UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE

Service d'oto-rhino-laryngologie et chirurgie cervico-faciale, CHUV

Pattern and clinical significance of cancer-testis gene expression in head and neck squamous cell carcinoma

THESE

préparée sous la direction du Docteur Luc Bron, PD
et du Professeur Philippe Monnier

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Originaire de Champéry (VS)

Lausanne

2012

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale
Doctorat en médecine

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Directeur de thèse	*Monsieur le Docteur* ***Luc Bron***
Co-Directeur de thèse	
Expert	*Monsieur le Professeur* ***Serg Leyvraz***
Directrice de l'Ecole doctorale	*Madame le Professeur* ***Stephanie Clarke***

la Commission MD de l'Ecole doctorale autorise l'impression de la thèse de

Monsieur Cyril Cuffel

intitulée

Analyse du taux d'expression des antigènes associés aux tumeurs chez des patients atteints d'un carcinome épidermoïde des voies aérodigestives supérieures

Lausanne, le 17 avril 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Madame le Professeur Stephanie Clarke
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RAPPORT DE SYNTHESE

Profil antigénique et caractéristiques cliniques de l'expression d'antigènes tumoraux spécifiques dans les cancers ORL : prélude à une vaccination.

Les cancer-testis antigènes appartiennent à la famille des antigènes tumoraux spécifiques. Ils ont montré un pouvoir immunogène chez les patients porteurs de différents cancers. En effet, ils stimulent sélectivement les lymphocytes cytotoxiques, et leur expression spécifique dans les tissus tumoraux en fait une cible idéale pour une vaccination antitumorale.
Le but de cette étude est d'identifier l'expression de certains de ces antigènes, d'analyser leur valeur pronostique et de déterminer la meilleure cible antigénique pour permettre une immunothérapie spécifique dans les carcinomes épidermoïdes des voies aérodigestives supérieures.

Le profil et le taux d'expression de 12 cancer-testis antigènes (MAGE-A1, MAGE-A3, MAGE-A4, MAGEA10, MAGE-C2, NY-ESO-1, LAGE-1, SSX-2, SSX-4, BAGE, GAGE-1/2, GAGE-3/4) et de 3 autres antigènes tumoraux spécifiques (PRAME, HERV-K-MEL, NA-17A) ont été évalués par RT-PCR sur 57 échantillons de cancers ORL primaires.
Les paramètres tumoraux et cliniques ont été prospectivement collectés afin de corréler ces données avec le résultat de nos investigations immunobiologiques.

Quatre-vingt-huit pour cent des tumeurs expriment au moins 1 antigène. Une co-expression de 3 gènes ou plus est détectée chez 59% des patients. MAGE-A4 (60%), MAGE-A3 (51%), PRAME (49%) et HERV-K-MEL (42%) sont les gènes le plus fréquemment exprimés. Ils sont totalement absents des muqueuses saines avoisinantes.
La présence de MAGE-A et NY-ESO-1 à la surface des cellules a été vérifiée par immunohistochimie.
Nos analyses statistiques ont permis d'identifier une diminution de la survie liée au cancer chez les patients porteurs d'une tumeur exprimant de multiples cancer-testis antigènes et notamment MAGE-A4 dont l'expression indépendante d'autres éléments cliniques s'associe statistiquement à un taux de survie diminué.

Nos résultats ont permis d'identifier un rôle pronostique de l'expression des gènes associés aux tumeurs dont l'expression est apparemment liée à un phénotype de malignité plus élevé. Cette constatation, corroborée par l'identification parallèle d'un infiltrat lymphocytaire spécifique confirme l'utilité potentielle de certains cancer-testis antigènes comme cible pour une immunothérapie ciblée dans les carcinomes des voies aérodigestives supérieures.

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Pattern and clinical significance of cancer-testis gene expression in head and neck squamous cell carcinoma

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Cancer-testis (CT) antigens comprise families of tumor-associated antigens that are immunogenic in patients with various cancers. Their restricted expression makes them attractive targets for immunotherapy. The aim of this study was to determine the expression of several CT genes and evaluate their prognostic value in head and neck squamous cell carcinoma (HNSCC). The pattern and level of expression of 12 CT genes (*MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, MAGE-C2, NY-ESO-1, LAGE-1, SSX-2, SSX-4, BAGE, GAGE-1/2, GAGE-3/4*) and the tumor-associated antigen encoding genes *PRAME, HERV-K-MEL*, and *NA-17A* were evaluated by RT-PCR in a panel of 57 primary HNSCC. Over 80% of the tumors expressed at least 1 CT gene. Coexpression of three or more genes was detected in 59% of the patients. *MAGE-A4* (60%), *MAGE-A3* (51%), *PRAME* (49%) and *HERV-K-MEL* (42%) were the most frequently expressed genes. Overall, the pattern of expression of CT genes indicated a coordinate regulation; however there was no correlation between expression of *MAGE-A3/A4* and *BORIS*, a gene whose product has been implicated in CT gene activation. The presence of MAGE-A and NY-ESO-1 proteins was verified by immunohistochemistry. Analysis of the correlation between mRNA expression of CT genes with clinico-pathological characteristics and clinical outcome revealed that patients with tumors positive for *MAGE-A4* or multiple CT gene expression had a poorer overall survival. Furthermore, *MAGE-A4* mRNA positivity was prognostic of poor outcome independent of clinical parameters. These findings indicate that expression of CT genes is associated with a more malignant phenotype and suggest their usefulness as prognostic markers in HNSCC.

Key words: head and neck squamous cell carcinoma (HNSCC), cancer-testis antigens, tumor-associated antigens, immunotherapy, gene expression

Additional supporting information may be found in the online version of this article.

