Heterogeneity in Interleukin-13 Receptor Expression and Subunit Structure in Squamous Cell Carcinoma of Head and Neck: Differential Sensitivity to Chimeric Fusion Proteins Comprised of Interleukin-13 and a Mutated Form of Pseudomonas Exotoxin

Bharat H. Joshi, Koji Kawakami, Pamela Leland, and Raj K. Puri

Laboratory of Molecular Tumor Biology, Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

ABSTRACT

Squamous cell carcinoma of the head and neck (SCCHN) is characterized by a high proliferation index and marked propensity for local invasion resulting in poor prognosis for these patients. To develop tumor-targeted novel therapeutic agents, here we demonstrate that SCCHN cell lines express receptors for an immune regulatory cytokine, interleukin (IL) 13. By reverse transcription-PCR (RT-PCR), we found that 16 SCCHN cell lines express equally strong RT-PCR positive bands for mRNA of IL-13Rα1 and IL-4Rα chains. However, only three cell lines, HN12, YCUM911, and KCTT873, expressed a strong band for transcripts for IL-13Rα2 chain and five cell lines, YCUL891, KCTTC871, KCL871, KCTTC901, and RPMI 2650 expressed faint bands. Transcripts for IL-2Rγc chain were absent in all of the cell lines tested. Indirect immunofluorescence analysis for four different receptor chains confirmed RT-PCR results and showed pronounced expression of IL-13Rα2 protein in three high IL-13 expressing cell lines. All of the cell lines were equally positive for IL-13Rα1 and IL-4Rα chains. Receptor-binding studies demonstrated that IL-13Rα2-positive cell lines expressed a high density of IL-13 receptors. Using two chimeric proteins composed of IL-13 and mutated forms of Pseudomonas exotoxin (IL-13-PE38 or IL-13-PE38QQR), we found that these two fusion toxins were highly and equally cytotoxic to IL-13Rα2-positive SCCHN, whereas IL-13Rα2-negative cell lines showed low or no sensitivity to IL-13 toxins. To additionally substantiate the critical role of the IL-13Rα2 chain in IL-13R-mediated cytotoxicity, two head and neck tumor cell lines (YCUMS861 and KB), devoid of the transcripts of this chain, were transfected with IL-13Rα2 cDNA and then tested for cytotoxicity. Transient transfection of the IL-13Rα2 chain highly sensitized these cells to IL-13 toxin as compared with mock-transfected control cells. Thus, our results indicate that IL-13Rα2 is present in 50% SCCHN tumor cell lines; of these, 19% are high expresser for this chain and respond to IL-13 cytotoxin. Thus, IL-13 cytotoxin may be a useful agent for high IL-13R-expressing SCCHN.

INTRODUCTION

Epithelial carcinomas derived from the mucosa of the oral cavity and oropharynx account for 30,000 new cases of cancer each year in the United States (1). This class of tumors forms a subset within a broader category of SCCHN. SCCHN is the sixth most common malignancy and is a major cause of cancer morbidity and mortality worldwide. Despite extensive research into the pathogenesis and management of SCCHN, the 5-year survival rate for patients with these tumors has not improved in the last 25 years, remaining at 53% (1). The treatment of primary or advanced human SCCHN in the upper aerodigestive tract remains a major therapeutic challenge, despite advances in surgical and radiotherapeutic techniques (2, 3).

