Cancer-Testis Genes Are Coordinately Expressed and Are Markers of Poor Outcome in Non–Small Cell Lung Cancer

Ali O. Gure,[1] Ramon Chua,[1] Barbara Williamson,[1] Mithat Gonen,[2] Cathy A. Ferrera,[3] Sacha Gnjatic,[1] Gerd Ritter,[1] Andrew J.G. Simpson,[1] Yao-T. Chen,[4] Lloyd J. Old,[1] and Nasser K. Altorki[3]

Abstract

Purpose: Cancer-testis genes mapping to the X chromosome have common expression patterns and show similar responses to modulators of epigenetic mechanisms. We asked whether cancer-testis gene expression occurred coordinately, and whether it correlated with variables of disease and clinical outcome of non–small cell lung cancer (NSCLC).

Experimental Design: Tumors from 523 NSCLC patients undergoing surgery were evaluated for the expression of nine cancer-testis genes (*NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT7/MAGE-C1, SSX2*, and *SSX4*) by semiquantitative PCR. Clinical data available for 447 patients were used to correlate cancer-testis expression to variables of disease and clinical outcome.

Results: At least one cancer-testis gene was expressed by 90% of squamous carcinoma, 62% of bronchioloalveolar cancer, and 67% of adenocarcinoma samples. Statistically significant coexpression was observed for 34 of the 36 possible cancer-testis combinations. Cancer-testis gene expression, either cumulatively or individually, showed significant associations with male sex, smoking history, advanced tumor, nodal and pathologic stages, pleural invasion, and the absence of ground glass opacity. Cox regression analysis revealed the expression of NY-ESO-1 and MAGE-A3 as markers of poor prognosis, independent of confounding variables for adenocarcinoma of the lung.

Conclusions: Cancer-testis genes are coordinately expressed in NSCLC, and their expression is associated with advanced disease and poor outcome.

Cancer-testis gene expression is observed at different frequencies in all tumors regardless of tissue of origin. Of the 40 or so cancer-testis genes/gene families, more than half map to chromosome X (http://www.cancerimmunity.org/CTdatabase/). These X chromosome cancer-testis genes (CT-X) and BAGE genes that map to juxtacentromeric regions are typically multigene families that have arisen through chromosomal duplications. Most cancer-testis genes have been initially identified through immunologic assays (1). In normal tissues, CT-X genes are consistently expressed in spermatogonia, oogonia, and the trophoblast cells (1). In testicular germ cells, the expression of most CT-X genes decrease as cells enter meiosis (2), and regain genomic methylation (3, 4), coinciding with the loss of both Suv39h2 expression and H4 hyperacetylation (5, 6). Cancer-testis genes mapping to somatic chromosomes, on the other hand, tend to have fewer homologues and can be expressed in meiotic gametes (7–9). Based on these differences and the common epigenetic mechanisms associated with the regulation of their expression, it has been proposed that CT-X genes might constitute a distinct group (10).

Ectopic hypomethylation of genomic DNA has been associated with CT-X gene expression and the demethylation of critical CpG residues within their promoter regions (11). All CT-X genes that are expressed in tumors or testis can be induced *in vitro* by DNA demethylation or by inhibitors of histone deacetylation (1). Despite the evidence suggesting that CT-X genes share common epigenetic regulatory mechanisms, the evidence regarding CT-X coexpression and whether CT-X expression associates with clinical variables of disease and outcome is inconclusive.

In this study, we analyzed tumors from 523 non–small cell lung cancer (NSCLC) patients for the expression of nine CT-X genes. We show coordinate expression among all CT-X genes tested. In 447 patients for whom clinical data were available, we evaluated the association of CT-X expression with variables of disease and outcome. CT-X expression was found to be sporadically associated with advanced disease and other variables that indicate worse prognosis in all major histologic types of NSCLC. In addition, Cox regression analysis revealed

Authors' Affiliations: [1]Ludwig Institute for Cancer Research, [2]Department of Statistics and Epidemiology, Memorial Sloan Kettering Cancer Center; Departments of [3]Cardiothoracic Surgery and [4]Pathology, Weill Medical College of Cornell University, New York, New York

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Requests for reprints: Ali O. Gure, Ludwig Institute for Cancer Research, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY, 10021. Phone: 212-746-6450; Fax: 212-746-4483; E-mail: agure@med.cornell.edu.

