.8% expressed MAGE-A protein, with no distinction between the subtypes of MAGE-A by the antibody used for the IHC. Of 52 randomly selected tumors expressing MAGE-A protein, 28 (53.8%) displayed unmethylation of the *MAGE-A3* promoter [24]. In the present study, 20 of the 29 (69.0%, recalculated from **Table 4**) tumors

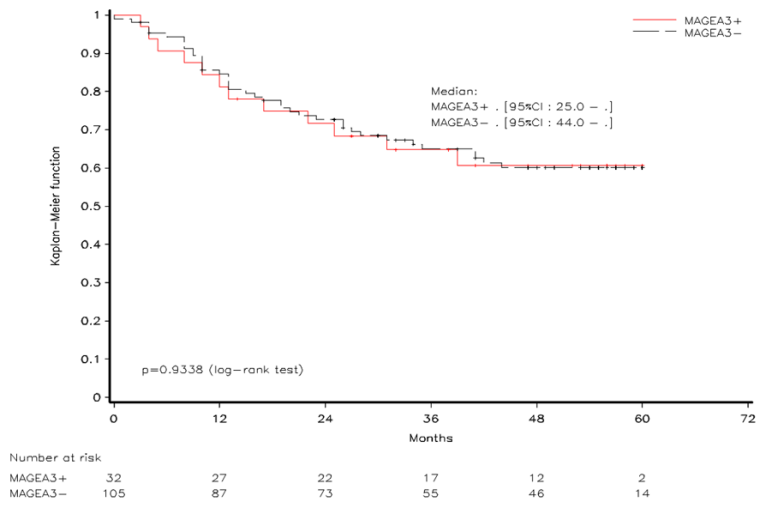

Figure 4. Kaplan-Meier curves for overall survival by mRNA expression of MAGE-A3 (Total cohort) HR for MAGE-A3-positive: 1.03 (95% CI: 0.538-1.963; P = 0.93) (Wald test from Cox regression model).

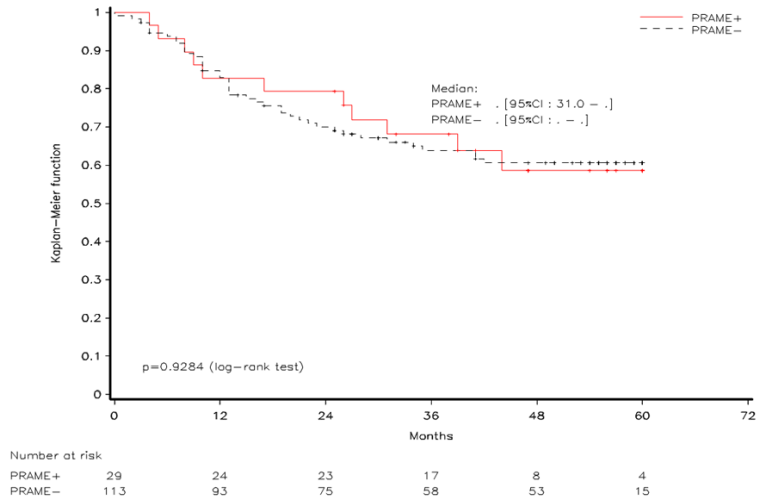

Figure 5. Kaplan-Meier curves for overall survival by mRNA expression of PRAME (Total cohort) HR for PRAME-positive: 0.97 (95% CI: 0.499-1.885; P = 0.93) (Wald test from Cox regression model).

expressing the MAGE-A protein displayed unmethylation of the *MAGE-A3* promoter, whereas this was displayed by 14 of the 107 (13.1%) tumors not expressing the MAGE-A protein. These results indicate that unmethylation of the *MAGE-A3* promoter may be (partly) responsible for the expression of the MAGE-A protein in gastric cancer.

Honda et al. [13] detected *MAGE-A3* promoter demethylation in 66% of 84 GC specimens, tumors displaying *MAGE-A3* promoter demethylation were in a more advanced state and were associated with a higher prevalence of lymph-node metastases than those without demethylation. In the present study, 23.0% of gastric tumors displayed an unmethylated *MAGE-A3* promoter.

We did not observe any relation between OS and mRNA TAA expression of the FFPE specimen, for either MAGE-A3 or PRAME. Similarly, there was no statistically significant association between OS and MAGE-A protein expression (P = 0.11). In contrast, Jung et al. [24] reported a significantly worse prognosis for patients with tumors expressing the MAGE-A proteins (P < 0.001).