To develop novel targeted therapeutic agents, we have identified previously expression of high numbers of receptors for an immune regulatory cytokine, IL-4, on different human cancer cell lines and primary tumors (4–10). To target these receptors (IL-4R), we have developed a recombinant fusion protein comprised of circularly permuted IL-4 and a mutated form of a bacterial toxin, PE (PE38KDEL; Ref. 11). This molecule [IL-4 (38–37) PE38KDEL] is highly cytotoxic to SCCHN cell lines in vitro and in vivo (12, 13). Because IL-13, a Th2-cell derived cytokine, has similar biological activity to IL-4, we have tested and reported that human solid tumor cell lines e.g., renal cell carcinoma, malignant glioma, ovarian carcinoma, and AIDS-associated Kaposi’s sarcoma express high numbers of IL-13R (14–21). The structure of IL-13R has been studied extensively on various cell types. We and others have reported that IL-13R may be expressed as three different types...
in different cell lines. Type I IL-13R consists of IL-13Rα1 (also known as IL-13R α'), IL-13Rα2 (also known as IL-13Rα), and IL-4Rα (also known as IL-4Rβ) chains, whereas type II IL-13 receptor complex consists of IL-4Rγ and IL-13Rα1 chains (14, 16, 22–25). Type III IL-13R is similar to type II IL-13R except cells that express this type of receptor also express IL-2Rγ (γc) chain, which is shared by IL-4R system (14, 22). The role of γc in the formation of IL-13R complex is not clearly understood. It has been shown that the introduction of γc could decrease IL-13 and IL-4 binding, and interfere in functioning of both receptors in cells that usually do not express this chain (26, 27). These and other studies have additionally revealed that IL-4Rα and IL-13Rα1 subunits are shared, and are required for signal transduction through IL-4 and IL-13 (21–24, 28–30). Different configurations of receptors may render cells a peculiar biological function.

To target IL-13R, a chimeric fusion protein comprised of human IL-13 and a mutated form of PE (IL-13-PE38QQR) has been produced (31, 32). The mutated form of PE (PE38QQR) consists of substitutions of three lysine residues at positions 590 and 606 by glutamine, and at 613 by arginine. IL-13-PE38QQR is highly cytotoxic to IL-13R-positive cancer cells in vitro and in vivo (15, 18, 19, 32–35). Here we examined whether SCCHN cell lines express IL-13R and if IL-13-PE38QQR is cytotoxic to these cell lines. In addition, we have examined the subunit structure of IL-13R in 16 SCCHN cell lines to evaluate possible heterogeneity of receptor expression. Finally, we have examined the role of mutations of three amino acids at the COOH terminus of PE. For this, we expressed, purified, and tested an IL-13-PE38 without the QQR mutation at the COOH terminus of the molecule.

MATERIALS AND METHODS

Cell Lines and Antibodies. SCCHN cell lines KB, A253, and RPMI 2650 were purchased from the American Type Culture Collection (Manassas, VA). The WSU-HN12 (HN12) cell line was a kind gift from Dr. Andrew Yeudall (National Dental and Craniofacial Research Institute, NIH, Bethesda, MD; Ref. 36). Twelve head and neck cell lines were established in the Department of Otolaryngology, Yokohama City University School of Medicine Research Institute, Kanagawa Cancer Center, Yokohama, Japan (37). PM-RCC, a renal cell carcinoma cell line, was developed in our laboratory (4). These cell lines were maintained in Eagle’s Modified Essential Medium (KB, A253, RPMI 2650, and HN12), DMEM (PM-RCC) or RPMI 1640 (12 cell lines from Yokohama University, Yokohama, Japan) containing 10% fetal bovine serum (Bio-Whittaker Inc., Walkersville, MD), 1 mM HEPES, 1 mM nonessential amino acids, 100 µg/ml penicillin, and 100 µg/ml streptomycin (Bio-Whittaker Inc.). Monoclonal antibodies for IL-13Rα1 and IL-13Rα2 were obtained from Diaclone (Besancon, France). Monoclonal antibody for IL-4Rα (M-57) was a kind gift from Immunex Corporation (Seattle, WA), and polyclonal rabbit anti IL-2Rγc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RNA Extraction. SCCHN cells in the logarithmic phase were detached with trypsin-EDTA, washed with 1× PBS, and RNA was extracted using RNAeasy RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Briefly, 10 × 10⁶ cells were pelleted and lysed in guanidinium thiocyanate lysis buffer provided in the kit. The total cell lysate was mixed with an equal volume of 70% ethanol and loaded on silica spin columns. After a brief centrifugation for 20 s, the columns were washed and RNA eluted with RNase-free water. RNA was quantitated.