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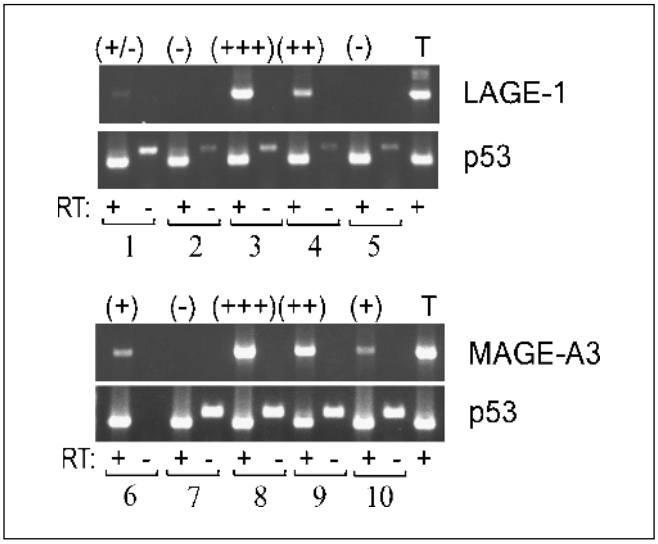

Fig. 1. Semiquantitative RT-PCR. Examples of reverse transcription-PCR (*RT-PCR*) reactions resulting in different quantities of products (− to +++). Tumor samples are indicated by numbers. T, testis. Control amplifications without reverse transcriptase (*RT* −). p53 amplification was done to ensure RNA integrity. The larger p53 band in lanes without RT is due to amplification of contaminating genomic DNA.

that expression of *NY-ESO-1* and *MAGE-A3* were independent markers of worse outcome in adenocarcinomas of the lung. CT-X expression was not found to affect outcome in squamous carcinomas, bronchioloalveolar carcinoma, or adenocarcinoma with bronchioloalveolar features.

Patients and Methods

Patients. A total of 523 patients undergoing curative surgical resection for primary NSCLC at the Department of Cardio-Thoracic Surgery, Weill Medical College of Cornell University, from 1991 to July 2004, were included in this study. Informed consent was obtained from all patients. The study was approved by the Institutional Review Board of Weill Medical College of Cornell University.

Expression analysis. Tumor tissues were obtained during surgery. Following gross dissection, tissues were immediately frozen on dry ice. Total RNA was prepared following homogenization by the guanidium isothiocyanade method followed by CsCl gradient centrifugation. Alternatively, the Ribopure kit (Ambion, Austin, TX) was used according to manufacturer's instructions. Remaining tumor tissue was maintained either frozen or in RNAlater (Ambion). Total RNA (2 μg) was reverse-transcribed with 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, in the presence of 2 μg random hexamers (Applied Biosystems, Foster City, CA), 20 units RNaseOUT (Invitrogen), and 5 mmol/L DTT in a total volume of 20 μL. Each reverse transcription reaction was done in the presence or absence of Moloney murine leukemia virus-RTase to test for contamination. For individual PCR reactions, 250 ng of cDNA were amplified with gene-specific oligonucleotides (2 ng per 25 μL reaction) in the presence of 1 unit AmpliTaq Gold (Applied Biosystems) and 5 μmol/L of each deoxynucleotidetriphosphates (Applied Biosystems). Gene-specific primers used to amplify individual CT-X transcripts are shown in Supplementary Table S1. The integrity of cDNA obtained was tested by amplification of *p53* transcripts (p53-F, 5′-TACTCCCCTGCCCTCAACAAG; p53-R, 5′-CTCAGGCGGCTCATAGGG). For semiquantitative PCR analysis, reverse transcription-PCR products were categorized after separation on ethidium bromide–stained agarose gels as either −, +/−, +, ++, or +++, reflecting the intensity of the product when compared with a standardized testis sample (Fig. 1). Real-time reverse transcription-PCR analysis (ABI Prism, Applied Biosciences) of a representative number of tumor RNA suggested the different intensities corresponded to ~0.1-1 fg (+/−), 1-5 fg (+), 5-100 fg (++), and <100 fg (+++) of transcript per 2 μg of mRNA (12). Expression levels of <5 fg (−, +/−, +) were classified as low ($CT\text{-}X_{[low]}$), and those with >5 fg (++ and +++) as high ($CT\text{-}X_{[high]}$).

Statistical analysis. To analyze coordinate cancer-testis gene expression, the frequency of the expression of a given cancer-testis in a tumor sample expressing a second cancer-testis gene was compared with the frequency observed among all tumors. Additionally, expression data was analyzed as a categorical value where the strength of coexpression was represented by κ (13). The strength of coexpression is proportionate to κ, with higher values corresponding to more frequent coexpression. κs can range from −1.0 to 1.0. Values of >0 indicate agreement better than chance. For example, $\kappa = 0.5$ means that the expression of two given CT-X genes occurs simultaneously 50% of the time over and above that expected by chance alone (14).

