



HHS Public Access

Author manuscript

Cancer Res. Author manuscript; available in PMC 2015 April 15.

Published in final edited form as:

Cancer Res. 2014 September 1; 74(17): 4694–4705. doi:10.1158/0008-5472.CAN-13-3725.

A search for novel cancer/testis antigens in lung cancer identifies VCX/Y genes expanding the repertoire of potential immunotherapeutic targets

Ayumu Taguchi^{1,*}, Allen D. Taylor², Jaime Rodriguez¹, Müge Çelikta³, Hui Liu¹, Xiaotu Ma⁴, Qing Zhang², Chee-Hong Wong², Alice Chin², Luc Girard^{5,6}, Carmen Behrens⁷, Wan L. Lam⁸, Stephen Lam⁸, John D. Minna^{5,6,9}, Ignacio I. Wistuba¹, Adi F. Gazdar^{5,10}, and Samir M. Hanash³

¹Department of Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

³Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

⁷Department of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

²Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., Seattle, WA 98109, USA

⁴Department of Molecular and Cell Biology, Center for Systems Biology, The University of Texas Southwestern Medical Center at Dallas, 800 W. Campbell Road, Dallas, TX 75080, USA

⁵Hamon Center for Therapeutic Oncology Research, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390, USA

⁶Department of Pharmacology, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390, USA

⁹Department of Internal Medicine, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390, USA

¹⁰Department of Pathology, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390, USA

⁸Department of Integrative Oncology, British Columbia Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC V521L3, Canada

Abstract

Cancer/testis (CT) antigens are potential immunotherapeutic targets in cancer. However, the expression of particular antigens is limited to a subset of tumors of a given type. Thus, there is a need to identify antigens with complementary expression patterns for effective therapeutic

* **Corresponding Author:** Correspondence should be addressed to Ayumu Taguchi, Department of Translational and Molecular Pathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA; ataguchi@mdanderson.org; fax: 713-563-5746; phone: 713-563-8069.

Disclosure: The authors have no conflicts of interest to disclose.

intervention. In this study, we searched for genes that were distinctly expressed at a higher level in lung tumor tissue and the testes compared to other non-tumor tissues and identified members of the VCX/Y gene family as novel CT antigens. VCX3A, a member of the VCX/Y gene family, was expressed at the protein level in approximately 20% of lung adenocarcinomas and 35% of squamous cell carcinomas, but not expressed in normal lung tissues. Among CT antigens with concordant mRNA and protein expression levels, four CT antigens, XAGE1, VCX, IL13RA2, and SYCE1, were expressed, alone or in combination, in about 80% of lung adenocarcinoma tumors. The CT antigen VCX/Y gene family broadens the spectrum of CT antigens expressed in lung adenocarcinomas for clinical applications.

Keywords

Cancer/testis antigen; VCX/Y; lung cancer; immunotherapy

INTRODUCTION

Many aberrantly expressed antigens are currently being targeted as therapeutic cancer vaccines (1, 2). Cancer/testis (CT) antigens are specifically expressed in immune-privileged tissues, notably the testis and placenta, and exhibit aberrant expression in various types of cancer (3). Thus, they are highly immunogenic, without self-tolerance, and are considered promising targets for cancer immunotherapy with fewer adverse effects. However, the expression of particular CT antigens is limited to only a subset of patients with a particular tumor type. This is the case for members of the MAGE family and NY-ESO-1 which are expressed in 10%-50% and 10%-30% of non-small cell lung cancers (NSCLCs), respectively (4, 5), and are currently being targeted in clinical trials of vaccine immunotherapy (6). Moreover, the effectiveness of immunotherapy targeting of MAGEA3 or NY-ESO-1 is so far limited (7, 8).

In this study, we identified VCX/Y genes as novel CT antigens. VCX/Y genes, mostly located on the X chromosome, may have arisen from a common ancestor to known CT antigens SPANX and CSAG2 (9). VCX may be involved in mRNA stability (10). The expression levels of VCX/Y mRNAs were reduced in normal, immortalized lung cell lines compared to lung cancer cell lines. Reactivity against the protein product of the VCX3A gene was observed in approximately 20% of lung adenocarcinoma and 35% of squamous cell carcinoma (SCC) tumors using tissue microarrays, whereas no expression was detected in normal lung tissues. We further examined the expression patterns of multiple CT antigens in lung adenocarcinomas. We found that one or more of four CT antigens (XAGE1, IL13RA2, SYCE1, and VCX) were expressed in about 80% of lung adenocarcinoma tumors.

MATERIALS AND METHODS

Cell culture and transfection

All lung cancer cell lines used in this study were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin cocktail (Gibco). Human bronchial epithelial cells (HBEC30-KT) were cultured with keratinocyte serum-free medium (Life

Technologies, Inc.) that contained 50 µg/mL bovine pituitary extract (Life Technologies) and 5 ng/mL epidermal growth factor (EGF) (Life Technologies). Human small airway epithelial (HSAEC1-KT) cells were cultured with SAGM (Lonza) according to the manufacturer's protocol. For the small interfering RNA (siRNA) transfection experiments, two siRNAs that targeted VCX (#1: Hs_VCX_8 FlexiTube siRNA, cat. #SI04173568, and #2: Hs_VCX_10 FlexiTube siRNA, cat. #SI04187295) were obtained from Qiagen. PC-9 cells were transfected at a final concentration of 50 nM siRNA using Lipofectamine RNAiMAX (Life Technologies), according to the manufacturer's instructions. Seventy-two hours after transfection, protein was harvested using RIPA buffer for Western blotting, and an MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) was performed according to the manufacturer's protocol. Three independent experiments for the MTS assay were performed in triplicate. For treatment with 5-aza-2-deoxycytidine (5-Aza-dC; Sigma), cells were incubated with 2 µM 5-Aza-dC. Media were changed every 24 h. After 5 days, total RNA was extracted from cell lines using the RNeasy Plus Mini Kit (Qiagen).

Quantitative real-time reverse transcription–polymerase chain reaction

Complementary DNA samples were prepared using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). A TaqMan polymerase chain reaction (PCR) assay was performed with a 7500 Fast Real-Time PCR System, TaqMan PCR master mix, commercially available primers, FAM-labeled probes for the VCX3A gene (Hs04191277_gH), and VIC-labeled probes for 18S (Hs99999901_S1), according to the manufacturer's instructions (Life Technologies). Each sample was run in triplicate. Ct values for the VCX3A gene were calculated and normalized to Ct values for 18S (Ct). The Ct values were then calculated by being normalized to the Ct value for the control.

The Cancer Genome Atlas dataset

The Cancer Genome Atlas (TCGA) cancer methylomes, measured using Illumina Human Methylation 450 BeadChips, were downloaded from the TCGA website, where the methylation levels of 485577 CpG sites are represented as a β value of 0 to 1 ($\beta = 1$ for fully methylated sites and $\beta = 0$ for fully unmethylated sites). RNA-seq data of TCGA cancer samples were also downloaded. The normalized count from RSEM (11) for each gene was extracted from files with the extension “rsem.genes.normalized_results”. Between-sample quantile normalization was performed using the R software package preprocessCore. The samples were classified as tumors (primary solid tumor, code: 01) or non-tumors (solid tissue normal, code: 11) according to the sample naming conventions specified in the code tables of the TCGA and corresponding barcodes.

Tissue samples

Eighty-three pairs of treatment-naive lung adenocarcinoma tissues and adjacent non-tumor lung tissues had been obtained with informed consent (12, 13). EGFR and KRAS mutations were screened as previously described (13). Tumor tissues were considered TITF1/NKX2-1 negative when mRNA expression levels were < 0.5-fold lower than those in adjacent normal tissues.

Gene expression analysis

Total RNA was extracted from cell lines and tissues using the RNeasy Plus Mini Kit (Qiagen). RNA quality and concentration were checked using the Experion Automated Electrophoresis System (Bio-Rad) according to the manufacturer's protocol. Expression data were obtained using Illumina Human WG-6 v3.0 Expression BeadChips (Illumina) in the Genomics Core at The University of Texas Southwestern Medical Center at Dallas (Dallas, Texas). Bead-summarized data were obtained using Illumina BeadStudio software and pre-processed using the R software package MBCB (Model-based Background Correction for Beadarray) for background correction and probe summarization. Pre-processed data were quantile normalized and log transformed.

