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Cannabinoids promote progression of HPV positive head and neck squamous cell carcinoma via p38 MAPK activation

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Abstract

Purpose: Human papilloma virus (HPV) related head and neck squamous cell carcinoma (HNSCC) is associated with daily marijuana use and is also increasing in parallel with increased marijuana use in the United States. Our study is designed to define the interaction between cannabinoids and HPV positive HNSCC.

Experimental Design: The expression of cannabinoid receptors *CNR1* and *CNR2* was analyzed using The Cancer Genome Atlas (TCGA) HNSCC data. We used agonists, antagonists, siRNAs or shRNA based models to explore the roles of *CNR1* and *CNR2* in HPV positive HNSCC cell lines and animal models. Cannabinoid downstream pathways involved were

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Acquisition of data (provided RNA sequencing data, acquired HNSCC cells, etc.): C. Liu, A. Sakai, T. Guo, J.S. Gutkind

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Other (additional experiments): S. Sadat, K. Ebisumoto, Y. Saito

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

This study has no potential conflict of interest that should be disclosed.

determined by Western blotting and analyzed in a primary HPV HNSCC cohort with single sample gene set enrichment analysis (ssGSEA) and the OncoGenome Positioning System (Onco-GPS).

Results: In TCGA cohort, the expression of *CNR1* and *CNR2* was elevated in HPV positive HNSCC compared with HPV negative HNSCC, and knockdown of *CNR1*/*CNR2* expression inhibited proliferation in HPV positive HNSCC cell lines. Specific CNR1 and CNR2 activation as well as non-selective cannabinoid receptor activation in cell lines and animal models promoted cell growth, migration, and inhibited apoptosis through p38 MAPK pathway activation. CNR1/CNR2 antagonists suppressed cell proliferation and migration, and induced apoptosis. Using whole genome expression analysis in a primary HPV HNSCC cohort, we identified specific p38 MAPK pathway activation signature in tumors from HPV HNSCC patients with objective measurement of concurrent cannabinoid exposure.

Conclusion: Cannabinoids can promote progression of HPV positive HNSCC through p38 MAPK pathway activation.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Globally approximately 30% of HNSCC patients demonstrate human papillomavirus (HPV) infection, with higher rates of HPV in oropharyngeal cancer (1). Despite the overall declining or unchanged incidence of HNSCC, the incidence of HPV positive HNSCC is still increasing (2,3). Initial reports do not find the relationship between marijuana and all head and neck cancers, however, for HPV positive HNSCC, case control studies and other reports have shown that daily marijuana use is associated with HPV oral infection and with development of HPV positive HNSCC (4–7). Marijuana use has undergone dramatic expansion in the United States over the past few decades attributable to cultural, legal, and other forces, which coupled with high rates of early exposure to oral sex and lagging HPV vaccination rates, may potentially contribute to the increase in HPV positive HNSCC (8–10).

Cannabinoids, the major constituents of marijuana, have been primarily used for palliative care in cancer patients (11). The biological effects of cannabinoids are mediated by the endocannabinoid system, including cannabinoid specific G protein-coupled receptors (GPCR), CNR1 and CNR2, and their endogenous ligands endocannabinoids like anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (12,13). CNR1 is expressed mainly in the central nervous system such as brain, while CNR2 is predominantly expressed in the immune system (14). Recent studies have implicated the role of cannabinoid receptors in tumor initiation and progression, with the expression of CNR1 or CNR2 up-regulated in hepatocellular carcinoma, renal cancer and breast cancer, and associated with the severity of disease and poor prognosis (15–17). However, contradictory roles for cannabinoid receptor pathways have been reported, with some groups indicating cannabinoid receptors exert anti-tumoral effects, and others demonstrating a tumor-promoting role for cannabinoids (18,19). For examples, Hijiya et al showed that CNR1 overexpression was independently associated with poor prognosis in esophageal squamous cell carcinoma (20), and Klein-Nulent et al found that CNR2 was highly expressed in HNSCC and associated with reduced survival

(21). Other investigators found that CNR1 and CNR2 was elevated in mobile tongue squamous cell carcinoma, but this higher expression was associated with favorable prognosis (22). However, the physiologic role of cannabinoids in HPV positive HNSCC has been largely unexplored.

In our current study, we investigated the role of classic cannabinoid receptors on the progression of HPV positive HNSCC and identified the possible molecular mechanisms by which these effects occur. Our results demonstrate that knockdown of *CNR1* and *CNR2* inhibit the growth of HPV positive HNSCC cells and CNR1 and CNR2 agonists promote the proliferation and migration of cancer cells and inhibit apoptosis, while the antagonists suppress cell growth and migration and induce apoptosis. In particular, cannabinoid receptor activation produces specific activation of p38 MAPK pathway and this pathway activation is a prominent feature in HPV positive HNSCC primary tumors from patients with cannabinoid exposure. These results provide critical data that indicate a role for cannabinoids in HPV positive HNSCC progression.

MATERIALS AND METHODS

The Cancer Genome Atlas (TCGA) dataset

The mRNA expression of *CNR1* and *CNR2* (Illumina Hiseq RNAseq V2, RSEM normalized) in HNSCC patients were downloaded from the TCGA Research Network (TCGA Provisional version updated in 2016, http://cancergenome.nih.gov/). These TCGA data included 90 HPV positive HNSCC samples, 407 HPV negative HNSCC samples and 44 normal tissues. mRNA expression levels were log2-transformed.

Patient samples

Plasma samples of HPV positive HNSCC patients (n=32) were obtained from the cohort described previously and gene expression data were generated by RNA sequencing (23). All these samples were collected from the Johns Hopkins Tissue Core under an Institutional Review Board approved protocol (#NA_00036235). The study was conducted in accordance with the Declaration of Helsinki. Written informed consents were obtained from all the patients prior to participation in the study.

