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

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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 ≤ 0.00391). 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 BLOTTO-Tween (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′-CACAAAGTTTATATTCAGT; and SC, 5′-ACTAGCTATACTAGCTAT.

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3The 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.
ROLE OF K-ras EXPRESSION IN HNSCC PROLIFERATION

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 < 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 two or three cell divisions. The strength of the proliferative response gives weight to the ability of K-ras to enhance proliferation and survival of the keratinocytes in vitro.

Finally, we also wanted to perform the converse experiment. If overexpression of K-ras induces proliferation, then it can be predicted

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.

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

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Mutated</th>
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<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>
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The statistical probability that a mutant allele is expressed: P ≤ 0.00391.

We also wanted to perform the converse experiment. If overexpression of K-ras induces proliferation, then it can be predicted

Fig. 1. K-ras expression is elevated in HNSCC. Western blot analysis (50 μg protein/ lane) of K-ras expression in HNSCC cell lines SCC-9, SCC-15, SCC-25, and FaDu compared with primary keratinocytes. β-Actin serves as loading control.

![Fig. 1](image1.png)

![Fig. 2](image2.png)

![Fig. 3](image3.png)
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 were also transfected with SC oligodeoxynucleotides or mock-transfected. Data are expressed as the mean ± SD. *P ≤ 0.0022 (by Student’s t test analysis) as compared with mock transfection.

Cell number (% of untreated control)

Mock SC AS

Fig. 4. Transfection of FaDu cells with K-ras AS but not SC oligodeoxynucleotides (5 μM) or mock transfection results in strong growth inhibition. Viable cell numbers were determined 5 days after transfection by MTS assay. Cell number is calculated relative to mock-transfected control (arbitrarily set at 100%). Shown is a representative experiment done in triplicate and repeated twice with identical results. Data are expressed as the mean ± SD. *P ≤ 0.0022 (by Student’s t test analysis) as compared with mock transfection.

 ROLE OF K-ras EXPRESSION IN HNSCC PROLIFERATION

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.

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References


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