Grant sponsors: Conrad N. Hilton Foundation (Ludwig Institute for Cancer Research, Ltd.), Swiss National Science Foundation, Swiss Cancer League

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DOI: 10.1002/ijc.25607

History: Received 19 Apr 2010; Accepted 22 Jun 2010; Online 16 Aug 2010

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The incidence of squamous cell carcinoma of the head and neck (HNSCC) is greater than 40,000 new cases per year in the United States, and ~500,000 cases annually world wide. Despite significant advances in early detection and treatment of this cancer, the survival rate for patients with HNSCC has not changed dramatically over the last decades. The majority of patients present with advanced disease and prognosis is usually poor. Loco-regional recurrences are the most frequent cause of treatment failure even after large resections and adjuvant therapy, both of which carry severe long term morbidity for the patient. Current staging criteria, including TNM staging, grading of differentiation, size and site of the neoplasm, are not sufficient for predicting outcome. It is therefore mandatory to identify new prognostic markers to select high-risk patients who may benefit from more aggressive therapy and search for novel therapeutic approaches to reduce the need for mutilating surgery and morbid adjuvant therapy.

The role of cell mediated immunity against cancer has been established for two decades. Numerous antigens coding for immunogenic sequences have been identified in different tumor types (reviewed in[1]; a peptide database of T-cell-defined tumor antigens can be found at http://www.cancerimmunity.org/

peptidedatabase/Tcellepitopes.htm), leading to the development of new strategies for targeted immunotherapy of cancers. Among various classes of tumor associated antigens identified, cancer-testis (CT) antigens are particularly interesting targets for specific immunotherapy. CT genes comprise a large number of genes or gene families, such as *MAGE, BAGE, GAGE, SSX,* and *NY-ESO-1*, many of which are mapped to chromosome X (X-CT) (reviewed by Simpson et al.[2]). They are expressed by human tumors of different histological types but not by normal somatic tissues, with the exception of male germ cells and placenta. Epigenetic mechanisms are at the base of their restricted expression pattern.[3,4] Among the X-linked CT antigens, the MAGE-A family, encoded by 12 highly homologous genes, and NY-ESO-1 family, consisting of NY-ESO-1 and LAGE-1, are the best studied antigens and have been shown to generate both spontaneous and vaccination-induced T-cell mediated responses. In addition to X-CT gene products, tumor associated proteins like PRAME (preferentially expressed antigen on melanoma), HERV-K-MEL, a product related to the env-gene of the endogenous human retrovirus K (HERV-K), and NA-17A, the product of an alternatively spliced N-acetylglucosaminyltransferase V mRNA, also contain epitopes recognized by cytolytic T cells on tumor cells.[5–8]

A small number of studies have reported a relatively frequent expression of selected CT genes in HNSCC.[9–13] However, the small patient number or short follow up time did not allow evaluation of their impact of their expression on survival. In this study, we investigated the correlation of expression of 15 tumor associated antigen-encoding genes, including 12 CT genes, in a cohort of HNSCC patients with known follow up. The genes were chosen based on the capability of their products to generate epitopes recognized by CD8 and/or CD4 T cells. In addition, we sought to determine the impact of individual and combined CT gene expression on clinical outcome. Our findings show frequent coexpression of CT genes in HNSCC of different primary site. Expression of *MAGE-A4* and coexpression of several CT genes was associated with poor overall survival. In addition, Cox regression analysis indicated that *MAGE-A4* was an independent marker of worse outcome in HNSCC.

Material and Methods

Patients

Fifty-seven tumor samples from 52 patients treated for primary HNSCC were prospectively collected at the Department of Otolaryngology and Head and Neck surgery of Lausanne University Hospital (CHUV), Switzerland. Tumor specimens were collected during initial pretherapeutic endoscopy and immediately snap-frozen. For 11 patients, samples of nearby normal mucosa were collected at the same time. Presence of tumor cells was confirmed in each biopsy sample by a standard haematoxylin-eosin and keratin staining on formalin fixed/paraffin embedded material. Tumor site, histological grade and clinical stage (according to the 2002 IUCC staging system) were prospectively recorded. Following diagnosis, patients were treated by a combination of surgery and chemoradiotherapy, when required, according to standard international treatment guidelines. This study was conducted after approval by the Research Ethics Committee of Lausanne University and conformed to the 1975 Declaration of Helsinki. All patients provided informed consent.

RNA extraction and RT/PCR

Total RNA was isolated from frozen tissue samples using a Nucleospin RNA II kit (Macherey-Nagel) and a Fast-Prep device (Bio 101 Savant; Savant Instruments). RNA (2 μg) was primed with an oligo $(dT)_{18}$ oligonucleotide and reverse-transcribed with MMLV-RT (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Aliquots of cDNA corresponding to 100 ng input RNA were used for different PCR reactions using a Qiagen Hotstar Taq polymerase Master Kit, except for *LAGE-1, NY-ESO-1* and actin PCR, performed with Qiagen Taq polymerase (Qiagen GmbH, Hilden, Germany). cDNA quality was tested by amplification of β-actin in a 21-cycle PCR reaction. The primers, annealing temperature and number of cycles performed are described in Supporting Information Table S1. The number of cycles chosen for BORIS amplification allowed the detection of a 1:500 dilution of a testis sample (0.2%), but not background transcription (not shown).[14] For amplification, after an initial denaturation for 15 min (Hotstar Taq polymerase) or 5 min (for other PCR) at 94°C, PCR cycles were performed as follows: denaturation at 94°C for 1 min, 30 sec at the indicated annealing temperature, 1 min at 72°C. A final elongation step was performed at 72°C for 10 minutes. Aliquots of each reaction were size-fractionated on a 1.5% agarose gel and visualized by ethidium bromide staining. Sequence identity of representative PCR products was confirmed by automated sequencing (Microsynth, Switzerland). RNA from SK-Mel-37 cells (a gift from Y. T. Chen, New York), a melanoma cell line expressing high levels of a broad range of CT genes, and NA8-MEL (a gift from F. Jotereau, Nantes, France) was used as positive and negative control, respectively. Quantitative assessment was performed as previously described, using 1:10 dilutions of SK-Mel-37 RNA as reference.[15] A threshold level of CT gene expression by tumor cells appears necessary for antigen presentation and recognition by T cells.[15,16] Taking this in consideration, the threshold for sample positivity was set at 1% the expression level of the reference cell line. A tumor "CT score" was calculated by integrating scores of individual CT genes (from 0 to 4+) obtained from semiquantitative analyses. Median X-CT antigen score was 6 (mean 8.6). Tumors with a "CT score" ≥7 were defined as "high CT Score".

