Impaired T lymphocyte function increases tumorigenicity and decreases tumor latency in a mouse model of head and neck cancer

TONY K.S. KU and DAVID L. CROWE

University of Illinois Cancer Center, Chicago, IL 60612, USA

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Abstract. Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide. SCC is the most common malignant tumor of the oral cavity with over 35,000 cases and 8,000 deaths reported in the United States each year. Previous case studies have reported increased incidence of HNSCC in patients on immunosuppressive therapy for organ transplantation. The results of these studies indicate that effective immune surveillance is important for preventing emergence of HNSCC. HNSCC may also inhibit immune response to tumor cells, which may be responsible for progression. We previously reported induction of metastatic HNSCC in p53 null mutant mice. Despite induction with the potent carcinogen dimethylbenzanthracene, each mouse developed only 1-2 primary tumors with a relatively long induction period of 22 weeks. We hypothesized that immune surveillance might eliminate early tumor cells resulting in the relatively small number of primary tumors and long induction time. To test this hypothesis we performed the induction protocol in nude mice which have defective T lymphocyte function. Decreased T lymphocyte function resulted in reduced tumor latency and increased tumor formation. Immunohistochemical studies showed that expression of cell cycle regulatory proteins is similar in mouse and human HNSCC. However, distinct differences exist between primary and metastatic tumors from nude and wild-type mice. We also determined that lymphocytes react to metastatic tumor cells by upregulating immunoglobin gene expression but are prone to apoptosis via decreased expression of survival factors and upregulation of cell death genes.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most frequent cancer worldwide (1). HNSCC is a major cause of morbidity and mortality in developing nations, comprising up to 50% of all malignant tumors. SCC is the most common malignant tumor of the oral cavity with over 35,000 cases and 8,000 deaths reported in the United States each year (2). Tobacco carcinogens are the primary etiologic agents of the disease with age and genetic background as contributory factors. The overall 5-year survival rate is low among the major cancers and has not declined significantly in recent years.

Previous case studies have reported increased incidence of HNSCC in patients on immunosuppressive therapy for organ transplantation (3-7). Induction of HNSCC was second to that of liver cancer in patients with bone marrow transplant (8). The results of these studies indicate that effective immune surveillance is important for preventing emergence of HNSCC. HNSCC may also inhibit immune response to tumor cells, which may be responsible for progression (9). It has been proposed that immune surveillance consists of three phases: elimination, equilibrium, and escape (10,11). Tumor elimination is the goal of immunosurveillance, but during equilibrium and escape the interactions between the immune system and tumor cells may result in development of cells which can gradually inactivate or kill immune effector cells. Later stages of tumor development may include cells which are less immunogenic. Natural killer and cytotoxic T lymphocytes which have crucial effector functions in immune defense against tumor cells are inactivated in HNSCC (12,13). Regression of tumor grafts is characterized by NK and cytotoxic T lymphocyte attack, and patients with metastatic HNSCC have low NK and cytotoxic T lymphocyte activity (14,15). Spontaneous apoptosis of circulating T lymphocyte populations in patients with HNSCC is an indicator of diminished immune function (16). Cytokine induced stimulation of the immune system and transfer of effector cells have not been effective in head and neck cancer therapy (17,18). Tumor infiltrating lymphocytes are not cytotoxic to autologous tumor cells and exhibit reduced clonogenicity (19). Human HNSCC have been shown to induce minimal cell mediated anti-tumor immune responses (20). Diminished numbers of cytotoxic T lymphocytes correlated with reduced survival in cancer patients (21). A variety of cellular defects such as signaling abnormalities, spontaneous apoptosis, and reduced proliferation have been reported in natural killer, T lymphocyte, and dendritic cells in HNSCC patients (16,22). The results of these studies indicate that cell mediated immune responses are critically important in tumor initiation and progression.

Correspondence to: Dr David L. Crowe, University of Illinois Cancer Center, 801 S. Paulina Street, Room 530C, Chicago, IL 60612, USA
E-mail: dlcrowe@uic.edu

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We previously reported induction of metastatic HNSCC in p53 null mutant mice (23). These tumors ranged from well to poorly differentiated histopathology and showed many molecular features of human HNSCC. These tumors progressed rapidly which resulted in euthanasia criteria being met in 13 weeks. Despite induction with the potent carcinogen dimethylbenzanthracene, each mouse developed only 1-2 primary tumors with a relatively long induction period of 22 weeks. We hypothesized that immune surveillance might eliminate early tumor cells resulting in the relatively small number of primary tumors and long induction time. To test this hypothesis we performed the induction protocol in nude mice which have defective T lymphocyte function. Our results show that defective T lymphocyte function reduces tumor latency period and significantly increases tumor number. Global gene expression profiling demonstrated important differences between HNSCC induced in nude and wild-type mice. We also profiled gene expression in lymphocytes exposed to metastatic tumor cells *in vivo*. Tumor cell exposed lymphocytes upregulate immunoglobin gene expression but are prone to apoptosis via decreased expression of survival factors and upregulation of cell death genes. The results of our study indicate that T lymphocyte function is an important regulator of tumor development in HNSCC.

Materials and methods

Mouse procedures. This study was approved by the Institutional Animal Care and Use Committee before any experiments were performed. The Foxn1 mutant (nude) mouse strain was purchased from The Jackson Laboratory (Bar Harbor, ME). C57Bl6J mice were used as control animals. Mice were housed in approved environmentally controlled facilities on 12-h light-dark cycles with unlimited access to food and water. Twenty male and female 1-month old nude and wild-type mice were dosed orally twice weekly using 25 μl dimethylbenzanthracene (DMBA) dissolved in 20 μl ethanol. The time course and number of tumors were recorded for each animal. Mice were euthanized when any institutional criterion for experimental neoplasia in rodents was met. Complete necropsies were performed on each mouse. A portion of each tumor specimen was flash frozen in liquid nitrogen or fixed in 4% buffered formaldehyde for 16 h at room temperature.

Histopathology and immunohistochemistry. Tumor tissue was dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin. Five micrometer sections were prepared and mounted on poly-L-lysine coated slides. Representative sections were stained with hematoxylin and eosin and histologically evaluated by a pathologist. Immunohistochemical analysis was performed using a commercially available kit (Invitrogen, Carlsbad, CA). Sections were incubated at 60˚C for 30 min and deparaffinized in xylene. Endogenous peroxidase activity was quenched by incubation in a 9:1 methanol/30% hydrogen peroxide solution for 10 min at room temperature. Sections were rehydrated in PBS (pH 7.4) for 10 min at room temperature. Sections were blocked with 10% normal serum for 10 min at room temperature followed by incubation with anti-EGFR, cyclin A, cyclin B, cyclin D, cyclin E, p16, c-myc, HGF, TGFα, c-met, and PCNA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 16 h at room temperature. After three washes in PBS, the sections were incubated with secondary antibody conjugated to biotin for 10 min at room temperature. After additional washes in PBS, the sections were incubated with streptavidin conjugated horseradish peroxidase for 10 min at room temperature. Following final washes in PBS, antigen-antibody complexes were detected by incubation with hydrogen peroxide substrate solution containing aminoethylcarbazole chromogen reagent. Slides were rinsed in distilled water, coverslipped using aqueous mounting medium, and allowed to dry at room temperature. The relative intensities of the completed immunohistochemical reactions were evaluated using light microscopy by independent trained observers who were unaware of the mouse genotypes. A scale of 0-3 was used to score relative intensity, with 0 corresponding to no detectable immunoreactivity and 1, 2, and 3 equivalent to low, moderate, and high expression respectively. Non-parametric data was analyzed by Fisher exact test.

