Establishment and characterization of an orthotopic sinonasal squamous cell carcinoma mouse model

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Accepted 29 June 2014
Published online 25 September 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/hed.23832

ABSTRACT: *Background.* Despite therapeutic improvements, patients with sinonasal squamous cell carcinoma (SCC) still face an unfavorable prognosis and there is great need for alternative treatments.

Methods. SCCNC4 cells, originally derived from a T2N1M0 primary and untreated sinonasal SCC, were inoculated in the maxillary sinus of immunodeficient mice. Histology, invasive behavior, and genetic features were evaluated and compared with the original primary tumor.

Results. The mice developed tumors that invaded bone, surrounding tissues, and brain, showing the same poor differentiation as the original primary tumor. Genetic analysis revealed an almost identical pattern of copy number alterations, except for the deletion and loss of expression of the genes *CDKN2A* and *PTEN*.

Conclusion. This article shows the feasibility of an orthotopic mouse model of SCC of the maxillary sinus. Completed by genome-wide genetic profiling data, this model will be useful for preclinical testing of specific gene-targeted anticancer drugs. © 2014 Wiley Periodicals, Inc. *Head Neck* **37:** 1769–1775, 2015

KEY WORDS: sinonasal squamous cell carcinoma, orthotopic mouse model, tumor invasion, immunohistochemistry, genetic profiling

INTRODUCTION

Malignant sinonasal tumors represent 3% of all head and neck neoplasms.[1] They are etiologically related to occupational exposure to wood, leather dust, and other industrial substances.[2] These tumors arise in a complex anatomic area, close to structures, such as the eyes and the brain, which is of special relevance for treatment because mutilation and esthetic deformities are difficult to avoid. The major sinonasal epithelial tumor type, representing approximately 80% of all sinonasal tumors, is sinonasal squamous cell carcinoma (SCC).[1] The most frequent location is the maxillary sinus (50% to 80%) followed by the nasal cavity, however, the exact origin of advanced sinonasal tumors is often difficult to determine. Most patients present with locally advanced stage disease. Surgical resection and postoperative radiation therapy is the standard therapeutic approach, in some cases, followed by chemotherapy. The prognosis of patients with sinonasal SCC is still poor, with a 5-year survival rate of approximately 40% and local recurrence being the main cause of death.[3]

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Contract grant sponsor: This work was supported by grants PI08-1599, PI11-929, and EMER07–048 of Fondos de Investigación Sanitaria (FIS) and RD12/0036/0015 of Red Temática de Investigación Cooperativa en Cáncer (RTICC), Spain

The fact that sinonasal SCC are rare tumors, with an incidence of approximately 1 case per 100,000 inhabitants per year,[4] has hampered the testing of alternative treatment strategies. There are very few specific clinical trials, and chemotherapy or chemoradiotherapy protocols are usually administered by extrapolation of approaches in similar but more frequent tumors, such as laryngeal SCC. Genetic profiling and in vitro cell lines and animal models are important tools for the testing of future targeted anticancer therapies, taking advantage of the experience in other more frequent tumors with the same druggable genetic targets.

In recent years, our knowledge on the genetic changes in sinonasal tumors has increased; however, few animal models have been developed so far for preclinical testing of alternative therapies for sinonasal tumors. The animal model that best mimics the clinical behavior of human tumors is obtained by orthotopic xenografting. The purpose of this study was to develop a reliable orthotopic mouse model for sinonasal SCC using a validated sinonasal SCC cell line. The results were evaluated by comparing the histology, invasive behavior, and the protein expression and genetic aberration profiles of the xenograft tumors with the original primary tumor from which the cell line was derived.