Immunohistochemistry (IHC)

Four-micrometer thick serial sections of formalin-fixed, paraffin-embedded tissue samples were obtained. Expression of proteins of the MAGE-A family was assessed using the anti–

pan-MAGE-A antibodies 57B (a gift from G. Spagnoli, Basel, Switzerland) and 6C1.[17] Clone 57B, originally raised against MAGE-A3, cross-reacts with several of the homologous MAGE-A proteins, and has been reported to primarily detect MAGE-A4 in melanoma.[17–19] NY-ESO-1 and LAGE-1 were detected with the monoclonal antibody D8.38 (a gift from G. Spagnoli).[18,20] Antigen retrieval was performed with microwave treatment in 0.1 M sodium citrate, pH 6.0. Detection was performed with the DAKO EnVision™+ system and DAB as chromogen (DAKO). Nonimmune mouse IgG was used as negative control and sections of testis were used as a positive control. Slides were then analyzed by one of the authors (W.S.) and by a second independent pathologist as control.

Statistical analyses

Statistical analysis was performed with STATA 10 software. The chi-square test and Fisher's exact test were used to evaluate the associations between CT antigen expression and clinico-pathological features, as appropriate. The Kaplan-Meier method was used to estimate overall survival of patients, and differences between groups were compared using the log-rank test. Multivariate analyses were performed using the Cox proportional hazard model to determine the independent contribution of each variable. Covariates with $p < 0.08$ by univariate analysis were entered in the multivariate analysis. Probability values ≤0.05 were regarded as significant. In case of multiple tumors, a patient was considered CT antigen positive if at least one of the tumors analyzed tested positive.

Results

Over a period of 19 months, 57 primary HNSCC and 11 samples of normal mucosa were collected from 52 untreated patients (39 male and 13 female). Median age was 61 years (range 42–84 years). Table 1 summarizes the clinical and histological characteristics of patients and tumors. Most common localizations of the primary tumors were oral cavity and oropharynx, followed by hypopharynx and larynx. Half of the tumors were moderately differentiated. According to TNM classification, 11 patients had early stage (i.e. I and II) and 41 advanced (i.e. III and IV) cancers.

Semiquantitative RT/PCR was performed to analyze tumor expression of the cancer testis genes *MAGE-A1/3/4/10, MAGE-C2, LAGE-1 and NY-ESO-1, SSX-2* and *4, BAGE, GAGE-1/2* and *3/4*. In addition, we studied the expression of the genes coding for the tumor-associated antigens HERV-K-MEL, PRAME, and NA17. Tumor samples were considered as positive when they expressed a given gene at the level of at least 1% that of a reference cell line (see "Material and Methods" section). Representative PCR analyses are shown in Supporting Information Figure S1. Frequency of expression of various genes in tumors is summarized in Figure 1*a*. *MAGE-A4, MAGE-A3, PRAME,* and *HERV-K-MEL* were the most frequently expressed genes and were detected in over 40% of the samples. *MAGE-A3* (51%) and *MAGE-A4* (60%) were coexpressed in 35% of tumors, and 75% of tumors expressed either gene. *MAGE-A1, MAGE-A10, LAGE-1, SSX-4,* and *GAGE* were expressed in 16–30% of tumors, while other genes had lower expression frequency. *BAGE* and *NA-17A* were detected only in 1 of the 57 tumors analyzed. Eighty-nine percent of the tumors showed expression of at least one gene in our panel and 81% expressed at least one X-CT gene. Tumors expressed up to 11 of the 12 X-CT genes tested. Frequency of coexpression of CT genes on a patient basis is shown in Figure 1*b*. Patients had tumors expressing an average of 2.9 X-CT and 3.8 tumor associated antigen-encoding genes. None of the genes tested was expressed in 11 normal mucosa biopsies collected as controls (not shown).

Table 1. Clinico-pathological characteristics of patients and tumors studied

Variable	*n*	(%)
Patients ($n = 52$)		
Sex		
F	13	(25)
M	39	(75)
Tumors ($n = 57$)		
Localization		
Oral cavity	21	(37)
Oropharynx	20	(35)
Hypopharynx	12	(21)
Larynx	4	(7)
TNM stage		
T1	13	(23)
T2	20	(35)
T3	12	(21)
T4	12	(21)
N0	21	(37)
N1	11	(19)
N2	23	(40)
N3	2	(4)
Clinical stage		
I	9	(15)
II	5	(9)
III	14	(25)
IV	29	(51)
Differentiation grade		
1 (High)	11	(19)
2 (Moderate)	29	(51)
3 (Low)	17	(30)

CT gene expression is independent of BORIS

BORIS (Brother of the Regulator of Imprinted Sites), a testis specific paralog of the DNA binding protein CTCF, has been

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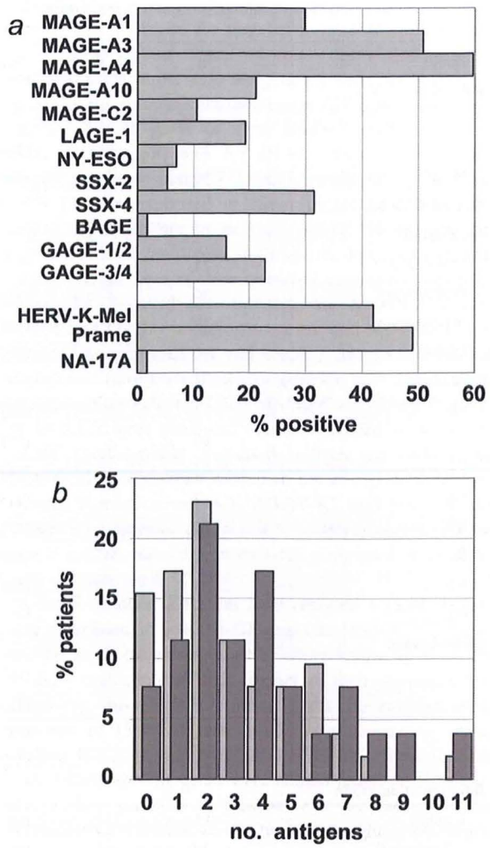