RNA extraction and gene expression profiling. Total RNA was extracted from microdissected primary and metastatic tumor tissue using a commercially available kit (RNasy, Qiagen, Valencia, CA). Individually matched well differentiated primary and metastatic tumor tissue was used in microarray analysis. Three independent samples from each group were used in gene expression analysis. The integrity of rRNA bands was confirmed by Northern gel electrophoresis. Total RNA (10 μg) with spike in controls was reverse transcribed using a T7-oligo(dT) promoter primer in the first strand cDNA synthesis reaction. Following RNase H mediated second strand synthesis, the double stranded cDNA was purified and served as template in the subsequent *in vitro* transcription reaction. The *in vitro* transcription reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analogue/ribonucleotide mix for complementary RNA amplification and biotin labeling. The biotinylated complementary RNA targets were then purified, fragmented, and hybridized to Affymetrix GeneChip Expression arrays (Santa Clara, CA). The murine genome 430 2.0 microarray was used to interrogate 39,000 possible transcripts in each sample. After washing, hybridization signals were detected using streptavidin conjugated phycoerythrin. Affymetrix GCOS software was used to generate raw gene expression scores and normalized to the relative hybridization signal from each experiment. All gene expression scores were set to a minimum value of 2 times background determined by GCOS software in order to minimize noise associated with less robust measurements of rare transcripts. Normalized gene expression data was imported into dChip sotware for hierarchical clustering analysis using the average linkage algorithm. Raw data was analyzed for quality control and the significance of differential gene expression determined by t-test ($p<0.05$) and ratio analysis (>2-fold).

Results

We used the chemical carcinogenesis protocol described in Materials and methods to induce head and neck cancer in the nude mouse. As shown in Fig. 1, nude mice developed tumors by 14 weeks induction on average compared to 22 weeks for wild-type animals ($p<0.0002$). This latency period was 4 weeks

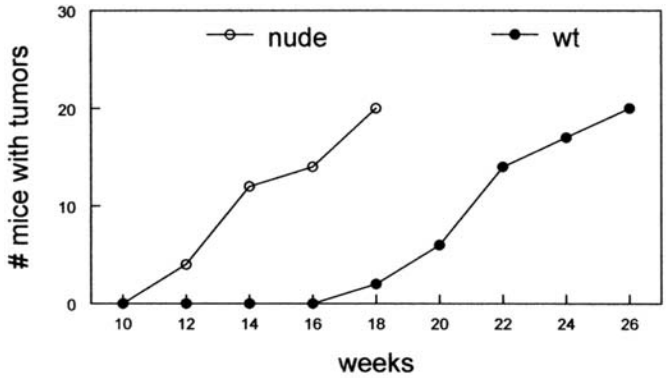

Figure 1. Decreased tumor latency in a nude mouse model of head and neck cancer. Squamous cell carcinoma was induced as described in Materials and methods. The total number of mice which developed tumors each week was recorded.

shorter than tumor formation in p53+/- mice as previously reported (23). There also was a dramatic increase in the number of tumors in nude mice (Fig. 2A). Nude mice developed 5.1 tumors per mouse while wild-type animals had only 1.4 lesions/mouse ($p<10^{-12}$). There were no significant differences in primary tumor growth rates or number of metastatic lymph nodes in nude and wild-type mice (Fig. 2B and C). These results indicate that decreased T lymphocyte function results in reduced tumor latency and increased tumor formation.

We examined the histopathology of head and neck tumors in both nude and wild-type mice. In nude mice, primary tumors were well differentiated SCC which developed primarily on labial and buccal mucosa (Fig. 3A). In wild-type mice, 60% of SCC were well differentiated, 30% were moderately differentiated, and 10% were poorly differentiated as previously reported (23). We examined expression of several cell cycle regulatory proteins in tissue sections of primary tumors by immunohistochemistry. We previously demonstrated correlations between expression of these proteins in human HNSCC (24). Epidermal growth factor receptor, PCNA, cyclin A, cyclin B, cyclin D, cyclin E, HGF, c-met, and TGFα were overexpressed in 40-50% of SCC in nude mice (Fig. 3B-H and J-L). c-myc was overexpressed in 25% of primary SCC (Fig. 3I). These percentages were similar to those observed in wild-type mice. These results indicate that expression of cell cycle regulatory proteins is similar in SCC in nude and wild-type mice.

The average number of cervical lymph nodes containing metastatic tumor cells was 4.1 per mouse, which was not significantly different between nude and wild-type mice. At necropsy, there was no evidence of tumor extension through the lymph node capsule or distant metastasis to any organ. Metastatic tumor in cervical lymph nodes were uniformly moderately differentiated SCC in both nude and wild-type mice (Fig. 3M). These tumors showed decreased stratification and keratinization, loss of intercellular junctions, increased nuclear/cytoplasmic ratio, nuclear pleiomorphism, and occasional mitotic figures. We examined expression of cell cycle regulatory proteins in metastatic tumors in nude and wild-type mice. PCNA was overexpressed in 50% of metastatic tumors in nude mice compared to 10% in wild-type animals