To evaluate whether cancer-testis gene expression was related to sex, smoking status, tumor size, pleural invasion, disease stage, and other clinical variables, univariate analysis using the Wilcoxon rank sum test

Table 1. Frequency of CT-X gene expression in NSCLC

	All histologies			Adenocarcinoma			BAC + AdenoBAC			Squamous cell carcinoma			Other		
	n	CT^+ (%)	$CT_{[high]}$ (%)	*n*	CT^+ (%)	$CT_{[high]}$ (%)	*n*	CT^+ (%)	$CT_{[high]}$ (%)	*n*	CT^+ (%)	$CT_{[high]}$ (%)	*n*	CT^+ (%)	$CT_{[high]}$ (%)
Any CT-X	523	72	49	221	67	42	95	62	34	97	90	70	110	76	56
NY-ESO-1	518	27	15	220	22	14	95	18	5	95	43	19	108	30	19
LAGE-1	443	32	20	183	27	19	83	31	11	85	45	33	92	32	19
MAGE-A1	475	46	30	200	41	26	90	34	19	87	71	52	98	46	30
MAGE-A3	520	45	40	219	46	27	95	43	27	96	80	65	110	64	48
MAGE-A4	314	35	22	118	29	15	75	16	5	57	61	49	64	44	31
MAGE-A10	250	27	19	84	25	19	73	15	10	42	50	41	51	29	16
CT7	323	18	10	147	16	8	57	12	5	58	28	17	61	20	12
SSX2	231	10	5	102	9	4	52	2	0	48	10	8	29	24	14
SSX4	215	14	7	89	9	5	47	6	2	42	19	10	37	27	14

Abbreviations: BAC, bronchioloalveolar cancer; AdenoBAC, adenobronchioloalveolar cancer; CT, cancer-testis.

(or t test) and Fisher's exact test (or χ^2 test) were used for continuous and categorical variables, respectively. The effect of cancer-testis gene expression on survival was evaluated using the Kaplan-Meier method, and differences between two groups were compared using the log-rank test. All survival curves were calculated from the date of surgery. All statistical analyses were two sided with a 5% type I error rate and were computed using SAS (version 9.0) software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant. Covariates with $P < 0.05$ by univariate analysis were subjected to multivariate analysis. Cox regression analysis was done to assess the effects of CT-X expression on survival while controlling for confounding clinical covariates.

Results

Frequency of X chromosome cancer-testis genes in non–small cell lung cancer. A total of 523 cases of NSCLC were typed for CT-X expression. The comparative level of CT-X expression was estimated by semiquantitative PCR and recorded as +/−, +, ++, and +++ based on the intensity of the amplification product (Fig. 1). Among tumors tested, 377 (72.1%) expressed at least one of the nine CT-X genes tested. The most frequently observed CT-X was *MAGE-A3*, present in 55.2% of samples followed by *MAGE-A1* (46.3%), *MAGE-A4* (34.7%), *LAGE-1* (32.1%), *MAGE-A10* (27.2%), *NY-ESO-1* (26.6%), *MAGE-C1/CT7* (18.8%), *SSX4* (13.5%), and *SSX2* (9.6%; Table 1). The expression frequency of CT-X genes showed striking differences between histologic subtypes. Sixty-two percent (59 of 95) of the bronchioloalveolar carcinoma cases (including adenocarcinomas with bronchioloalveolar features) expressed at least one CT-X gene. This frequency was 67% (148 of 221) for adenocarcinomas and 90% (87 of 97) for squamous cell

