Role of NOTCH family receptors in head and neck squamous cell carcinoma (HNSCC)

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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.[1] In recent genomic analyses, *NOTCH1* mutations were identified in 10-15% of HNSCC tumors,[2-3] and evidence of activation of the NOTCH pathway has also been shown.[4] NOTCH has been shown to play a complex role in cancer showing evidence of activity both as a tumor suppressor and oncogene.[4]

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.[6] Tumors with any mutations in NOTCH family receptors 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 *HEY1* 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). Invasion assays were performed over 24-48 hours using Matrigel reagent (Corning) in multiple cell lines. NOTCH blocking antibodies (Genentech)[5] against NOTCH1 (aN1), NOTCH2 (aN2) and NOTCH3 (aN3) were cultured in vitro and growth was assessed over 72 hours.

Notch Pathway Genes	HES activated vs. non-activated tumors (p-value)	HEY activated vs. non-activated tumors (p-value)
DTX1	0.857	0.999
JAG1	0.857	0.295
JAG2	0.662	0.999
DLL1	0.545	0.341
DLL3	0.857	0.103
DLL4	0.090	3.96E-05
NOTCH1	0.731	0.000509
NOTCH2	0.731	0.0406
NOTCH3	0.857	0.000292
NOTCH4	0.197	2.13E-06
OCT4	0.857	0.0952
SOX2	0.857	3.50E-14
NANOG	0.910	0.258
CD44	0.662	0.128
LMO2	0.857	0.999

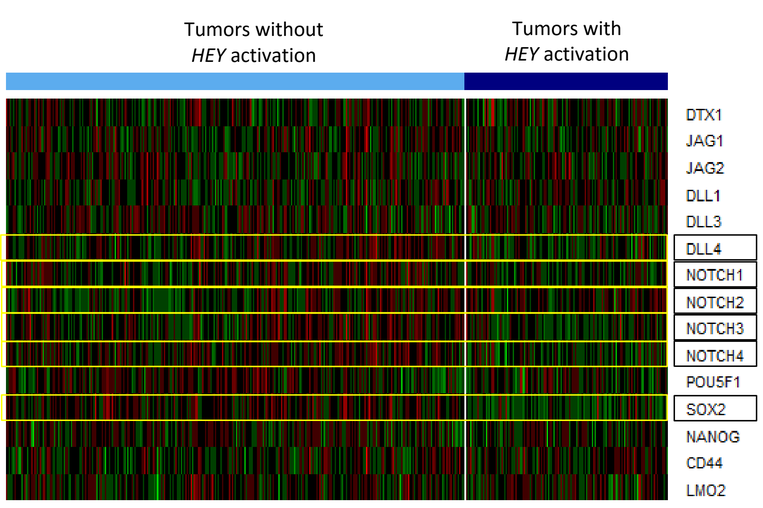

Figure 1. TCGA analysis of NOTCH pathway. Table (left) shows comparison of gene expression of NOTCH pathway members in tumors with and without HES activation or comparing tumors with and without HEY activation. HEY activation was significantly associated with changes in gene expression changes in *NOTCH1*, *NOTCH2, NOTCH3, NOTCH4, DLL4* and *SOX2*. The heatmap (right) depicts gene expression of NOTCH pathway genes in tumors with and without *HEY* activation. Increased expression is shown in green, and decreased expression is shown in red.

TCGA Gene expression analysis

Gene expression was analyzed for NOTCH pathway members (Figure 1, table, left). Genes were compared between tumors with and without HES activation, and tumors with and without HEY activation. Gene expression of *NOTCH1-4, DLL4* and *SOX2* were significantly associated with HEY activation. Further analysis (Figure 1, heatmap, right) showed that HEY activation was associated with increased expression of *NOTCH1, NOTCH3, NOTCH4, SOX2*, and *DLL4*; in addition, HEY activation was associated with decreased *NOTCH2* expression.

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 an HPV-positive cell line (SCC090) with knockdown of *NOTCH2* and trending inhibition in *NOTCH1* knockdown (Figure 2). Gene expression shows specificity of siRNA knockdown for each NOTCH family member. Relative gene expression of *HES1* and *HEY1* 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 *NOTCH1-3* were incubated with SCC090, they inhibited growth compared to PBS control. Treatment with blocking antibodies to *NOTCH2* (aN2) and *NOTCH3* (aN3) decreased *HEY* expression, correlating with growth inhibition (Figure 4).