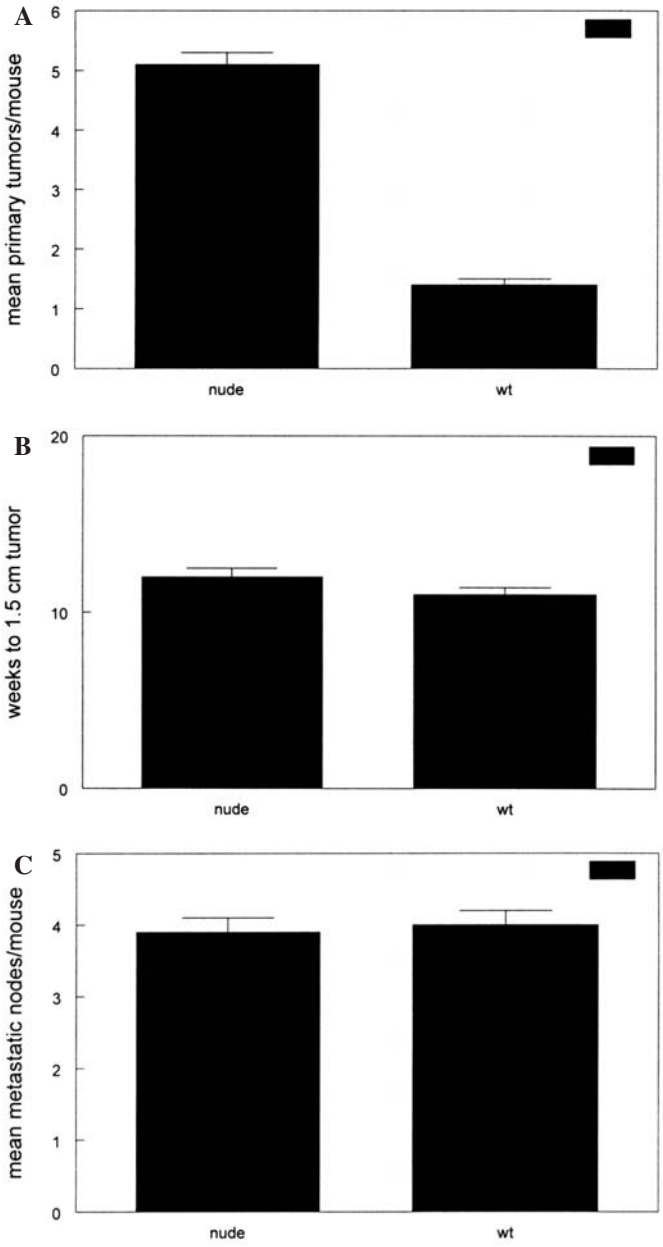

Figure 2. Increased number of tumors in a nude mouse model of head and neck cancer. Squamous cell carcinoma was induced as described in Materials and methods. (A), The total number of tumors which developed per mouse was recorded. (B), Similar growth rates in tumors arising in nude and wild-type mice. The number of weeks to achieve a 1.5-cm tumor was recorded. (C), Similar numbers of metastatic lymph nodes in nude and wild-type mice. Cervical lymph nodes were evaluated by hematoxylin and eosin staining of serial sections. Error bars indicate SEM.

(Fig. 3N). Cyclins B and D were overexpressed in 13% of metastatic tumors in nude mice, and c-met was overexpressed in 25% of metastatic tumors in these animals which was similar to percentages observed in wild-type animals (data not shown). These results indicate that distinct differences exist between primary and metastatic tumors in the mouse model.

To characterize differences in gene expression between primary well differentiated SCC in nude and wild-type mice, we performed global gene expression profiling by microarray analysis. As shown in Table I, proteins initially characterized in salivary gland differentiation were upregulated in squamous cell carcinoma from nude mice (demilune cell and parotid

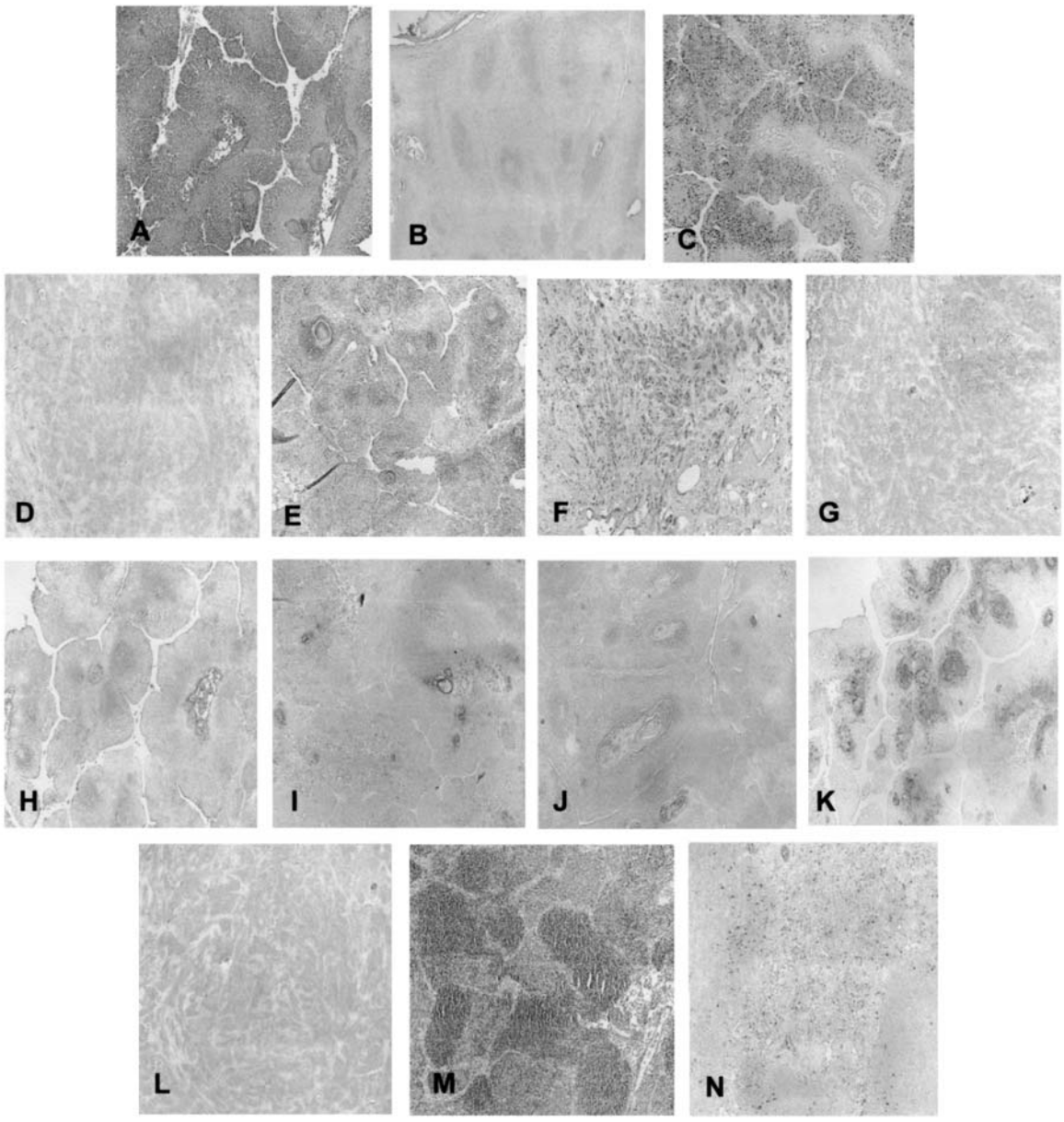

Figure 3. Expression of cell cycle regulatory proteins in squamous cell carcinoma derived from the nude mouse model. (A), Hematoxylin and eosin stained section of well differentiated SCC in the nude mouse. (B), Expression of epidermal growth factor receptor, proliferating cell nuclear antigen (C), cyclin A (D), cyclin B (E), cyclin D (F), cyclin E (G), p16 (H), c-myc (I), hepatocyte growth factor (J), transforming growth factor α (K), and c-met (L) in primary SCC derived from the nude mouse model is shown by immunohistochemistry. (M), Hematoxylin and eosin stained section showing metastatic squamous carcinoma cells invading a cervical lymph node in the nude mouse model of head and neck cancer. (N), Proliferating cell nuclear antigen expression in metastatic tumor cells in a cervical lymph node from the nude mouse model is shown by immunohistochemistry. These experiments were performed at least three times with tissue from different tumors. Representative sections are shown.