Mass spectrometry analysis

A mass spectrometry analysis of cell lines was performed as previously described (14). In brief, all cell lines were grown in RPMI 1640 (Pierce) that contained 10% of dialyzed fetal bovine serum (Invitrogen), 1% penicillin and streptomycin cocktail, and ^{13}C -lysine instead of regular lysine for seven passages, according to the standard SILAC protocol (15). The same batch of cells was used to analyze the whole cell extract (WCE), conditioned media, and cell surface proteins. WCEs, conditioned media, and cell surface proteins were fractionated by reverse-phase chromatography using 2 mg, 1 mg, and 0.5 mg of total protein, respectively. Cell extracts were reduced and alkylated with iodoacetamide prior to chromatography. Protein digestion and identification by LC-MS/MS was performed as described previously (16, 17). The tandem mass spectra were searched against a composite database of IPI human (v3.57) and IPI bovine (v3.43) proteins. A fixed modification of 6.020129 mass units was added to lysine residues for database searching to account for incorporation of the ^{13}C -lysine isotope. To estimate the significance of the peptide and protein matches, we used the tools PeptideProphet and ProteinProphet. We selected all peptides that had been initially identified with a PeptideProphet probability minimum of 0.05 and not shared between humans and bovines and submitted them to ProteinProphet. Protein Prophet infers a minimal set of proteins that can explain the peptide evidence, assigning a probability to each protein on the basis of the combined peptide probabilities. The derived protein identifications were filtered at a maximum 5% error. The total number of spectral counts for each protein group in ProteinProphet was used in the semi-quantitative analysis. Each dataset was normalized to the total number of spectral counts of each compartment.

Tissue microarrays

The tissue microarrays used in this study comprised 463 surgically resected NSCLC tumor specimens (305 adenocarcinomas and 158 SCCs) collected under an institutional review board protocol and archived as formalin-fixed, paraffin-embedded specimens in The University of Texas Specialized Program of Research Excellence thoracic tissue bank at The University of Texas MD Anderson Cancer Center (Houston, Texas). Tissue microarrays were prepared with a manual tissue arrayer (Advanced Tissue Arrayer ATA100, Chemicon International, Temecula, CA) using 1-mm diameter cores in triplicate for tumors, as

described previously (18). Histological sections that were 4 μm in thickness were then prepared for the subsequent immunohistochemical analysis.

Western blot analysis and immunohistochemical analysis

Monoclonal anti-mouse antibody raised against human VCX3A (Abnova) was used for the immunohistochemical and Western blot analyses. β -tubulin (Cell Signaling Technology) was used as a loading control for the Western blot analysis. Tissue microarray slides and lung tissue sections were immunostained in a Dako Autostainer Plus automated machine. The slides were deparaffinized and hydrated, and antigen retrieval was performed using a decloaker with a Dako pH 6.0 target retrieval solution (Dako North America, Inc., Carpinteria, CA). Intrinsic peroxidase activity was blocked using 3% methanol and hydrogen peroxide for 10 minutes, and serum-free protein block (Dako) was used for 5 minutes to block non-specific antibody binding. Slides were then incubated with antibodies against human VCX3A (1:1000 dilution) for 30 min at ambient temperature. After being washed three times in Tris-buffered saline, slides were incubated for 30 minutes with Dako Envision + Dual Link at ambient temperature. They were then washed three more times, incubated with Dako chromogen substrate for 5 minutes, and counterstained with hematoxylin. Formalin-fixed, paraffin-embedded whole-section specimens, except for the omission of the primary antibodies, were used as negative controls. For the immunostaining scoring, two board certified surgical pathologists participated in the analysis of this TMA (H.L. and J.R.). The scoring system evaluates the intensity of staining (0: negative, 1+: weakly positive, 2+: moderately positive, and 3+: strongly positive) and percentage of staining distribution in the tumor cells (from 0 to 100% of the cells) for each TMA core. Three TMA cores from the same patient were evaluated and the percentage of staining distribution was averaged to provide the final scoring. In this study tumors in which 20% or more of the cells with 2+ or 3+ staining intensity were considered as positive. Before the TMA scoring, the two pathologists evaluated together at a double head microscope the immunostaining pattern for VCX3A in control slides and in the TMA slides to agree on the criteria for intensity and distribution. Then one of the pathologists (H.L.) performed the scoring of the TMA slides. Finally, the two pathologists (H.L. and J.R.) meet again to review the TMA scoring together in the double head microscope and agreed on any question or discrepancy about the evaluation.

Serological immunoreactivity against arrayed VCX3A protein

A recombinant protein microarray (Version 5 Protoarrays, Invitrogen) containing VCX3A protein was utilized to determine Ig based immunoreactivity in plasmas from 96 female subjects that included 12 and 17 subjects subsequently diagnosed with lung and colon adenocarcinoma, respectively. One control was matched to colon adenocarcinoma cases and two to five controls were matched to individual lung adenocarcinoma cases. The array study was performed as part of discovery studies of biomarker candidates for the early detection of epithelial tumor types (19, 20). Plasma samples were randomly allocated to the arrays within batches to eliminate potential bias, and were each hybridized with an individual protein microarray at a dilution of 1/500 in probe buffer (Synthetic Block in 150 mM PBS, 0.1% Tween-20) (PB) for 1.5 hours at 4 $^{\circ}\text{C}$. IgG reactivity was assessed quantitatively using an indirect immune-fluorescence protocol, and local background-subtracted median spot

intensities for downstream statistical analysis were generated using GenePix Pro 6.1. Background-subtracted median spot intensities from individual slides were quantile normalized, and individual clones were normalized to the mean and standard deviation of the normal samples prior to analysis.

RESULTS

Identification of potential CT antigens in lung cancer

To discover potential CT antigens in lung cancer, we identified genes that were overexpressed in the testis compared to normal tissues using BioGPS (<http://biogps.org/>) (GSE1133) (21) (Fig. 1A for the workflow). We focused on CT antigens on the X chromosome (CT-X antigens) because their gene expression is more testis-restricted than that of non-X CT antigens (22). We compared the mRNA expression profiles of 1,013 probes for 632 genes on the X chromosome between five testis and 47 other normal tissues, excluding the placenta, ovary and neuronal tissue, as CT antigens may be expressed in those tissues (22). Forty-five probes that corresponded to 37 genes had more than two-fold higher mRNA expression levels in testis than in non-testis tissues ($P < 0.05$, Mann-Whitney U test) (Supplementary Table S1). A subset of 23 of these genes had not been previously recognized as CT antigens (CTDatabase; <http://www.cta.lncc.br/>) (23).

Four gene families, BEX1, NXF3, TCEAL3, and VCX2, were considered plausible candidates for encoding CT antigens because their family members were predominantly localized to the X chromosome, which is a typical characteristic of CT-X antigens (Supplementary Table S1) (3). To determine whether these genes are expressed in lung cancer, we profiled the mRNA expression of four gene families (BEX, NXF, TCEAL, and VCX/Y) in 113 NSCLC (78 adenocarcinoma, 3 adenosquamous cell carcinoma, 10 SCC, 12 large cell carcinoma, and 10 other NSCLC), 29 SCLC, 2 carcinoid, 4 mesothelioma, and 59 normal lung immortalized cell lines (30 normal bronchial epithelial cells and 29 normal small airway epithelial cells). VCX/Y family genes were co-expressed at a higher level in a subset of NSCLC and SCLC cell lines than in normal bronchial epithelial cell lines or normal small airway epithelial cell lines (Fig. 1B). VCX, VCX3A, and VCX3B were predominantly expressed compared to VCX2 and VCX4. BEX1, BEX2, and BEX4, and BEX5 were highly expressed in some NSCLC cell lines and most SCLC cell lines but were also expressed in normal cell lines, except for BEX5 (Supplementary Fig. S1). These data suggested that BEX5 is a potential CT antigen in lung cancer, particularly SCLC. Two members of the NXF gene family, NXF2 and NXF2B, are known CT antigens (CT39), and mRNA expression levels of the other NXF family genes were not significantly different between cancer and non-tumor cell lines. Of the TCEAL family genes, TCEAL2 was strongly expressed in most SCLC cell lines, compared to non-tumor cell lines indicating that it is a potential cancer testis antigen in SCLC, together with BEX5.