Figure 1. Expression of CT and other genes in HNSCC. (*a*) Frequency of expression of the indicated genes, determined by RT-PCR, calculated on a tumor basis ($n = 57$). (*b*) frequency of coexpression of the indicated numbers of genes (in any combination) in patients. Light gray, X-CT genes only; dark gray, all genes.

proposed as a mediator of the induction/derepression of other CT genes in lung cancer cells and dermal fibroblasts.[21,22] We therefore analyzed the expression of *BORIS*, itself considered a non-X linked CT gene because of its expression pattern, in the HNSCC tumors. *BORIS* expression was detected in 17% of tumors. To visualize the correlation between expression of various CT genes and *BORIS* in individual tumors, we grouped the latter into two groups according to *BORIS* expression (Fig. 2). Within these groups, tumors were further ordered according to the number of CT genes expressed. A correlation was observed between expression of the more frequently expressed *MAGE-A3* and *A4* and that of various X-linked CT genes ($p < 0.05$), but not *BORIS* ($p > 0.2$). Expression of *BORIS* however significantly correlated with expression of multiple CT genes (≥ 4) and *MAGE-A10* ($p < 0.05$). Figure 2 also shows that tumors expressing multiple CT genes generally displayed also quantitatively high expression levels. *PRAME*, but not *HERV-K-MEL*, was more frequently expressed in tumors expressing multiple CT antigens ($p < 0.05$ and $p = 0.81$, respectively).

Expression of MAGE-A and NY-ESO-1 proteins

To verify that *MAGE-A* genes are also expressed at the protein level, RT-PCR positive tumors were analyzed by IHC using two anti-panMAGE antibodies (clones 57B and 6C1). Eighty-eight percent (38/43) of *MAGE-A3* and/or *A4* RT-PCR positive tumors showed a positive IHC staining with antibody 57B. Although heterogeneous, ~¾ of the tumors showed positive staining in over 80% of the cells (not shown). At the cellular level, the staining was both cytoplasmic and nuclear, but a nuclear localization was slightly predominant (not shown). Twenty-one of the 43 tumors tested were also positive with clone 6C1. Using an antibody that recognizes both NY-ESO-1 and LAGE-1, NY-ESO-1 protein family was detected in 7 of the 12 *NY-ESO-1* and/or *LAGE-1* RT-PCR-positive samples. This staining was predominantly cytoplasmic. In five cases the staining was extensive, with over 75% positive tumor cells. Examples of immunostainings are shown in Figure 3.

Correlation between expression of CT genes, PRAME, and HERV-K-MEL and clinico-pathological parameters

There was no statistically significant correlation between CT gene expression (tested as individual genes, gene combinations, or number of coexpressed genes) and gender, clinical stage, tumor localization, differentiation grade, or tumor recurrence (not shown). One exception was the *NY-ESO-1* family, which was expressed in moderately and poorly, but not well differentiated tumors ($p = 0.0345$) and more frequently in hypopharynx/larynx compared to oral cavity/oropharynx ($p = 0.014$). In addition, *MAGE-A4* tended to be preferentially expressed in advanced stage tumors ($p = 0.0782$). No correlation was found between *PRAME* or *HERV-K-MEL* mRNA positivity and any clinico-pathological parameter.

Tumor CT gene expression and survival

The patients in this study had a median follow-up of 27.5 months (range 1–53, mean 26.4), and overall survival at 4 years was 52%. Univariate analysis showed that overall survival significantly correlated with clinical stage, nodal status, and tumor stage, but not tumor localization, differentiation grade, or sex of the patient (Supporting Information Table S2). We investigated the correlation of the mRNA expression of various genes, individually or as combinations, with overall survival. Patients with *MAGE-A4* mRNA positive tumors had a significantly poorer outcome compared to those with *MAGE-A4* mRNA negative tumors ($p = 0.0493$, Fig. 4). No significant correlation was observed between survival and

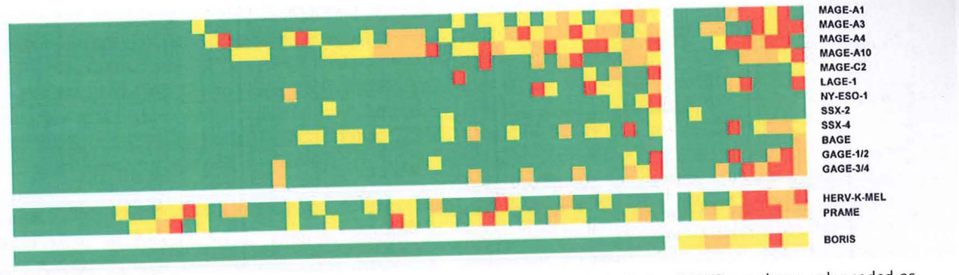

Figure 2. Correlation between expression of *BORIS* and other CT genes. Results from semiquantitative RT-PCR are shown color-coded as follows: green, negative; yellow to red, low to high levels, respectively, determined as indicated in "Material and Methods" section relative to the reference melanoma cell line SK-Mel-37. Tumor samples are clustered into two groups according to *BORIS* expression (negative, left; positive, right).

