Amplification of Wild-Type K-ras Promotes Growth of Head and Neck Squamous Cell Carcinoma

Michael Hoa, Shannon L. Davis, Sarah J. Ames, and Remco A. Spanjaard

Departments of Otolaryngology—Head and Neck Surgery [M. H., S. L. D., S. J. A., R. A. S.] and Biochemistry [R. A. S.], Cancer Research Center, Boston University School of Medicine, 715 Albany Street R903, Boston, MA 02118. Phone: (617) 638-5854; Fax: (617) 638-5837; E-mail: rspan@bu.edu

Abstract

In contrast to many other tumors of different lineage, oncogenic ras mutations are rarely found within head and neck squamous cell carcinoma (HNSCC). On the other hand, increased expression of wild-type K-ras in HNSCC tumor material has been noticed, but the potential physiological consequences of this observation have not yet been experimentally assessed. The current study addresses this issue by modulating K-ras expression in HNSCC cell lines and primary keratinocytes and determining its effects on cell growth and survival in vitro. Consistent with earlier reports using patient tumor material, Western blot analysis of four HNSCC lines (SCC-9, SCC-15, SCC-25, and FaDu) revealed varying but universally increased protein expression of K-ras relative to keratinocytes. All HNSCC lines expressed wild-type K-ras mRNA based on a random sequencing of eight K-ras cDNA samples obtained by reverse transcription-PCR from each HNSCC line (P ≤ 0.0039). Transfection of keratinocytes with a plasmid expression vector containing wild-type K-ras cDNA resulted in dramatically increased proliferation and survival compared with control-transfected or untransfected keratinocytes. Conversely, transfection of FaDu cells, which express the highest level of endogenous K-ras, with K-ras antisense oligonucleotides but not control oligonucleotides significantly reduced cellular proliferation (P ≤ 0.0022). These results show that the level of K-ras protein expression is a major determinant of proliferation of HNSCC cells and keratinocytes and suggest that amplification of nonmutated K-ras in HNSCC contributes to tumor growth. These novel findings may have important ramifications for potential K-ras-targeted interventions in the treatment of HNSCC.

Introduction

Ras genes, which include H-ras, K-ras, and N-ras, encode a M, 21,000 protein that is located on the cytoplasmic face of the plasma membrane and that transmits mitogenic signals in response to a variety of physiological stimuli by binding and hydrolyzing GTP (1). Mutations in Ras gene family members resulting in oncogenic activation have been extensively studied and implicated in the pathogenesis of various cancers including lung, pancreatic, and colorectal carcinoma (2, 3). In contrast, Ras genes are rarely mutated in HNSCC at a frequency of <5% in the Western world (4, 5). Some studies have indicated that K-ras and other members of the Ras family are overexpressed in oral cancers (6, 7), but the biological consequences of these observations have never been experimentally assessed. Interestingly, a recent study by Oft et al. (8) suggested that overexpression of H-ras contributes to progression to an invasive motile form of squamous carcinoma. We hypothesized that amplification of wild-type K-ras in oral cancer results in an overactive mitogenic signal, in turn resulting in an increased proliferative and/or survival response that contributes to the etiology of HNSCC. In this study, we characterize the biological role of K-ras amplification in a cell culture model for HNSCC and provide evidence that K-ras plays a previously unrecognized growth-promoting role in HNSCC.

Materials and Methods

Cell Culture. Human oral squamous cell carcinoma cell lines (SCC-9, SCC-15, SCC-25, and FaDu) were obtained from American Type Culture Collection (Manassas, VA). Keratinocytes were obtained from BioWhittaker Inc. (Walkersville, MD). Cells were grown and maintained at 37°C/5% CO2 in humidified air in DMEM and Ham’s F-12 medium in a 1:1 ratio supplemented with 400 ng/ml hydrocortisone and antibiotic-antimycotic (100X; Life Sciences) and 10% fetal bovine serum. Passages were made 1–2 times/week, and all cells were harvested at similar confluence.

Protein Extraction. Protein extracts from tissue culture cells were prepared by lysing cells in a whole cell buffer composed of 50 mM Tris (pH 8), 150 mM NaCl, 1 mM EDTA, 0.1% NP40, 50 mM NaF, 2 μg/ml aprotinin/leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM NaVO4. Extracts were stored at −80°C when necessary.

Western Blot Analysis. Western blot assay was performed as described in Ref. 9. Briefly, protein samples were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with Biotto-Twee (5% nonfat milk and 0.05% Tween 20 in PBS) and incubated with the primary antibodies against K-ras and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). A secondary IgG antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was incubated with the membranes and developed according to Amersham’s enhanced chemiluminescence protocol (Amersham, Piscataway, NJ).

