Inducible Activation of Oncogenic K-ras Results in Tumor Formation in the Oral Cavity

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Abstract

Mouse models for cancer represent powerful tools to analyze the causal role of genetic alterations in cancer development. We have developed a novel mouse model that allows the focal activation of mutations in stratified epithelia. Using this system, we demonstrate that activation of an oncogenic K-rasG12D allele in the oral cavity of the mouse induces oral tumor formation. The lesions that develop in these mice are classified as benign squamous papillomas. Interestingly, these tumors exhibit changes in the expression pattern of keratins similar to those observed in human premalignant oral tumors, which are reflective of early stages of tumorigenesis. These results demonstrate a causal role for oncogenic K-ras in oral tumor development. The inducible nature of this model also makes it an ideal system to study cooperative interactions between mutations in oncogenes and tumor suppressor genes that are similar to those observed in human tumors. To our knowledge, this is the first reported inducible mouse model for oral cancer.

Introduction

Oral cancer ranks among the worst forms of cancer in mortality and morbidity. Although significant progress has been made in early detection, diagnosis, and treatment, the 5-year survival rates for patients with oral cancer have not improved in the past 25 years and remain ~50%. This high lethality is ascribed to the advanced state of malignancy exhibited by oral cancers when they are detected. However, when detected early, they can be cured in 80–90% of the cases. Understanding the molecular mechanisms involved in initiation and progression to malignancy of oral cancer will help to improve the prognosis of the disease and to develop novel therapeutic strategies. Genetic alterations commonly found in oral cancer are thought to have a causal role in the disease. Approximately 30% of all of the human tumors carry mutations in ras genes (1). Interestingly, a tremendous variation in the mutation rates of ras genes has been observed in human oral cancer. Although some studies have found ras mutations in ~20% of the patients from Western countries (2, 3), the consensus is that mutations in ras genes occur in ~5% of the cases (4). However, ras mutations are found in >50% of the patients from south Asian populations (5), where oral cancer accounts for ~50% of all of the cancers and is associated with the habit of chewing tobacco. Although the difference in mutation rates may be related to exposure to different carcinogens, these data suggest that acquisition of ras mutations may predispose to oral cancer. Previous reports have used conditional transgenic mouse models to determine the causal role of K-ras mutations in tumor development in different tissues. These models have proven that activation of an oncogenic K-ras allele can initiate a variety of cancers, including lung, glioblastoma, and pancreatic cancer (6–8). We have developed a novel inducible mouse model that allows oncogene activation and/or tumor suppressor inactivation exclusively in stratified epithelia of the oral cavity. To determine whether ras mutations can induce oral cancer formation, we used this system to induce the activation of an oncogenic K-ras allele in the oral cavity. Our results demonstrate that oncogenic K-ras has a causal role in oral tumor development.

Materials and Methods

Mouse Generation. The generation of K14.CrePR1 mice has been described previously (9). K5.Cre*PR1 mice were generated by cloning the Cre*PR1 cDNA (10) into a vector containing the bovine keratin (K) 5 promoter (11). Tyler Jacks and David Tuveson provided Lox-stop-Lox (LSL)-K-rasG12D mice (6). Mice were genotyped for the presence of the LSL-K-rasG12D allele as described previously (6). Primer sequence and PCR conditions for detection of K14.CrePR1 and K5.Cre*PR1 transgenes are available on request. Excision of the stop cassette from the LSL-K-rasG12D allele in R1486-treated tissue was determined as described previously (6).

Histologic Analysis. Oral tissues or tumors were fixed in 10% neutral-buffered formalin at room temperature overnight. Samples then were transferred to 75% ethanol, embedded in paraffin, sectioned, and stained with H&E.

Western Blot Analysis. Total protein lysates of oral tissue and tumors were prepared by homogenization in protein loading buffer. Equal amounts of protein from each sample were separated by electrophoresis on an SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes. Western blot analysis was performed using specific antibodies for K-ras2A (sc-522) and actin (sc-1616) from Santa Cruz Biotechnology (Santa Cruz, CA) and the ECL-Plus chemiluminescent detection system (Amersham, Piscataway, NJ).