Cell culture and reagents

HNSCC cell lines UM-SCC-47, UD-SCC-2, 93VU147T and UPCI:SCC090 were obtained from the Gutkind Laboratory at the University of California San Diego, Moores Cancer Center and were fingerprinted and confirmed using short tandem repeat analysis (24). UM-SCC-104 cells were purchased from EMD Millipore Corp (Billerica, MA, USA). All these cell lines were HPV positive HNSCC cells and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) plus penicillin (50 U/ml) and streptomycin (50 μg/ml). For the UM-SCC-104 cells, the medium was also supplemented with 1 × non-essential amino acids and 2 mM fresh L-glutamine (Gibco, Gaithersburg, MD, USA). All cells were incubated at 37°C under an atmosphere of 5% CO_2.

Selective CNR1 agonist ACEA and antagonist SR141716A (Rimonabant), selective CNR2 agonist Hu308 and antagonist SR144528 were synthesized by Tocris Bioscience (Ellisville, MO, USA). Non-selective cannabinoid receptor agonist THC was obtained from Actavis Pharma, Inc. (Parsippany, NJ, USA). Specific p38 inhibitor SB203580 was purchased from Sigma Aldrich. ACEA, Hu308 and THC were dissolved in ethanol. Rimonabant, SR144528 and SB203580 were dissolved in DMSO.

siRNA transfection

HPV positive HNSCC cell lines were transfected with siRNA reagents using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The pooled siRNAs for knockdown the expression of *CNR1* and *CNR2* were purchased from GE Dharmacon (Lafayette, CO, USA), utilizing ON-TARGETplus SMART-pool for *CNR1* (L-004711-00-0005) and *CNR2* (L-005469-00-0005), and ON-TARGETplus Non-targeting pool siRNA (i.e. scrambled siRNA, D-001810-10-20) was used as a negative control (NC). After transfection for 48 hours, the expression of *CNR1* and *CNR2* mRNA was assessed by qRT-PCR.

Inducible stable shRNA transfection

Doxycycline inducible shRNA expression vectors of CNR1 and CNR2 and SMARTvector Inducible Non-targeting control vector (VSC11653) were purchased from GE Dharmacon. Lentiviral particles were prepared for CNR1 and CNR2 shRNA and non-targeting shRNA expression using HEK293T cells as the packaging cells. UM-SCC-47 and UD-SCC-2 cells were infected with viral supernatants containing CNR1 or CNR2 shRNA with Polybrene, followed by selection using 1 μg/ml Puromycin (InvivoGen, San Diego, CA, USA). After infection by virus, the cells were cultured in DMEM with 10% Tet-System-Approved FBS (Takara Bio USA, Inc., Mountain View, CA, USA). The shRNA expression of CNR1 and CNR2 were induced with 1 μg/ml doxycycline (Sigma Aldrich).

Quantitative real-time PCR

Total RNA was extracted from cells using RNeasy plus mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). The primers and probes were obtained from TaqMan Gene Expression Assays (Thermo Fisher Scientific). Each gene ID was described as follows: *CNR1*: Hs01038522_s1; *CNR2*: Hs00952005_m1; *β-actin*: Hs01060665_g1. PCR quantification was conducted using the $2^{-\Delta\Delta CT}$ method and normalized to *β-actin*.

Western blotting analysis

Western blotting analysis was conducted as previously described (25). Cells were lysed with RIPA lysis buffer, and total protein concentrations were quantified using Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated on Mini-PROTEAN TGX gels (Bio-Rad) and transferred onto PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA at room temperature for 30 min, the membranes were incubated with the relevant primary antibody followed by an appropriate secondary antibody. The primary antibody of p38, p-p38,

MAPKAPK2, p-MAPKAPK2, HSP27 and p-HSP27 were obtained from Cell Signaling Technology (1:2000, Danvers, MA, USA). CNR1 and CNR2 antibody were purchased from Abcam (1:800, Cambridge, MA, USA). Anti-GAPDH (1:10000, Cell Signaling Technology) was used as the loading control. Western blots were developed using ECL reagent (Pierce ECL Western Blotting Substrate, Thermo Scientific).

Viability assay

Cells were seeded into 96-well plates at the concentration of 3,000 to 9,000 cells/well. After cells were starved in serum free media for 24 h, different doses of CNR1/CNR2 agonists and antagonists were added and cell viabilities were measured using Vita Blue Cell Viability Reagent (Bimake, Houston, TX, USA). After incubation for 1 hour at 37°C in the assay solution, fluorescence was measured by microplate reader (BioTek, Winooski, VT, USA). All the experiments were repeated three or more times.

Transwell migration assay

Transwell migration assay was conducted with 8 μm pore size Corning Transwell migration chambers (Corning Inc., Corning, NY, USA) following manufacturer's instruction. Briefly, 1 to 4 × 10^5 HPV positive HNSCC cells in serum-free medium were treated with CNR1/CNR2 agonist or antagonist and seeded to the upper chamber. After incubation for 48 h, non-migrating cells were removed from the surface of the upper chamber with a cotton-tipped swab. The migrated cells on the lower surface were fixed in 4% paraformaldehyde, stained with crystal violet, and then counted in five random fields under microscope. The number of cells in the membrane of bottom chamber represents the ability of cell migration.

Cell apoptosis assay

HPV positive HNSCC cells were plated into 6-well plates (3–9 × 10^5 cells/well). Cells were starved in serum free media and treating with CNR1/CNR2 agonists or antagonists for 72 hours. For the controls, the cells were treated with corresponding vehicle. Then flow cytometry was used to detect apoptosis of the cells with Annexin V-FITC Apoptosis Detection Kit (Sigma Aldrich).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The cannabinoid metabolites in the plasma samples of 32 HPV positive HNSCC were detected using LC-MS/MS method. Briefly, plasma samples were spiked with internal standard, cannabinoids were precipitated out using a cold acetonitrile crash, and diluted preparations underwent solid phase extraction (Oasis PRiME HLB 96-well plate, 30 mg, Waters). Samples were then evaporated, reconstituted, and injected on to a 2.1 × 50 mm Acquity UPLC BEH C18 column packed with 1.7 μm sized particles (Waters). A gradient elution was performed on an Acquity i-class UPLC system (Waters) using a 5 mM ammonium formate buffer with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The LC was coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer (Waters) interfaced with an electrospray ionization probe in positive ionization mode. The transition ions of Δ9-tetrahydrocannabinol (THC), cannabinol (CBN), cannabidiol (CBD), 11-hydroxy-THC (11-OH-THC), (±)-11-nor-9-carboxy-Δ9-

THC (THC-COOH), cannabigerol (CBG), and tetrahydrocannabivarin (THC-V) were collected using multiple reaction monitoring (controlled by Masslynx V4.1, Waters) and peaks were processed using TargetLynxs XS. The lower limit of quantification (LLOQ) for CBN, CBD, THC, and THC-V was 1 ng/mL whereas the LLOQ for 11-OH-THC and THC-COOH was 2 ng/mL.