MATERIALS AND METHODS

Cell line

Human cell line SCCNC4 was established in our laboratory from a T2N1M0 poorly differentiated SCC of the right maxillary sinus.[5] The patient, a 73-year-old woman,

underwent a right medial maxillectomy and a right modified radical neck dissection and subsequently received postoperative radiotherapy. A local recurrence developed 5 months later and the patient was treated by total maxillectomy. However, after 10 months, the patient had an unresectable local recurrence and died at 22 months after diagnosis. Written informed consent was obtained from the patient and experiments were performed in accordance with the approved guidelines of the Ethics Committee of the Hospital Universitario Central de Asturias. At the time of writing this article, SCCNC4 had been in culture for over 18 months and had been passaged more than 100 times; for xenografting the nude mice, cells between passages 50 and 69 were used. SCCNC4 has been described and genetically characterized by García–Inclán et al[5] and used in previous studies on centromeric methylation and anticancer drug testing.[5–7] SCCNC4 was maintained as adherent monolayer cultures in minimal essential medium culture, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 200 μg/mL streptomycin, 2 m*M* L-glutamine and nonessential amino acids 100 μM (PAA Laboratories GmbH, Pasching, Austria), and incubated in 5% CO_2 at 37°C. Possible contamination by mycoplasma was checked using the LONZA MycoAlert Mycoplasma Detection Kit (LONZA, Rockland, ME). In preparation for the inoculation of the mice, cells were grown in several 75 cm culture flasks to a 70% to 80% confluence. Then the cells were harvested by trypsinization, collected in 10 mL tubes, centrifuged 5 minutes at 1000 rpm, and the supernatant was discarded. The cells were washed by resuspending the pellet in 10 mL phosphate-buffered saline and centrifuging 5 minutes at 1000 rpm. Finally, the pellets were resuspended at the right concentration in phosphate-buffered saline, collected in 1 Eppendorf tube, and kept on ice until the moment of inoculation.

Animal care

Male athymic nu/nu mice (ages 4–5 weeks; Charles River Laboratories, Barcelona, Spain) were maintained in a pathogen-free environment and fed irradiated mouse chow (Panlab S.L.U., Barcelona, Spain) and autoclaved water, in accord with current regulations and standards for animal care and use of the University of Oviedo.

Implantation of tumor cells

All experimental procedures were performed in accordance with a protocol approved by the Ethics Committee for Medical Research and Animals Used in Experiments of the University of Oviedo. Before inoculation, the mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (75 g/Kg body weight) and medetomidine (0.5 mg/Kg body weight). In order to test the correct inoculation in the mucosa of the right maxillary sinus, 5 immunocompetent animals (Charles River Laboratories), were injected with 50 μL of methylene blue vital stain (Sigma–Aldrich, Madrid, Spain) using a 0.5 mL syringe of insulin with a 30 G needle (U-100 insulin micro-fine needle; BD Biosciences, San Jose, CA). The mice were killed with CO_2 20 minutes after inoculation and the heads were processed as described below. Using this experience, a total of 20 mice were inoculated with 60 μL of a suspension of SCCNC4 cells containing a total of 2,000,000 cells. The needle was introduced in the nasal cavity and then turned slightly toward the lateral wall of the nasal cavity, proceeding to inoculate the cells at the level of the medial wall of maxillary sinus. The mice were observed until the effects of anesthesia had resolved. Absence of a fluid leak from the nasal cavity confirmed a successful injection. The animals were checked daily for hypomotility, absence of grooming behavior, and body weight was measured twice a week to detect weight loss. In accordance with Animal Care and Use Guidelines of The University of Oviedo, mice would be euthanized with CO_2 when they lost more than 20% of body weight, had ulcerated tumors, or became moribund. In this study, all animals lived until the maximum time of 12 weeks, at which point they were killed.

Necropsy and tissue preparation

The full heads of the mice were divided into 1 median and 2 paramedian sagittal sections, fixed in a periodate-lysine-paraformaldehide solution for 24 hours, and decalcified in ethylenediaminetetraacetic acid for 6 days. Finally, all head samples were embedded in paraffin blocks. Serial cuts of 4 μm were made for hematoxylin-eosin staining to determine the tumor size and the degree of invasion into surrounding structures, and then evaluated by an experienced pathologist (S.F.). More tissue sections were prepared for use in immunohistochemical analyses. One mouse was used to obtain a fresh tumor sample for DNA extraction.