Reverse Transcription-PCR and Restriction Fragment Length Polymorphism Analysis. Total RNA was isolated from oral tissue and tumors according to the TRIzol reagent (Invitrogen, Carlsbad, CA) protocol. cDNA was generated from RNA samples by reverse transcription using the One-Step Reverse Transcription-PCR kit (Qiagen, Valencia, CA). Total RNA from oral tissue and tumors was amplified with the One-Step Reverse Transcription-PCR kit (Qiagen, Valencia, CA), using primers K-ras15S, 5'-GAGTTCTCCAGGACCCGACGCGAAGGA and K-ras1A, 5'-CCTACAGGCCCGCCGGGATCGCGAAGGA. RNA expression of wild-type K-ras and mutant K-rasG12D was determined by digestion of 5 μl of the reverse transcription-PCR products with HindIII for 1 h at 37°C. The restriction products were resolved in a 2% agarose gel. The mutant K-rasG12D allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele. Therefore, digestion of the 448-bp product generates 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type PCR product.

Immunofluorescence. Biopsies from oral tissue and tumors were embedded and frozen in OCT compound at ~70°C, sectioned, and subjected to double-label immunofluorescence using polyclonal antibodies for K13, K6, and K14 (12). OCT sections were fixed in cold 75% acetone-25% ethanol for 15 min and then blocked in 5% BSA and incubated with the primary antibodies overnight at room temperature. After washing with PBS, the sections were incubated for 45 min with secondary antibodies conjugated to 488- or 594-Alexa Fluor dyes (Molecular Probes, Eugene, OR). Images were captured with the Nikon Eclipse E600 microscope (Tokyo, Japan) using the MetaVue soft-
were taken at age 5 months and analyzed for and biopsies of oral tissue from bigenic K5.CrePR1/ROSA26 mice exposed to RU486 again. Mice were allowed to develop to adulthood, /H9262 was induced K5.CrePR1 mice with ROSA26 reporter mice (15). Activation of Cre stratified epithelia of the oral cavity in these mice, we crossed K5.CrePR1 with K14 promoter, which are expressed in the basal cells of stratified generated mice that express CrePR1 under the control of the K5 or /H9252/g/kg RU486 at 14.5 days of gestation. After birth, litters were never exposed to RU486. This fusion protein (CrePR1) is sequestered in the cytoplasm. After activation with RU486, CrePR1 translocates to the nucleus, where it mediates the excision of DNA sequences flanked by LoxP sites (13). We generated mice that express CrePR1 under the control of the K5 or K14 promoter, which are expressed in the basal cells of stratified epithelia (9, 14). To determine whether Cre activation occurs in the stratified epithelia of the oral cavity in these mice, we crossed K5.CrePR1 mice with ROSA26 reporter mice (15). Activation of Cre was induced in utero after injection of pregnant females with 100 μg/kg RU486 at 14.5 days of gestation. After birth, litters were never exposed to RU486 again. Mice were allowed to develop to adulthood, and biopsies of oral tissue from bigenic K5.CrePR1/ROSA26 mice were taken at age 5 months and analyzed for β-galactosidase (β-Gal) activity. We found β-Gal staining throughout the oral epithelia (Fig. 1A). Because the rate of renewal of oral epithelium is ~5 days (16), persistent β-Gal expression 5 months after the last exposure to RU486 confirms that CrePR1 was activated in stem cells that regenerate the oral epithelium. We also observed persistent β-Gal activity in the oral epithelia of K14.CrePR1/ROSA26 mice 5 months after exposure to RU486 (Fig. 1B). These results, combined with our previous reports (9, 14), document that K5.CrePR1 and K14.CrePR1 are expressed and activated in stem cells that regenerate the epidermis, sebaceous glands and hair follicles, and the oral cavity. Therefore, K5.CrePR1 and K14.CrePR1 can be used to activate conditional alleles in the oral cavity.

To determine whether activation of an oncogenic K-ras allele plays a causal role in oral tumor formation, we crossed LSL-K-rasG12D mice developed by Jackson et al. (6) with K14.CrePR1 mice. LSL-K-rasG12D mice were generated by substitution of a wild-type K-ras allele for a mutant K-ras allele (K-rasG12D) using homologous recombination. The LSL-K-rasG12D allele carries the glycine to aspartic acids mutation in codon 12 and a stop cassette flanked by LoxP sites that prevents the expression of mutant K-ras (6). Activation by Cre recombinase induces the excision of the stop cassette and expression of K-rasG12D. Four-week-old bigenic K14.CrePR1/LSL-K-rasG12D mice (K14Cre-Ras) and control littermates were treated with RU486 in the oral cavity. Activation of K-rasG12D induced severe hyperplasia in the oral cavity. By age 5 months, the mice had to be euthanized because of the size of the oral tumors (Fig. 1, C and D). Biopsies of oral tumors were taken, and histologic examination showed marked hyperplasia and hyperkeratosis typical of benign squamous lesions of the oral mucosa and tongue (Fig. 1, G and H).