Single sample gene set enrichment analysis (ssGSEA)

ssGSEA is an extension of GSEA, which calculates separate enrichment scores for each pairing of a sample and gene set. Dysregulated pathways were identified by ssGSEA (26,27) and data was compared between the HPV positive HNSCC patients whose plasma samples with and without cannabinoid metabolites expression. $P < 0.01$ was considered to be significant.

The OncoGenome Positioning System (Onco-GPS)

Onco-GPS is a computational method designed to decompose groups of transcriptional signatures into summary components that are used in order to define functional tumor subtypes aptly referred to as “oncogenic states” (28). The resulting Onco-GPS “map” provides a robust framework to examine the complex, wide-ranging functional differences and similarities, originating from differences in molecular features including gene & pathway expression among tumor samples. The Onco-GPS approach used here is similar to the one described previously (28) but we used the ssGSEA enrichment profiles of MSigDB gene sets (29) as input to the methodology instead of KRAS signature genes. Our purpose in applying this classification technique was to investigate if our cannabinoid positive samples stratified to the same oncogenic state, which would suggest that cannabinoid exposure in HPV positive HNSCC is associated with a distinct, functional tumor subclass discernible by a predictable pattern of oncogenic transcriptional activity.

Specifically, pathway expression scores from each of the HPV positive HNSCC samples corresponding to the gene sets were used as the criteria for the Onco-GPS map. The list of gene sets is unfiltered, and they collectively represented a broad array of biological processes that allowed us to identify the transcriptomic relationships among our tumor samples in a strictly unbiased fashion. Non-negative matrix factorization (NNMF) was applied to the collection of tumor sample pathway expression scores in order to identify discrete patterns of transcriptional activity, each of which formed a “component” or “node” in our Onco-GPS map. Each pathway’s correlation with a node is represented statistically by an information coefficient (IC) and P-value. Tumor samples were then assigned scores corresponding to their transcriptional similarity to each of the nodes. Tumor samples with similar relative relationships to each component stratify into oncogenic states, each of which characterizes a functionally distinct molecular subtype of our tumor cohort. The number of nodes (five) and states (three) comprising our Onco-GPS map was explicitly chosen to produce a model with an appropriate degree of granularity to decisively illustrate the molecular heterogeneity of our cohort. Pathways distinguishing each oncogenic state and node were investigated and herein reported.

Tumor Xenografts

Four or five weeks old female nude mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained under specific pathogen-free conditions with the approval of the Institutional Animal Care and Use Committee of University of California San Diego. Tumor xenografts were induced by subcutaneous injection in the flank of nude mice with 2×10^6 UD-SCC-2 cells or UM-SCC-47 shRNA CNR1 and CNR2 cells. For the UD-SCC-2 xenografts, when tumors had reached an average size of 50 mm^3, animals were divided randomly into various groups and injected intraperitoneally with THC (3 mg/kg) and control vehicle every day, or Rimonabant (1 mg/kg), SR144528 (1 mg/kg), SB203580 (5 mg/kg) and control vehicle every other day. For the UM-SCC-47 shRNA xenografts, all the mice in empty vector (EV), shCNR1 and shCNR2 group were fed with doxycycline food. Tumors were measured with external caliper, and the volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Statistical analysis

All experiments were performed at least in triplicate. The statistical comparisons of two groups were determined with a two-sided unpaired Student's t test (for equal variances) or Mann–Whitney U test (for unequal variances) using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

CNR1 and *CNR2* are up-regulated in HPV positive HNSCC

To investigate the role of *CNR1* and *CNR2* in HPV positive HNSCC, we first examined the expression of *CNR1* and *CNR2* within the TCGA dataset. This analysis showed that the *CNR1* and *CNR2* expression were both up-regulated in HPV positive HNSCC compared with HPV negative HNSCC (Fig. 1A, B, $P < 0.001$). In addition, the expression of *CNR2* was higher in HPV positive HNSCC compared to normal samples (Fig. 1B, $P < 0.001$).

CNR1 and *CNR2* knockdown inhibits HPV positive HNSCC cell growth

To evaluate the role of cannabinoid receptors in HPV positive HNSCC, we knocked down the expression of *CNR1* and *CNR2* in HPV positive HNSCC cells using pooled siRNA. qRT-PCR results showed that the expression of *CNR1* and *CNR2* was successfully down-regulated by siRNA (Fig. 2A, B). Following knockdown the expression of *CNR1* in HPV positive HNSCC cell lines such as UD-SCC-2, UM-SCC-47 and 93VU147T, the proliferation of cancer cells were significantly decreased (Fig. 2C). Meanwhile, downregulation of *CNR2* also inhibited the growth of most of HPV positive HNSCC cell lines such as UPCI:SCC090, UM-SCC-47 and 93VU147T (Fig. 2D). To further confirm the effect of *CNR1* and *CNR2* on cell growth, we established doxycycline (Doxy) inducible shCNR1 and shCNR2 stable UD-SCC-2 cell lines. qRT-PCR and western blotting assay confirmed that after doxycycline induction, the expression of CNR1 or CNR2 was successfully down-regulated in shRNA group (Supplemental Fig. S1A, B). Compared with the empty vector group, the proliferative ability of cancer cells in both shCNR1 and shCNR2 group were significantly inhibited with doxycycline induction (Supplemental Fig. S1C, D).