Immunohistochemistry

The following antibodies were used: anti-p53 clone DO-7 (DAKO, Glostrup, Denmark), anti-p16 clone E6H4 (CINTEC, MTM Laboratories, Madrid, Spain), anti-cyclin D1 clone DCS-6 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PTEN clone 6H2.1 (DAKO), anti-cortactin clone 30 (BD Biosciences), anti-Rb clone IF8 (Santa Cruz Biotechnology), anti-E-cadherin clone HECD-1 (Zymed Laboratories, San Francisco, CA), anti-c-myc clone C33 (Santa Cruz Biotechnology), and anti-EGFR clone 2–18C9 (DAKO). Immunohistochemistry was performed using an automatic staining workstation (DakoCytomation, DAKO) with the Envision system with diaminobenzidine chromogen used as the substrate. All immunostainings were evaluated by 2 independent observers (M.C. and S.F.). P16 immunostaining was scored as negative (0), weak to moderate staining (1+), or moderate to strong diffuse cytoplasmic and nuclear staining (2+). P53 immunostaining was evaluated as positive when >10% of the malignant cells showed nuclear staining. Epidermal growth factor receptor (EGFR) immunostaining was evaluated as positive when strong membranous and/or cytoplasmic staining was observed in at least 10% of tumor cells. PTEN was scored according to the percentage of positive cells (0: <1%; 1: 1% to 10%; 2: 11% to 50%; 3: 51% to 80%, and 4: >80%) and the intensity of staining (0: negative; 1: weak; 2: moderate; and 3: strong). The other immunostainings were scored as the percentage of stained cells: weak expression (score 1:

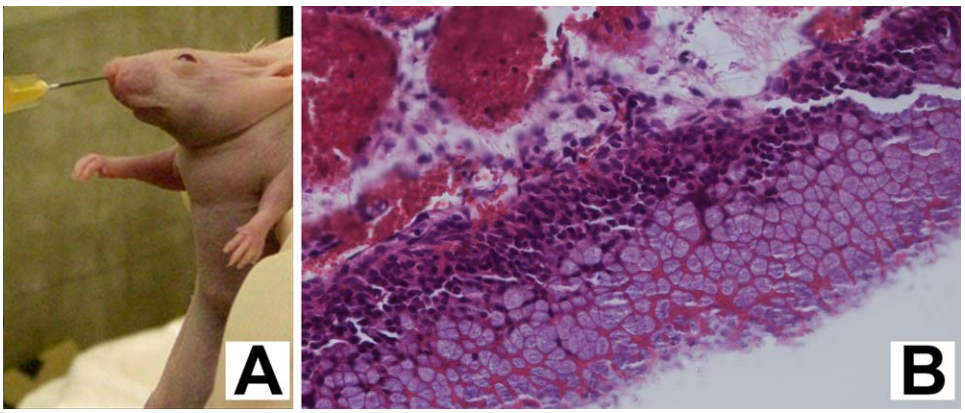

FIGURE 1. Inoculation of SCCNC4 cells in the maxillary sinus reached through the nasal cavity of the mouse (A). Tissue section of a normal mouse depicts methylene blue vital staining of apical cells of pseudostratified respiratory epithelium of the sinonasal cavity (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

0% to 35% of stained cells), moderate expression (score 2: 35% to 70% of stained cells), and strong expression (score 3: >70% of stained cells), and the intensity of the staining (score 1: weak; score 2: moderate; and score 3: strong).

Genetic characterization

Tumor DNA was extracted with Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) using fresh frozen material from cell line SCCNC4, one of the orthotopic xenograft tumors and from the primary tumor and peripheral blood lymphocytes of the patient from which the cell line had been derived. Microarray comparative genomic hybridization (CGH) analysis was performed, as described previously,[8] using a 180k oligonucleotide array (SurePrint G3 Human CGH Microarray Kit 4 × 180K; Agilent Technologies, Palo Alto, CA). Images were acquired using a Microarray scanner G2505B (Agilent Technologies). Analysis and data extraction were quantified using feature extraction software, version 9.1 (Agilent Technologies). Gains and losses were defined as deviations of 0.2 or more from log2 ratio = 0.0. High-level amplification was considered when at least 2 neighboring clones reached a log2 ratio of >2.0 and homozygous deletions were defined as 2 or more consecutive oligonucleotides with a log2 ratio of <-3. The possibility of copy number variations (rather than copy number alterations) was excluded by using normal DNA of the same patient as a reference. Multiplex ligation-dependent probe amplification (MLPA) was performed, as described in detail previously,[9] using kit P105 (MRC-Holland, Amsterdam, The Netherlands), containing 5 probes for CDKN2A, 11 for EGFR, 2 for ERBB2, 11 for PTEN, and 6 for TP53, apart from 8 reference probes. The polymerase chain reaction products were analyzed on an ABI Prism 3100 sequencer using GeneScan software version 3.7 (Applied Biosystems, Warrington, UK). For every gene, the relative copy number was calculated by dividing the average relative peak area of the tumor by the median relative peak area of the normal reference samples. Relative copy numbers values <0.8 were interpreted as losses, >1.2 as gains, and >2.0 as amplifications.