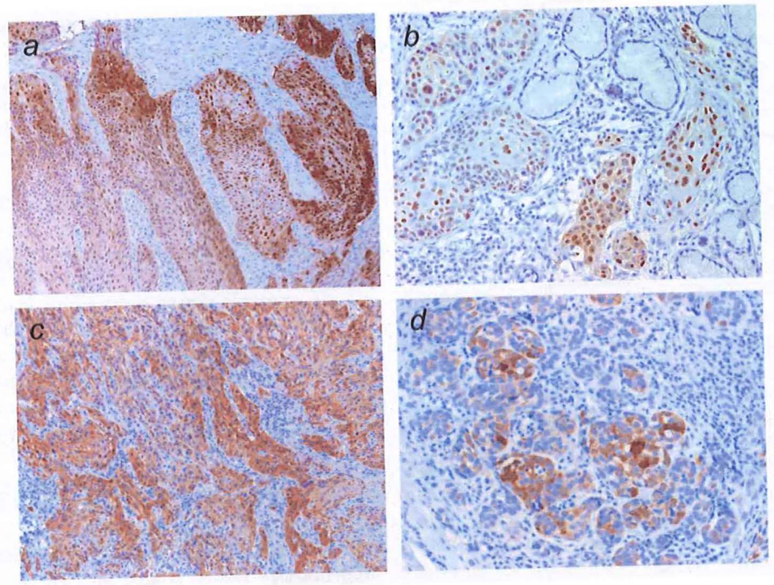

Figure 3. Immunohistochemical detection of MAGE-A and NY-ESO-1 family proteins in HNSCC. (*a*) representative staining with anti-MAGE-A antibody 57B. The extensive, heterogeneous staining is both cytoplasmic and nuclear. (*b*), heterogeneous staining, mainly nuclear, with anti-MAGE-A antibody 6C1. Tumors shown in A and B were *MAGE-A1/3/4/10* positive by RT-PCR. (*c*) and (*d*), staining with anti-NY-ESO-1/LAGE-1 antibody D8.38. Tumor in C shows diffuse staining; D, area with focal staining in a mostly negative tumor. Both tumors were *LAGE-1* positive by RT-PCR.

positivity for expression of other individual CT genes (Table 2 and Fig. 4*b*, showing survival curve relative to *MAGE-A3* as example). Interestingly, curves of patients with tumors mRNA-positive for *NY-ESO-1* family genes leaned towards a poorer outcome ($p = 0.161$). Expression of *HERV-K-MEL* and *PRAME* (42 and 49% positive patients, respectively) had no impact on survival. We next asked whether expression of multiple X-CT genes (in any combination) or quantitative high levels of expression (assessed by calculating a "CT score", as described in "Material and Methods" section) had an impact on outcome. Coexpression of four or more X-CT genes ($n = 17$, 33% patients) did indeed correlate with a significantly poorer survival ($p = 0.045$, Fig. 4*c*). In addition, a high CT score ($n = 24$, 46%) was associated with 18% difference in overall survival at 40 months ($p = 0.117$). Interestingly, patients with tumors negative for all tested X-CT genes

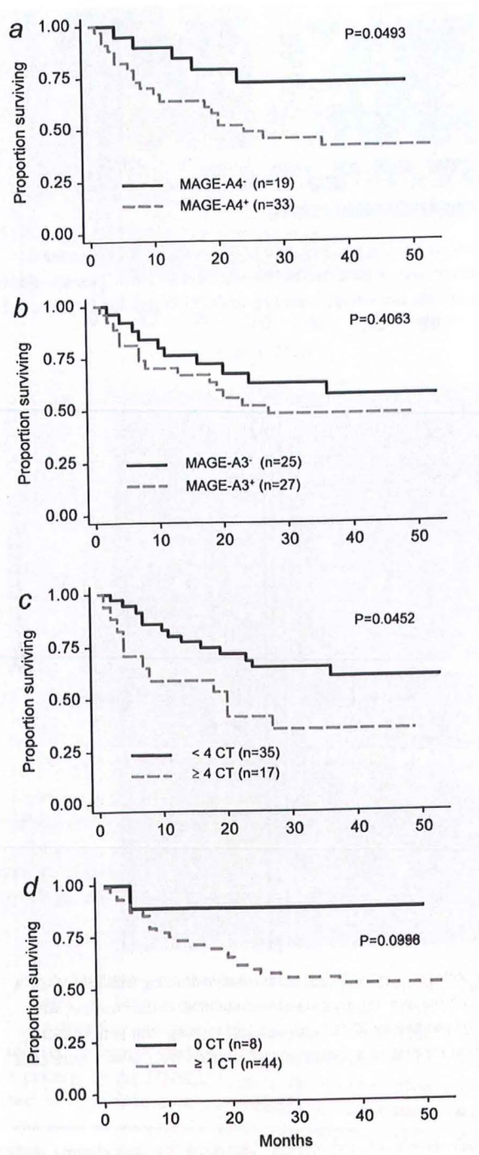

Figure 4. Correlation between X-CT gene expression and overall survival. Kaplan-Meier survival estimates of patients were performed according to RNA expression of (*a*), *MAGE-A4*; (*b*), *MAGE-A3*; (*c*), multiple X-CT genes (≥4). (*d*), survival curves for patients with tumors positive or negative for 1 or more X-CT genes.

Table 2. Correlation of expression of CT genes with overall survival

		Survival estimates		
Variable		4 years (%)	Median (months)	*p*-value[1]
MAGE-A1	–	55.4	–[2]	0.380
	+	43.8	26.9	
MAGE-A3	–	56.8	–	0.406
	+	48.2	26.9	
MAGE-A4	–	72.6	–	**0.049**
	+	41.6	24.0	
MAGE-A10	–	55.3	–	0.380
	+	41.7	26.9	
MAGE-C2	–	52.3	–	0.866
	+	50.0	–	
NY-ESO-1	–	54.4	–	0.242
	+	25.0	26.9	
LAGE-1	–	53.6	–	0.378
	+	44.4	20.3	
SSX-2	–	51.1	–	0.523
	+	66.7	–	
SSX-4	–	58.2	–	0.318
	+	41.2	23.4	
GAGE-1/2	–	56.9	–	0.180
	+	22.2	20.0	
GAGE-3/4	–	54.2	–	0.537
	+	46.2	26.9	
PRAME	–	56.7	–	0.767
	+	48.5	36.2	
HERV-K-MEL	–	54.3	–	0.464
	+	48.5	36.2	
≥1 CT	No	87.5	–	0.100
	Yes	46.7	26.9	
≥4 CT	No	60.4	–	**0.045**
	Yes	35.3	20.0	
High CT score[3]	No	60.7	–	0.117
	Yes	41.7	23.4	
MAGE-A3 and/or A4⁺	No	80.8	–	0.083
	Yes	45.3	26.9	
NY-ESO-1 and/or LAGE-1⁺	No	56.4	–	0.161
	Yes	36.4	20.3	

[1]Log-rank. *p*-values ≤0.05 are indicated in bold; [2]Fifty % survival not yet reached; [3]CT gene score (as defined in "Material and Methods" section) ≥7.