CNR1 and CNR2 agonists or antagonists affect the proliferation of HPV positive HNSCC cells

To drive specific cannabinoid pathways in HPV positive HNSCC, cell lines were treated with a selective CNR1 agonist ACEA, CNR2 agonist Hu308 and non-selective cannabinoid receptor agonist THC. We specifically chose dose ranges consistent with cannabinoid concentrations commonly found during recreational marijuana exposures (30,31). Compared with control cells, proliferation in cells treated with different concentrations of ACEA and Hu308 (1 nM, 10 nM, 100 nM and 1 μM) was increased in UD-SCC-2, UPCI:SCC090 and UM-SCC-104 cells, but not in UM-SCC-47 and 93VU147T cells (Fig. 3A, B, and F). In addition, higher concentrations of ACEA and Hu308 (10 μM) could inhibit cell growth in UM-SCC-47 cells (Supplemental Fig. S2A, B). 1 nM to 1 μM THC enhanced the proliferative ability of UD-SCC-2, UPCI:SCC090 and UM-SCC-47 cells (Fig. 3C, F). Following knockdown the expression of *CNR1*, the effect of selective CNR1 agonist ACEA on cell growth was attenuated, and down-regulation of *CNR2* also reversed the cell proliferative changes caused by selective CNR2 agonist Hu308 in UD-SCC-2 cells (Supplemental Fig. S3A, B). Only one cell line and condition was not consistent with a pro-proliferative action of CNR1 and CNR2 activation, as 1 μM THC suppressed tumor growth in 93VU147T cells (Fig. 3F). Taken together these results reveal that, in general, activation of cannabinoid receptors promotes the proliferation of HPV positive HNSCC cells.

In parallel, selective CNR1 antagonist Rimonabant and CNR2 antagonist SR144528 were used to inhibit cannabinoid receptors in HPV positive HNSCC. As shown in Fig. 3D and F, the number of cells treated with Rimonabant (1 nM, 10 nM, 100 nM and 1 μM) was significantly decreased compared to untreated cells in UD-SCC-2, UM-SCC-47 and 93VU147T cells. In UD-SCC-2, UPCI:SCC090 and 93VU147T cells, 1 nM to 1 μM SR144528 also suppressed cell growth (Fig. 3E, F). These results confirm that cannabinoid receptor antagonists inhibit the proliferation of HPV positive HNSCC cells.

CNR1 and CNR2 agonists or antagonists affect the apoptosis and migration of HPV positive HNSCC cells

To study whether cannabinoid receptors impact cell apoptosis, we performed flow cytometric assessment of Annexin V expression to quantify apoptotic cells. We observed that CNR1 or CNR2 agonist ACEA (1 μM), Hu308 (1 μM) and THC (1 μM) inhibited apoptosis of HPV positive HNSCC cells (Fig. 4A–C), while CNR1 or CNR2 antagonist Rimonabant (1 μM) and SR144528 (1 μM) could induce cell apoptosis in HPV positive HNSCC (Fig. 4D, E).

A transwell migration assay was used to quantify cell migration ability in HPV positive HNSCC cells treated with 1 μM CNR1 and CNR2 agonists or antagonists. Our results demonstrated that CNR1 or CNR2 agonist ACEA, Hu308 and THC could increase cell migration ability across multiple cell lines (Fig. 4F–H, Supplemental Fig. S4A–C), while the number of migrating cells was significantly reduced after treating with CNR1 antagonist Rimonabant and CNR2 antagonist SR144528 (Fig. 4I, J, Supplemental Fig. S4D, E).

Activation of cannabinoid receptor is associated with p38 MAPK pathway activation

It has been previously reported that a variety of GPCR agonists can activate the p38 MAPK pathway (32,33). Thus, we hypothesized that p38 MAPK pathway may be involved in the process of promoting cell progression caused by cannabinoid receptor agonists. Western blotting results showed that treatment with 1 μM cannabinoid receptor agonists ACEA, Hu308 and THC for 5 min, 15 min and 30 min in HPV positive HNSCC UPCI:SCC090 cells resulted in increased levels of the active, phosphorylated form of p38, p-p38, and in general, MAPK downstream targets p-MAPKAPK2 and p-HSP27 were elevated in 15 min and 30 min (Fig. 5). These were also validated in UM-SCC-104 cells with increased levels of p-p38 after treating with 1 μM ACEA, Hu308 and THC (Supplemental Fig. S5A). In UD-SCC-2 cells, the levels of p-p38 were elevated in treating with 1 μM ACEA and THC for 15 min and 30 min, albeit more obvious for THC at 10 μM (Supplemental Fig. S5B). These results indicate that stimulation of cannabinoid receptor results in p38 MAPK pathway activation.

To further confirm the interaction between the p38 MAPK pathway and cannabinoid receptor activation, we used 10 μM SB203580 (a p38 MAPK specific inhibitor) to inactive p38 MAPK in HPV positive HNSCC cells (34). As expected, in UD-SCC-2, UPCI:SCC090 and UM-SCC104 cells, inactivation of p38 MAPK pathway significantly inhibited the proliferative ability triggered by CNR1 agonist ACEA (Supplemental Fig. S6A) and CNR2 agonist Hu308 (Supplemental Fig. S6B). In UD-SCC-2, UPCI:SCC090 and UM-SCC-47 cells, inhibition of p38 MAPK could also attenuate the pro-proliferative effect caused by non-selective cannabinoid receptor agonist THC (Supplemental Fig. S6C). These data indicate that CNR1 and CNR2 promoted proliferation in HPV HNSCC cells is mediated in part by p38 MAPK activation.