Table 2. Coexpression of CT-X genes in NSCLC

CT-X_1	CT-X_2	Samples tested for both CT-X_1 and CT-X_2	% Samples expressing CT-X_1 among CT-X_2(+) samples	% Observed/% expected ratio for CT-X_1 among CT-X_2 (+) samples	% Samples expressing CT-X_2 among CT-X_1(+) samples	% Observed/% expected ratio for CT-X_2 among CT-X_1 (+) samples	κ	P
NY-ESO-1	LAGE-1	442	57	2	64	2	0.43	<0.0001
NY-ESO-1	MAGE-A1	474	44	1.7	78	1.7	0.34	<0.0001
NY-ESO-1	MAGE-A3	516	40	1.5	83	1.5	0.28	<0.0001
NY-ESO-1	MAGE-A4	313	49	1.8	63	1.8	0.36	<0.0001
NY-ESO-1	MAGE-A10	249	53	1.8	51	1.9	0.33	<0.0001
NY-ESO-1	CT7	323	51	2.1	40	2.2	0.30	<0.0001
NY-ESO-1	SSX2	229	68	2.3	22	2.2	0.23	<0.0001
NY-ESO-1	SSX4	214	72	2.1	29	2.1	0.27	<0.0001
LAGE-1	MAGE-A1	409	51	1.7	80	1.6	0.39	<0.0001
LAGE-1	MAGE-A3	442	45	1.5	81	1.4	0.27	<0.0001
LAGE-1	MAGE-A4	302	49	1.4	51	1.8	0.24	<0.0001
LAGE-1	MAGE-A10	250	54	1.6	44	1.6	0.26	<0.0001
LAGE-1	CT7	269	56	1.9	37	2	0.28	<0.0001
LAGE-1	SSX2	189	75	2.1	22	2.2	0.22	0.0005
LAGE-1	SSX4	174	56	1.4	21	1.3	0.11	0.09
MAGE-A1	MAGE-A3	473	70	1.5	84	1.5	0.52	<0.0001
MAGE-A1	MAGE-A4	309	72	1.4	49	1.4	0.30	<0.0001
MAGE-A1	MAGE-A10	250	93	1.8	48	1.8	0.43	<0.0001
MAGE-A1	CT7	322	71	1.6	30	1.7	0.22	<0.0001
MAGE-A1	SSX2	209	90	1.9	18	1.8	0.16	0.0001
MAGE-A1	SSX4	194	88	1.6	22	1.7	0.18	0.0001
MAGE-A3	MAGE-A4	313	76	1.3	47	1.3	0.26	<0.0001
MAGE-A3	MAGE-A10	250	91	1.7	43	1.6	0.33	<0.0001
MAGE-A3	CT7	321	90	1.7	31	1.7	0.26	<0.0001
MAGE-A3	SSX2	228	91	1.7	16	1.6	0.13	0.0001
MAGE-A3	SSX4	213	97	1.7	23	1.6	0.19	<0.0001
MAGE-A4	MAGE-A10	247	76	2.3	62	2.3	0.55	<0.0001
MAGE-A4	CT7	218	55	1.6	28	1.7	0.18	0.006
MAGE-A4	SSX2	181	72	1.8	18	1.8	0.15	0.0068
MAGE-A4	SSX4	162	75	1.9	28	1.9	0.25	0.0003
MAGE-A10	CT7	166	54	2	34	2	0.27	0.0015
MAGE-A10	SSX2	142	72	2.6	25	2.5	0.27	0.0017
MAGE-A10	SSX4	128	68	2.4	36	2.4	0.35	0.0002
CT7	SSX2	136	56	2.8	19	2.1	0.10	0.04
CT7	SSX4	108	36	1.5	19	1.5	0.10	0.3
SSX2	SSX4	194	35	3.5	50	3.3	0.33	0.0007

Table 3. Demographics and clinical characteristics

	n*	Mean survival (SE)	P
Age			
>60	318	59.2 (3.4)	0.6
≤60	129	52.3 (1.9)	
Sex			
Male	208	56 (2.7)	0.53
Female	239	54.9 (2.3)	
Smoking history			
No	42	56 (6.4)	0.78
Yes	342	59.9 (2.1)	
Tumor size (cm)			
≤3	247	60.9 (2.4)	0.08
>3	189	51.8 (2.7)	
Tumor stage			
I	169	63 (2.8)	0.0001†
II	216	55.7 (2.5)	
III	17	22.5 (4.1)	
IV	34	36.7 (4.2)	
Nodal stage			
0	308	62.6 (2.1)	<0.0001
I + II	127	45.5 (3.1)	
Metastasis stage			
0	419	58.5 (1.9)	0.03
I	19	11 (0.9)	
Pathologic stage			
I	280	65 (2.2)	<0.0001†
II	81	46.4 (4)	
III	90	34.6 (2.9)	
IV	18	11 (0.9)	
Pleural invasion			
No	272	62.4 (2.2)	0.01
Yes	126	51.3 (3.5)	
Ground glass opacity			
No	187	62.2 (2.9)	0.2
Yes	45	39.4 (2)	

*Patients for whom both CT-X typing and clinical data were available are shown.
† Wilcoxon.

carcinomas; the frequency in the latter group being significantly different than in that of the other two ($P < 0.0001$). This difference was preserved when the frequency of only tumors of the different histologic types expressing high levels of CT-X genes ($CT\text{-}X_{[high]}$) were compared.

X chromosome cancer-testis gene expression is coordinated. Of the *NY-ESO-1* positive tumors, 64% also expressed *LAGE-1*, which is twice the frequency of 32% expected if the expression of these genes were independent events. Similarly, the possibility of finding any one of the nine CT-X genes expressed in a tumor with a second CT-X gene was consistently and significantly higher than expected (Table 2). Coexpression of CT-X genes was tested using κ, a statistical measure that indicates the degree of coexpression above that expected due to chance. When applied to *NY-ESO-1* and *LAGE-1* expression, κ statistics result in a value of 0.43, indicating coexpression is 43% above that expected by chance ($P < 0.0001$). Only 2 of the 36 CT-X combinations showed no statistically significant coexpression (*SSX4* and *LAGE-1*, and *SSX4* and *CT7/MAGE-C1*). However, these coexpression rates are still higher than expected, and their κ values are similar to those of the other CT-X combinations analyzed. Overall, our results conclusively show coordinate expression of all nine CT-X genes in NSCLC.