Recent studies investigating the molecular basis of GC have reported associations between aberrant DNA methylation of several gene promoters and the prognosis for GC patients, generally suggesting that hypermethylation of the identified gene promoters is an indicator of poor prognosis (reviewed in [15]). Our observations indicate that *MAGE-A3* is not to be added to this list of genes, as the methylation status of the *MAGE-A3* promoter had no association with patient OS (P = 0.82). In contrast, Honda et al. [13] described a trend for worse survival for patients with tumors displaying demethylated *MAGE-A3*, in particular if their tumor also showed demethylated *MAGE-A1* (P = 0.18).

Circulating tumor cell DNA can be detected in serum or blood even at early stages of the disease as shown by Kounalakos et al. [25] for melanoma patients. This DNA may be derived from lysed cells of the primary tumor or from circulating tumor cells releasing their DNA. With

a prior understanding of a potential correlation between gene promoter methylation status and gene expression, methylation testing of circulating DNA could be useful in detecting the expression of specific genes in tumor cells. Such non-invasive detection methods that are not dependent on the availability of tumor tissue could increase the number of patients that can be screened for expression of tumor antigens.

A rough assessment of the possibility of relying on such non-invasive methods for gene expression screening based on the observations of the present study was performed. Due to the lack of MAGE-A3 specific antibodies, IHC cannot distinguish MAGE-A3 from other members of the MAGE-A family. MAGE-A3 expression was thus generally screened for using RT-PCR assays. Here we found that 86.7% (91/105) of the FFPE specimens with no mRNA MAGE-A3 expression showed methylation of the *MAGE-A3* promoter, whereas 64.5% (20/31) of specimens with mRNA MAGE-A3 expression were unmethylated in the *MAGE-A3* promoter. Arguably, the concordance between the results obtained with the two methods may be considered too low to rely on testing of *MAGE-A3* promoter methylation status for patient screening. According to these observations, screening based on methylation testing of the *MAGE-A3* promoter would miss more than a third of the patients whose tumors show mRNA MAGE-A3 expression.

The overall concordance between the three MAGE-A3 assays was relatively low, compared to previous findings (75% vs nearly 100%) reported in certain studies (e.g., [26]). The qRT-PCR assay is specific for MAGE-A3 and the cut-off value was defined as 1% of the GERL control. By contrast, the IHC assay could also detect other members of the *MAGE-A* family (in particular *MAGE-A6*) and the cut-off values for these assays are linked to their detection limits. Additional investigations are needed to assess the impact of *MAGE-A3/A6* expression and to homogenize the cut-off values of the assays.

In summary, the rate of mRNA expression of each of these TAAs in tumors from GC patients was found to be relatively low, with 23.4% expressing MAGE-A3, 20.4% expressing PRAME, and 33.8% expressing at least one of them. Neither MAGE-A3 nor PRAME would serve as a prognostic biomarker for OS of GC patients.

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Disclosure of conflict of interest

Olivier Gruselle and Bart Spiessens are employees of the GSK group of companies. An de Creus, Nicole Kusuma and Aung Myo were employees of the GSK group of companies at the time of the study and manuscript development. Bart Spiessens own stock/stock options in GSK group of companies. Woo Ho Kim, Eun Ji Jung and Kim Hee Sung have no conflict of interest.

Authors' contribution

BS, AM, AdeC and EJJ were involved in study design; AM, AdeC, NK were involved in study supervision; OG, AM, AdeC, NK, WHK, EJJ were involved in data acquisition; HSK, EJJ, NK, AM, WHK and OG were involved in data collection; OG, AM, NK and WHK were involved in data extraction and quality check (AdeC in quality check only and EJJ in data extraction only); OG, WHK and EJJ were involved in provision of study material; WHK and EJJ were involved in laboratory testing. BS (biostatistician), OG, AM, AdeC, WHK and HSK performed or supervised the analyses; BS, OG, AM, AdC, NK, WHK and HSK were involved in results interpretation; OG, AdeC, NK, EJJ were involved in administrative/technical/logistic support; EJJ and AM were involved in centers recruitment; AM and NK were involved in center coordination; AM was involved in acquisition of funding.

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Table S1. Primer sequences for methylation specific PCR

Methylation status	Type	Sequence
Unmethylated	Anti-sense	5'-TGTTAGGATGTGATGTTATTGATTTGT-3'
	Sense	5'-CCTCACCAAACCTAAACCAA-3'
Methylated	Anti-sense	5'-CCATCTGACGTTATTGATTTGC-3'
	Sense	5'-CTCACCGAACCTAAACCGAC-3'

Table S2. Distribution of immunohistochemistry MAGE-A3 results in gastric cancer

IHC score	0-50	51-100	101-150	151-200	201-250	251-300	Total
# of cases	201	19	8	4	3	19	250
% of total	80.4	7.6	3.2	1.6	1.2	7.6	100
IHC result (Score > 50)	Negative	Positive					
	80.4%	19.6%					