Activation of cannabinoid receptor promotes proliferation of HPV positive HNSCC cells xenografts

To evaluate the role of cannabinoid receptor *in vivo*, we tested the effects of THC on HPV positive HNSCC growth in a subcutaneous xenograft model with UD-SCC-2 cells. Tumor-bearing nude mice were treated with the control vehicle or 3 mg/kg THC daily. As shown in Fig. 6A–C, compared with the vehicle treated group, the growth of tumor was significantly increased in the THC treated group. In addition, in THC treated tumor xenografts, western blotting confirmed that p38 MAPK pathway was activated with increased levels of p-p38, p-MAPKAPK2 and p-HSP27 correspondingly (Fig. 6D). In a complementary experiment tumor-bearing nude mice were treated with CNR1 antagonist Rimonabant (1 mg/kg), CNR2 antagonist SR144528 (1 mg/kg) and p38 MAPK inhibitor SB203580 (5 mg/kg) every other day. The results revealed that compared with the control group, the growth of tumor was significantly decreased in the Rimonabant, SR144528, and SB203580 treated group (Fig. 6E–G).

We also generated xenografts in mice with the established UM-SCC-47 doxycycline inducible shRNA CNR1 and CNR2 cells. qRT-PCR and western blotting assay validated that the expression of CNR1 or CNR2 was decreased in UM-SCC-47 shRNA cells (Supplemental Fig. S7A, B). Tumor-bearing nude mice were fed with doxycycline food to

induce appropriate shRNA mediated inhibition. The growth of tumor was significantly decreased in the shCNR1 and shCNR2 group than control group (Fig. 6H–J). These results demonstrate that the cannabinoid receptor CNR1 and CNR2 promote proliferation of HPV positive HNSCC cells *in vivo*, and that cannabinoid receptor blockade inhibits tumor growth *in vivo*.

HPV positive HNSCC tumors from patients with cannabinoid exposure activate the p38 MAPK pathway

To define if patients exposed to cannabinoids with HPV positive HNSCC activate similar pathways to those activated in *in vitro* and *in vivo* animal studies, we analyzed a previously reported cohort of HPV positive oropharynx cancer patients who have undergone whole genomic RNA sequencing that had available plasma samples to define systemic exposure to cannabinoids (23). The plasma samples of 32 HPV positive HNSCC patients were assayed for cannabinoid metabolites and five patients were noted to have cannabinoids present in plasma samples obtained the same day as tumor biopsy (Supplemental Table S1). In this cohort, there was no significance in age, gender, tobacco smoking and alcohol status between patients with and without cannabinoid exposure (Supplemental Table S2, all $P > 0.9999$) and no significance in overall survival ($P = 0.9928$) and disease free survival ($P = 0.1436$) between these two groups (Supplemental Fig. S8). ssGSEA was performed using RNAseq data to define differential pathway activation between the tumor samples corresponding to patients with and without the presence of cannabinoid metabolites in their plasma. 261 up-regulated pathways and 274 down-regulated pathways were determined in patients with cannabinoid metabolites (Supplemental Fig. S9 and Supplemental Table S3). Importantly, the pathway “P38 MAPK PATHWAY” was upregulated and “APOPTOSIS PATHWAY” was downregulated in the patients with cannabinoid metabolites, consistent with *in vitro* and *in vivo* data above showing MAPK activation by cannabinoids. In addition, we also found numerous FGFR1 associated pathways were up-regulated, which indicated that in addition to the p38 MAPK pathway, FGFR1 and other pathways might be involved with HPV positive HNSCC in patients with cannabinoid exposure.

Onco-GPS is an effective approach to explore the complex landscape of oncogenic cellular states across cancers (28), and we used this method to discern oncogenic network states within the cohort of 32 HPV positive HNSCC samples, with the explicit purpose of defining common oncogenic networks in HPV HNSCC patients with cannabinoid exposure. The relatively enriched pathways for each transcriptional component and oncogenic state forming the Onco-GPS map are listed in Supplemental Table S4 and Table S5. The Onco-GPS map suggested the 32 samples aggregate into three distinct oncogenic states, with almost of all (4/5) cannabinoid positive samples stratifying to the same State 0 group with component nodes defined by MAPK and FGFR1 signaling in the map (Fig. 7A, B), and all tumors with higher enrichment in FGFR1 signaling activity are present in State 0 group, with the majority of these samples deriving from cannabinoid positive patients (Fig. 7C). A heatmap also shows FGFR1 and MAPK signaling (high) and apoptosis pathways (low) represent robust oncogenic characteristics of State 0 (Fig. 7D).

Taken together, these data indicate that patients with cannabinoid exposure present with similar oncogenic network characteristics involving MAPK and FGFR1 pathway activation and apoptosis pathway inhibition.

DISCUSSION

Previous epidemiologic data have showed that marijuana use is associated with the development of HPV positive HNSCC (4,5,7). Initial studies that did not find an association between marijuana and head and neck cancers did not examine HPV related head and neck cancers specifically and epidemiologic data do not implicate a causative relationship between marijuana use and HPV negative HNSCC (5,35,36). In this study, we focused the role of cannabinoids in HPV positive HNSCC and defined the oncogenic interaction between cannabinoids and HPV positive HNSCC progression.

Although the cannabinoid receptor pathway has been investigated in multiple tumor types, there are conflicting publications regarding the role of cannabinoid receptor in tumor proliferation (18,37). Recently, *in vitro* and *in vivo* experiments suggest that CNR1 and CNR2 agonists are potential options for treatment of a variety of cancer types (37,38). However, reports of tumor inhibition via CNR1 and CNR2 agonists often use cannabinoid concentrations in the 5–20 μM range, which exceed the binding constants between cannabinoids and their cognate receptors, and are not reflective of traditional exposures, as peak plasma concentrations of cannabinoids rarely exceed the 1 μM level in marijuana smokers (15,30,31,39). For example, a recent report of the endocannabinoid anandamide in HNSCC cell lines reported an antitumor effect but employed 20 μM concentrations, and defined this mechanism as receptor independent (40). In this study, data show cannabinoids driven proliferation of HPV positive HNSCC cells at concentrations of 1 nM to 1 μM, consistent with levels from recreational marijuana use. Of note, when we increased the concentration of cannabinoid receptor agonists to 10 μM, the growth of cancer cells was largely inhibited (Supplemental Fig. S2). These results were consistent with similar findings in gastric cancer (41), colon cancer (42), glioblastoma and lung carcinoma (39). In addition, cannabinoids may exert activity via immune modulation, as THC enhances breast cancer growth and metastasis by suppressing antitumor immune response in *in vitro* and *in vivo* models (43).