The expression of CT-X genes is associated with promoter de-methylation and more broadly with genomic hypo-methylation (3, 6). Thus, we determined whether mRNA expression levels of VCX/Y genes were associated with hypo-methylation. The methylation status of the five CpG sites in VCX, three in VCX2, four in VCX3A, and five in VCX3B was analyzed in TCGA data (Supplementary Fig. S2A). Methylation data were available for

four, one, three, and two CpG sites in VCX, VCX2, VCX3A, and VCX3B respectively, for lung adenocarcinomas (406 tumor and 32 non-tumor tissues) and lung squamous cell carcinomas (252 tumor and 43 non-tumor tissues). The methylation levels of all CpG sites were significantly lower in cancer tissues compared to non-tumor tissues (Fig. 2A and Supplementary Fig. S2B). We further evaluated the correlation between mRNA and methylation levels of VCX/Y family genes in cancer tissues. mRNA levels of VCX/Y genes were also obtained from the TCGA RNA seq data (326 adenocarcinomas and 217 SCCs for VCX; 377 adenocarcinomas and 248 SCCs for VCX2; and 378 adenocarcinomas and 248 SCCs for VCX3A and VCX3B). mRNA levels were significantly and inversely correlated with methylation for all VCX/Y genes in lung adenocarcinomas (VCX, Spearman correlation coefficient = -0.22 , $P < 0.0001$; VCX2, Spearman correlation coefficient = -0.16 , $P = 0.0025$; VCX3A, Spearman correlation coefficient = -0.21 , $P < 0.0001$; and VCX3B, Spearman correlation coefficient = -0.18 , $P = 0.0004$), whereas the correlation was not significant in squamous cell carcinoma, except for VCX3A (VCX, Spearman correlation coefficient = -0.03 , $P = 0.6983$; VCX2, Spearman correlation coefficient = 0.02 , $P = 0.8127$; VCX3A, Spearman correlation coefficient = -0.16 , $P = 0.0099$; and VCX3B, Spearman correlation coefficient = -0.07 , $P = 0.2598$) (Fig. 2B), suggesting that VCX/Y gene expression is epigenetically regulated in lung adenocarcinoma and that additional regulatory mechanisms for VCX/Y gene expression may occur in SCC that are not associated with the CpG sites tested. In addition, VCX3A gene expression levels were determined in eight NSCLC cell lines after treatment with the DNA demethylating agent 5-aza-2-deoxycytidine (5-Aza-dC). VCX3A gene expression was clearly induced with 5-Aza-dC treatment in all eight NSCLC cell lines, further supporting the epigenetic regulation of VCX/Y gene expression (Fig. 2C). VCX3A protein expression was confirmed by Western blotting in PC-9 cells, in which VCX/Y genes were highly overexpressed (Fig. 1B and 2D). Of note, cell proliferation was inhibited in PC-9 cells by treatment with VCX siRNA (Fig. 2E and F), indicating an oncogenic role for VCX/Y family genes in NSCLC.

Tissue microarray-based analysis of VCX3A protein expression in NSCLC

Next, we determined VCX3A protein expression in NSCLC tumors using a tissue microarray. VCX3A protein was strongly expressed in normal testis (data not shown) but was negative or very weakly positive in normal lung tissue (Fig. 3A). VCX3A expression was predominantly found in the nucleus (Fig. 3B). A tumor was considered positive if 20% or more of tumor cells were moderately or strongly stained. While non-tumor adjacent tissues from 20 adenocarcinomas and 8 SCCs in the tissue microarray were not stained with VCX3A (data not shown), 118 (25.5%) of 463 tumors were positively stained, unrelated to sex, age ($>$ or $<$ 65 years), smoking status, stage, or mutational status for EGFR and KRAS. Reactivity was significantly higher in SCC than in adenocarcinoma (37.3% and 19.3%, respectively; $P < 0.0001$, Fisher's exact test) (Table 1).

CT antigen expression in NSCLC cell lines

Given that no single CT antigen is widely expressed in NSCLC, we assessed whether a combination of CT antigens exhibited complementary expression and would thus in principle, provide a more effective approach to immunotherapy than would single antigens. We first determined CT antigen expression at both the mRNA and protein levels in 38

NSCLC cell lines, given the potential for discordance between RNA and protein expression (24). Of 255 known CT antigens in the CTDatabase (23), 182 genes were profiled in 38 NSCLC cell lines using Illumina WG-6 v3.0 gene expression microarrays (Supplementary Table S2), including 87 (47.5%) CT-X antigens and 96 (52.5%) non-X CT antigens. Next, we determined the protein expression levels of CT antigens in the same NSCLC cell line set with quantitative in-depth proteomic profiling of three compartments: WCE, conditioned media, and cell surface proteins (14, 25). One hundred fifteen (45.1%) of 255 CT antigens were identified in at least one cell line, and 83, 69, and 83 CT antigens were identified in WCE, media, and the cell surface, respectively (Fig. 4A and Supplementary Table S3), consisting of 47 (40.9%) CT-X antigens and 68 (59.1%) non-X CT antigens. One hundred nine CT antigens were profiled at both the mRNA and protein levels. The mRNA expression levels of 10 CT antigens (CT45A5, GAGE7, IL13RA2, MAGEA10, MAGEA4, MAGEB2, NY-ESO-1, PAGE5, SYCE1, and XAGE1) were significantly and positively correlated with protein expression levels in at least one compartment ($P < 0.05$, Spearman correlation) (Fig. 4B). In addition, mRNA levels were significantly higher in cell lines with protein expression of these CT antigens compared to cell lines without protein expression of these CT antigens ($P < 0.05$, Mann–Whitney U test). Protein expression levels were more widely distributed than were mRNA levels, suggesting translational regulation of CT antigens. All CT antigens, except for SYCE1, with significant correlation between mRNA and protein levels were CT-X antigens. Peptides encoded in the VCX gene family (VCX, VCX2, VCX3A, and VCX3B) are largely indistinguishable and were identified by mass spectrometry in the WCE of PC-9 cells (Fig. 4C). VCX/Y proteins are highly homologous and have a paucity of arginine and lysine residues in their sequences, resulting in few detectable tryptic peptides for identification by mass spectrometry. A search for VCX peptides among other cell lines only yielded identification of VCX3A in SW620 colon adenocarcinoma cell line.

mRNA expression profiles of CT antigens in lung cancer tissues

We determined the mRNA expression profiles of 10 CT antigens with concordant mRNA and protein expression levels, together with VCX as a representative gene for the VCX/Y family genes and MAGEA3, which is a current target for vaccine immunotherapy. We evaluated 83 lung adenocarcinomas and adjacent non-tumor tissues (Supplementary Table S4). Unsupervised hierarchical clustering of gene expression in tumors revealed distinct CT antigen expression patterns. Interestingly, lung adenocarcinomas yielded three clusters on the basis of XAGE1 mRNA levels: high, intermediate, and low (upper heatmap in Fig. 5A). In the intermediate and low clusters, VCX, IL13RA2, or SYCE1 was highly expressed in most tumors. Thus, we selected XAGE1, VCX, IL13RA2, and SYCE1 to form a CT antigen panel and compared the mRNA expression levels of these four genes in tumor and adjacent non-tumor tissues. The number of tumors with CT antigen overexpression (more than two-fold higher mRNA expression levels in cases vs. controls) was 55 (66.3%) for XAGE1, 26 (31.3%) for VCX, 10 (12.0%) for IL13RA2, and 9 (10.8%) for SYCE1 (lower heatmap in Fig. 5A and Table 2). One or more of the CT antigens in the panel were overexpressed in 66 (79.5%) lung adenocarcinomas. XAGE1 overexpression was significantly associated with TITF1/NKX2-1-positive lung adenocarcinomas ($P = 0.0054$, Fisher's exact test) (Table 2 and Supplementary Table 4). While overexpression of VCX and SYCE1 was not associated

with the adenocarcinoma patients' clinical characteristics, IL13RA2 overexpression was significantly less common in current smokers ($P = 0.0436$, chi-square test).

The CT antigen panel was more broadly expressed than were NY-ESO-1 and MAGEA3 (lower heatmap in Fig. 5A), which are currently being studied in clinical trials as vaccine immunotherapy. Therefore, we determined the immunogenicity of the four CT antigens. We used two epitope prediction algorithms, BIMAS (<http://www.bimas.cit.nih.gov/>) (26) and SYFPEITHI (<http://www.syfpeithi.de/>) (27). Five peptides that consisted of nine amino acid residues for XAGE1, 22 for VCX, 31 for IL13RA2, and 20 for SYCE1 were predicted to bind HLA molecules, with high scores, in both algorithms (100 in BIMAS and 20 in SYFPEITHI) (Fig. 5B and Supplementary Table S5). Interestingly, VCX contains identical immunogenic 9-mer peptides in different locations: EPLSQESEV at starting amino acid positions 112, 132, and 152; QESEVEEPL at 116, 136, and 156; EPLSQESQV at 122, 142, 162, and 182; and QESQVEEPL at 126, 146, and 166. HLA binding of EPLSQESEV and EPLSQESQV was predicted to be HLA-B*51:01 and restricted, and HLA binding of QESEVEEPL and QESQVEEPL was predicted to be HLA-B*40:01 restricted (Supplementary Table S5). Thus, these repeating amino acid sequences may amplify the immune response against VCX in patients with HLA-B*51:01 or HLA-B*40:01. As part of prior studies that were aimed at identifying tumor antigens associated with autoantibodies in epithelial tumors types, we conducted discovery studies using recombinant and natural protein microarrays (19, 20). Given our discovery of novel CT antigens we examined their association with an autoantibody response using protein microarrays. VCX3A, which we have found to be overexpressed in both lung and colon cancer cell lines, was represented in recombinant protein microarrays. We assessed its Ig based reactivity in pre-diagnostic sera from 29 plasmas from subjects diagnosed with lung or colon adenocarcinoma and 29 pools of matched controls. Serum Ig autoantibody reactivity against VCX3A was significantly higher among cases compared to controls ($P = 0.020$, Mann–Whitney U test) and a receiver operating characteristic (ROC) analysis yielded an area under the curve (AUC) of 0.678, suggesting the occurrence of an immune response against VCX3A in a subset of subjects with lung and colon adenocarcinoma and potential usefulness of VCX3A autoantibodies as part of a diagnostic biomarker panel (Figure 5C).