RT-PCR. Sixteen RNA samples from SCCHN cells were subjected to RT-PCR analysis. β-Actin mRNA amplification from these samples served as an internal control. RT-PCR conditions for each chain and the primers used in the amplification protocols have been published previously (22). Total RNA (500 ng) from these cell lines were reverse-transcribed using a RNA-PCR kit according to the manufacturer’s instructions (Perkin-Elmer Corp., Norwalk, CT). Reverse-transcribed products (10 µl) were amplified for 30 cycles using the GeneAmp PCR system 9700 (Applied Biosystem-Perkin-Elmer, Norwalk, CT; Ref. 22). The amplified products were electrophoresed on 2% agarose gel, stained with ethidium bromide, visualized in a transilluminator, and photographed. The band intensities of RT-PCR products for IL-13Rα2 and β-actin were evaluated using a fluorescence densitometer (Molecular Dynamics, Sunnyvale, California). The relative fluorescence intensity was determined by dividing the intensity of α2 chain mRNA band by density of β-actin band and expressed as ratio of RFUs.

Immunofluorescence Analysis. Cells (20,000) were cultured in a chambered glass slide (Lab-Tek-Nagle Nunc International, Naperville, IL) for 48 h. The cells were washed twice with 1× PBS and fixed with cold methanol:acetone (1:1, v/v), and incubated at −20°C for 2 h. The cells were then washed and rehydrated with PBS, and subjected to immunofluorescence analysis. The optimal conditions for immunofluorescence analysis were described previously (20). Briefly, the rehydrated cells were incubated with 1% BSA and 5% goat or horse serum in PBS to block nonspecific binding of antibody. The slides were washed with PBS twice and incubated for 2 h with either the specified primary antibody (1:1500), or mouse IgG1 or rabbit IgG as isotype control. Slides were then washed three times and incubated for 1 h with a secondary antibody that had either tetramethylrhodamine isothiocyanate or FITC tag after diluting in PBS containing 0.1% BSA per manufacturer’s instructions. The slides were washed with PBS three times, air dried, and layered with Vectashield antifluorescence fading mounting medium (Vector Laboratories, Burlingame, CA) and a coverslip. The slides were viewed in a Nikon fluorescent microscope using appropriate filters.

IL-13 Receptor Binding Studies. Recombinant human IL-13 was labeled with 125I (Amersham Research Products) by using IODO-GEN reagent (Pierce, Rockford, IL) according to the manufacturer’s instructions. The specific activity of the radiolabeled cytokine was estimated to range between 40 and 120 µCi/µg of protein. Binding experiments were performed as described elsewhere (14). Typically, 1 × 10⁶ cells were incubated at 4°C for 4 h with 125I-IL-13 (100–500 mCi) in the absence or presence of 200-fold unlabeled IL-13. Duplicate samples of the cells associated with 125I-IL-13 were separated from free 125I-IL-13 by centrifugation through cushion of phthalate oils. The cell pellets were counted in a gamma counter.
(Wallac, Gaithersburg, MD). The binding sites were calculated using specific activity of IL-13.

Construction of IL-13PE Chimeric Genes. The IL-13 PE38 and IL-13 PE38QQR chimeric genes were constructed in the laboratory. Briefly, the human IL-13 gene (pMPL13) was cloned in its matured form from stimulated human peripheral blood mononuclear cells. Total RNA was extracted from peripheral blood mononuclear cells and reverse-transcribed to cDNA with Moloney murine leukemia virus reverse transcriptase. PCR-based amplification of cDNA was performed to produce the IL-13 gene with Nde I and Hind III sites at 5' and 3' of the open reading frame of gene by using sequence specific primers. A 336-bp long DNA fragment was purified from the PCR product and digested with the appropriate restriction enzymes. The digested DNA fragment was subcloned into the vector obtained from plasmid YR39 or pRKL438QQR (kindly provided by Dr. Ira Pastan, National Cancer Institute, Bethesda, MD) digested previously with the same restriction endonuclease enzyme pair to yield IL-13-PE38 (pBII13PE38) and IL-13-PE38QQR (pRPI13PE38QQR). The junctions of the chimeric genes as well as IL-13 genes were sequenced to confirm correct DNA sequence.