Associations of X chromosome cancer-testis expression with prognostic indicators in non–small cell lung cancer. The patient characteristics of this series are shown in Table 3. Univariate analyses confirmed associations between overall survival and tumor stage, nodal stage, metastasis stage, and pathologic stage. We then investigated the possible correlation between CT-X expression and these clinical variables (Table 4). "Any CT-X" included patients whose tumors expressed at least one CT-X gene. In addition, tumors with high levels of CT-X expression ($CT\text{-}X_{[high]}$), as judged by semiquantitative PCR, were compared with tumors with no or low cancer-testis

Table 4. CT-X expression and clinical characteristics

	n*	CT+ (%)	P	$CT_{[high]}$ (%)	P
Age					
>60	318	72	0.5	48	0.9
≤60	129	74		47	
Sex					
Male	208	77	0.02	56	0.0009
Female	239	68		41	
Smoking history					
No	42	64	0.2	26	0.003
Yes	342	74		51	
Tumor size (cm)					
≤3	247	70	0.06	41	0.0005
>3	189	77		58	
T stage					
I	169	66	0.04	40	0.004
II	216	77		54	
III	17	94		77	
IV	34	77		44	
N stage					
0	308	73	0.9	46	0.14
I + II	127	78		53	
M stage					
0	419	73	0.26	49	0.6
I	19	84		42	
Pathologic stage					
I	280	72	0.43	46	0.3
II	81	78		59	
III	90	69		49	
IV	18	83		44	
Pleural invasion					
No	272	70	0.06	43	0.009
Yes	126	79		57	
Ground glass opacity					
No	187	72	0.34	49	0.01
Yes	45	64		31	

Abbreviation: CT, cancer-testis.
*Patients for whom both CT-X typing and clinical data were available are shown.

Table 5. Cox proportional hazards model for CT expression in adenocarcinoma of the lung

	+ vs −		high vs low	
	Hazard ratio (95% confidence interval)	*P*	Hazard ratio (95% confidence interval)	*P*
Any CT	1.6 (0.9-3)	0.1	1.6 (1-2.8)	0.06
NY-ESO-1	1.6 (0.9-2.8)	0.1	2.3 (1.3-4.2)	0.008
LAGE-1	1.5 (0.6-2.2)	0.7	1.1 (0.5-2.4)	0.9
MAGE-A1	1.7 (1-2.9)	0.07	1.5 (0.8-2.6)	0.24
MAGE-A3	1.6 (0.9-2.6)	0.1	2.1 (0.2-3.5)	0.007
MAGE-A4	0.9 (0.4-2)	0.9	1.2 (0.5-3)	0.7
MAGE-A10	0.8 (0.2-2.5)	0.6	0.9 (0.3-3)	0.9
CT7/MAGE-C1	1.6 (0.8-3.4)	0.2	2.1 (0.8-5.2)	0.1
SSX2	2.2 (0.8-6.3)	0.1	1.9 (0.3-13.9)	0.5
SSX4	0.7 (0.2-3)	0.6	0.8 (0.1-6.1)	0.9

Abbreviation: CT, cancer-testis.

expression (CT-$X_{[low]}$). Clinical associations of CT-X expression were studied either as a group (Any CT-X), or individually. CT-X expression generally correlated with larger tumor size, presence of pleural invasion, a lack of ground glass opacity, and advanced-stage disease, by univariate analysis. Any CT-X expression was found to be associated with male sex ($P = 0.02$) and advanced tumor stage ($P = 0.04$). In addition, high level expression of any CT-X gene (Any-$CT_{[high]}$) associated with male sex ($P = 0.0009$), smoking history ($P = 0.003$), larger tumors (>3 cm; $P = 0.0005$), tumor stage ($P = 0.004$), the presence of pleural invasion ($P = 0.009$), and the absence of ground glass opacity ($P = 0.014$). Expression of individual cancer-testis genes showed similar trends (Supplementary Table S2).

When the patients were stratified according to histology similar associations were observed. These correlations included those listed above, but in addition, CT-X expression was found to be associated with later nodal and pathologic stages in all histologic groups. Statistically significant associations are shown in Supplementary Table S3. CT-X gene expression consistently associated with advanced disease or indicators of worse outcome except for *SSX2* expression in adenocarcinomas, which was more frequent among nonsmokers.