protein, 56.1-fold; cysteine-rich secretory protein 1, 43.7-fold; submaxillary gland androgen regulated protein 1, 35.1-fold). A number of the forkhead family of transcription factors were differentially regulated in SCC from nude mice (forkhead box A1, 33.8-fold; forkhead box G1, 7.6-fold; forkhead box C1, 5.7-fold; forkhead box P4, -6.3-fold). Keratins found in simple epithelia were upregulated in SCC in nude mice (keratin 19, 26.6-fold; keratin 8, 19.8-fold; keratin 18, 15.9-fold). Genes involved in terminal differentiation were downregulated in SCC from nude mice (transglutaminase 3, -26.9-fold; desmoglein 1ß, -7.8-fold; corneodesmosin, -5.4-fold; keratin complex 1, acidic, gene 5, -5.1-fold). Inhibitory growth factor pathways were downregulated in SCC from nude mice (transforming growth factor ß receptor III, -9.3-fold; bone morphogenic protein receptor, type II, -6.8-fold; transforming growth factor ß2, -5.6-fold). The growth promoting epidermal growth factor receptor also was downregulated by 5.1-fold. Genes involved in epigenetic regulation were downregulated [Jumonji, AT rich interactive domain 2, -7.6-fold; jumonji, AT rich interactive domain 1C (Rbp2 like), -7.1-fold; p300/CBP-associated factor, -6.9-fold; histone deacetylase 8, -6.2-fold; CREB binding protein, -5.3-fold]. Genes involved in embryonic development were differentially regulated in SCC from nude mice (SRY-box containing gene 2, 18.2-fold; homeobox B3, 6.6-fold; SRY-box containing gene 9, 6.1-fold; distal-less homeobox 5, -5.3-fold). These results suggest that differentiation of HNSCC from nude mice is altered compared to those arising in wild-type mice.

Table I. Gene expression changes between primary SCC in wild-type and nude mice (831 genes).

Accession	Symbol	Gene name	Fold change
C86550	Dcpp	Demilune cell and parotid protein	56.1
AV360029	Vnn1	Vanin 1	54.9
NM_009638	Crisp1	Cysteine-rich secretory protein 1	43.7
NM_011422	Smr1	Submaxillary gland androgen regulated protein 1	35.1
NM_008259	Foxa1	Forkhead box A1	33.8
AI323288	Id4	Inhibitor of DNA binding 4	29.2
NM_007812	Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 4	29.1
NM_008471	Krt1-19	Keratin complex 1, acidic, gene 19	26.6
NM_031170	Krt2-8	Keratin complex 2, basic, gene 8	19.8
U31967	Sox2	SRY-box containing gene 2	18.2
NM_010664	Krt1-18	Keratin complex 1, acidic, gene 18	15.9
AW554594	Prlr	Prolactin receptor	12.1
AK010826	Jam2	Junction adhesion molecule 2	9.2
BG862223	Camk2b	Calcium/calmodulin-dependent protein kinase II, beta	9.0
BB543291	Ccrl1	Chemokine (C-C motif) receptor-like 1	9.0
AF333251	Il24	Interleukin 24	8.8
BQ176424	Clic6	Chloride intracellular channel 6	8.7
AF319173	Psca	Prostate stem cell antigen	8.5
NM_011478	Sprr3	Small proline-rich protein 3	8.3
AW611462	Cldn3	Claudin 3	8.2
NM_008241	Foxg1	Forkhead box G1	7.6
C80220	Rab2	RAB2, member RAS oncogene family	7.2
NM_008827	Pgf	Placental growth factor	7.1
AI172943	Gsta3	Glutathione S-transferase, alpha 3	6.9
BG073383	Hoxb3	Homeobox B3	6.6
NM_023785	Cxcl7	Chemokine (C-X-C motif) ligand 7	6.4
BC024958	Sox9	SRY-box containing gene 9	6.1
NM_011146	Pparg	Peroxisome proliferator activated receptor gamma	5.8
BB759833	Foxc1	Forkhead box C1	5.7
C80147	Hdgf	Hepatoma derived growth factor	5.3
NM_008935	Prom1	Prominin 1	5.2
NM_011452	Serpinb9b	Serine (or cysteine) peptidase inhibitor, clade B, 9b	-5.0
AW538733	Adam17	A disintegrin and metallopeptidase domain 17	-5.0
X65506	Krt1-5	Keratin complex 1, acidic, gene 5	-5.1
AK014017	Egfr	Epidermal growth factor receptor	-5.1
BG147188	Muc6	Mucin 6, gastric	-5.2
BM217698	Tgfbr1	Transforming growth factor, beta receptor I	-5.2
NM_010056	Dlx5	Distal-less homeobox 5	-5.3
BB475090	Crebbp	CREB binding protein	-5.3
BC019788	Plcb2	Phospholipase C, beta 2	-5.4
BM231053	Cdsn	Corneodesmosin	-5.4
BB477214	Masp1	Mannan-binding lectin serine peptidase 1	-5.5
BB296763	Tgfb2	Transforming growth factor, beta 2	-5.6
Y17709	Fzd9	Frizzled homolog 9 (Drosophila)	-5.7
AB012278	Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	-5.8
BB136012	Capg	Capping protein (actin filament), gelsolin-like	-5.8
AV372589	Itga1	Integrin alpha 1	-6.0
AK004739	Dll4	Delta-like 4 (Drosophila)	-6.1
AK011332	Hdac8	Histone deacetylase 8	-6.2
BQ286886	Foxp4	Forkhead box P4	-6.3
NM_011691	Vav1	Vav1 oncogene	-6.4
AK020411	Bmp7	Bone morphogenetic protein 7	-6.6
BB185152	Bmpr2	Bone morphogenic protein receptor, type II	-6.8
BM240241	Pcaf	P300/CBP-associated factor	-6.9
BB050663	Rora	RAR-related orphan receptor alpha	-6.9
BB362489	Jarid1c	Jumonji, AT rich interactive domain 1C (Rbp2 like)	-7.1
BM293452	Jarid2	Jumonji, AT rich interactive domain 2	-7.6
NM_008781	Pax3	Paired box gene 3	-7.6
AY028607	Krt2-16	Keratin complex 2, basic, gene 16	-7.7
AV253195	Dsg1b	Desmoglein 1 beta	-7.8
AF039601	Tgfbr3	Transforming growth factor, beta receptor III	-9.3
BB324744	Esrra	Estrogen related receptor, alpha	-12.1
BB790825	Efna2	Ephrin A2	-13.9
AI893889	Tgm3	Transglutaminase 3, E polypeptide	-26.9