Expression and Purification of the Chimeric Proteins. Expression and purification of IL-13-PE38 and IL-13-PE38QQR was carried out using Escherichia coli BL21(ADE3)pLys for transformation. The bacterial culture was induced with 1 mM of IPTG and plated in a bacterial shaker for 6 h. The chimeric proteins were produced in inclusion bodies. After washing, the inclusion bodies were denatured with guanidinium-hydrochloride containing Tris-HCl buffer (pH 8.0) overnight. Soluble inclusion bodies were refolded by diluting 1:150 with Tris-HCl buffer containing arginine and oxidized glutathione. The renatured preparation was dialyzed against 10 mM Tris-Cl (pH 7.4) buffer containing 60 mM of urea. The chimeric protein was purified by Fast Protein Liquid Chromatography using Q Sepharose, mono Q and sephacryl S-100 gel exclusion columns (Amersham Pharmacia, Piscataway, NJ). The purified protein was electrophoresed on 10% SDS-PAGE and stained with Coomassie Blue. The gel was destained with destaining solution that contained 7% acetic acid and 5% methanol (v/v).

Both IL-13-PE38QQR and IL-13-PE38 proteins appeared to be refolded and demonstrated high purity of the protein products. The chimeric proteins migrated approximately at Mr 50,000 as expected (Fig. 1, A and B).

Protein Synthesis Inhibition Assay. The cytotoxicity of chimeric toxins IL-13-PE38 and IL-13-PE38QQR was determined as described previously (38). Briefly, 1 × 10^6 cells were plated in leucine-free medium (Biofluids, Rockville, MD) for 6 h to allow adherence to flat-bottomed microtiter plates. Various concentrations of either cytotoxin were added to the cells and incubated for 20 h at 37°C. For blocking experiments, cells were preincubated with IL-13 or IL-4 (2 µg/ml) for 45 min before addition of IL-13 toxin. [3H]Leucine (1 µCi; NEN Research Products, Boston, MA) was then added to each well, and the cells were incubated for an additional 4 h. Cells were harvested and labeled leucine incorporation into cells was measured by a β plate counter (Wallac). The inhibition of protein synthesis is directly correlated to cell death as determined by viable cell count as demonstrated previously (6, 32, 35).

Transient Transfection of IL-13Rα2 DNA. Human IL-13Rα2 cDNA (39, 40) was cloned into a pME18S expression vector for transient transfection experiments. For this purpose, two low IL-13Rα2 expressor SCCHN cell lines (YCUMS861 and KB) were plated onto 100-mm Petri dish and grown until the plate was 60% confluent. Then, plasmid DNA (12 µg/100-mm Petri dish) was transfected with Gene Porter transfection reagent (Gene Therapy System, San Diego, CA) according to the manufacturer’s instructions. In brief, 3 × 10^6 cells were cultured with DNA-GenePorter mixture for 5 h in DMEM. DMEM containing 20% FBS was added, and the culture was maintained for an additional 48 h with one change of medium.

Colony Formation Assay. In vitro cytotoxic activity of IL-13PE38 on HN12, YCUM911, and KCCT873 cells was also evaluated by colony formation assay. The cells were harvested from culture, washed, and resuspended in complete medium. The cells were plated in quadruplicate in 100-cm^2 tissue culture Petri dishes (Falcon; Becton Dickinson, Lakeridge, NJ) and cultured overnight to attach the bottom of the plates. The number of the cells/plate was selected such that >100 colonies were obtained in the control group. The cultures were then incubated with IL-13PE38 (0–1000 ng/ml) for 10 days at 37°C in a humidified CO2 incubator. The medium was removed, and the colonies were washed with PBS and stained with 0.025% crystal
RESULTS

Subunit Structure and Characterization of IL-13R.

The molecular configuration of IL-13 and IL-13R in 16 SCCHN cell lines was examined by RT-PCR analysis for different receptor chains. Three of 16 SCCHN cell lines strongly expressed mRNA for IL-13Rα2 chain, whereas 5 other cell lines (YCUL891, KCCCL871, KCCCTM8901, and RPMI 2650) expressed IL-13Rα1 chain, and IL-4Rα chain was expressed in all cell lines. RT-PCR analysis for different receptor proteins by indirect immunofluorescence analysis showed that IL-13Rα2 chain was strongly expressed in three cell lines (Fig. 4). The IC50 value for the presence of γc mRNA that is abundantly present in H9 T-lymphoma cells that served as a positive control (data not shown).

Immunofluorescence Analysis for Receptor Subunit Protein on SCCHN Cell Lines.

