Introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide, with approximately 60,000 new diagnoses in the United States annually. In recent genomic analyses, NOTCH2 mutations were identified in 10-15% of HNSCC tumors, and evidence of activation of the NOTCH pathway has also been shown. NOTCH has been shown to play a complex role in cancer showing evidence of activity both as a tumor suppressor and oncogene.

Based on these findings, we sought to better understand the role of other NOTCH family member receptors in head and neck cancers, including NOTCH2 and NOTCH3. Therefore we performed genomic analysis of using TCGA and functional studies to elucidate their potential role in the carcinogenesis of HNSCC.

Materials & Methods

Gene expression analysis was performed using TCGA RNA-seq data based on RSEM calculations using provisional data of 520 HNSCC tumors and 46 normal tissue samples. Tumors with any mutations in NOTCH family members were excluded to eliminate variation due to mutations, leaving 447 tumors for analysis. To identify tumors with activation of NOTCH pathway, tumors with HES and HEY activation were identified. Tumors with over expression of HES1 or HES5 greater than 1 standard deviation above normal tissue expression were categorized as HES activated (n=115). Tumors with HEY4 expression greater than 1 standard deviation above normal tissue expression were categorized as HEY activated (n=138). Gene expression of NOTCH family genes was compared between tumors with and without HES activation or with and without HEY activation using standard t-test. Significant associations were identified after correction for multiple hypothesis testing.

For functional in vitro assays, siRNA were obtained for specific knockdown of NOTCH1, NOTCH2, and NOTCH3 (Dharmacon, GE Healthcare). Transient knockdown was achieved through overnight transfection using lipofectamine delivery (Thermo Fisher Scientific). Cell growth was assessed over 72 hours following transfection using alamarBlue reagent (AbD Serotec, BioRad). Matrigel invasion assay with siRNA knockdown of NOTCH (p<0.05) showed specific knockdown of NOTCH1, NOTCH2 and NOTCH3 in each cell line. Gene expression analyses and gene expression analyses were performed using standard t-test. Significant associations were identified after correction for multiple hypothesis testing.

Results

Functional in vitro assays showed that silencing of NOTCH1, NOTCH2 and NOTCH3 using siRNA inhibited growth in two HPV-negative cell lines (SKN3 and SCC61). Growth inhibition was also seen on an HPV-positive cell line (SCC908) with knockdown of NOTCH2 and HEY4 knockdown (Figure 2). Gene expression shows specificity of siRNA knockdown for each NOTCH family member. Relative gene expression of HES1 and HEY4 were evaluated but did not correlate with NOTCH inhibition or growth. Matrigel invasion assay showed that silencing of NOTCH1 and NOTCH3 with siRNA inhibited invasion potential in multiple cell lines.

When blocking antibodies against NOTCH3 were incubated with SCC908, they inhibited growth compared to PBS control. Treatment with blocking antibodies to NOTCH2 (n=2 and NOTCH3 (n=3) decreased HEY expression, correlating with growth inhibition (Figure 4).

Discussion

NOTCH1 is one of the more commonly mutated genes in HNSCC. However, bioinformatics analysis of TCGA HNSCC data suggests that other NOTCH family members may also play an important role in downstream activation of the NOTCH pathway (indicated by HES and HEY activation). Indeed, functional assays show that knockdown of NOTCH2 and NOTCH3 inhibit growth, and knockdown of NOTCH2 and NOTCH3 inhibits invasion potential. Blocking antibodies to NOTCH1, NOTCH2 and NOTCH3 represent potential therapeutics which show inhibition of HEY and growth inhibition with blocking antibodies to NOTCH3. These results point to the potential role for the inhibition of not only NOTCH1, but also NOTCH2 and NOTCH3 in the treatment of HNSCC.

Conclusions

Prior research has shown that NOTCH1 plays an important role in the HNSCC. Both bioinformatics analysis of HNSCC gene expression and functional analysis suggest that other NOTCH family members, particularly NOTCH3, may also contribute to oncogenic potential of HNSCC.

References