We also examined gene expression changes between metastatic SCC in wild-type and nude mice. As shown in Table II, 2144 differentially expressed genes were identified between metastatic SCC in wild-type and nude mice. A number of kinases were upregulated in metastatic SCC from nude mice (phosphatidylinositol-4-phosphate 5-kinase, 25.9-fold; nemo like kinase, 21.8-fold; casein kinase 1, 10.5-fold). Growth factors and receptors were upregulated in SCC from nude mice (desert hedgehog, 7.4-fold; nerve growth factor receptor, 6.9-fold; transforming growth factor ß2, 6.4-fold; fibroblast growth factor 4, 6.2-fold; hepatoma derived growth factor, 5.3-fold; frizzled homolog 3, 5.2-fold). Telomere associated proteins were upregulated in SCC from nude mice (telomeric repeat binding factor 2, 9.2-fold; tankyrase, 6.0-fold). Transcription factors were also upregulated in metastatic SCC from nude mice (CCAAT/enhancer binding protein δ, 9.0-fold; signal transducer and activator of transcription 3, 5.5-fold; forkhead box F1a, 5.5-fold; transformation related protein 63, 5.4-fold). Histone demethylases were differentially regulated in metastatic SCC from nude mice (Jumonji domain containing 1C, 7.4-fold; Jumonji domain containing 3, 5.8-fold; Jumonji, AT rich interactive domain 1A, -9.0-fold; Jumonji, AT rich interactive domain 1C, -10.3-fold). Certain growth factors and receptors were downregulated in metastatic SCC from nude mice (Jagged 1, -5.2-fold; δ like 3, -5.8-fold; transforming growth factor receptor ß1, -6.4-fold; wingless related MMTV integration site 10a, -6.9-fold). Tumor suppressor expression was inhibited in metastatic SCC from nude mice (BRCA1 associated protein, -5.1-fold; retinoblastoma binding protein 6, -7.0-fold; large tumor suppressor 2, -12.7-fold). Markers of epithelial differentiation were downregulated in metastatic SCC from nude mice (suprabasin, -6.6-fold; mucin 10, -7.4-fold; desmoplakin, -8.1-fold). Cell cycle regulatory genes were inhibited in metastatic SCC from nude mice (cyclin D2, -7.5-fold; cyclin dependent kinase inhibitor 1B, -14.0-fold). Specific transcription factors also were downregulated in metastatic SCC from nude mice (retinoid X receptor γ, -5.2-fold; forkhead box Q1, -5.6-fold; Jun proto-oncogene related gene D1, -6.1-fold; Vav3 oncogene, -10.4-fold; lymphoid enhancer factor 1, -12.7-fold; myeloblastosis oncogene, -57.8-fold). These gene expression changes suggest that metastatic SCC from nude mice may be more biologically aggressive than their counterparts in wild-type mice.

In previously published study, we examined differential gene expression between primary and metastatic tumors in wild-type mice (23). In the present study, we examined gene expression differences between primary and metastatic tumors in nude mice. As shown in Table III, chemokine ligands and receptors were upregulated in metastatic SCC compared to primary tumors (Cxcl9, 37.5-fold; Ccl19, 27.2-fold; Cxcl13, 17.0-fold; Ccl8, 13.5-fold; Ccl5, 11.2-fold; Cxcl10, 7.6-fold; Ccr6, 5.7-fold; Cxcl12, 5.0-fold). Signal transduction genes also were upregulated in metastatic SCC (Ras related C3 botulinum substrate 2, 20.3-fold; signal transducer and activator of transcription 1, 12.6-fold; growth factor receptor bound protein 2, 6.3-fold; c-src tyrosine kinase, 5.8-fold). Growth factors and receptors were downregulated in metastatic SCC compared to primary tumors in nude mice (heparin binding EGF like growth factor, -5.0-fold; epidermal growth factor, -5.1-fold; smoothened homolog, -5.3-fold; v-erb-b2 erythroblastic leukemia viral oncogene, -6.5-fold; bone morphogenetic protein 1, -7.0-fold; amphiregulin, -7.8-fold; growth hormone receptor, -8.9-fold; Jagged 1, -10.1-fold; connective tissue growth factor, -24.8-fold). Epithelial differentiation markers were downregulated in metastatic SCC (vitamin D receptor, -6.7-fold; loricrin, -13.1-fold; keratin 8, -15.8-fold; desmoglein 3, -20.0-fold; keratin 18, -21.7-fold; keratin 16, -23.0-fold; suprabasin, -39.1-fold; desmoplakin, -45.0-fold; keratin 10, -50.5-fold; keratin 6a, -90.5-fold; keratin 5, -94.6-fold; small proline rich protein 2A, -110.9-fold; keratin 14, -123.1-fold). Peptidase inhibitor gene expression was down-regulated in metastatic SCC (serpin E1, -5.4-fold; serine peptidase inhibitor 5, -6.7-fold; tissue inhibitor of metalloproteinase 1, -12.0-fold). These gene expression changes are consistent with metastatic phenotype in the nude mouse model of SCC.

We wondered how lymphocytes in cervical lymph nodes responded to metastatic tumor cells. We performed differential gene expression on wild-type lymphocytes from non-tumor bearing mice and those in contact with metastatic tumor cells in lymph nodes. As shown in Table IV, the major class of genes upregulated in lymphocytes in contact with metastatic tumor cells was immunoglobin chains [immunoglobulin κ light chain 17-1A, 80.5-fold; immunoglobulin κ chain variable 21, 70.3-fold; Ig rearranged heavy chain mRNA VH-DH-JH1 region, 50.4-fold; Ig κ chain, 47.3-fold; immunoglobulin heavy chain γ polypeptide, 42.8-fold; immunoglobulin heavy chain J558 family, 33.1-fold; immunoglobulin κ chain variable 32, 29.3-fold; similar to immunoglobulin light chain, 23.1-fold; immunoglobulin κ chain constant region, 18.6-fold; immunoglobulin κ chain variable 28, 15.8-fold; immunoglobulin heavy chain 4 (serum IgG1), 11.3-fold; immunoglobulin λ chain, variable 1, 5.8-fold; immunoglobulin heavy chain 1a (serum IgG2a), 5.5-fold]. A number of other lymphocyte genes were upregulated in tumor cell exposed lymphocytes (lymphocyte antigen 9, 17.8-fold; pre-B lymphocyte gene 1, 11.3-fold; lymphocyte enhancer binding factor 1, 9.6-fold). Toll like receptor signaling was upregulated in tumor cell exposed lymphocytes (toll like receptor 4, 9.8-fold; toll like receptor 12, 6.3-fold; toll like receptor adaptor molecule 1, 6.2-fold; toll interleukin 1 receptor domain adaptor protein, 5.2-fold). Cell death genes were upregulated in tumor cell exposed lymphocytes (BH3 interacting domain death agonist, 11.2-fold; programmed cell death 1, 10.3-fold; caspase 7, 7.0-fold; caspase recruitment domain 4, 5.7-fold). A number of lymphocyte survival factors were downregulated in tumor cell exposed lymphocytes (BCL2 like 12, -20.2-fold; myeloproliferative leukemia virus oncogene, -19.1-fold; plasmacytoma expressed transcript 2, -14.2-fold; B-cell CLL/lymphoma 7A, -14.1-fold; B cell leukemia/lymphoma 11B, -11.3-fold; early B cell factor 3, -8.3-fold; pre B cell leukemia transcription factor 4, -8.2-fold; B cell translocation gene 4, -7.1-fold; pre B cell leukemia transcription factor 1, -6.1-fold; T cell lymphoma invasion and metastasis 2, -5.9-fold; inhibitor of Bruton agammaglobulinemia tyrosine kinase, -5.8-fold; B cell CLL/lymphoma 9 like, -5.4-fold; B cell leukemia/lymphoma 2 related protein A1a, -5.1-fold). These results indicate that lymphocytes react to metastatic tumor cells by upregulating immunoglobin gene expression but are prone to apoptosis via decreased expression of survival factors and upregulation of cell death genes.