As the main cannabinoid receptor in central nervous system, CNR1 was also found to be elevated in a series of cancers, which indicated that CNR1 may be pro-tumorigenic. In support of this, CNR1 immunoreactivity was associated with disease severity and outcome in esophageal cancer (20), prostate cancer (44), pancreatic cancer (45), ovarian cancer (46) and others. Furthermore, CNR1 knockout mice developed smaller and fewer tumors in a diethylnitrosamine induced hepatocellular carcinoma model (15), and overexpression of CNR1 in esophageal squamous cell carcinoma and gastric cancer revealed that CNR1 activation could promote cell proliferation and invasion (20,47). In this study, although TCGA data showed that the expression of *CNR1* mRNA was downregulated in HNSCC compared with normal samples, *CNR1* mRNA was highly expressed in HPV positive HNSCC compared with HPV negative HNSCC (Fig. 1A), which suggests that *CNR1* plays a specific role in HPV positive HNSCC. This was confirmed by knockdown the expression of

CNR1 in HPV positive HNSCC cells lines and mice xenografts, whose proliferation ability was inhibited. The highly selective CNR1 agonist ACEA, despite the bimodal effects on tumor proliferation, was reported to stimulate the proliferation of gastric cancer cells at concentrations under 1 μM (41). The CNR1 antagonist Rimonabant also inhibited growth of breast and colon cancer xenografts in mice and induced G1 arrest and cell apoptosis to enhance anticancer immunity in glioma (48–50). Consistent with these findings, our current results demonstrated that ACEA and Rimonabant affected the proliferation, apoptosis and migration of HPV positive HNSCC cancer cells, and support the oncogenic role of CNR1 in HPV positive HNSCC.

Similar with CNR1, the cannabinoid receptor CNR2 was also found to be highly expressed and correlated with worse prognosis in tumors such as breast cancer (16), renal cancer (17), lung cancer (51) and HNSCC (21). In our study, the expression of *CNR2* mRNA was upregulated in HNSCC compared with normal samples, and much higher in HPV positive HNSCC samples. Meanwhile, knockdown the expression of *CNR2* inhibited the proliferation of HPV positive HNSCC *in vitro* and *in vivo*. In addition, selective CNR2 agonist Hu308 increased the growth, migration and inhibited the apoptosis of HPV positive HNSCC cells while the selective CNR2 antagonist SR144528 exhibited an opposite effect. These phenomena were also observed in other tumors like colon cancer, in which CNR2 activation promoted cancer progression via AKT/GSK3β signaling pathway when cannabinoids employed in concentrations similar to those in marijuana exposure (42).

Interestingly, cannabinoid receptors can couple to Gi/o family of G-proteins with subsequent inhibition of adenylyl cyclase activity and stimulation of MAPK pathways (18,52). Dysregulations within MAPK pathways are also consistently observed throughout most of HNSCC cases, which play critical roles in multiple cellular processes including cell growth, apoptosis and differentiation, and ultimately contributes to HNSCC progression (34,53). Indeed, when cannabinoid receptors were activated by the agonists ACEA, Hu308 and THC, the expression of p-p38 MAPK was elevated and the downstream molecules of p38 MAPK signaling pathway, such as p-MAPKAPK2 and p-HSP27, increased correspondingly. These were further confirmed by inhibition of p38 MAPK with SB203580, while the pro-proliferative effect caused by cannabinoid receptors agonists were largely attenuated. Through gene set analysis between 32 HPV positive HNSCC patients with and without cannabinoid metabolites in plasma, we confirmed that the p38 MAPK pathway was significantly activated and apoptosis pathway was inhibited in HPV positive HNSCC patients with cannabinoid exposure. However, additional dysregulated pathways were identified (Supplemental Table S3), including the FGFR1 pathways. Using the Onco-GPS method, the 32 HPV positive HNSCC samples were stratified into three functional oncogenic states, each demonstrating unique patterns of pathway expression. The finding that almost all (4/5) cannabinoid positive samples aggregated in a single state (State 0) suggests that cannabinoid exposure has important, discerning effects on oncogenesis of HPV positive HNSCC. Notably, upregulation of FGFR1 and MAPK signaling represent robust oncogenic characteristics of State 0, and the majority of samples with high FGFR1 activity included cannabinoid positive patients. Genes involved in apoptosis were significantly downregulated among all samples in State 0 relative to those in other states, suggesting that patients with cannabinoid exposure exemplify a broader oncogenic state in which apoptosis

is differentially suppressed. These data are consistent with our *in vitro* and *in vivo* findings, and indicate that additional dysregulated pathways may be driven by cannabinoids in HPV positive HNSCC.

Using *in vitro* and animal models, as well as patient cohorts, these data show that cannabinoids promote oncogenic activation of a p38 MAPK pathway in HPV positive HNSCC and may represent a novel therapeutic target for HPV positive HNSCC. These findings are consistent with epidemiologic data that daily marijuana use may serve as a biologic cofactor role in development of HPV positive HNSCC. Additional studies are warranted to further define the mechanism by which cannabinoids exert these effects in HPV positive HNSCC, as well as define therapeutic strategies based on these pathways. These data may have broader public health implications, given the rapid increase in HPV related HNSCC, as well as the increase in marijuana use and legalization in the US.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

HPV positive HNSCC represents a distinct clinical and molecular entity associated with daily marijuana use. However, the functional role of the cannabinoid receptor pathway in HPV positive HNSCC is undescribed. In this study, we demonstrated that cannabinoids promote progression of HPV positive HNSCC via p38 MAPK activation using *in vitro* and animal models, as well as in patient cohorts, which is consistent with epidemiologic data that daily marijuana use may serve as a biologic cofactor role in development of HPV positive HNSCC. This expands the understanding of HPV positive HNSCC carcinogenesis and may also have broader public health implications in marijuana use in the US.