($n = 8$) had a remarkably good survival, though the log-rank test only indicated a trend ($p = 0.100$, Fig. 4*d*). We next performed a multivariate Cox regression analysis to assess whether CT gene expression was prognostic of poor survival independent of clinico-pathological parameters, including clinical and TNM stage. This analysis showed that RT/PCR positivity for *MAGE-A4* was an independent prognostic indicators in HNSCC (hazard ratio, 2.949; 95% confidence interval 1.085–8.020; $p = 0.034$). The association of coexpression of four or more X-CT genes with a higher risk of death did not persist in the multivariate analysis (hazard ratio, 2.057; 95% confidence interval 0.919–4.602; $p = 0.079$).

Discussion

The identification of tumor specific antigens capable of inducing a specific immune response has raised interest for novel antitumor therapies in various tumor types. CT antigens are amongst the most promising targets for immunotherapy. Our finding that CT gene expression confers a higher risk of poor outcome in HNSCC further strengthens the choice of their products as therapeutic targets in this type of cancer.

We found expression of one or more of the 12 X-CT genes tested in 90% and three or more in over 40% of HNSCC tumor samples. Overall, ~60% of the patients had tumors expressing at least three of the whole panel of genes investigated in this study. The X-linked CT genes *MAGE-A4, MAGE-A3*, together with *PRAME* and *HERV-K-MEL*, were the most frequently expressed. Of the genes studied, only four were detected at a frequency below 10%. *NY-ESO-1*, encoding one of the most immunogenic CT antigens, was among the latter. However, together with its homolog *LAGE-1*, which codes for identical HLA-A2 restricted epitopes, it was expressed in approximately a quarter of tumors. The frequencies of expression of individual genes in HNSCC were similar to those reported in previous studies.[10–12] Expression of MAGE-A and NY-ESO-1 protein families was confirmed by IHC staining in the majority of RT-PCR positive tumors. Heterogeneous and scattered staining pattern for CT proteins has been frequently observed in tumors and could have implications for targeted immunotherapy. In this study, most tumors showed an extensive staining for both MAGE-A and NY-ESO-1 family proteins, indicating that CTA positive cells are not rare. Kienstra et al. found that less than half of the *MAGE-A1* and *A3* RT-PCR positive tumors were also positive by immunohistochemistry, however, the antibodies used were not specified.[12] Concordant with our results, a recent immunohistochemical study has reported the expression of MAGE-A (detected by clone 57B) and NY-ESO-1 family proteins in 70% and 30% of pharyngeal tumors, respectively.[23] These results are consistent with the relatively high CTA expression levels detected by RT-PCR, similar to those observed in melanoma. Altogether our study, to our knowledge the larger, in terms of number of genes coding for immunogenic products and patients, confirms that a large proportion of patients with HNSCC of different site could receive specific immunotherapy targeting multiple antigens.

Three quarters of the tumors expressed *MAGE-A3* and/or *A4*, and expression of other X-linked CT genes was significantly correlated with these genes. A similarly coordinated expression of CT genes has been reported for nonsmall cell lung cancer.[24] Interestingly, coexpression of multiple CT genes also associated with high mRNA levels (Fig. 2), suggesting that high transcriptional activity is associated with the extent of DNA demethylation. The exact mechanism underlying this observation is not clear at present. Expression of CT genes, especially those encoded in the X-chromosome, is strictly confined to germline and placenta. Methylation of CpG islands in CT gene promoters is the primary silencing mechanism in healthy somatic tissues. Activation of CT gene expression in tumors is thought to result from demethylation of these sequences.[3,4] BORIS, the product of a CT gene located on chromosome 20, has been recently suggested by Vatolin et al. as an essential mediator of CT gene derepression, particularly *MAGE-A1*.[22] In addition, BORIS has been implicated in *NY-ESO-1* expression in lung cancer cells.[21] In HNSSC, the frequency of *BORIS* expression was only 17% and contrasted with the frequent expression of X-linked CT genes. As a comparison, a parallel *BORIS* analysis applied to metastatic melanoma samples yielded a frequency of ~50% (Rimoldi D., unpublished observation). Expression of CT genes, particularly *MAGE-A3* and *4*, but also *MAGE-A1* and *NY-ESO-1*, was observed in the absence of *BORIS*. Conversely, *BORIS* positive tumors did not necessarily express high levels of other CT genes. Thus, *BORIS* expression does not seem to be sufficient or necessary for the expression of other CT genes in HNSCC, although we cannot rule out that a transient expression of *BORIS* may precede their activation. Similar to our results, a lack of association between *BORIS* expression and *MAGE-A1* activation has been reported in cutaneous melanoma,[14] and thus the "gate keeper" for the expression of CT genes in these cancers still remains to be identified.

Overall, we did not find a correlation between expression of CT genes, either individually or in combination, and clinical parameters (including TNM staging) or characteristics of the primary tumor. This is in agreement with results of most previous studies on HNSCC.[9,12,23,25] A positive correlation between expression of two or more of a panel of nine genes, including *MAGE-A* and *NY-ESO-1* families, and higher tumor stage was reported by Figueiredo et al. in a study on 33 HNSCC patients.[11] There was no correlation in our cohort between CT gene expression and tumor or clinical stage, except for a marginal association of *MAGE-A4* with advanced TNM stage. It should be noted however that in our, as well as previous HNSCC studies, late stage patients were a majority, thus firm conclusions on correlations with stage await results from larger studies. Eura et al ($n = 83$) found that the expression levels of individual *MAGE-A* genes varied with tumor localization and degree of differentiation, though no common pattern could be drawn.[10] The only significant correlation of CT gene expression and tumor grade in our cohort was the lack of *NY-ESO-1* and *LAGE-1* in well differentiated tumors. This is at odds with a recent report that shows similar expression of these proteins in grade 1 and 2 pharyngeal tumors, but lower frequency in grade 3 ones.[23]

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This discrepancy may be due to the different localizations of tumors studied (grade 1 tumors in our study were mostly from the oral cavity). A lower frequency of *NY-ESO-1* expression in low histological grade tumors has also been observed in urinary tract cancer,[26] while the opposite has been reported for esophageal cancer.[27] Altogether, the correlation between differentiation grade and expression of *NY-ESO-1* family genes remains unclear.