RESULTS

Orthotopic xenograft model

The inoculation of methylene blue vital stain into the lateral nasal wall by way of the nasal cavity of immunocompetent mice showed that orthotopic implantation is technically feasible. The staining was localized in the apical cytoplasm of ciliated respiratory epithelial cells of maxillary and ethmoid sinuses (Figure 1). Successful implantation of orthotopic tumors from SCCNC4 cells was achieved in 7 of 17 mice (41%). Three animals died of unknown causes 1 day after the injection of cells. Exophytic growing tumors were observed on the right side or on top of the nose in all 7 mice (Figure 2).

Histological and immunohistochemical analysis

Histopathological evaluation indicated poorly differentiated SCC in all xenograft tumors, similar to the original primary tumor (Figure 3). The tumors showed anterior and superior exophytic growth, 5 developed from the ethmoidal cavity and 2 from the right maxillary sinus. Bone destruction and invasion into surrounding tissues was observed, and in 2 cases also intracranial extension. Protein expression of cyclin D1, cortactin, pRb, E-cadherin, and EGFR, and absence of p53 expression was observed both in the primary tumor and in the orthotopic tumor. C-myc expression was stronger in the xenograft, both in intensity as in percentage positivity. Weak and moderate immunopositivity of p16 and PTEN, respectively, was present in the primary, but lost in the xenograft tumor (Table 1 and Figure 3).

Genetic characterization

Microarray CGH analysis showed an almost identical pattern of copy number alterations in primary tumor, cell line, and xenograft tumor. However, the amplitude of the gains and losses was higher in the cell line and in