Because antigen presentation occurs in an HLA-restricted manner, we determined the allelic distribution of HLA molecules with potential binding to our CT antigen panel which yielded 17 HLA class I molecules. The allelic frequencies of 13 HLA class I molecules were available for two US populations in the Allele*Frequencies database (<http://www.allelefreqencies.net/>) (28) (Supplementary Table S6). For African-Americans, the sum of the allele frequency of HLA-A molecules was 36.1% and that of HLA-B was 20.1%; and for Caucasians, it was 65.8% for HLA-A and 37.6% for HLA-B.

DISCUSSION

We identified the VCX/Y gene family as encoding novel CT antigens initially by mining gene expression data. These genes were initially identified as testis specific and were associated with X-linked non-specific mental retardation (29, 30). They exhibited in our study several characteristics of CT-X antigens: testis-specific expression; a multigene family

on the X chromosome; overexpression in a subset of NSCLC cell lines and tumor tissues, as confirmed by a tissue microarray analysis; no overexpression in non-tumor lung cell lines or tissues; and co-expression with family genes (3, 6). Our findings also suggest epigenetic regulation of VCX/Y gene expression, which is another typical characteristic of CT-X antigens. There is also independent evidence that VCX gene expression is epigenetically regulated (31). Moreover, the results of a recent genomic study suggested that VCX/Y and the known CT antigens SPANX and CSAG2 are derived from a common ancestor and that VCX/Y and SPANX are paralogs with a similar protein structure that consists of C-terminal acidic repeats of variable lengths (9). To our knowledge, the relevance of VCX/Y to cancer has not been previously investigated. VCX/Y proteins are localized in the nucleus and may be involved in regulating ribosome assembly during spermatogenesis (32), inhibiting mRNA decapping and regulating mRNA stability (10). In our study, VCX siRNA inhibited cell proliferation in PC-9 cells, suggesting that VCX/Y genes have oncogenic roles. Therefore, further functional studies are warranted.

An integrative mRNA analysis and proteomic profiling of 38 NSCLC cell lines revealed that mRNA and protein expression levels were significantly correlated with each other for 10 CT antigens. We analyzed 83 lung adenocarcinomas samples to determine the gene expression profiles of these 10 CT antigens, VCX, and MAGEA3, which is currently being targeted in clinical trials. Our analysis resulted in a panel of four CT antigens (XAGE1, VCX, IL13RA2, and SYCE1); one or more of these was overexpressed in 80% of lung adenocarcinomas, highlighting the potential of this combination for immunotherapy in lung cancer.

XAGE1 expression and an associated autoantibody response have been observed in NSCLC (33), and several XAGE1 transcript variants have been identified. Most XAGE1 peptides identified by mass spectrometry in our study were common in various XAGE1 isoforms. However, consistent with the findings of a previous study (34), XAGE1b-unique peptides were most commonly identified among XAGE1 isoform-unique peptides in 38 NSCLC cell lines (data not shown). Five 9-mer XAGE1 peptides were identified as highly immunogenic on the basis of two distinct epitope prediction algorithms, BIMAS and SYFPEITHI, and several additional immunogenic peptides have been identified (35).

IL13RA2 is known to be overexpressed in solid tumors, particularly glioblastoma multiforme. Although IL13RA2 has been associated with inflammation in lung epithelial cells, its role in lung cancer has not been investigated (36). Okada et al. recently reported the results of a phase I/II trial using α -type 1 polarized dendritic cells loaded with synthetic peptides for glioma-associated antigen epitopes, including EphA2, interleukin-13 receptor- α 2, YKL-40, gp100, and polyinosinic-polycytidylic acid for HLA-A2(+) patients with recurrent malignant glioma. Of 22 patients in the study, two experienced objective clinical tumor regression. One patient with recurrent glioblastoma experienced a complete response (37).

SYCE1 is a constituent of the synaptonemal complex during meiosis (38); however, its role in cancer has not been well studied. Approximately 20% of lung adenocarcinomas did not exhibit expression of the CT antigens in our panel (Fig. 5A). XAGE1 (39) and IL13RA2

(40) are known to be induced by 5-Aza-dC. Our data indicate that similar epigenetic regulation occurs in VCX/Y genes. Thus, for patients whose tumors do not express CT antigens, a combination of CT antigen-targeted therapy and DNA demethylating agents may be promising (6).

In conclusion, we found that VCX/Y family genes are novel CT antigens in lung cancer on the basis of an analysis of gene expression data; we further confirmed VCX3A expression at the protein level in a tissue microarray analysis. Importantly, we identified a panel of CT antigens, consisting of XAGE1, VCX, IL13RA2, and SYCE1, which were expressed in most lung adenocarcinomas. This finding suggests that targeting this four-antigen panel will be a promising immunotherapy approach for lung adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Support: This work was supported by the Canary Foundation, the Lungevity Foundation, the National Cancer Institute Early Detection Research Network, The Department of Defense W81XWH-09-LCRP-CTRA, Lung Cancer SPORE (P50CA70907), and MD Anderson's Moon Shots Program.

REFERENCES

- Schlom J. Therapeutic cancer vaccines: current status and moving forward. *Journal of the National Cancer Institute*. 2012; 104:599–613. [PubMed: 22395641]
- Dougan M, Dranoff G. Immune therapy for cancer. *Annual review of immunology*. 2009; 27:83–117.
- Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nature reviews Cancer*. 2005; 5:615–25.
- Suri A. Cancer testis antigens--their importance in immunotherapy and in the early detection of cancer. *Expert Opin Biol Ther*. 2006; 6:379–89. [PubMed: 16548764]
- Chiriva-Internati M, Pandey A, Saba R, Kim M, Saadeh C, Lukman T, et al. Cancer testis antigens: a novel target in lung cancer. *International reviews of immunology*. 2012; 31:321–43. [PubMed: 23083344]
- Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, et al. The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol*. 2011; 5:164–82. [PubMed: 21376678]
- Vansteenkiste J, Zielinski M, Linder A, Dahabreh J, Gonzalez EE, Malinowski W, et al. Adjuvant MAGE-A3 immunotherapy in resected non-small-cell lung cancer: phase II randomized study results. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2013; 31:2396–403. [PubMed: 23715567]
- Kakimi K, Isobe M, Uenaka A, Wada H, Sato E, Doki Y, et al. A phase I study of vaccination with NY-ESO-1 peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *International journal of cancer Journal international du cancer*. 2011; 129:2836–46. [PubMed: 21448901]
- Hansen MA, Nielsen JE, Retelska D, Larsen N, Leffers H. A shared promoter region suggests a common ancestor for the human VCX/Y, SPANX, and CSAG gene families and the murine CYPT family. *Molecular reproduction and development*. 2008; 75:219–29. [PubMed: 17342728]
- Jiao X, Wang Z, Kiledjian M. Identification of an mRNA-decapping regulator implicated in X-linked mental retardation. *Molecular cell*. 2006; 24:713–22. [PubMed: 17157254]

11. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics*. 2011; 12:323. [PubMed: 21816040]
12. Selamat SA, Chung BS, Girard L, Zhang W, Zhang Y, Campan M, et al. Genome-scale analysis of DNA methylation in lung adenocarcinoma and integration with mRNA expression. *Genome research*. 2012
13. Thu KL, Vucic EA, Chari R, Zhang W, Lockwood WW, English JC, et al. Lung adenocarcinoma of never smokers and smokers harbor differential regions of genetic alteration and exhibit different levels of genomic instability. *PLoS one*. 2012; 7:e33003. [PubMed: 22412972]
14. Taguchi A, Politi K, Pitteri SJ, Lockwood WW, Faca VM, Kelly-Spratt K, et al. Lung cancer signatures in plasma based on proteome profiling of mouse tumor models. *Cancer cell*. 2011; 20:289–99. [PubMed: 21907921]
15. Ong SE, Mann M. A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protoc*. 2006; 1:2650–60. [PubMed: 17406521]
16. Faca V, Pitteri SJ, Newcomb L, Glukhova V, Phanstiel D, Krasnoselsky A, et al. Contribution of protein fractionation to depth of analysis of the serum and plasma proteomes. *Journal of Proteome Research*. 2007; 6:3558–65. [PubMed: 17696519]
17. Faca VM, Ventura AP, Fitzgibbon MP, Pereira-Faca SR, Pitteri SJ, Green AE, et al. Proteomic Analysis of Ovarian Cancer Cells Reveals Dynamic Processes of Protein Secretion and Shedding of Extra-Cellular Domains. *PLoS One*. 2008:3.
18. Sun M, Behrens C, Feng L, Ozburn N, Tang X, Yin G, et al. HER family receptor abnormalities in lung cancer brain metastases and corresponding primary tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009; 15:4829–37. [PubMed: 19622585]
19. Ladd JJ, Chao T, Johnson MM, Qiu J, Chin A, Israel R, et al. Autoantibody signatures involving glycolysis and spliceosome proteins precede a diagnosis of breast cancer among postmenopausal women. *Cancer research*. 2013; 73:1502–13. [PubMed: 23269276]
20. Ladd JJ, Busald T, Johnson MM, Zhang Q, Pitteri SJ, Wang H, et al. Increased plasma levels of the APC-interacting protein MAPRE1, LRG1, and IGFBP2 preceding a diagnosis of colorectal cancer in women. *Cancer Prev Res (Phila)*. 2012; 5:655–64. [PubMed: 22277732]
21. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:6062–7. [PubMed: 15075390]
22. Hofmann O, Caballero OL, Stevenson BJ, Chen YT, Cohen T, Chua R, et al. Genome-wide analysis of cancer/testis gene expression. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:20422–7. [PubMed: 19088187]
23. Almeida LG, Sakabe NJ, deOliveira AR, Silva MC, Mundstein AS, Cohen T, et al. CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic acids research*. 2009; 37:D816–9. [PubMed: 18838390]
24. Schwanhauser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. *Nature*. 2011; 473:337–42. [PubMed: 21593866]
25. Faca VM, Ventura AP, Fitzgibbon MP, Pereira-Faca SR, Pitteri SJ, Green AE, et al. Proteomic analysis of ovarian cancer cells reveals dynamic processes of protein secretion and shedding of extra-cellular domains. *PLoS one*. 2008; 3:e2425. [PubMed: 18560578]
26. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol*. 1994; 152:163–75. [PubMed: 8254189]
27. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999; 50:213–9. [PubMed: 10602881]
28. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic acids research*. 2011; 39:D913–9. [PubMed: 21062830]
29. Fukami M, Kirsch S, Schiller S, Richter A, Benes V, Franco B, et al. A member of a gene family on Xp22.3, VCX-A, is deleted in patients with X-linked nonspecific mental retardation. *American journal of human genetics*. 2000; 67:563–73. [PubMed: 10903929]

30. Lahn BT, Page DC. A human sex-chromosomal gene family expressed in male germ cells and encoding variably charged proteins. *Human molecular genetics*. 2000; 9:311–9. [PubMed: 10607842]
31. Fukutomi S, Seki N, Koda K, Miyazaki M. Identification of methylation-silenced genes in colorectal cancer cell lines: genomic screening using oligonucleotide arrays. *Scandinavian journal of gastroenterology*. 2007; 42:1486–94. [PubMed: 17994469]
32. Zou SW, Zhang JC, Zhang XD, Miao SY, Zong SD, Sheng Q, et al. Expression and localization of VCX/Y proteins and their possible involvement in regulation of ribosome assembly during spermatogenesis. *Cell research*. 2003; 13:171–7. [PubMed: 12862317]
33. Nakagawa K, Noguchi Y, Uenaka A, Sato S, Okumura H, Tanaka M, et al. XAGE-1 expression in non-small cell lung cancer and antibody response in patients. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2005; 11:5496–503. [PubMed: 16061866]
34. Sato S, Noguchi Y, Ohara N, Uenaka A, Shimono M, Nakagawa K, et al. Identification of XAGE-1 isoforms: predominant expression of XAGE-1b in testis and tumors. *Cancer immunity : a journal of the Academy of Cancer Immunology*. 2007; 7:5.
35. Ohue Y, Eikawa S, Okazaki N, Mizote Y, Isobe M, Uenaka A, et al. Spontaneous antibody, and CD4 and CD8 T-cell responses against XAGE-1b (GAGED2a) in non-small cell lung cancer patients. *International journal of cancer Journal international du cancer*. 2012; 131:E649–58. [PubMed: 22109656]
36. Nakashima H, Husain SR, Puri RK. IL-13 receptor-directed cancer vaccines and immunotherapy. *Immunotherapy*. 2012; 4:443–51. [PubMed: 22512637]
37. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2011; 29:330–6. [PubMed: 21149657]
38. Costa Y, Speed R, Ollinger R, Alsheimer M, Semple CA, Gautier P, et al. Two novel proteins recruited by synaptonemal complex protein 1 (SYCP1) are at the centre of meiosis. *Journal of cell science*. 2005; 118:2755–62. [PubMed: 15944401]
39. Lim JH, Kim SP, Gabrielson E, Park YB, Park JW, Kwon TK. Activation of human cancer/testis antigen gene, XAGE-1, in tumor cells is correlated with CpG island hypomethylation. *International journal of cancer Journal international du cancer*. 2005; 116:200–6. [PubMed: 15800911]
40. Takenouchi M, Hirai S, Sakuragi N, Yagita H, Hamada H, Kato K. Epigenetic modulation enhances the therapeutic effect of anti-IL-13R(alpha)2 antibody in human mesothelioma xenografts. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011; 17:2819–29. [PubMed: 21357681]

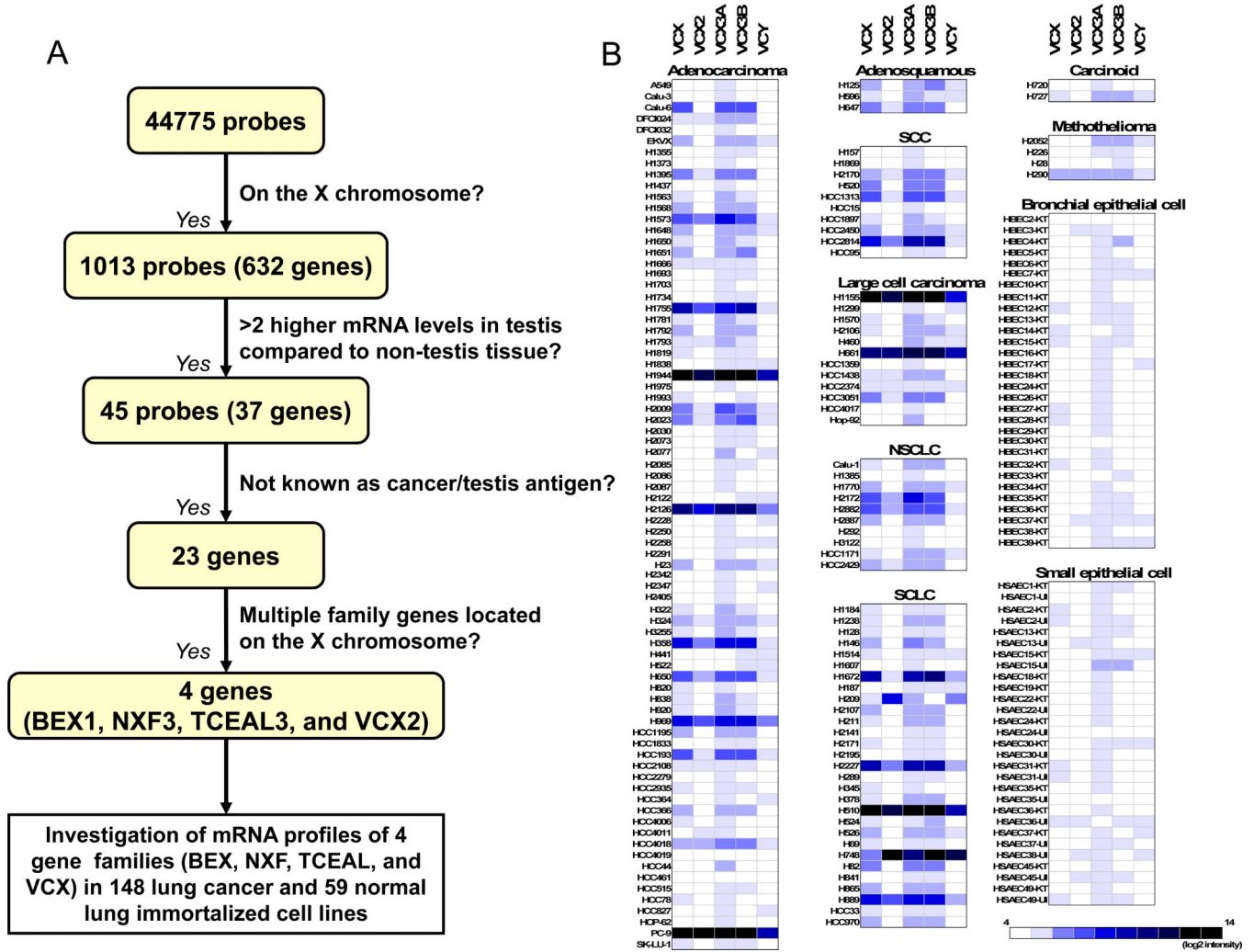


Figure 1. Identification of VCX/Y genes as novel CT antigens in lung cancer.