We next examined the expression of different receptor proteins by indirect immunofluorescence analysis in high and low IL-13Rα2 chain-expressing SCCHN cell lines. Fig. 3A shows a representative ×400 magnified immunofluorescence staining for IL-13Rα2 protein in one of the high expresser cell lines, YCUM911. These cells grew as colonies and showed strong staining with anti-IL-13Rα2 antibody. Similar results were also observed in two other high-expressor cell lines, HN12 and KCCCT873 cells (data not shown). On the other hand, IL-13Rα1 mRNA and IL-4Rα chain expression was demonstrated that these two chains are expressed in SCCHN cell lines. However, similar to RT-PCR results, none of these cell lines expressed γc protein (data not shown).

Expression of IL-13 in SCCHN Cells.

On the basis of RT-PCR and immunofluorescence results, we predicted that radiolabeled IL-13 would specifically bind to SCCHN cell lines. Therefore, binding studies were performed using 125I-IL-13 in three IL-13Rα2-expressing cell lines. As shown in Table 2, these three cell lines expressed a high number of IL-13 binding sites on their cell surface. The number of IL-13 binding sites ranged between 5800 and 8600/cell in these cell lines.

Cytotoxic Activity of IL-13 Toxins in SCCHN Cell Lines.

We first tested the cytotoxic activity of IL-13-PE38QQR on SCCHN cell lines by protein synthesis inhibition assays. IL-13-PE38QQR was highly cytotoxic to three IL-13Rα2-positive SCCHN cell lines (Fig. 4). The IC50 (concentration of IL-13 toxin causing 50% inhibition of protein synaptic

Table 1 mRNA expression for different receptor subunits in SCCHN cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Origin</th>
<th>Receptor subunit</th>
<th>RFU</th>
<th>RFLa,b</th>
<th>α2/β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>YCUMS861</td>
<td>Maxillary sinus</td>
<td>α2</td>
<td>±</td>
<td>0.60</td>
<td>±</td>
</tr>
<tr>
<td>KCCT871</td>
<td>Tongue</td>
<td>α1</td>
<td>++</td>
<td>1.10</td>
<td>±</td>
</tr>
<tr>
<td>KCCT891</td>
<td>Hyophranyx</td>
<td>IL-4Rα</td>
<td>++</td>
<td>1.50</td>
<td>±</td>
</tr>
<tr>
<td>KCCCL871</td>
<td>Larynx</td>
<td>γc</td>
<td>++</td>
<td>0.50</td>
<td>±</td>
</tr>
<tr>
<td>KCCCTM8901</td>
<td>Metastasis to the chest fluid</td>
<td>γc</td>
<td>++</td>
<td>1.60</td>
<td>±</td>
</tr>
<tr>
<td>KCCT873</td>
<td>Tongue</td>
<td>α2</td>
<td>±</td>
<td>0.50</td>
<td>±</td>
</tr>
<tr>
<td>A253</td>
<td>Submandibular gland</td>
<td>α1</td>
<td>++</td>
<td>1.40</td>
<td>±</td>
</tr>
<tr>
<td>HN12</td>
<td>Lymph node</td>
<td>α1</td>
<td>++</td>
<td>1.40</td>
<td>±</td>
</tr>
<tr>
<td>KB</td>
<td>Mouth</td>
<td>α1</td>
<td>++</td>
<td>1.40</td>
<td>±</td>
</tr>
<tr>
<td>RPMI2650</td>
<td>Nasal septum</td>
<td>α1</td>
<td>++</td>
<td>0.56</td>
<td>±</td>
</tr>
</tbody>
</table>

α Positivity of the RT-PCR product was ascertained by fluorescence intensity after staining with ethidium bromide. --, negative; ±, weakly positive; ++, strongly positive; +++, more strongly positive.

RFUs were determined by fluorescence densitometry analysis of RT-PCR bands of α2 and β-actin and expressed as mean ± SD of two experiments performed in duplicate.
sis) ranged between 4 and 9 ng/ml. The PM-RCC cell line that has been shown to express high numbers of IL-13R was extremely sensitive to IL-13-PE38QQR (IC50 < 0.1 ng/ml; data not shown; Ref. 32). The other 12 cell lines that lacked or expressed very low levels of IL-13R by RT-PCR were considerably less sensitive to IL-13-PE38QQR. The IC50 in these cell lines ranged between 100 and 1000 ng/ml (Table 3). The specificity of IL-13 toxin-mediated cytotoxicity was confirmed by neutralization assays in the presence of excess of IL-13 or IL-4. In all three of the cell lines, IL-13 was able to neutralize cytotoxic activity (Fig. 4A), whereas IL-4 (Fig. 4B) did not indicate specificity.