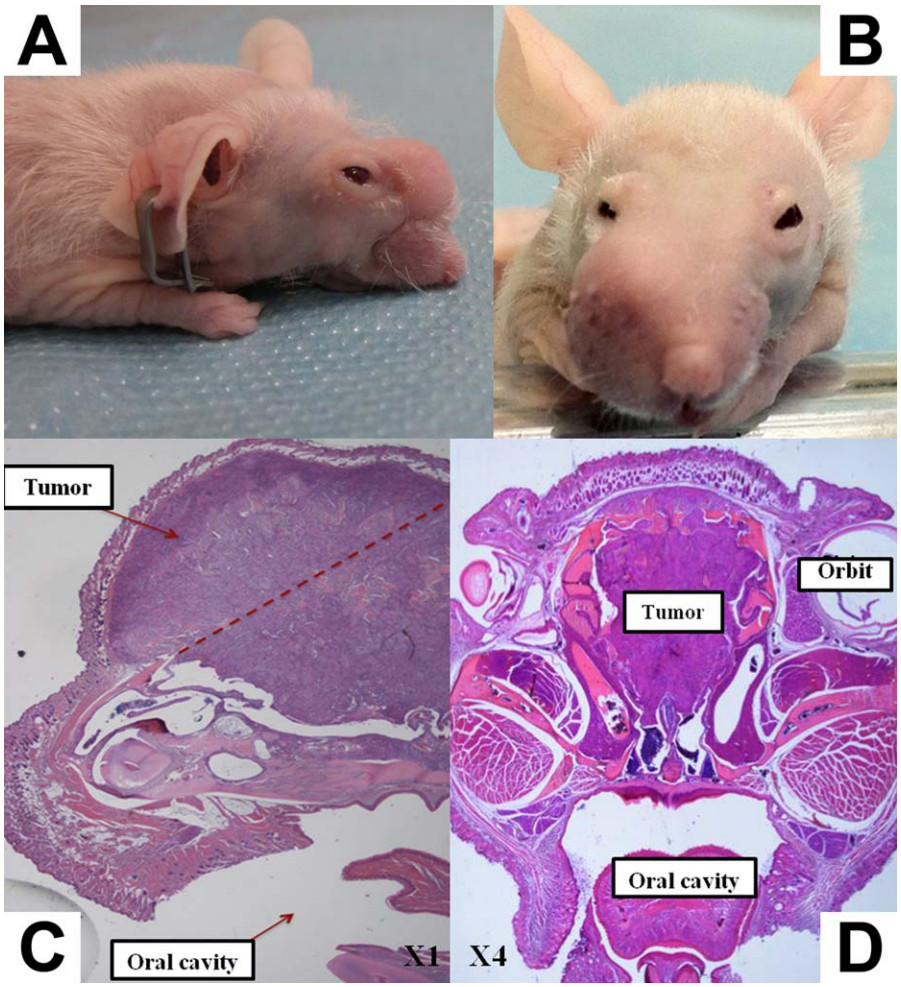

FIGURE 2. Orthotopic mouse model of sinonasal squamous cell carcinoma (SCC) of the maxillary sinus showing exophytic tumors growing on the right side and on top of the nose of the animals (A and B). Hematoxylin-eosin staining of a sagittal section through the cranial midline of the mouse showing the xenograft tumor localized in the right maxillary sinus with extension toward the cranial vault. The dotted arrow marks the normal margin of the head (original magnification ×4) (C). Hematoxylin-eosin staining of a frontal section showing the xenograft tumor between the orbits and occupying the ethmoid sinuses (original magnification ×4) (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

orthotopic primary tumor than in primary tumor sample, probably because of the contamination of the latter with normal cells, such as stroma and infiltrating lymphocytes. Two chromosomes showed different copy numbers: in the xenograft tumor, a loss was observed at chromosome arms 9p and 10q that were not present in the original primary tumor. MLPA analysis showed loss of 2 genes, *CDKN2A* localized at 9p21 and *PTEN* at 10q24 in the xenograft tumor, but not in the original primary tumor (Figure 4).

DISCUSSION

The clinical course of patients with sinonasal SCC depends on previous treatment, tumor stage, and invasion into surrounding structures, especially the brain.[10] Partly because of the unspecific clinical symptoms, patients with

TABLE 1. Protein expression of cancer-related genes in the original primary tumor and in the orthotopic xenograft.

		Primary tumor		Orthotopic xenograft	
Protein	Expression site	E	I	E	I
Cyclin-D1	Nucleus	3	2	2	2
Cortactin	Cytoplasm and membrane	3	3	3	3
PTEN	Cytoplasm and nucleus	4	2	0	0
Rb	Nucleus	2	2	2	1
E-cadherin	Cytoplasm and membrane	3	3	3	3
C-myc	Nucleus	1	1	2	2
p16	Nucleus	Positive 1+		Negative 0	
p53	Nucleus	Negative 0		Negative 0	
EGFR	Cytoplasm and membrane	Positive		Positive	

Abbreviations: E, extension, expressed as score of percentage of positive cells; I, intensity score (see Materials and Methods); EGFR, epidermal growth factor receptor.