A. Workflow for selecting novel CT antigen candidates in BioGPS.

B. Heatmap for gene expression levels of VCX/Y family genes in 148 lung cancer and 59 normal lung immortalized cell lines: adenocarcinoma (n = 78), adenosquamous cell carcinoma (adenosquamous) (n = 3), SCC (n = 10), large cell carcinoma (n = 12), other NSCLC (n = 10), SCLC (n = 29), carcinoid (n = 2), mesothelioma (n = 4), bronchial epithelial (n = 30), and small airway epithelial (n = 29).

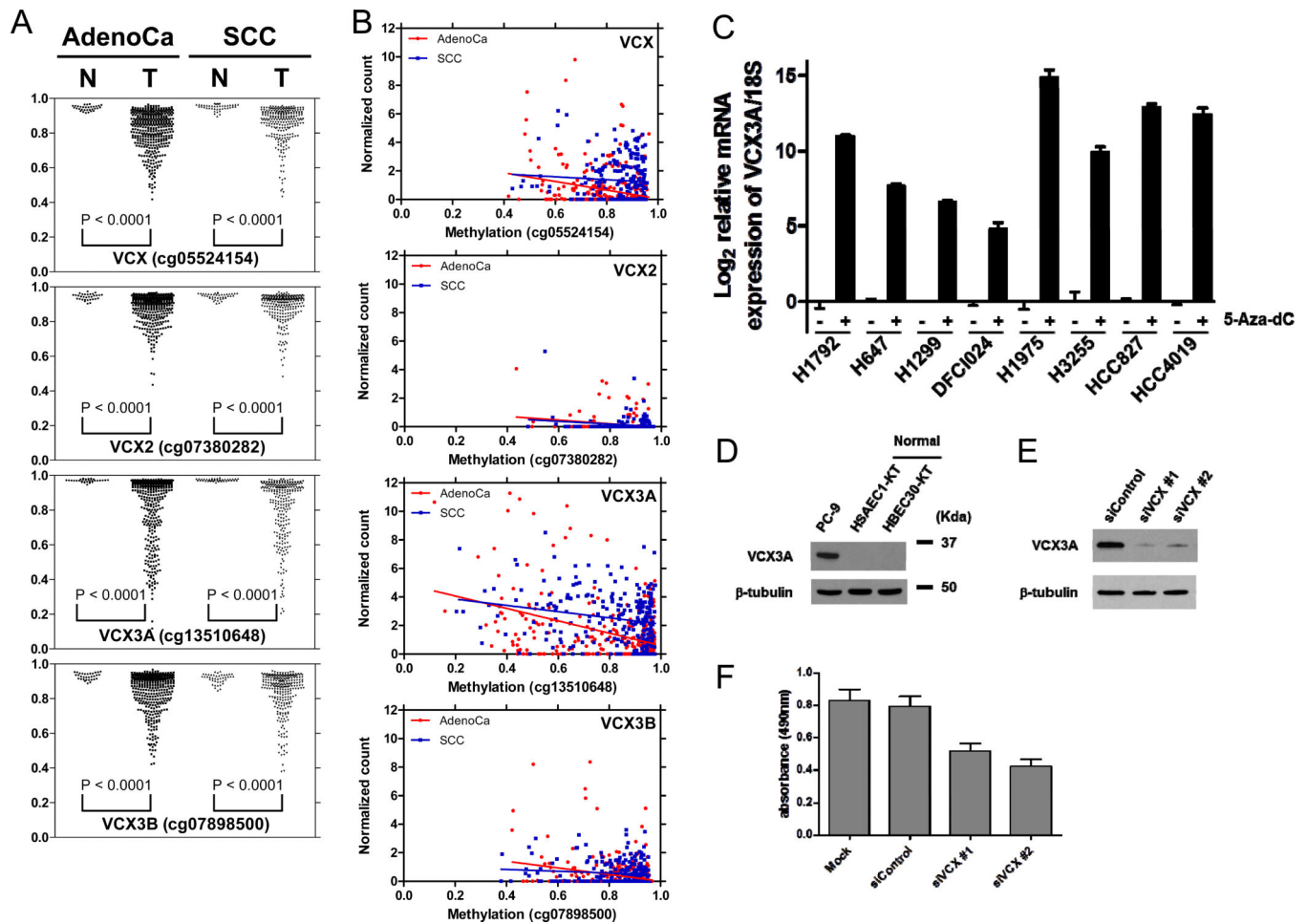


Figure 2. Regulatory mechanism and functional relevance of VCX/Y in NSCLC

A. Methylation levels of representative CpG sites for VCX/Y genes. P values were calculated using the Mann-Whitney U test. N, non-tumor tissue; T, tumor tissue.

B. Correlation of methylation levels and mRNA expression levels in tumor tissue.

C. Induction of VCX3A mRNA with 5-Aza-dC treatment in eight NSCLC cell lines.

D. Western blot analysis of VCX3A protein in PC-9 and normal immortalized lung cell lines. β -tubulin was served as a loading control.

E. Knockdown of VCX with siRNA in PC-9 cells was verified by Western blotting. β -tubulin was served as a loading control.

F. MTS assay of PC-9 cells treated with negative control siRNA or siRNAs against VCX. Columns indicate the average of triplicate samples from a representative experiment, and bars indicate SD.