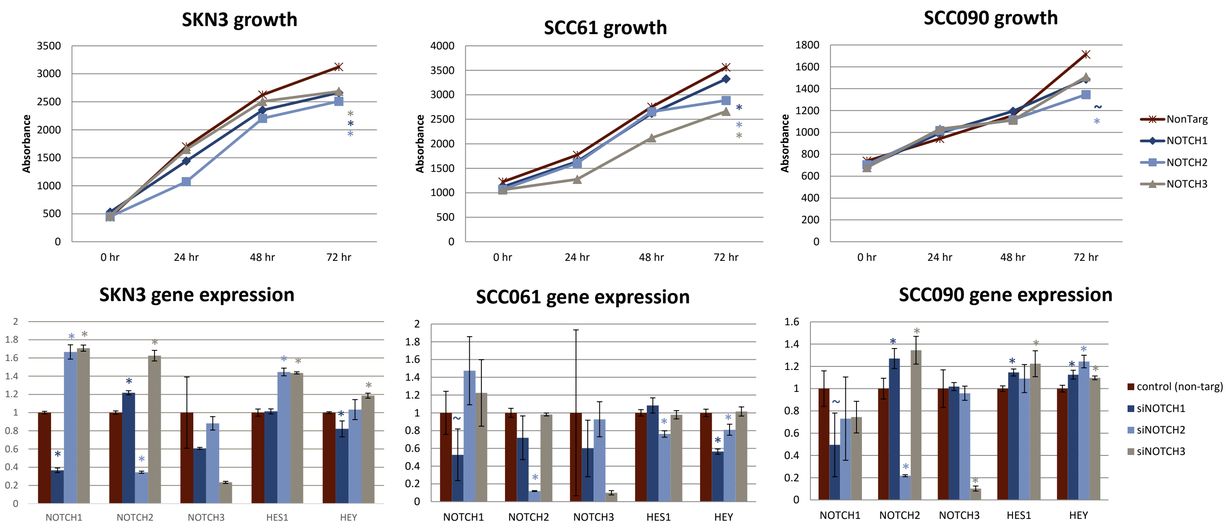

Figure 2. Growth with siRNA knockdown of *NOTCH1, NOTCH2* and *NOTCH3* in three independent head and neck squamous cell carcinoma cell lines. Line plots (above) show growth over 72 hours compared to non-targeting siRNA control. Bar graphs (below) show gene expression analyzed by qRT-PCR showing specific knockdown of *NOTCH1, NOTCH2* and *NOTCH3* in each cell line. Gene expression *NOTCH1-3*, *HES* and *HEY* relative to GAPDH are also shown. (* denotes p<0.05, ~ p<0.10)

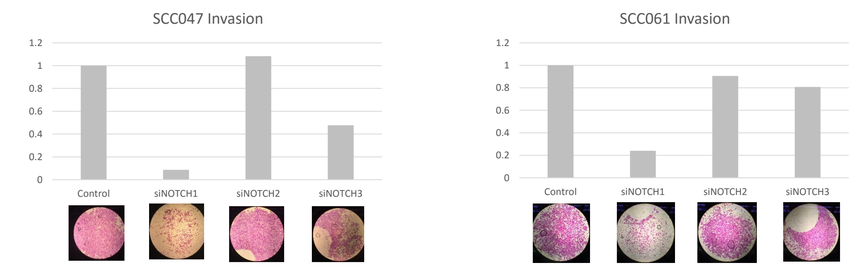

Figure 3. Matrigel invasion assay with siRNA knockdown of *NOTCH1, NOTCH2*, and *NOTCH3*. Invasion was quantified in Adobe photoshop and normalized to non-targeting siRNA control.

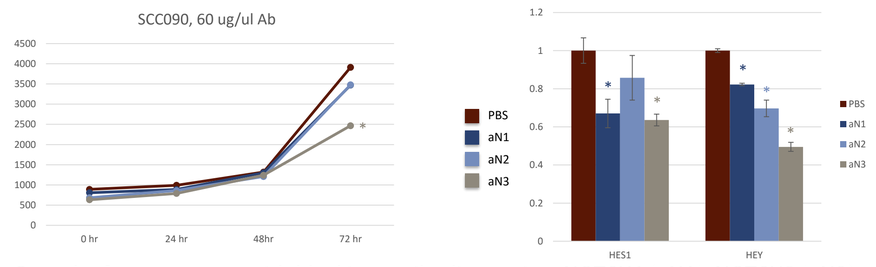

Figure 4. Growth after treatment with blocking antibodies against NOTCH1 (aN1), NOTCH2 (aN2) and NOTCH3 (aN3). Relative gene expression of *HES* and *HEY* are shown after antibody treatment (right).

Discussion

NOTCH1 is one of the more commonly mutated genes in HNSCC.[2-3] 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 *NOTCH1* 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 in vitro assays suggest that other NOTCH family receptors, particularly *NOTCH3,* may also contribute to oncogenic potential of HNSCC.

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