Plasmid Transfections. Keratinocytes and FaDu cells were cotransfected with K-ras-encoding pZCR plasmid and pEGFP-N3 vector plasmid (BD Biosciences/Clontech, Palo Alto, CA) in a 9:1 ratio using the Qiagen Effectene Transfection Reagent protocol (Qiagen, Valencia, CA) or with the same total amount of pEGFP-N3 vector plasmid alone or no DNA at all. A total of 10 μg of DNA was used in each transfection per 6-cm plate. Forty-eight h after transfection, cells were examined using both fluorescence and light microscopy, and digital images were captured as TIFF format files.

Oligodeoxynucleotide Transfections and Cell Proliferation Assay. Phosphorothioate-modified AS oligodeoxynucleotides against K-ras and a SC were designed as described previously (10). Sequences were as follows: AS, 5’-CACAAAGTTTATACAGT; and SC, 5’-ACTAGCTATACTAGCTAT. SC and AS oligodeoxynucleotides (5 μM) were transfected in triplicate into FaDu cells on 24-well plates using Oligofectamine reagent (Invitrogen, Carlsbad, CA). Mock transfections were performed using distilled water. Five days after transfection, cell proliferation was determined by Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), which measures bioreduction of MTS (Owen’s reagent) into a soluble formazan that was determined in a microplate reader at A490nm as described previously (11).

RT-PCR and cDNA Sequence Analysis. Total RNA was extracted from a 10-cm plate of each HNSCC line using Trizol reagent (Life Technologies, Inc., Grand Island, NY). Superscript One-Step RT-PCR (Invitrogen) was used to specifically obtain K-ras cDNA. PCR conditions were as follows: forward

Received 8/26/02; accepted 10/24/02.

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1 Supported in part by a Summer Research Fellowship and Cancer Research Center Summer Fellowship from Boston University School of Medicine (M. H.) and by the NIH Cancer Research Center Lung and Airway Cancer Research Program (R. A. S.).

2 To whom requests for reprints should be addressed, at Department of Otolaryngology—Head and Neck Surgery, Cancer Research Center, Boston University School of Medicine, 715 Albany Street R903, Boston, MA 02118. Phone: (617) 638-5854; Fax: (617) 638-5837; E-mail: rspan@bu.edu

3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; RT-PCR, reverse transcription-PCR; eGFP, enhanced green fluorescent protein; AS, antisense; SC, scrambled control.
school of medicine core sequencing facility. into pGEM-T (Promega) and completely sequenced by the Boston University. C. Amplified cDNA was cloned 2 min, followed by a 6-min extension at 72 °C for 30 s, 50 °C. Protocol was as follows: 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a 6-min extension at 72 °C. Amplified cDNA was cloned into pGEM-T (Promega) and completely sequenced by the Boston University. School of Medicine core sequencing facility. 

Table 1. HNSCC cells express wild-type K-ras as determined by DNA sequence analysis of 8 randomly selected K-ras cDNAs obtained by RT-PCR. The statistical probability that a mutant allele is expressed: \( P \leq 0.00391 \).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wild type</th>
<th>Mutated</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC-9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>SCC-15</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>SCC-25</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>FaDu</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
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primer, 5'-ATGACTGAATATAAACTTTGTTGATAG; and reverse primer, 5'-TTACATAATTACACACTTTGTCTTTGAC. cDNA PCR amplification protocol was as follows: 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min, followed by a 6-min extension at 72°C. Amplified cDNA was cloned into pGEM-T (Promega) and completely sequenced by the Boston University School of Medicine core sequencing facility.

Results and Discussion

Earlier reports showed that HNSCC tumors express elevated levels of wild-type K-ras relative to nonmalignant mucosal cells (10). To determine whether these observations would also apply to HNSCC cell culture models, whole cell lysates were prepared from four different HNSCC cell lines (SCC-9, SCC-15, SCC-25, and FaDu), as well as primary keratinocytes as a nonmalignant control. K-ras expression was established by Western blot analysis shown in Fig. 1. Consistent with the in vivo data, FaDu and SCC-15 cells showed markedly elevated expression of K-ras relative to keratinocytes, whereas SCC-9 and SCC-25 cell lines had moderately increased levels.