We next compared the cytotoxic activity of IL-13-PE38QQR with IL-13-PE38, which was also produced and purified by an identical technique. As shown in Table 3, IL-13-PE38 was equally cytotoxic to IL-13Rα2-positive cell lines when compared with IL-13-PE38QQR (IC50 = 8 ng/ml), whereas it was less cytotoxic or not cytotoxic to the other 11 SCCHN cell lines tested (IC50, 100 to >1000 ng/ml).

When IC50 of IL-13 toxins was compared with RFU of α2 chain mRNA expression, 3 highly sensitive SCCHN cell lines showed RFU >1 compared with 5 other α2-weakly positive cell lines, which showed RFU of <0.6. Because IL-13Rα2 mRNA band intensity of weakly positive cells was dim, no additional comparisons could be made between relative cytotoxicity and α2-mRNA expression.

**Inhibition of SCCHN Colony Formation by IL-13PE38.**

To confirm whether IL-13-PE38-mediated protein synthesis inhibition of SCCHN cells correlates with cell death, we performed a colony formation assay. SCCHN cells were plated in 100-cm² Petri dishes and treated with various concentrations of the IL-13PE38. After a 10-day culture period, the percentage of colonies formed in control and cytotoxin-treated groups was

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**Table 2** IL-13 receptor expression on SCCHN cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IL-13 binding sites/cell</th>
<th>IL-13-PE38QQR IC50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN12</td>
<td>5800 ± 203</td>
<td>7.5 ± 1.2</td>
</tr>
<tr>
<td>YCUM911</td>
<td>8600 ± 112</td>
<td>4.5 ± 0.32</td>
</tr>
<tr>
<td>KCCTC873</td>
<td>6185 ± 282</td>
<td>8.6 ± 1.8</td>
</tr>
</tbody>
</table>

*Number of binding sites for each cell type was calculated by radio receptor binding as described in “Materials and Methods.” The data are shown as the mean number of molecules/cell ± SE of three experiments performed in quadruplicate.

*IC50, the concentration of IL-13 toxin at which 50% inhibition of protein synthesis is observed compared with untreated cells. The data are shown as the mean ± SD of two experiments performed in quadruplicate.*

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**Fig. 3** Immunofluorescence analysis of various receptor chains on SCCHN cells. A, YCUM911 cells; B, RPMI 2650 cells (×400). Left panels, cells stained with mouse IgG control; right panels, cells stained with monoclonal anti-IL-13Rα2, anti-IL-13Rα1, or anti-IL-4Rα antibody.
compared. As shown in Table 4, the number of colonies decreased in IL-13PE38 treated cells in a concentration dependent manner. The IC50 of IL-13PE38 by colony formation assay corroborated with the IC50 determined by protein synthesis inhibition assays.

Increased Sensitivity of SCCHN Cell Lines on Gene Transfer of IL-13Rα2 Chain. Because only 19% of SCCHN cell lines were highly sensitive and the majority of the cell lines were modestly sensitive or not sensitive at all, we examined whether or not sensitive or modestly sensitive cell lines could be sensitized to high cytotoxic effect of IL-13 cytotoxin. We transiently introduced IL-13Rα2 chain cDNA into 2 SCCHN cell lines that did not show PCR positivity for IL-13Rα2 chain, and cytotoxicity of IL-13-PE38QQR was determined. As shown in Fig. 5, transfection of IL-13Rα2 chain in YCUM861 and KB cell lines improved their sensitivity to IL-13-PE38QQR suggesting that IL-13Rα2 chain was expressed on the cell surface. The IC50 in YCUM861 SCCHN cell line decreased by 12-fold from 1000 ng/ml to 80 ng/ml and from 125 ng/ml to 10 ng/ml in KB cell line as compared with mock-transfected control cells.