A major finding of this study is the correlation between CT gene expression (assessed by RT/PCR) and clinical outcome. Both *MAGE-A4* expression and coexpression of multiple X-linked CT genes (at least 4 of the 12 analyzed) significantly correlated with poor survival. More importantly, the former emerged as a potential new prognostic indicator. These findings further strengthen the choice of CT antigens as immunotherapy targets in this type of cancer. A previous report on a smaller group of patients found no correlation between mRNA expression of CT genes, including the *MAGE-A* family, and tumor recurrence or metastasis, though clinical parameters and follow-up were not specified.[11] Our study is the first one to evaluate the effect of individual CT genes on survival of HNSCC patients. Association of expression of MAGE-A proteins with a poorer disease-free survival in patients with pharyngeal squamous cell carcinoma, has been shown in a recent immunohistochemical study, although the difference was not statistically significant.[23]

While coexpression of multiple X-linked CT genes correlated with poor overall survival, it appears that the different genes may not equally contribute to outcome. This was evident for the *MAGE-A* family. *MAGE-A4* and *A3* were expressed at similar frequencies, yet only expression of the former had an impact on the patients' survival (Figs. 4*a* and 4*b*). This is similar to results by Shigematsu et al.[28] showing that expression of *MAGE-A4,* but not *MAGE-A3* or *NY-ESO-1*, as determined by RT/PCR, was predictive of poor survival in nonsmall cell lung cancer patients.[29,30] Gure et al. identified *MAGE-A3* and *NY-ESO-1*, but not *MAGE-A4*, as independent markers of poor prognosis for adenocarcinoma of the lung.[24] These discrepancies may be related to the different patient populations. Expression of MAGE-A4 protein, as detected by antibody 57B, has been reported to be an independent marker of poor survival for serous ovarian cancer patients[31] and to associate with progression of noninvasive bladder cancer to muscle invasive tumors.[32] Caution however must be applied in interpreting positive 57B staining as MAGE-A4 positivity, as the antibody can also recognize other MAGE-A proteins.[17,18] Expression of other CT genes has also been generally associated with poor prognosis,[24,33,34] with only few studies revealing a positive effect.[27,35] None of the CT genes tested in this study was associated with better survival. Noteworthy, the small group of patients whose tumors tested negative for RNA of all 12 X-linked CT genes tested appeared to have a particularly good outcome, though this did not reach statistical significance. The severe prognosis of patients with tumors expressing *MAGE-A4* or multiple CT genes suggests that these patients may require more intense follow-up and aggressive therapy.

At present, how the expression of *MAGE-A4* and other CT genes translates into poor clinical outcome can only be a subject of speculation. Products of CT genes may confer a highly malignant phenotype to the tumor or resistance to chemo/radiotherapy. Alternatively, expression of these genes may be coinduced with that of others in a subset of tumors with a more aggressive behavior. Although the function of CT proteins remains poorly understood, different MAGE-A proteins have been reported to associate with p53 containing complexes and inhibit DNA damage-induced apoptosis, lending support to the former hypothesis.[36–38] However, other reports indicated that MAGE-A4 may actually promote apoptosis.[39,40] Further studies are clearly needed to establish the direct contribution, if any, of MAGE-A4 and other CT gene products to a more malignant phenotype in HNSCC.

In addition to X-linked CT genes, *PRAME* and *HERV-K-MEL*, both coding for *in vivo* generated CTL epitopes,[5,7,8,41,42] are interesting candidates for specific immunotherapy of HNSCC. Because of its restricted expression and its epigenetic regulation, *PRAME*, a gene located on chromosome 22 (reviewed in[43,44]), is sometimes considered as a non-X-linked CT gene (e.g. in CTdatabase, at http://www.cta.lncc.br/). In this regard, it is interesting that *PRAME* expression correlated with that of multiple X-linked CT genes in our tumor series. The frequency of expression of *PRAME* in this study (49%) confirms frequencies reported in smaller studies (39 and 42%).[8,11] While *PRAME* expression has been reported as a predictor of both poorer and better patient outcome (e.g. in breast cancer and promyelocytic leukemia, respectively),[34,45,46] it had no influence on survival in our cohort of HNSCC patients. We are the first to report the extent of *HERV-K-MEL* expression in a large series of HNSCC tumors. *HERV-K-MEL* is a spliced env sequence from a *HERV-K* pseudogene expressed in over 80% of benign and malignant melanocytic lesion.[5] Normal tissue expression has been reported to be confined to testis and, at a low level, normal skin. Spliced env and rec mRNAs from *HERV-K* genes have been detected in other cancers.[47] While promoter demethylation has been implicated in some tumors in the activation of related *HERV-K* sequences,[48] the mechanism of activation of *HERV-K-MEL* is not known. In HNSCC tumors, expression of *HERV-K-MEL* was independent of CT gene expression, suggesting that different mechanisms are involved in the activation of CT genes and endogenous viral sequences. Similar conclusions have been drawn in melanoma. As *HERV-K* products can elicit immune responses, they may have biological implications in HNSCC.

In conclusion, this study showed a coordinated activation of different CT genes in HNSCC and established an association between expression of *MAGE-A4* and multiple X-CT antigens with poor survival. The value of *MAGE-A4* as an independent prognostic marker should be confirmed in a larger prospective study.

Acknowledgements

The authors thank Dr. G. Spagnoli for the gift of antibodies and Dr. M. Faouzi for help with statistical analyses. D.R. was supported by a grant from the Conrad N. Hilton Foundation to the Ludwig Institute for Cancer Research, Ltd. P.R. and L.B. are supported by grants from the Swiss National Science Foundation and the Swiss Cancer League, respectively.