Table II. Gene expression changes between metastatic SCC in wild-type and nude mice (2144 genes).

Accession	Symbol	Gene name	Fold change
AW495875	Ptp4a2	Protein tyrosine phosphatase 4a2	26.6
AI447325	Pip5k2b	Phosphatidylinositol-4-phosphate 5-kinase, type II, beta	25.9
AU022700	Arnt	Aryl hydrocarbon receptor nuclear translocator	23.3
BB636266	Nlk	Nemo like kinase	21.8
NM_021518	Rab2	RAB2, member RAS oncogene family	17.0
BE446893	Igfbp7	Insulin-like growth factor binding protein 7	15.1
BB543291	Ccrl1	Chemokine (C-C motif) receptor-like 1	13.5
AI451488	Csnk1g1	Casein kinase 1, gamma 1	10.5
AK017392	Ppp1cb	Protein phosphatase 1, catalytic subunit, beta isoform	9.4
BE954012	Terf2	Telomeric repeat binding factor 2	9.2
AI642132	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	9.0
AV257260	Plcd4	Phospholipase C, delta 4	7.5
AV367068	Dhh	Desert hedgehog	7.4
BB667902	Jmjd1c	Jumonji domain containing 1C	7.4
BB790825	Efna2	Ephrin A2	6.9
BI151406	Ngfr	Nerve growth factor receptor	6.9
BB296763	Tgfn2	Transforming growth factor, beta 2	6.4
NM_007939	Epha8	Eph receptor A8	6.3
BM943059	Pten	Phosphatase and tensin homolog	6.2
BB709552	Fgf4	Fibroblast growth factor 4	6.2
BB645478	Tnks	Tankyrase	6.0
BG228765	Jmjd3	Jumonji domain containing 3	5.8
BC003806	Stat3	Signal transducer and activator of transcription 3	5.5
NM_010426	Foxf1a	Forkhead box F1a	5.5
X99143	Krt2-10	Keratin complex 2, basic, gene 10	5.4
AF075436	Trp63	Transformation related protein 63	5.4
C80147	Hdgf	Hepatoma-derived growth factor	5.3
AU020229	Fzd3	Frizzled homolog 3 (Drosophila)	5.2
BM238599	Vcl	Vinculin	5.2
BF467164	Mta1	Metastasis associated 1	5.1
NM_028227	Brap	BRCA1 associated protein	-5.1
NM_009107	Rxrg	Retinoid X receptor gamma	-5.2
NM_008239	Foxq1	Forkhead box Q1	-5.6
AB013440	Dll3	Delta-like 3 (Drosophila)	-5.8
AJ318863	Ccrl2	Chemokine (C-C motif) receptor-like 2	-5.9
BM122502	Jund1	Jun proto-oncogene related gene d1	-6.1
AA880220	Jag1	Jagged 1	-6.2
BM217698	Tgfbr1	Transforming growth factor, beta receptor I	-6.4
M64429	Braf	Braf transforming gene	-6.5
AI507307	Sbsn	Suprabasin	-6.6
BB475090	Crebbp	CREB binding protein	-6.8
NM_009518	Wnt10a	Wingless related MMTV integration site 10a	-6.9
AI428101	Sox4	SRY-box containing gene 4	-7.0
BB367420	Rbbp6	Retinoblastoma binding protein 6	-7.0
BB284583	Itga4	Integrin alpha 4	-7.1
NM_008644	Muc10	Mucin 10, submandibular gland salivary mucin	-7.4
NM_009829	Ccnd2	Cyclin D2	-7.5
BM947855	Plk3	Polo-like kinase 3 (Drosophila)	-7.5
AV297961	Dsp	Desmoplakin	-8.1
AK002609	Sirt5	Sirtuin 5	-8.8
AK011603	Bcas	Breast carcinoma amplified sequence 3	-8.8
BB376407	Jarid1a	Jumonji, AT rich interactive domain 1A (Rbp2 like)	-9.0
NM_033573	Prcc	Papillary renal cell carcinoma (translocation-associated)	-9.2
AF146523	Malat1	Metastasis associated lung adenocarcinoma transcript 1	-9.9
BB362489	Jarid1c	Jumonji, AT rich interactive domain 1C (Rbp2 like)	-10.3
BC027242	Vav3	Vav3 oncogene	-10.4
BC003261	Aurkb	Aurora kinase B	-12.3
BE986745	Lats2	Large tumor suppressor 2	-12.7
NM_010703	Lef1	Lymphoid enhancer factor 1	-12.7
BF783030	Fzd4	Frizzled homolog 4 (Drosophila)	-12.7
NM_009875	Cdkn1b	Cyclin-dependent kinase inhibitor 1B (p27)	-14.0
M80360	Msh3	MutS homolog 3 (E. coli)	-24.7
NM_033597	Myb	Myeloblastosis oncogene	-57.8

Table III. Gene expression changes between primary and metastatic SCC in nude mice (1205 genes).