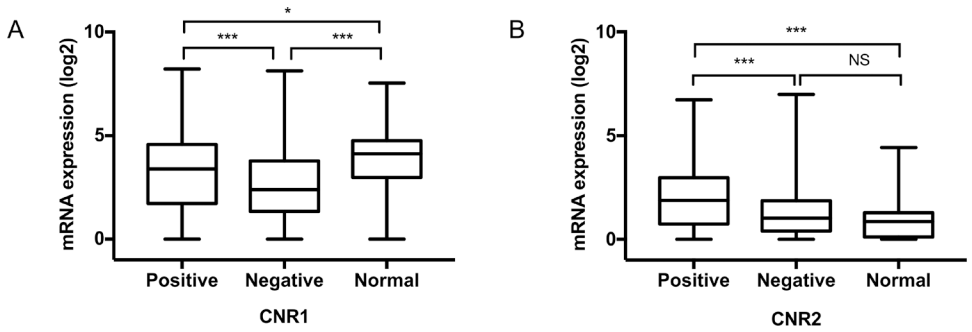

Figure 1. Expression of CNR1 and CNR2 in TCGA HNSCC data

(A) Compared with HPV negative HNSCC, the expression of *CNR1* is upregulated in HPV positive HNSCC samples. (B) Compared with HPV negative HNSCC and normal samples, the expression of *CNR2* is upregulated in HPV positive HNSCC samples. HPV positive: n = 98; HPV negative: n = 422; normal: n = 44. The statistical comparisons were determined with Mann–Whitney U test. *: $P < 0.05$, ***: $P < 0.001$, NS: not significant.

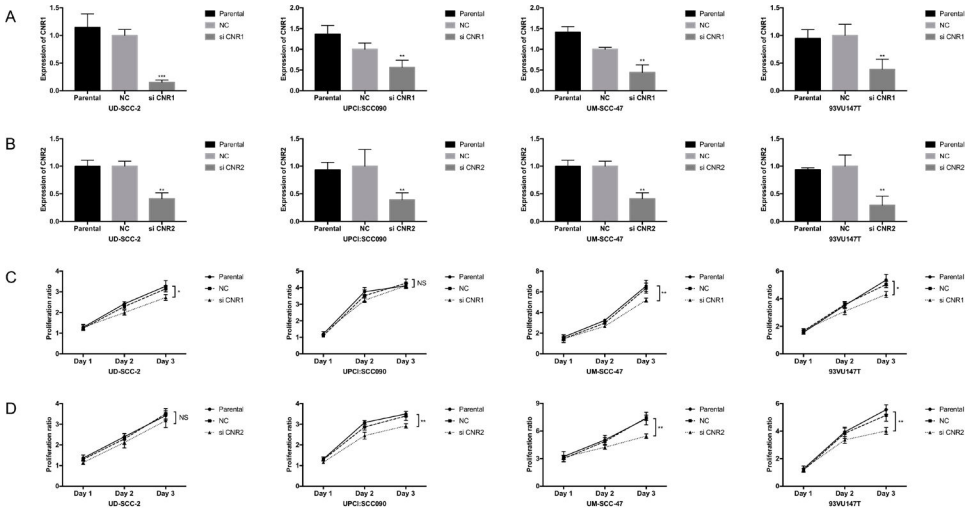

Figure 2. CNR1 and CNR2 knockdown inhibits HPV positive HNSCC cell growth

(A) qRT-PCR assays validate that the expression of *CNR1* is successfully down-regulated by pooled siRNA in HPV positive HNSCC UD-SCC-2, UPCI:SCC090, UM-SCC-47 and 93VU147T cells. (B) qRT-PCR assays validate that the expression of *CNR2* is successfully down-regulated by pooled siRNA in HPV positive HNSCC UD-SCC-2, UPCI:SCC090, UM-SCC-47 and 93VU147T cells. (C) Cell viability is measured after knockdown the expression of *CNR1* in HPV positive HNSCC cells. Growth is normalized to day zero and measured over 3 days, and proliferation ratio is calculated relative to day zero. Significant growth inhibition is seen with specific silencing of *CNR1* compared with parental and negative control (NC) group in UD-SCC-2, UM-SCC-47 and 93VU147T cells. (D) Cell viability assay shows significant growth decrease by knockdown of *CNR2* in UPCI:SCC090, UM-SCC-47 and 93VU147T cells. Experiments were performed in triplicate and the statistical comparisons were determined with Student's t test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: not significant.

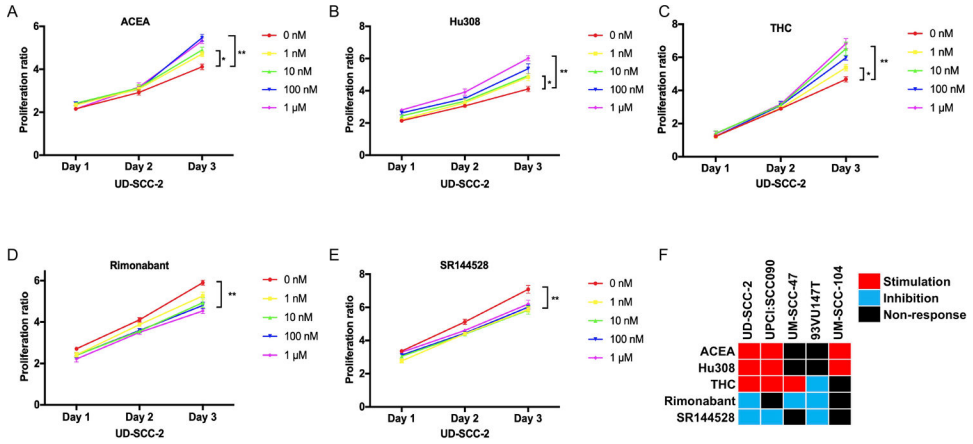

Figure 3. CNR1 and CNR2 agonists or antagonists affect the proliferation of HPV positive HNSCC cells