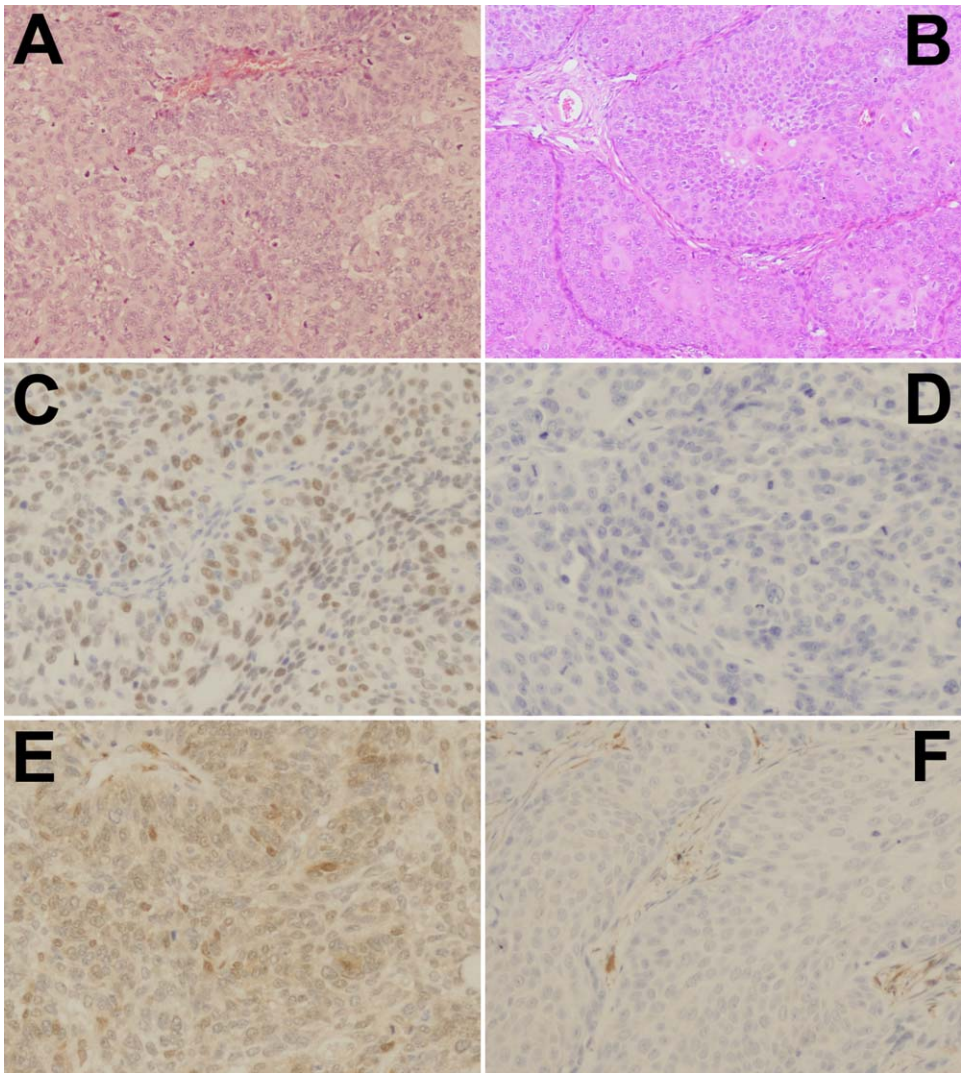

FIGURE 3. Hematoxylin-eosin staining of the original primary (A) and xenograft tumor (B), both showing poorly differentiated squamous cell carcinoma (SCC) (original magnification ×10). Weak p16 immunopositivity in the original primary tumor (C) and loss of p16 staining in the xenograft tumor (D) (original magnification ×40). Moderate PTEN expression in the primary tumor (E) and loss of PTEN staining in the xenograft tumor (F) (original magnification ×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sinonasal SCC often present with advanced stage tumors, and standard treatment (surgery and radiotherapy) would be greatly helped by additional chemotherapeutic options. The classical indication for chemotherapy in sinonasal malignancies is as palliative treatment (docetaxel, cisplatin, and fluorouracil) in locally advanced or metastatic tumors when surgery and radiotherapy are no longer effective.

Targeted anticancer therapy against specific molecular pathways could become a valuable alternative. Aberrant EGFR expression has been reported in 40% of sinonasal SCC, whereas KRAS and BRAF mutations do not occur.[11,12] The cell line SCCNC4, its primary tumor, and the xenograft tumor described in this study all showed EGFR copy number gain and protein expression. This means that targeted anti-EGFR therapy may be of benefit in sinonasal SCC, similar to the more prevalent and far more studied head and neck SCC. Other studies have indicated frequent alterations in NFkB, COX2, and FGFR1, that may also represent targets for molecular therapy, for example, solithromycin, dovitinib, and nonsteroidal anti-inflammatory drugs, respectively.[13,14]

In vitro and mouse models are important tools for the preclinical testing of such new treatment options. A considerable number of sinonasal tumor cell lines have been established in the past. However, only 4 have been derived from previously untreated, primary sinonasal SCC, and little is known on the genetic makeup of these cell lines.[15–18] Very recently, our group reported 6 new sinonasal SCC cell lines with different clinical (tumor stage, histological differentiation, and tobacco smoking status), functional (proliferation, invasion in matrigel), and genetic characteristics (TP53, EGFR, etc.) that reflect very well the clinical variety that is generally seen in sinonasal SCC.[5] These cell lines have been characterized by genome-wide genetic analyses. Although an estimated 15% to 20% of sinonasal SCC are suggested to be human papillomavirus (HPV)-positive,[19] none of the published cell lines represent this subset of sinonasal SCC.