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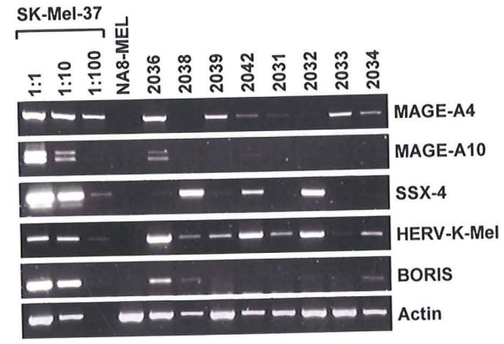

Supplementary Figure 1. Representative RT-PCR results. Semiquantitative RT-PCR was performed as described in Material and Methods. Tumor samples are indicated by numbers. The first three lanes show positive controls prepared with serially diluted RNA from the high CT gene expressing SK-Mel-37 cells, used as reference sample. NA8-MEL cells are CT antigen negative melanoma cells.

Supplementary Table S1. PCR primers and conditions.

Gene	Primers	Primer Sequence	Annealing	Cycles	*Ref.*
MAGE-A1	CHO14	5'-CGGCCGAAGGAACCTGACCCAG-3'	70°C	30	*Patard J.J. et al.*
	CHO12	5'-GCTGGAACCCTCACTGGGTTGCC-3'			*Int J Cancer 1995, 64: 60*
MAGE-A3	AB-1197	5'-TGGAGGACCAGAGGCCCCC-3'	68°C	30	*ibid.*
	BLE-5	5'-GGAACGATTATCAGGAGGCCTGC-3'			
MAGE-A4	MAGE-4s	5'-GAGCAGACAGGCCAACCG-3'	68°C	30	*ibid.*
	MAGE-4as	5'-AAGGACTCTGCGTCAGGC-3'			
MAGE-A10	MAGE10/3	5'-GGAACCCCTCTTTTCTACAGAC-3'	55°C	30	*Rimoldi D. et al.*
	MAGE10/4	5'-TCCTCTGGGGTGCTTGGTATTA-3'			*IntJCancer1999, 82:901*
NY-ESO-1	ESO-1A	5'-ATGGATGCTGCAGATGCGG-3'	60°C	35	*Rimoldi D. et al.*
	ESO-1B	5'-GGCTTAGCGCCTCTGCCCTG-3'			*J. Immunol. 2000, 165: 7253*
LAGE-1	BLE-71	5'-CTGGCCACTCGTGCTGGGA-3'	62°C	40	*Lethé B. et al.*
	BLE-72	5'-GCAGGATGGAAGGTGCCC-3'			*IntJCancer 1998, 76:903*
SSX-2	SSX-2A	5'-GTGCTCAAATACCAGAGAAGATC-3'	62°C	35	*Türeci O. et al.*
	SSX-2B	5'-TTTTGGGTCCAGATCTCTCGTG-3'			*IntJCancer 1998, 77: 19*
SSX-4	SSX-4A	5'-AAATCGTCTATGTGTATATGAAGCT-3'	60°C	35	*ibid.*
	SSX-4B	5'-GGGTCGCTGATCTCTTCATAAAC-3'			
MAGE-C2	SL102	5'-AGGCGCGAATCAAGTTAG-3'	56°C	32	*Wenbin M.A. et al.*
	SL103	5'-CTCCTCTGCTGTGCTGAC-3'			*Int J Cancer 2004, 109: 698*
BAGE	BAGE-1	5'-TGGCTCGTCT-CACTCTGG-3'	60°C	30	*van Baren et al.*
	BAGE-2	5'-CCTCCTATTGCTCCTGTTG-3'			*Blood 1999, 94: 1156*
GAGE-1/2	GAGE-1S	5'-GACCAAGACGCTACGTAG-3'	56°C	30	*ibid.*
	GAGE-1AS	5'-CCATCAGGACCATCTTCA-3'			
GAGE-3/6	GAGE-3S	5'-GACCAAGGCGCTATGTAC-3'	56°C	30	*ibid.*
	GAGE-1AS	5'-CCATCAGGACCATCTTCA-3'			
BORIS	Boris-a	5'-CAGGCCCTACAAGTGTAACGACTGCAA-3'	62°C	35	*Hong et al.*
	Boris-b	5'-GCATTCGTAAGGCTTCTCACCTGAGTG-3'			*Cancer Res 2005*
PRAME	PRAME-1	5'-CTGTACTCATTTCCAGAGCCAGA-3'	62°C	30	*van Baren N. et al.*
	PRAME-2	5'-TATTGAGAGGGTTTCCAAGGGGTT-3'			*Blood 1999, 94:1156-64*
HERV-K-MEL	OPC 646	5'-TGCAGAGGATATAAGGAGAT-3'	60°C	40	*Schiavetti F. et al.*
	OPC 600	5'-GGATCAAACTGCAAGGCA-3'			*Cancer Res 2002, 62:5510*
NA-17A	NA17-1	5'-GATGTGTTCATACGCTGTGTGGT-3'	62°C	30	*Guilloux Y. et al.*
	NA17-2	5'-CTCTACTTCCTCCTGATTGTTGAG-3'			*J Exp Med 1996, 183: 1173*
Actin	CHO15	5'-GGCATCGTGATGGACTCCG-3'	55°C	21	*Patard J.J. et al.*
	CHO16	5'-GCTGGAAGGTGGACAGCGA-3'			*Int J Cancer 1995, 64: 60*

Supplementary Table S2. Correlation of clinico-pathological parameters with overall survival.

		Survival estimates		
Variable		**4 years (%)**	**Median (months)**	**P-value[a]**
Sex	F	75.0	-[b]	0.061
	M	44.5	26.9	
Localization	Oral cavity	68.4	-	0.117
	Oropharynx	41.2	20.0	
	Hypopharynx	28.3	23.4	
	Larynx	75.0	-	
Grade	1	90.0	-	0.061
	2	38.5	24.0	
	3	51.3	-	
Clinical stage	I/II	80.1	-	**0.011**
	III	69.2	-	
	IV	32.5	18.3	
T-stage	1/2	66.9	-	**0.020**
	3/4	35.8	23.4	
N-stage	0	71.8	-	**0.018**
	1	70.0	-	
	>1	28.4	18.3	

[a] Log-rank. P-values ≤ 0.05 are indicated in bold
[b] Fifty % survival not yet reached.