Accession	Symbol	Gene name	Fold change
BC014718	Dnase	Deoxyribonuclease I	70.4
NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	37.5
NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	27.2
NM_009008	Rac2	RAS-related C3 botulinum substrate 2	20.3
AF030636	Cxcl13	Chemokine (C-X-C motif) ligand 13	17.0
NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	13.5
AW214029	Stat1	Signal transducer and activator of transcription 1	12.6
NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	11.2
NM_008404	Itgb2	Integrin beta 2	8.5
BE685667	Ccnd3	Cyclin D3	8.1
NM_009515	Was	Wiskott-Aldrich syndrome homolog (human)	7.6
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	7.6
BG064712	Grb2	Growth factor receptor bound protein 2	6.3
BG094076	Csk	C-src tyrosine kinase	5.8
NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	5.7
BC016492	Tgm2	Transglutaminase 2, C polypeptide	5.5
BM220820	Foxp1	Forkhead box P1	5.4
BC006640	Cxcl12	Chemokine (C-X-C motif) ligand 12	5.0
L07264	Hbegf	Heparin-binding EGF-like growth factor	-5.0
AV369812	Egfr	Epidermal growth factor receptor	-5.1
BB543979	Itgb5	Integrin beta 5	-5.1
BB364488	Foxo3a	Forkhead box O3a	-5.3
AW555326	Smo	Smoothened homolog (Drosophila)	-5.3
NM_008871	Serpine1	Serine (or cysteine) peptidase inhibitor, clade E, 1	-5.4
AV074236	S100a16	S100 calcium binding protein A16	-5.4
AF059567	Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15)	-5.6
NM_007631	Ccnd1	Cyclin D1	-5.7
NM_013599	Mmp9	Matrix metallopeptidase 9	-5.8
BC027242	Vav3	Vav 3 oncogene	-5.9
BC004663	Dsc2	Desmocollin 2	-5.9
BF147716	Mmp2	Matrix metallopeptidase 2	-6.2
BB759833	Foxc1	Forkhead box C1	-6.3
NM_009821	Runx1	Runt related transcription factor 1	-6.4
X66083	Cd44	CD44 antigen	-6.5
BF140685	Erbb3	V-erb-b2 erythroblastic leukemia viral oncogene	-6.5
AV241297	Spink5	Serine peptidase inhibitor, Kazal type 5	-6.7
AV290079	Vdr	Vitamin D receptor	-6.7
NM_007393	Actb	Actin, beta, cytoplasmic	-6.9
BI465857	Klf5	Kruppel-like factor 5	-6.9
L24755	Bmp1	Bone morphogenetic protein 1	-7.0
NM_013496	Crabp1	Cellular retinoic acid binding protein I	-7.1
BQ173967	Efna5	Ephrin A5	-7.5
NM_009704	Areg	Amphiregulin	-7.8
NM_010284	Ghr	Growth hormone receptor	-8.9
NM_007913	Egr1	Early growth response 1	-9.1
AV359819	Jag1	Jagged 1	-10.1
BG970109	Lamb1-1	Laminin B1 subunit 1	-10.2
BC024958	Sox9	SRY-box containing gene 9	-10.3
AV026617	Fos	FBJ osteosarcoma oncogene	-11.8
BC008107	Timp1	Tissue inhibitor of metalloproteinase 1	-12.0
NM_008508	Lor	Loricrin	-13.1
BM935811	Itga6	Integrin alpha 6	-14.3
M21836	Krt2-8	Keratin complex 2, basic, gene 8	-15.8
BB040443	Snai2	Snail homolog 2 (Drosophila)	-19.6
AV229522	Dsg3	Desmoglein 3	-20.0
NM_010664	Krt1-18	Keratin complex 1, acidic, gene 18	-21.7
NM_008470	Krt1-16	Keratin complex 1, acidic, gene 16	-23.0
NM_010217	Ctgf	Connective tissue growth factor	-24.8
AI844734	Sbsn	Suprabasin	-39.1
AV297961	Dsp	Desmoplakin	-45.0
AK014360	Krt1-10	Keratin complex 1, acidic, gene 10	-50.5
NM_008476	Krt2-6a	Keratin complex 2, basic, gene 6a	-90.5
BC006780	Krt2-5	Keratin complex 2, basic, gene 5	-94.6
AV371678	Sprr2a	Small proline-rich protein 2A	-110.9
BC011074	Krt1-14	Keratin complex 1, acidic, gene 14	-123.1

Table IV. Gene expression changes between normal node and metastatic SCC node lymphocytes (840 genes).

Accession	Symbol	Gene name	Fold change
L41881	LOC213481	Immunoglobulin kappa light chain 17-1A	80.5
Z95478	Igk-V1	Immunoglobulin kappa chain variable 21 (V21)	70.3
Z95476	-	Ig rearranged heavy chain mRNA VH-DH-JH1 region	50.4
U29768	IgM	Ig kappa chain	47.3
S69212	Ighg	Immunoglobulin heavy chain (gamma polypeptide)	42.8
AF065324	Igh-VJ558	Immunoglobulin heavy chain (J558 family)	33.1
NM_007646	Cd38	CD38 antigen	32.8
U25103	Igk-V32	Immunoglobulin kappa chain variable 32 (V32)	29.3
AF099052	Ccl20	Chemokine (C-C motif) ligand 20	23.7
U55641	-	Similar to immunoglobulin light chain	23.1
BF301241	Igk-C	Immunoglobulin kappa chain, constant region	18.6
NM_008534	Ly9	Lymphocyte antigen 9	17.8
AB007986	Igk-V28	Immunoglobulin kappa chain variable 28 (V28)	15.8
NM_010824	Mpo	Myeloperoxidase	13.8
NM_008920	Prg2	Proteoglycan 2, bone marrow	11.8
NM_016982	Vpreb1	Pre-B lymphocyte gene 1	11.3
AF466769	Igh-4	Immunoglobulin heavy chain 4 (serum IgG1)	11.3
NM_007544	Bid	BH3 interacting domain death agonist	11.2
NM_008798	Pdcd	Programmed cell death 1	10.3
AF185285	Tlr4	Toll-like receptor 4	9.8
NM_010703	Lef1	Lymphoid enhancer binding factor 1	9.6
NM_008840	Pik3cd	Phosphatidylinositol 3-kinase catalytic delta polypeptide	9.5
AF232024	Ly6i	Lymphocyte antigen 6 complex, locus I	9.4
NM_009892	Chi3l3	Chitinase 3-like 3	9.0
NM_007782	Csf3r	Colony stimulating factor 3 receptor (granulocyte)	8.2
NM_010780	Mcpt5	Mast cell protease 5	7.3
U67321	Casp7	Caspase 7	7.0
NM_011093	Pira1	Paired-Ig-like receptor A1	6.4
NM_010376	H13	Histocompatibility 13	6.4
NM_007572	C1qa	Complement component 1, q subcomponent, alpha	6.3
BB745017	Tlr12	Toll-like receptor 12	6.3
BB452539	Ticam1	Toll-like receptor adaptor molecule 1	6.2
NM_023517	Tnfsf13	Tumor necrosis factor (ligand) superfamily, member 13	6.2
BG072012	Thpok	T helper-inducing POZ/Krueppel factor	6.2
BB525754	Ikbkb	Inhibitor of kappaB kinase beta	5.9
BI676554	Igl-VI	Immunoglobulin lambda chain, variable 1	5.8
BB138330	Card4	Caspase recruitment domain 4	5.7
BC018365	Igh-1a	Immunoglobulin heavy chain 1a (serum IgG2a)	5.5
BC010602	H2-Q1	Histocompatibility 2, Q region locus 1	5.4
BB800282	Pfc	Properdin factor, complement	5.2
NM_054096	Tirap	Toll-interleukin 1 receptor domain adaptor protein	5.2
NM_008694	Ngp	Neutrophilic granule protein	5.1
AF240358	Clec4n	C-type lectin domain family 4, member n	5.1
BB667813	Mlf1ip	Myeloid leukemia factor 1 interacting protein	-5.0
AV245981	Igsf1	Immunoglobulin superfamily, member 1	-5.0
BI466363	Brca1	Breast cancer 1	-5.0
NM_009337	Tcl1	T-cell lymphoma breakpoint 1	-5.0
AF342896	Klrb1d	Killer cell lectin-like receptor subfamily B member 1D	-5.1
NM_010416	Hemt1	Hematopoietic cell transcript 1	-5.1
AF289078	Nfatc2	Nuclear factor of activated T-cells, cytoplasmic, 2	-5.1
AI326167	Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	-5.1
AA119055	Tcrg-V4	T-cell receptor gamma, variable 6	-5.2
AV294178	Klrb1d	Killer cell lectin-like receptor subfamily B member 1D	-5.2
X60958	Cd80	CD80 antigen	-5.2
AI481991	H2-BI	Histocompatibility 2	-5.3
BF464707	Bcl9l	B-cell CLL/lymphoma 9-like	-5.4
C81413	Igbp1	Immunoglobulin (CD79A) binding protein 1	-5.6
X03052	H2-T18	Histocompatibility 2, T region locus 18	-5.7
BE981385	Ibtk	Inhibitor of Bruton agammaglobulinemia tyrosine kinase	-5.8
AY059394	Igsf4c	Immunoglobulin superfamily, member 4C	-5.9
AI552177	Tiam2	T-cell lymphoma invasion and metastasis 2	-5.9
X03019	Csf2	Colony stimulating factor 2 (granulocyte-macrophage)	-6.0
NM_008783	Pbx1	Pre B-cell leukemia transcription factor 1	-6.1
AI847494	Leng9	Leukocyte receptor cluster (LRC) member 9	-6.1