Most mouse models use subcutaneous inoculation because it can be done reproducibly and the growing tumors are easy to detect and follow. However, they do not reproduce the patterns of invasion and metastasis of solid tumors, and, importantly, they do not show a drug

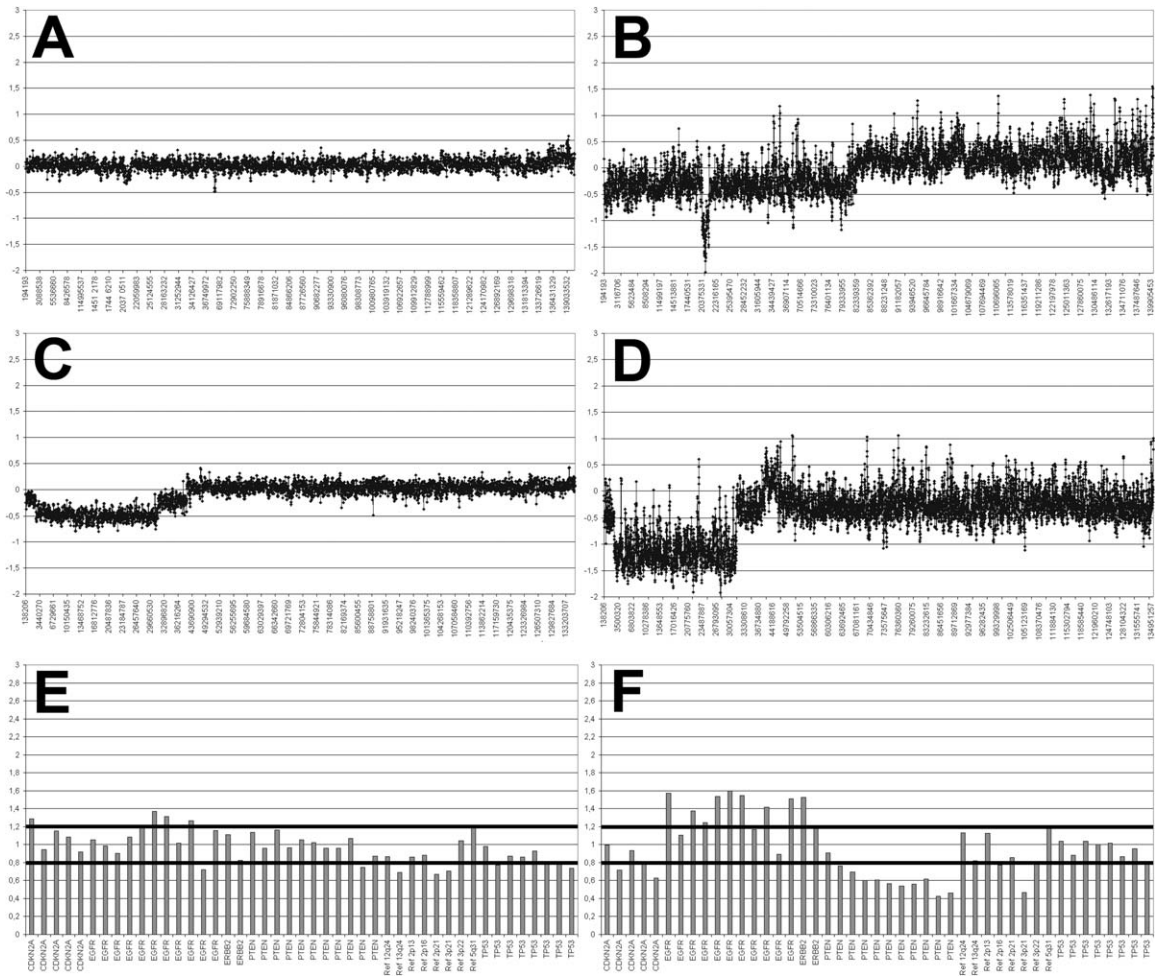

FIGURE 4. Microarray comparative genomic hybridization (CGH) analysis showing normal copy number of whole chromosome 9 in the primary tumor (A) and a loss of chromosome arm 9p in the xenograft tumor (B). Both tumors harbor a focal loss at band 9p21.3, but this does not include the gene *CDKN2A*. Microarray CGH analysis showing a loss of chromosome arm 10p and normal copy number of chromosome arm 10q in the primary tumor (C), whereas the xenograft tumor (D) harbors an additional loss of 10q11.21-qter and only a small region at 10q11 has a normal copy number. All microarray CGH data points are expressed as log2-ratios, ordered continuously from left to right according to the base pair positions. Multiplex ligation-dependent probe amplification (MLPA) analysis is in agreement with these findings; the primary tumor (E) shows a normal copy number for the genes *CDKN2A* (9p21) and *PTEN* (10q23), whereas the xenograft tumor (F) carries loss of both genes.

sensitivity comparable to human cancers of the same histology.[20] Orthotopic xenograft models may overcome these setbacks, however, in the case of sinonasal tumors, inoculation and mouse monitoring is technically challenging.

The first orthotopic sinonasal mouse model was described by Gelbard et al.[21] They implanted tumor cells in the maxillary sinus via transcutaneous injection underneath the infraorbital muscle groups or in the soft palate, and observed local invasion, intracranial extension, and lymph node or distant metastasis with both methods. Unfortunately, the applied cell lines were not originally derived from sinonasal tumors. Recently, an orthotopic skull base model using a cell line was established from a patient with undifferentiated carcinoma that had been treated previously with chemotherapy. Injecting the cells into the muscle of the soft palate, this model showed local invasion into muscle, bone, nerve, blood vessels, lymphatic vessels, and the brain.[22] The orthotopic sinonasal model presented in this article is the first one using a cell line derived from a previously untreated primary SCC of the maxillary sinus. The relatively low implantation rate (41%), compared to Gelbard et al,[21] may be related to the chosen approach of reaching the mouse maxillary sinus by way of the nasal cavity; it may be that the cell suspension was drained away and transported toward the pharynx. Leakage through the nose was never observed. Also, intrinsic cell line characteristics may play an important role in the success rate of implantation. Gelbard et al[21] used 2 aggressive tumor cell lines: DM14 is a highly metastatic clone of cell line TU167 of an SCC of the floor of the mouth, and ACC3 was, in fact, not an adenoid cystic carcinoma cell line, but HeLa.[23,24] Our cell line, SCCNC4, was derived from a T2N1M0 primary maxillary sinus SCC.