DISCUSSION

In this study, we demonstrate that 19% of human SCCHN cell lines express high density IL-13R at mRNA and protein levels. The high level of receptor expression correlated with the expression of the primary IL-13 binding protein, IL-13Rα2 chain. Cell lines that were weakly positive for this chain express few IL-13R. On the other hand, all 16 of the SCCHN cell lines expressed IL-13Rα1 and IL-4Rα chains. Because IL-13Rα1 and
IL-13 Receptor Targeting in SCCHN

IL-4Rα chains are required for IL-4- or IL-13-induced signal transduction (21, 23, 25, 29, 30, 41, 42), our results suggest that SCCHN cell lines express functional IL-13R. These results also indicate that SCCHN cell lines express two types of IL-13R. Nineteen percent of cell lines (3 of 16) expressed type 1 IL-13R, whereas 50% expressed predominantly type II IL-13R. Another 31% cell lines possibly also expressed type 1 IL-13R. Because none of the SCCHN cell lines expressed γc chain, no type III IL-13R were observed. These results indicate the phenotypic heterogeneity of SCCHN as defined by IL-13R expression.

It is of interest to note that 19% of SCCHN cell lines that expressed mRNA and protein for IL-13Rα2 chain were highly sensitive to the cytotoxic effect of IL-13PE38QQR. The other 81% of the cell lines showed low or no sensitivity. The difference in IC50 between IL-13Rα2-positive cell lines and negative cell lines ranged between 25-fold and 250 fold. IL-13PE38QQR has been shown to be highly cytotoxic to a variety of solid human tumor cell lines, e.g., renal cell carcinoma (32), AIDS-associated Kaposi’s sarcoma (19), and malignant glioma (15). Our current results support previous conclusions and extend the list of IL-13-PE38QQR-responsive tumors. In addition, our data suggest that inhibition of protein synthesis initiated by IL-13PE38 is directly proportional to cell death as evidenced by colony formation assay as the number of colonies decreased as the cytotoxic concentration increased in the assay system.

Additional confirmed this conclusion in our current study by transient gene transfer of IL-13Rα2 chain. SCCHN cells expressing IL-13Rα2 chain alone are sufficient to internalize the IL-13-IL-13R complex (43). In addition, this chain alone is sufficient to sensitize cancer cells to the cytotoxic activity of IL-13 cytotoxin (44, 45). We additionally confirmed this conclusion in our current study by transient gene transfer of IL-13Rα2 chain in two different IL-13Rα2-negative SCCHN cell lines. These transfectants acquired sensitivity to IL-13 cytotoxin in vitro. Taken together, IL-13R represents a new target for the therapy of SCCHN naturally expressing IL-13Rα2 chain or engineered to express IL-13Rα2 followed by IL-13 cytotoxin administration. Thus, our current study is important and will recommend testing of IL-13Rα2 chain expression in head and neck tumor samples before enroll-

### Table 3 Cytotoxic activity of IL-13-PE38 and IL-13-PE38QQR in SCCHN cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IC50 (ng/ml)</th>
<th>IC50 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-13PE38</td>
<td>IL-13PE38QQR</td>
</tr>
<tr>
<td>YCUMS861</td>
<td>ND</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>KCCT871</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>KCCL871</td>
<td>185</td>
<td>200</td>
</tr>
<tr>
<td>KCCOR891</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>YCU891</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>YCUMB62</td>
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<td>&gt;1000</td>
</tr>
<tr>
<td>YCUMM911</td>
<td>5</td>
<td>5</td>
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<tr>
<td>YCUT891</td>
<td>&gt;1000</td>
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<td>KCCTCM901</td>
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<td>A253</td>
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<td>HN12</td>
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<td>KB</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>RPMI 2650</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*IC50 concentration of IL-13 toxin at which 50% inhibition of protein synthesis is achieved compared to untreated cells.

### Table 4 In vitro inhibition of colony formation in SCCHN cell lines by IL-13PE38

<table>
<thead>
<tr>
<th>Colonies (% control)</th>
<th>HN12</th>
<th>YCUM911</th>
<th>KCCT873</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-13PE38 (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>70.0 ± 4.3</td>
<td>65.0 ± 1.6</td>
<td>80.0 ± 5.2</td>
</tr>
<tr>
<td>1.0</td>
<td>59.2 ± 2.8</td>
<td>58.2 ± 2.3</td>
<td>69.3 ± 3.6</td>
</tr>
<tr>
<td>10.0</td>
<td>26.1 ± 2.4</td>
<td>12.3 ± 0.9</td>
<td>59.4 ± 1.7</td>
</tr>
<tr>
<td>100.0</td>
<td>12.8 ± 0.6</td>
<td>9.7 ± 1.6</td>
<td>32.8 ± 1.9</td>
</tr>
<tr>
<td>1000.0</td>
<td>0.7 ± 0.3</td>
<td>5.6 ± 0.8</td>
<td>19.2 ± 2.2</td>
</tr>
<tr>
<td>IC50 (ng/ml)</td>
<td>6.5</td>
<td>2.5</td>
<td>11.5</td>
</tr>
</tbody>
</table>