Table IV. Continued.

Accession	Symbol	Gene name	Fold change
M16810	H2-DI	Histocompatibility 2, D region locus 1	-6.4
AW259474	Ighmbp2	Immunoglobulin mu binding protein 2	-6.6
BB333386	Igsf4d	Immunoglobulin superfamily, member 4	-6.7
NM_008463	Klra5	Killer cell lectin-like receptor, subfamily A, member 5	-6.9
NM_010702	Lect2	Leukocyte cell-derived chemotaxin 2	-7.0
BM225534	Btg4	B-cell translocation gene 4	-7.1
BB155514	-	H-12.C4 T cell receptor alpha chain (V alpha 13 family)	-7.3
BM941868	Csf2ra	Colony stimulating factor 2 receptor, alpha	-7.9
NM_030555	Pbx4	Pre-B-cell leukemia transcription factor 4	-8.2
AV244034	Ebf3	Early B-cell factor 3	-8.3
BM877183	Bcl11b	B-cell leukemia/lymphoma 11B	-11.3
BC017640	Bcl7a	B-cell CLL/lymphoma 7A	-14.1
NM_008821	Pet2	Plasmacytoma expressed transcript 2	-14.2
NM_010823	Mpl	Myeloproliferative leukemia virus oncogene	-19.1
AK013411	Bcl2l12	BCL2-like 12 (proline rich)	-20.2

Discussion

Our results indicate that decreased T lymphocyte function results in decreased tumor latency and increased numbers of tumors. These results suggest that T lymphocytes are key to elimination of tumorigenic clones. There is a large body of evidence that cytotoxic T lymphocytes are involved in the clinical course of cancer (reviewed in ref. 25). A variety of stimuli have been proposed to activate the immune response in cancer, including genotoxic stress induced by carcinogen exposure (reviewed in ref. 26). In early stage tongue cancer, tumors were infiltrated by cytotoxic T lymphocytes and natural killer cells (27). No intraepithelial tumor cells expressed the proliferation marker Ki-67 but did express the inhibitory receptor PD-1. These results indicate that lymphocytes which interact with tumor cells become suppressed or inactivated.

It was recently demonstrated in a mouse model that tumors do not escape recognition but induce immune tolerance (28). Vaccinated mice remained tumor-free but naïve mice developed a progressively growing tumor. Despite specific recognition by T lymphocytes, tumors did not lose immunogenicity and were rejected when transplanted to immunocompetent recipients. Tumor induced tolerance was associated with expansion of non-functional T lymphocytes. A variety of tumor derived factors contribute to immunosuppressive networks including VEGF, interleukin 10, TGFß, prostaglandin E, and Fas ligand (reviewed in ref. 29). These factors may be active at distant sites, thereby promoting invasion and metastasis. VEGF recruits bone marrow cells to become immature dendritic cells and macrophages. These immature cells may suppress roving dendritic cells and T lymphocytes. Soluble Fas ligand may help tumor cells escape lysis by cytotoxic T lymphocytes and natural killer cells by inducing apoptosis in the immune cells. In a mouse chemical carcinogenesis model, it was demonstrated that equilibrium was mechanistically distinguishable from elimination and escape (30). Neoplastic cells in this model were transformed but proliferated poorly *in vivo*. These cells were unedited in equilibrium but are edited when they escape immune control and become tumors. These results highlight the complex interplay between cancer cells and the immune system.

We determined that the largest class of upregulated genes in lymphocytes exposed to metastatic HNSCC was immunoglobulin genes. The antigens arising during the course of the disease may derive from somatic mutations in normal gene products, tumor specific antigens shared among patients with a particular type of cancer, normal tissue specific gene products, and normal proteins predominantly expressed by tumors (31-34). However, lymphocytes exposed to metastatic tumor cells were prone to apoptosis via decreased expression of survival factors and upregulation of cell death genes. These results suggest that lymphocytes are primed for elimination by interaction with metastatic tumor cells.

Primary and metastatic tumors arising in nude mice were significantly different from those in wild-type animals. Differentiation of HNSCC from nude mice is shifted towards secretory genes compared to those arising in wild-type mice. Comparison of gene expression in metastatic tumors in these two models indicated that metastatic SCC from nude mice may be more biologically aggressive than their counterparts in wild-type mice. However, these differences were not apparent in the number of lymph nodes containing metastatic tumor cells. Expression of cell cycle regulatory proteins examined by immunohistochemistry revealed important similarities with human HNSCC characterized in our previously published study (24). Future studies will determine how these signaling pathways regulate metastatic phenotype and immune escape in HNSCC.

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