X chromosome cancer-testis gene expression is an independent prognostic indicator in non–small cell lung cancer. The expression of CT-X was then compared with patient survival in various histologic groups. Kaplan-Meier analysis showed that CT-X expression conferred worse survival primarily in patients with adenocarcinomas (Table 5; Fig. 2). "Any CT-$X_{[high]}$" patients had worse survival when compared with "Any CT-$X_{[low]}$" patients with adenocarcinoma ($P = 0.06$). CT-X expression had no effect on the survival of patients with bronchioloalveolar carcinoma, adenocarcinoma with bronchioloalveolar characteristics, or for squamous cell carcinoma. When individually analyzed, the expression of *NY-ESO-1*, *MAGE-A1*, *SSX2*, and high levels of *MAGE-A3* was found to be associated with shorter survival in adenocarcinoma. When

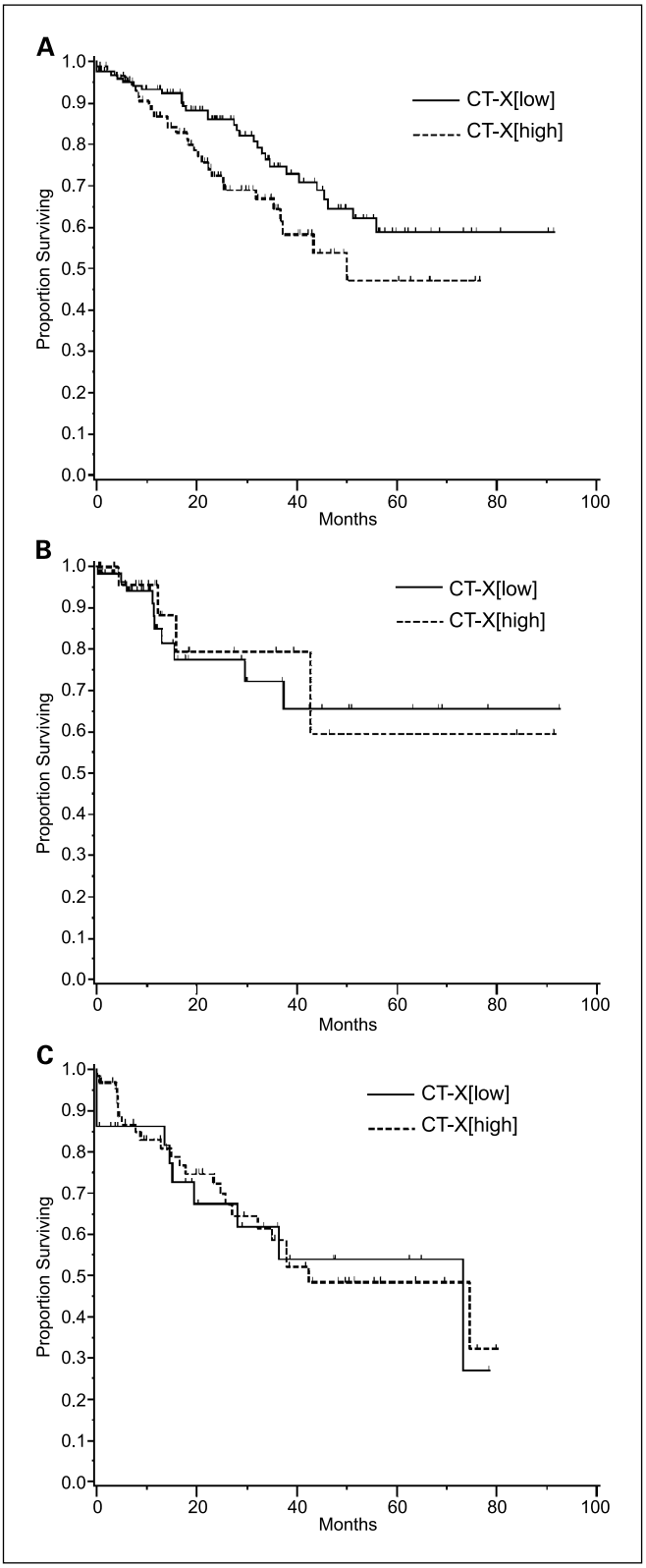

Fig. 2. Survival of patients with NSCLC stratified according to CT-X expression. Distributions were estimated using the Kaplan-Meier method. Tick marks represent the time of last follow-up for patients who remained alive. *A,* patients with adenocarcinoma with high level CT-X expression (Any CT group) had shorter survival than patients with no or low CT-X expression ($P = 0.06$). CT-X expression did not influence survival for patients with (*B*) bronchioloalveolar carcinoma or adenocarcinoma with bronchioloalveolar features ($P = 0.7$) or (*C*) squamous cell carcinoma ($P = 0.8$).