*Results are expressed as percentage of colonies formed by the treated cells compared with untreated control cells.

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**Fig. 5 Sensitization of SCCHN cells to IL-13PE38QQR by gene transfer of IL-13Rα2 chain.** YCUMS861 and KB tumor cell lines transiently transfected with vector alone (control; open symbols) or IL-13Rα2 chain (closed symbols) were cultured with various concentrations of IL-13PE38QQR (0–1000 ng/ml). The results are represented as mean of quadruplicate determination, and the assay was repeated two times; bars, ±SD.
ing any patient in future clinical trials for head and neck cancer therapy.

To translate our observations to clinical trials using IL-13-PE, we have performed several preclinical toxicology and pharmacology studies in mice, rats, and cynomolgous monkeys. These studies suggest that IL-13-PE38QQR is well tolerated up to 50 μg/kg dose injected i.v. or i.p. every alternate day for 3 days in mice or every day for 5 days in monkeys. The only toxicities observed were reversible hepatic enzyme elevations and injection site skin reactions in both mice and monkeys. In addition, up to 100 μg/ml dose is well tolerated when injected stereotactically in the frontal lobe cortex of rat brain. Because human IL-13 binds to murine and monkey cells, these studies in general may predict toxicity of this molecule in the clinic (16, 46). On the basis of these studies, IL-13PE38QQR is being tested in the clinic for the treatment of renal cell carcinoma and recurrent malignant glioma (47, 48). Because SCCHN is a localized disease, it is possible that IL-13-PE can be administered intratumorally or by combination of intratumor and i.v. routes for effective therapy.

In previous studies, we have used IL-13-PE38QQR in vitro and in vivo for targeting IL-13R-positive tumors (15, 19, 31, 32, 34). In this fusion molecule, the COOH terminus of the IL-13 molecule was fused to the NH2 terminus of domain II of the PE molecule. In addition, lysines at position 590 and 606 and lysine at position 613 in PE molecules were substituted by glutamines and arginine (PE38QQR). Because the role of these mutations in the IL-13-PE molecule has not been delineated, here we deleted these mutations and produced a molecule with PE38. IL-13-PE38 was expressed in E. coli in an identical manner to IL-13PE38QQR. On in vitro testing, IL-13-PE38 produced identical results as IL-13-PE38QQR, indicating that the 3 amino acid mutation at the COOH terminus of PE has no effect on IL-13PE38-mediated cytotoxicity. Therefore, we favor the use of a simpler molecule (IL-13-PE38) for future development.

In conclusion, we have shown that 19% of SCCHN cell lines express mRNA and protein for the IL-13Rα2 chain. Because IL-13-PE38 and IL-13-PE38QQR are highly cytotoxic to IL-13Rα2-positive SCCHN cell lines, we believe that IL-13R may serve as a target for delivery of cytotoxins to the certain type of SCCHN tumors. For SCCHN tumors that lack IL-13Rα2 chain in vivo, gene transfer of this chain may sensitize them to the cytotoxic effect of IL-13-PE. Various approaches of gene transfer have been tested in vivo (49–51). Among them, plasmid-mediated or virus-mediated gene transfer may be most desirable. Thus, the IL-13Rα2 chain could serve as a novel target for delivery of cytotoxins to SCCHN.

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Heterogeneity in Interleukin-13 Receptor Expression and Subunit Structure in Squamous Cell Carcinoma of Head and Neck: Differential Sensitivity to Chimeric Fusion Proteins Comprised of Interleukin-13 and a Mutated Form of Pseudomonas Exotoxin

Bharat H. Joshi, Koji Kawakami, Pamela Leland, et al.


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