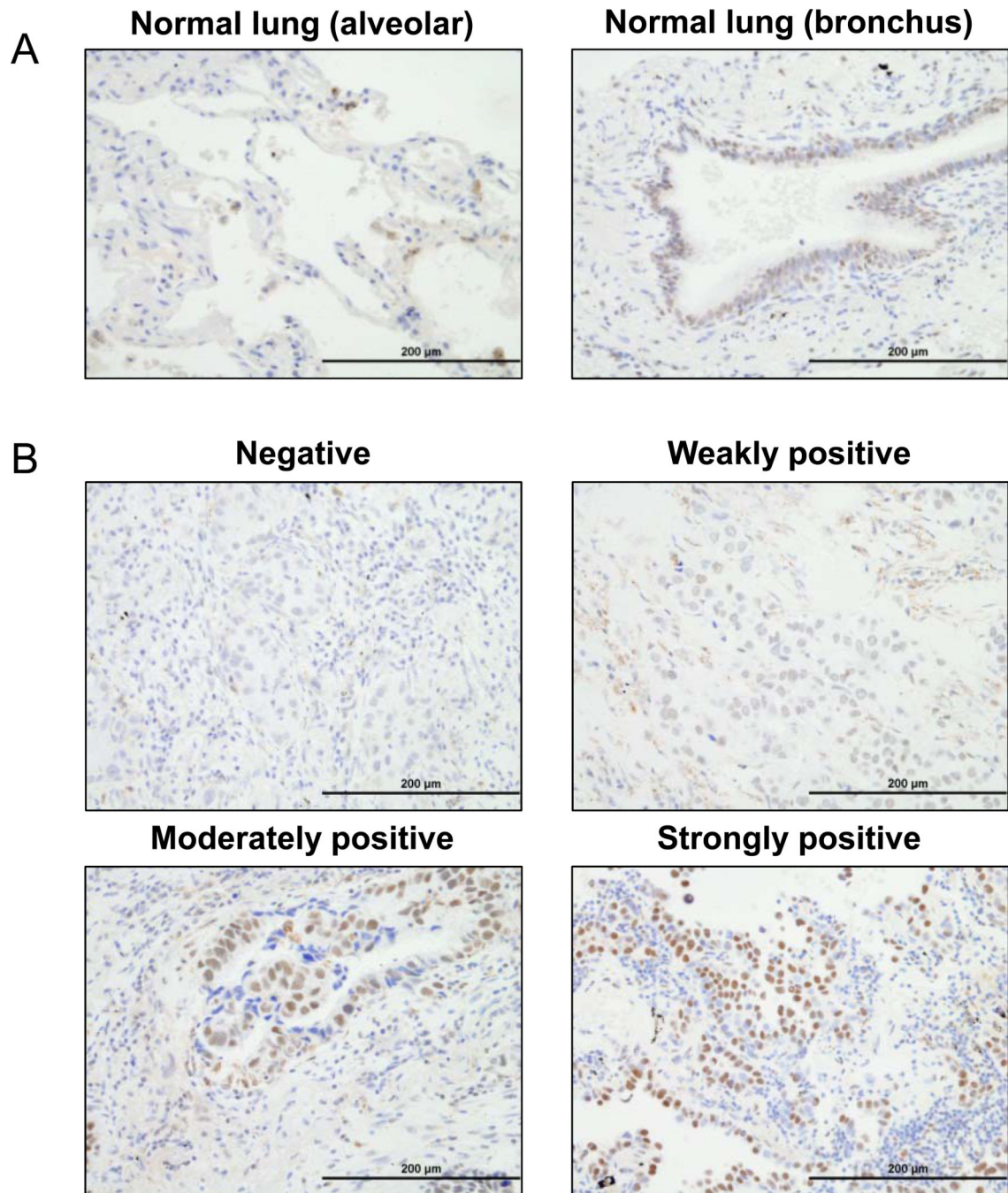
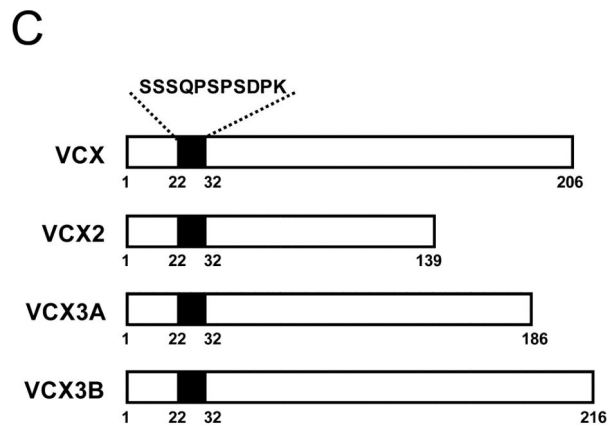
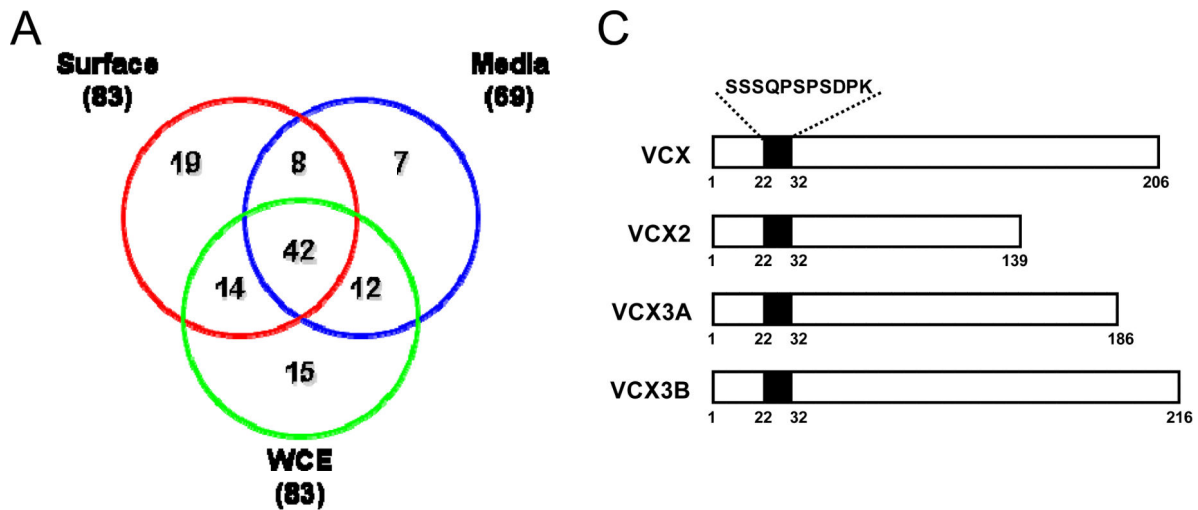


Figure 3. VCX3A protein expression in NSCLC tissue microarray

A. Immunohistochemical staining of VCX3A in normal lung (alveolar and bronchus) tissues. Scale bars represent 200 μm .

B. Assessment of VCX3A expression levels in the nucleus of NSCLC tumor tissues. Scale bars represent 200 μm .



B

Gene	Compartment	Spearman correlation	P value	Identified			Not identified		
				Protein (normalized MS2 counts) Mean ± SD	mRNA (log ₂ intensity)		mRNA (log ₂ intensity)		P value
					Cell lines	Mean ± SD	Cell lines	Mean ± SD	
CT45A5	Media	0.47	0.0031	2.7 ± 0.9	3	9.1 ± 0.1	35	4.6 ± 1.0	0.0002
	surface	0.35	0.0337	5.6 ± 4.6	2	8.5 ± 0.8	36	4.8 ± 1.4	0.0242
	WCE	0.39	0.0163	4.1 ± 1.8	2	9.2 ± 0.1	36	4.7 ± 1.2	0.0028
GAGE7	Media	0.47	0.0032	24.9 ± 12.9	3	14.5 ± 0.1	35	5.5 ± 1.9	0.0002
	surface	0.37	0.0218	8.1 ± 7.1	2	14.4 ± 0.1	36	5.8 ± 2.4	0.0071
	WCE	0.57	0.0002	20.3 ± 15.9	5	12.6 ± 2.4	33	5.3 ± 1.7	< 0.0001
IL13RA2	surface	0.47	0.0032	18.1 ± 4.4	3	12.5 ± 0.3	35	5.3 ± 2.0	0.0001
	WCE	0.36	0.0286	4.3 ± 2.2	2	12.2 ± 0.4	36	5.5 ± 2.3	0.0156
MAGEA10	Media	0.55	0.0004	9.3 ± 6.8	8	10.0 ± 1.4	30	4.7 ± 1.0	0.0009
	surface	0.38	0.0172	4.8 ± 2.5	10	8.9 ± 3.7	28	4.7 ± 1.4	0.0093
	WCE	0.60	< 0.0001	7.4 ± 3.5	6	11.8 ± 0.4	32	4.7 ± 1.5	< 0.0001
MAGEA4	Media	0.74	< 0.0001	44.0 ± 32.8	9	11.9 ± 0.7	29	4.5 ± 1.0	< 0.0001
	surface	0.69	< 0.0001	22.4 ± 22.6	17	8.7 ± 3.6	21	4.3 ± 0.3	0.0001
	WCE	0.77	< 0.0001	36.4 ± 27.6	10	11.6 ± 0.4	28	4.4 ± 0.3	< 0.0001
MAGEB2	surface	0.39	0.0163	3.0 ± 2.1	2	11.7 ± 0.4	36	4.9 ± 1.3	0.0028
	WCE	0.38	0.0197	1.4 ± 0.9	2	11.2 ± 1.0	36	4.7 ± 1.0	0.0057
NY-ESO-1	surface	0.49	0.0018	3.4 ± 1.9	5	9.1 ± 2.4	33	4.7 ± 1.2	0.0016
	WCE	0.37	0.0241	3.1 ± 2.2	4	8.7 ± 2.6	34	4.9 ± 1.5	0.0296
PAGE5	Media	0.47	0.003	16.7 ± 17.7	3	12.2 ± 4.6	35	4.6 ± 0.7	0.0002
	WCE	0.39	0.0163	24.5 ± 6.7	2	12.2 ± 4.8	36	4.8 ± 1.4	0.0014
SYCE1	WCE	0.39	0.0165	2.8 ± 0.3	2	9.8 ± 0.2	36	5.1 ± 0.7	0.0028
XAGE1	surface	0.50	0.0015	1.9 ± 1.4	6	13.8 ± 0.3	32	8.7 ± 4.1	0.0009
	WCE	0.60	< 0.0001	3.2 ± 4.0	9	13.8 ± 0.1	29	8.1 ± 4.0	< 0.0001

Figure 4. Transcriptomic and proteomic profiling of CT antigens in 38 NSCLC cell lines
 A. Venn diagrams of CT antigens identified by mass spectrometric analysis in WCE, cell surface (surface), and conditioned media (media) of 38 NSCLC cell lines.
 B. CT antigens with significant correlation of expression levels between mRNA and protein. P values for comparison of mRNA expression levels in cell lines with or without CT antigen protein expression were calculated by Mann–Whitney U test.