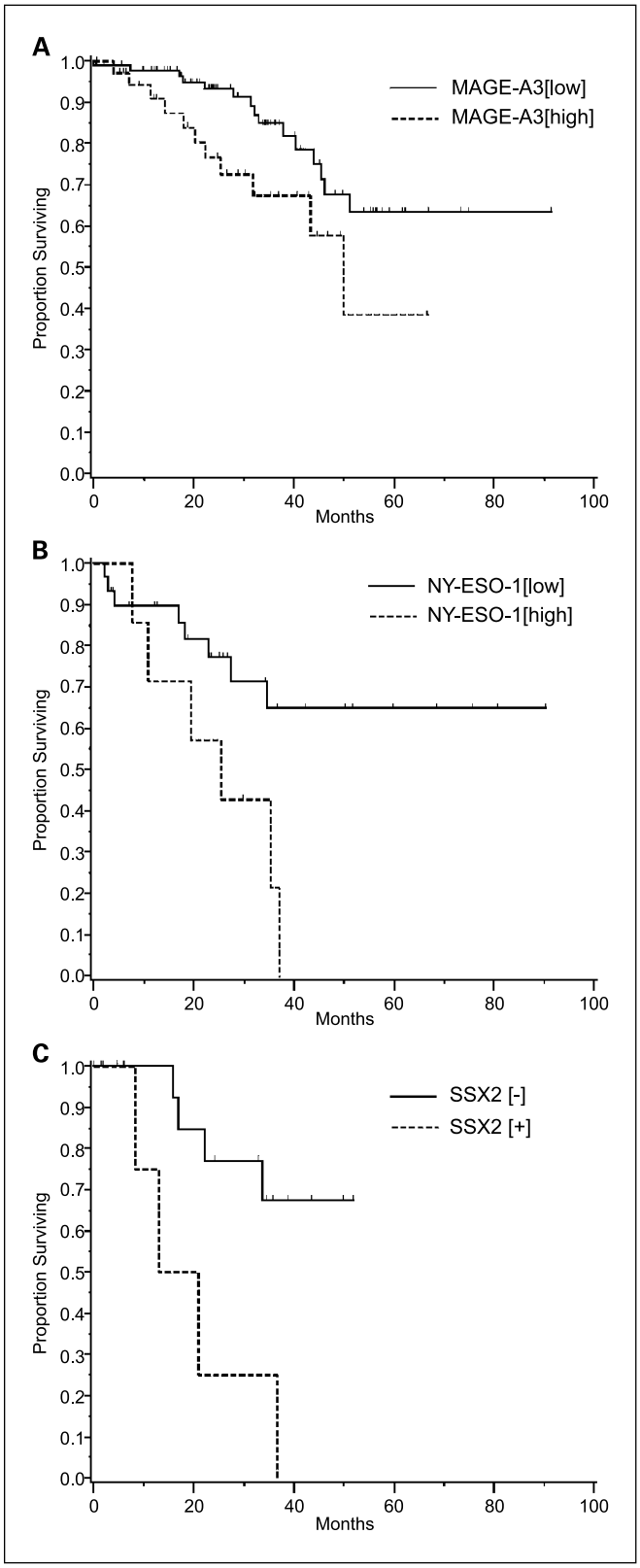

Fig. 3. Survival of patients with NSCLC stratified according to CT-X expression and pathologic stage. Distributions were estimated using the Kaplan-Meier method. Tick marks represent the time of last follow-up for patients who remained alive. Representative series. *A,* high-level *MAGE-A3* expression in adenocarcinoma patients of stage I ($P = 0.04$). *B,* high level *NY-ESO-1* expression in adenocarcinoma patients of stage II ($P = 0.02$). *C, SSX2* expression in patients with adenocarcinoma of stage III ($P = 0.05$).

stratified according to stage, CT-X expression was associated with worse survival at all stages. Examples for *MAGE-A3*, *NY-ESO-1*, and *SSX2* are shown in Fig. 3.

Cox regression analysis was done to assess whether CT-X expression was prognostic of survival independent of confounding criteria, including stage, histology, and adjuvant therapy (Table 5). This revealed that high level expression of *NY-ESO-1* or *MAGE-A3* were predictors of worse outcome in adenocarcinoma independent of confounding factors.

Discussion

The evaluation a large series of patients has made it possible to perform a rigorous analysis of CT-X expression and also to conduct correlative studies using multivariate analysis with greater reliability. We show that all the CT-X genes examined are expressed in a coordinate manner in NSCLC. Previous studies have argued both for and against coexpression (15–18). Such discrepancies might have been due to smaller sample sizes or due to the heterogeneous expression of CT-X genes within tumors, which might lead to false negative results in as many as 30% of samples, especially in immunohistochemical analyses (19). To avoid such caveats, this study was conducted with a very large sample size. We chose reverse transcription-PCR–based analysis due to its high sensitivity and because we could prepare total RNA from samples that were large enough to reflect CT-X gene expression in case the genes were heterogeneously expressed in the tumor tissues evaluated.