We also wanted to establish whether the HNSCC cells express wild-type or mutated (oncogenic) K-ras. By using RT-PCR, K-ras-specific cDNA was amplified, gel-purified, and cloned into an appropriate plasmid vector. After bacterial transformation, eight positive colonies from each cell line were randomly selected and prepared for DNA sequencing of the entire cDNA to be able to detect any mutation, particularly in oncogenic codons 12, 13, and 61 (10). The results (Table 1) show that in each case, eight of eight sequences were identical to wild-type K-ras, which shows that all HNSCC cell lines express wild-type K-ras (\( P \leq 0.00391 \)). Thus, our cell culture experiments are entirely in accordance with previous immunohistochemistry findings (8), and this validates the use of in vitro experiments to study the role of K-ras in HNSCC.

Next, we wanted to assess the effects of elevated K-ras expression on growth of primary keratinocytes. Cells were transfected by liposome techniques in two different ways. One set of keratinocytes was cotransfected with a plasmid driving expression of wild-type K-ras and another plasmid expressing eGFP in a 9:1 ratio. This ratio was chosen to ensure that each eGFP-positive cell has a high likelihood of also containing the K-ras expression vector. A second set of keratinocytes was transfected with the same amount of DNA but consisting only of the eGFP expression vector. Finally, a third set was not transfected at all. As shown in a representative field in Fig. 2, 48 h after transfection, untransfected and eGFP-transfected control plates contained only 46% (223,000 cells) and 20% (98,000 cells), respectively, of the number of cells on the K-ras-transfected plate (490,000 cells). Moreover, the K-ras-transfected cells have a relatively normal morphology, whereas the cells on the two control plates are largely rounded, indicative of cell death. Thus, K-ras expression enhanced both the growth and survival of the primary keratinocytes under our experimental culture conditions. To more clearly demonstrate that proliferation of the transfected keratinocytes was due to K-ras overexpression, we used light along with fluorescent microscopic visualization of a representative field in the K-ras transfectants. A typical field is shown in Fig. 3, where a cluster of about five eGFP-expressing cells can be seen, which, due to the skewed ratio between K-ras and eGFP expression vector, suggests that this most likely represents a single eGFP and K-ras-cotransfected keratinocyte that had undergone transformation (8). Light microscopic visualization of a group of field showing that single eGFP and thus likely K-ras-transfected keratinocyte had undergone proliferation as further evidenced by c. a merged image of A and B. Magnification, ×40.
that reduced levels cause slowing of cell division in HNSCC cells. To test this hypothesis, we decided to diminish K-ras expression using K-ras AS phosphorothioate-modified oligodeoxynucleotides that were previously shown to successfully inhibit growth and even stimulate regression of pancreatic tumor cells (12). As our model, we chose the FaDu cell line because it has the highest level of K-ras expression. FaDu cells were also transfected with SC oligodeoxynucleotides or mock-transfected. Five days later, cell proliferation was determined by MTS (Owen’s reagent)-assay (11). As shown in Fig. 4, FaDu cells treated with AS were significantly growth-inhibited, containing almost 40% fewer cells than mock- and SC-transfected cells ($P \leq 0.0022$). This result confirms that K-ras is a major growth regulator of HNSCC cells.

The relationship between the Ras gene family, particularly K-ras, and tumorigenesis has generated considerable interest. Much of the attention has been focused on the role of mutated, activated members of this family in various tumors (4, 10). Even though recent studies in both in vitro and in vivo systems indicated that increased expression levels of mutated Ras genes could potentially play an important role in the development and progression of HNSCC (4, 13–15), Ras mutations are rare events (6, 7). Rather, increased expression levels of wild-type Ras protein have been observed (16). Our studies using HNSCC cell lines are consistent with these latter observations. Increased expression of wild-type K-ras in primary keratinocytes, which have extremely low basal expression levels, resulted in a significant proliferative response and increased cell survival. This corroborates a previous report (4) that suggested that K-ras amplification could be an early event in the pathogenesis of HNSCC progression. Conversely, our AS oligonucleotide experiments confirm the importance of K-ras in HNSCC proliferation, at least in vitro. This result is consistent with an in vivo melanoma model, where it was demonstrated that sustained Ras protein expression is required for tumor growth and survival (17).

Our data suggest that enhanced K-ras expression contributes significantly to the etiology of HNSCC. This offers the potential of directed treatments against K-ras expression as a way of inhibiting the proliferation of HNSCC (2), a therapeutic strategy that deserves to be further explored.

**Acknowledgments**

We thank Dr. Douglas Faller (Department of Medicine, Cancer Research Center, Boston University School of Medicine) and Dr. Gregory Grillone (Department of Otolaryngology, Boston Medical Center) for valuable support.

**References**

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