Molecular analysis of the oral lesions confirmed that activation of the mutant allele K-rasG12D occurred in the oral tumors. Thus, DNA analysis demonstrated that the stop cassette was excised in the oral tumors but not in the spleen and liver of K14Cre-Ras mice that had been treated with RU486 in the oral cavity (Fig. 2B). We also examined tumors for expression of the wild-type and mutant K-ras alleles by reverse transcription-PCR and restriction fragment length polymorphism. Because the mutant K-rasG12D allele was engineered with a HindIII restriction site in exon 1, which is not present in the wild-type allele, the relative expression levels of the mutant and wild-type alleles can be easily evaluated in tissues exposed to RU486. Wild-type and mutant K-ras transcripts were detected (Fig. 2C), confirming that expression of the mutant allele is activated in these tumors. Interestingly, all of the oral tumors analyzed expressed similar levels of wild-type and mutant K-ras transcripts, indicating that neither increased expression of the mutant K-ras allele nor decreased expression of the wild-type allele is required for oral tumor formation (Fig. 2C). Finally, Western blot analysis shows that oral tumors and normal oral mucosa from wild-type mice express equal levels of the K-ras2A protein, which is expressed from the K-ras gene (Fig. 2D). Together, these results demonstrate that activation of oncogenic K-ras in the oral cavity is sufficient to induce benign oral tumor formation.

During the course of these experiments, we discovered that activation of CrePR1 could occur in the oral cavity in the absence of inducer. The level of leaky activation of K-rasG12D in K14Cre-Ras mice was sufficient to result in the development of a marked hyperproliferative phenotype in the oral cavity. Because leaky activation of conditional alleles could create a problem in attempt to generate compound mice with multiple conditional alleles, we have generated a new transgenic mouse model that expresses a variant of CrePR1 (designated Cre-P1). Cre-P1 was engineered to reduce unwanted

Fig. 1. Oral tumor formation in K14-Cre-Ras mice. A, β-galactosidase activity in the oral mucosa of a bigenic K5.CrePR1/ROSA26 mouse and (B) tongue of a K14.CrePR1/ROSA26 mouse 5 months after activation with RU486 in utero. C, frontal view of the gross appearance of the lesions developed in the oral cavity of bigenic K14Cre-Ras mice 16 weeks after activation with 1 mg/ml RU486. D, frontal view of the tumor shown in (C), showing the lesions developed in the oral cavity. E–H, H&E staining of sections corresponding to oral mucosa (E) and tongue (F) of wild-type mice and oral lesions developed in the lip area (G) and tongue (H) of a K14Cre-Ras mouse; magnification, 40×.
proteins. As a result of clonal expansion of a stem cell expressing an activated K-rasG12D allele. Histologically, these tumors were classified as benign squamous papillomas, showing marked hyperplasia and severe dysplasia (Fig. 3D). Invasion into the submucosal tissue was not detected, suggesting that the tumors had not progressed to malignancy. We have monitored untreated K5Cre*-Ras mice for >10 months and have not observed spontaneous tumor development or hyperplasia. Thus, treatment with RU486 is absolutely required for K-rasG12D activation and tumor formation in K5Cre*-Ras mice. In support of this conclusion, DNA analysis confirmed that Cre activation only occurs on treatment of K5Cre*-Ras mice with RU486. PCR analysis of DNA purified from the oral mucosa of K5Cre*-Ras mice demonstrated that RU486 treatment induced the excision of the LoxP-flanked stop cassette (Fig. 3E, Lane 2). The low level of activation of the mutant allele detected is because DNA was isolated from oral tissue, which includes the nonepithelial submucosal component, and Cre*PR1 activation occurs in a subset of cells of the stratified epithelium (data not shown). The stop cassette is not excised in the oral mucosa of untreated bigenic mice (Fig. 3E, Lane 1), proving that Cre*PR1 is tightly regulated in vivo. The oral tumors that developed in K5Cre*-Ras mice exhibit similar levels of activated mutant and wild-type K-ras alleles (Fig. 3E, Lane 3), indicating that the tumors were primarily comprised epithelial cells that exhibit activation of the mutant K-rasG12D allele. The total penetrance of the tumor phenotype, together with the rapid development of the oral tumors, suggests that no additional mutations are required for K-ras-induced oral tumor formation. Similarly, it has recently been reported that activation of the K-rasG12D allele can induce hyperplasia in lung and gastrointestinal epithelia (17) and initiate pancreatic tumors without additional genetic alterations (8).