C. Schema of human VCX/Y family proteins. Black bars indicate peptides identified in the PC-9 cell line. Presented numbers are based on NP_038480.2 (VCX), NP_057462.2 (VCX2), NP_057463.2 (VCX3A), and NP_001001888.2 (VCX3B).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

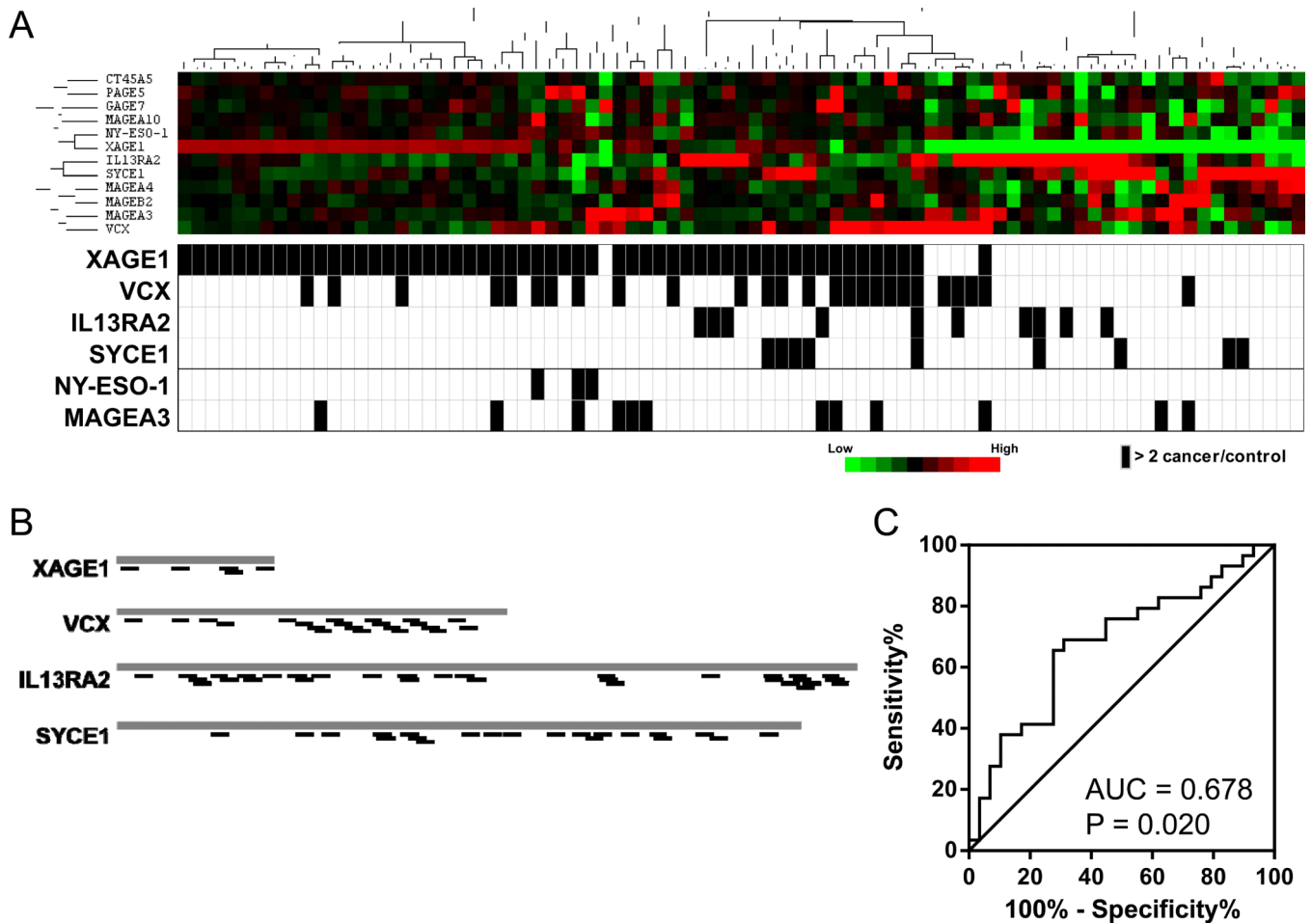


Figure 5. CT antigen panel in 83 lung adenocarcinoma patients

A. An unsupervised hierarchical clustering of 10 selected CT antigens, MAGEA3, and VCX. Black boxes in the lower heatmap indicate lung adenocarcinoma patients with CT antigen overexpression (more than two-fold higher mRNA expression levels in cases vs. controls).

B. Potential epitopes of XAGE1, VCX, IL13RA2, and SYCE1. Black bars indicate 9-mer peptides with high scores in both BIMAS and SYFPEITHI.

C. Receiver operating characteristic curves for autoantibodies to VCX3A in pre-diagnostic plasmas of lung and colon adenocarcinoma patients (n = 29) and matched controls (n = 29) from the WHI observational study. P values were calculated using the Mann-Whitney U test. AUC: area under the curve.

Table 1

VCX3A expression in NSCLC tissue microarray

Characteristic	Total	VCX3A immunostaining		P value ^c
		Positive, n (%)	Negative, n (%)	
Sex ^a				
Male	210	56 (26.7)	154 (73.3)	0.4335
Female	216	50 (23.1)	166 (76.9)	
Age ^a				
> 65 years	246	61 (24.8)	185 (75.2)	1.0000
65 years	180	45 (25.0)	135 (75.0)	
Smoking status ^a				
Current	176	41 (23.3)	135 (76.7)	0.5784
Former	203	55 (27.1)	148 (72.9)	
Never	47	10 (21.3)	37 (78.7)	
Histologic type				
Adenocarcinoma	305	59 (19.3)	246 (80.7)	< 0.0001
SCC	158	59 (37.3)	99 (62.7)	
Stage ^a				
I	219	48 (21.9)	171 (78.1)	0.2125
II	101	33 (32.7)	68 (67.3)	
III	87	20 (23.0)	67 (77.0)	
IV	19	5 (26.3)	14 (73.7)	
Mutation ^b				
KRAS	51	13 (25.5)	38 (74.5)	0.4901
EGFR	22	5 (22.7)	17 (77.3)	
KRAS and EGFR wild	103	18 (17.5)	85 (82.5)	

^aInformation was not available for 37 samples.^bMutations were not investigated in 287 samples.^cP values were calculated by Fisher's exact test or chi-square test.

Table 2
Association of CT antigen overexpression status and clinical characteristics of 83 lung adenocarcinoma patients.

Clinical Characteristic	All			XAGE1			VCX			IL13RA2			SYCE1			
	Total	Yes	No	P value ^c	Yes	No	P value ^c	Yes	No	P value ^c	Yes	No	P value ^c	Yes	No	P value ^c
Sex																
Male	24	20	4	0.7665	18	6	0.3183	9	15	0.4466	0	24	0.0562	2	22	1
Female	59	46	13		37	22		17	42		10	49		7	52	
Age																
> 65 years	54	46	8	0.0943	38	16	1	19	35	0.333	6	48	0.7339	8	46	0.1514
65 years	29	20	9		17	12		7	22		4	25		1	28	
Smoking status																
Current	39	30	9	0.7805	30	9	0.1542	12	27	0.9459	1	38	0.0436	3	36	0.6823
Former	14	12	2		8	6		4	10		3	11		2	12	
Never	30	24	6		17	13		10	20		6	24		4	26	
Mutation ^a																
KRAS	33	26	7	0.6638	22	11	0.9907	10	23	0.915	3	30	0.6745	5	28	0.3392
EGFR	20	17	3		13	7		7	13		3	17		2	18	
KRAS, EGFR wild	27	20	7		18	9		8	19		2	25		1	26	
TTF1/NKX2-1 expression																
Positive	68	57	11	0.0706	50	18	0.0054	22	46	0.7665	8	60	1	8	60	1
Negative	15	9	6		5	10		4	11		2	13		1	14	
Stage ^b																
I	36	30	6	0.7154	25	11	0.3337	13	23	0.7603	4	32	1	7	29	0.0816
II-IV	18	14	4		13	5		5	13		2	16		0	18	

^a Mutations were not studied in three patients.

^b Staging information was not available for 29 patients.

^c P values were calculated by Fisher's exact test or chi-square test.