The best documented epigenetic aberrations in tumors, regardless of tissue of origin, are genome-wide hypomethylation, region-specific hypermethylation, and loss of imprinting (20–23). Ectopic methylation has been associated with larger tumors, extensive disease, and ultimately poor prognosis (24–30). Current data suggests that the transcriptional regulation of all CT-X genes is governed by common epigenetic mechanisms (1). CT-X genes have in common a TATA-less promoter, which is heavily methylated and thus silent in normal tissues. Their expression is associated with genome wide hypomethylation, and they can be up-regulated by DNA methyl transferase inhibitors, histone deacetylase inhibitors, or by the absence of histone methyl transferase G9a (11, 31–35). Genome-wide hypomethylation has been shown to increase progressively in parallel to advanced grade in breast, ovarian, cervical, and neural cancers (26–30) and has been associated with lung cancer progression (36). Therefore, it is not surprising that CT-X gene expression has also been reported to be associated with less-differentiated, higher-grade tumors; later stages of cancer; and worse outcome (37–50). In fact, *MAGE-A3*, *MAGE-A10*, and *MAGE-A1* promoters were shown to undergo progressive demethylation in gastric cancer parallel to disease progression (43).

We found significant correlations between CT-X expression and larger tumors, pleural invasion, later stages of disease, and also male sex and a history of smoking. Moreover, multivariate analysis showed CT-X expression to be a marker of worse outcome independent of the known clinical prognostic indicators. We observed that high-level CT-X expression was even more closely related to later stages and worse disease outcome. This is similar to previous reports (37, 50, 51) and is likely to reflect more severe hypomethylation in these cancers. However, we have not tested for genome-wide hypomethylation

in tumor tissues and therefore can not conclude whether these are indeed parallel events. Although our observations are limited to CT-X genes, the expression of two other cancer-testis genes, *SCP-1* on chromosome 1 and *PRAME* that maps to chromosome 22, have been associated with worse survival in ovarian cancers and neuroblastoma, respectively, suggesting that cancer-testis genes located on somatic chromosomes might also show similar clinical associations (52, 53). Although CT-X expression could be a passive bystander in a more hypomethylated genome, recent evidence suggests these genes can have antiapoptotic qualities, thus conferring a survival advantage to some tumors (54, 55).

Although the expression of a number of CT-X genes did not reach the statistical significance to qualify as markers of worse outcome in this study, hazard ratios for most CT-X genes in adenocarcinoma or squamous cell carcinoma were >1, suggesting an association between the expression of almost all CT-X genes with worse prognosis.

In this series, squamous cell carcinomas were found to express all CT-X genes at significantly higher rates then adenocarcinoma and bronchioloalveolar carcinomas. In other studies, CT-X expression frequency has also been shown to be higher in squamous cell carcinomas of the lung, bladder, and esophagus compared with tumors of other histologic types in the same organs (50, 56, 57). Whether this directly reflects the degree of hypomethylation in these tumors is not known; however, squamous and spindle carcinoma of the skin have been shown to have more extensive hypomethylation compared with papilloma (58). The fact that expression of CT-X genes should associate with particular tumor types suggests that the underlying mechanisms controlling CT-X gene expression are complex and vary among tumor types. In this context, both aberrant CpG hypermethylation and imprinting defects have been shown to be tumor type specific (23, 59).

Most CT-X antigens are immunogenic, and their use as therapeutic cancer vaccines is being systematically evaluated (60–63). The DNA methyltransferase inhibitor 5-aza-2′ deoxycytidine (Desitabine) and various histone deacetylase inhibitors, including trichostain A, SAHA, and valproic acid, have been used as part of chemotherapy protocols, primarily to reverse hypermethylation of tumor suppressor gene promoters, and have been shown to be beneficial in hematologic and solid tumors (64–67). These drugs, if capable of inducing CT-X expression *in vivo*, could increase patient eligibility and treatment effectiveness in CT-X-targeted immunotherapy. Our results suggest that as tumors progress, the level and number of CT-X genes they express are likely to increase. This in turn suggests a potentially important therapeutic role for CT-X-based cancer vaccines in the management of later stages of malignancy. Because there is strong coexpression among CT-X genes, a multi-CT-X targeting strategy would be predicted to be most effective. Moreover, it could be argued that CT-X antigen immunization might be beneficial even to patients with CT-X -negative tumors, as an induced anti-CT-X immune response might prevent the emergence of CT-X expressing tumor cells, thus halting or slowing tumor progression.

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