These results show that the new K5.Cre*PR1 lines are appropriate to generate mouse models for oral cancer, especially to test the
consequences of accumulation of genetic alterations, as occurs in sporadic human cancer. Using these models, we have shown that focal activation of oncogenic K-ras is sufficient to induce tumor formation in oral epithelia. Although human oral cancers exhibit varying frequencies of mutations in ras genes, alterations in other genes involved in the ras signaling pathway are frequently observed. For example, amplification and/or overexpression of epidermal growth factor receptor, which activates ras signaling, occurs in >50% of oral tumors (18). The recently discovered RASSF1A gene, which is an inhibitor of the ras signaling pathway, is also frequently inactivated by promoter methylation in human oral cancer (19). Moreover, mutations in B-raf, a downstream effector of ras, also have been found at low frequencies in oral cancer (20). These data suggest that the ras signaling pathway is an important target in human oral cancer.

The oral epithelium is a self-renewing tissue that regenerates through a process that involves terminal differentiation. Alteration of the differentiation process may result in abnormal growth. Keratins constitute the major differentiation markers of stratified epithelia, and changes in the expression of keratins are associated with different stages of tumor progression in human oral tumors (21, 22). To determine whether the oral tumors that developed in K5Cre*-Ras mice exhibited changes in the differentiation patterns, we stained frozen sections of the K5Cre*-Ras tumors with antibodies specific for keratins expressed in oral epithelia. K13 is expressed in basal and suprabasal layers of the oral mucosa and tongue in wild-type mice (Fig. 4A), although K13 is not expressed in the basal layer in the posterior part of the dorsal side of the tongue (Fig. 4C). K14 is mainly expressed in the basal layers of the oral mucosa and tongue (Fig. 4, A and C), whereas K6 shows sparse expression in the suprabasal layers of the oral epithelia (Fig. 4, B and D). The oral tumors that developed in K5.Cre*-Ras mice express these keratins; however, they exhibit a significant change in the expression patterns. For example, K14 is expressed throughout the epithelial component of these tumors (Fig. 4, E and F), as well as K6, a keratin that also has been associated with hyperproliferation in processes such as wound healing and carcinogenesis (23, 24) (Fig. 4F). In addition, K13 expression appears restricted to the most differentiated cells of the tumors (Fig. 4E). These results indicate that a significant change in the expression pattern of these keratins occurs in oncogenic K-ras-induced oral tumors, even though these tumors are at an early stage and still well differentiated. The restricted expression of K13 and generalized expression of the basal keratin K14 and the hyperproliferation-associated K6 in the K5Cre*-Ras oral tumors suggest that decreased differentiation and increased proliferation may contribute to tumor formation in these mice. Interestingly, similar changes in keratin expression pattern have been observed in human benign oral lesions (21), suggesting that changes in differentiation associated with oral tumors that developed in K5Cre*-Ras resemble those observed in human patients. Therefore, this mouse model may be useful in the search for novel molecular markers for early detection of oral cancer.

In summary, we have developed a novel inducible mouse model that allows the activation of oncogenes or inactivation of tumor suppressor genes exclusively in the oral cavity. Using this system, we have demonstrated that activation of oncogenic K-ras in the oral cavity induces oral tumor formation. These tumors represent early stages of tumor progression, and their differentiation characteristics resemble those observed in benign human oral lesions. Because the mutations introduced in this model remain silent until activated by RU486, this inducible system will be invaluable for testing cooperation between different mutations in oral cancer formation and malignant progression and metastasis. These mouse models also should prove useful as preclinical models for testing preventive and therapeutic agents.

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References