The model in this article manifested marked resemblance to the histology and the invasive behavior of the primary tumor from which the cell line was derived,

including extension into surrounding structures and bone invasion. Also, with regard to the genetic characteristics, the model carried most of the aberrations of the original primary tumor. Two exceptions were tumor suppressor genes *CDKN2A* encoding p16 and *PTEN*. In the original primary tumor, these 2 genes were present in a normal copy number and showed a weak and moderate protein expression. In the xenograft model, however, these genes showed a copy number deletion accompanied by loss of protein expression. The most probable explanation for the discrepancy is that the established cell line is the result of a selection process of a minor subclone from a genetically heterogeneous tumor that best adapted to the in vitro growth conditions. The gene copy number deletions of CDKN2A and PTEN indeed were also observed in the cell line. Similar genetic differences among primary tumor and xenograft have been reported before.[25] As p16 expression was weak in the primary tumor and absent in the orthotopic xenograft tumors, HPV analysis was not performed. It can be safely assumed that both primary and xenograft tumors are HPV-negative, because strong p16 immunopositivity is a prerequisite for HPV testing.[26]

In conclusion, this article shows the feasibility of an orthotopic mouse model of SCC of the maxillary sinus. Depositing the cells in the right anatomic location requires some experience as is shown by the fact that not all of the inoculated mice developed tumors. In the animals that did not develop tumors, it may be that the cell suspension was drained away and transported toward the pharynx. In this study, the mice were monitored by regular observation; however, a better method would be to examine the animals by MRI if such infrastructure is available to the scientist. Together with the genome-wide genetic profiling data, this model will be useful for the study of specific molecular pathways associated with tumor progression, as well as for the preclinical testing of specific gene-targeted anticancer drugs for sinonasal SCC.

Acknowledgment

The authors thank Eva Allonca for the immunohistochemical stainings.

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