(A–C) Treatment with different concentrations (1 nM, 10 nM, 100 nM and 1 μM) of selective CNR1 agonist ACEA (A), selective CNR2 agonist Hu308 (B) or non-selective cannabinoid receptor agonist THC (C) promotes the proliferation of HPV positive HNSCC UD-SCC-2 cells. (D–E) Treatment with different concentrations (1 nM, 10 nM, 100 nM and 1 μM) of selective CNR1 antagonist Rimonabant (D) or selective CNR2 antagonist SR144528 (E) inhibits the proliferation of UD-SCC-2 cells. (F) Overview of the effects of cannabinoids on different HPV positive HNSCC cells growth: red, growth stimulation; blue, growth inhibition; black, non-response in growth. Experiments were performed in triplicate and the statistical comparisons were determined with Student's t test. *: $P < 0.05$, **: $P < 0.01$.

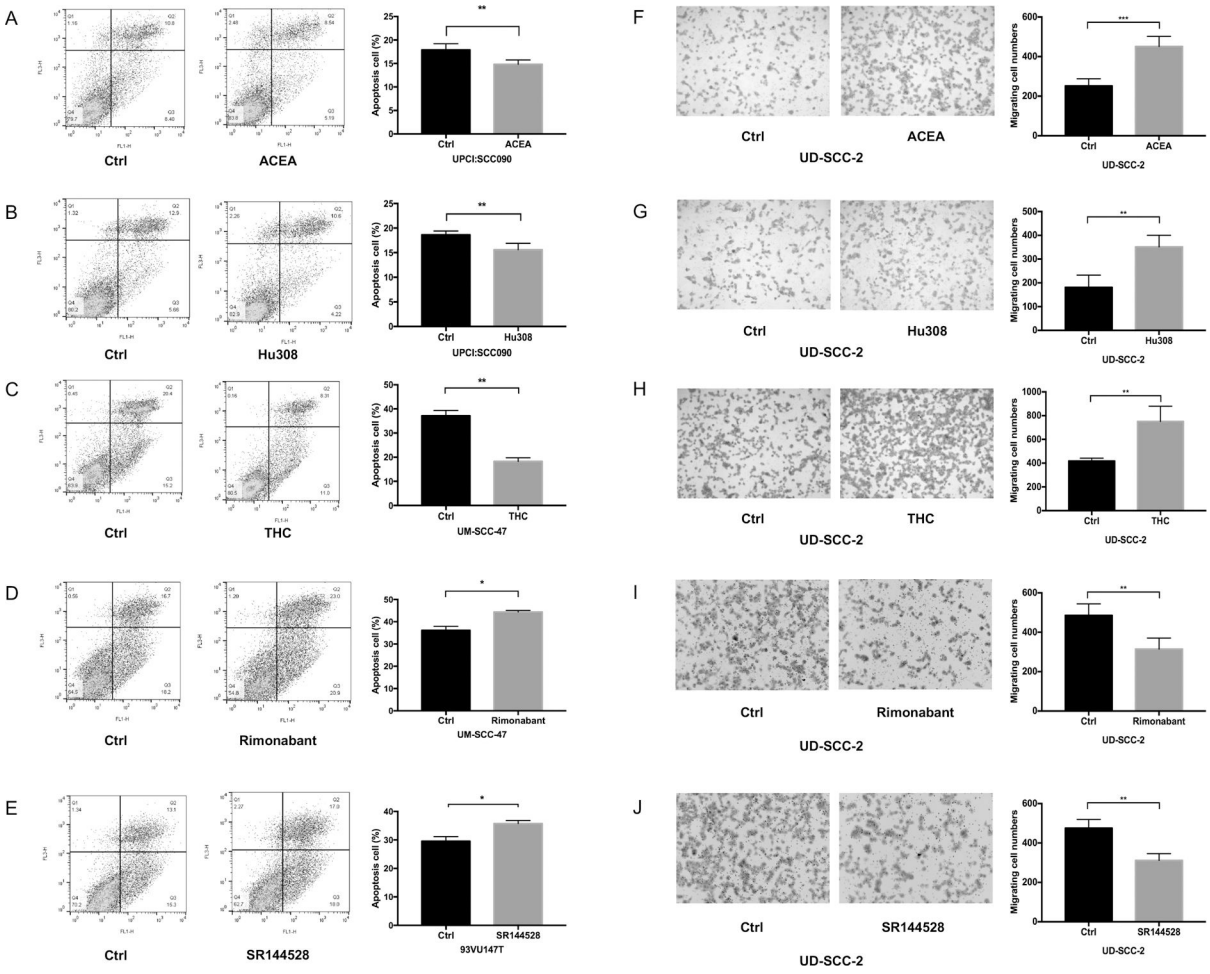

Figure 4. CNR1 and CNR2 agonists or antagonists affect the apoptosis and migration of HPV positive HNSCC cells

(A, B) Treatment with 1 μM ACEA (A) and Hu308 (B) inhibits apoptosis of HPV positive HNSCC UPCI:SCC090 cells. (C) Treatment with 1 μM THC inhibits the apoptosis of HPV positive HNSCC UM-SCC-47 cells. (D, E) Treatment with 1 μM Rimonabant (D) and SR144528 (E) induces the apoptosis of HPV positive HNSCC UM-SCC-47 and HNSCC 93VU147T cells, respectively. (F–H) Treatment with 1 μM ACEA (F), Hu308 (G) and THC (H) promotes the migration of HPV positive HNSCC UD-SCC-2 cells. (I–J) Treatment with 1 μM Rimonabant (I) and SR144528 (J) inhibits the migration of HPV positive HNSCC UD-SCC-2 cells. Experiments were performed in triplicate and the statistical comparisons were determined with Student's t test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

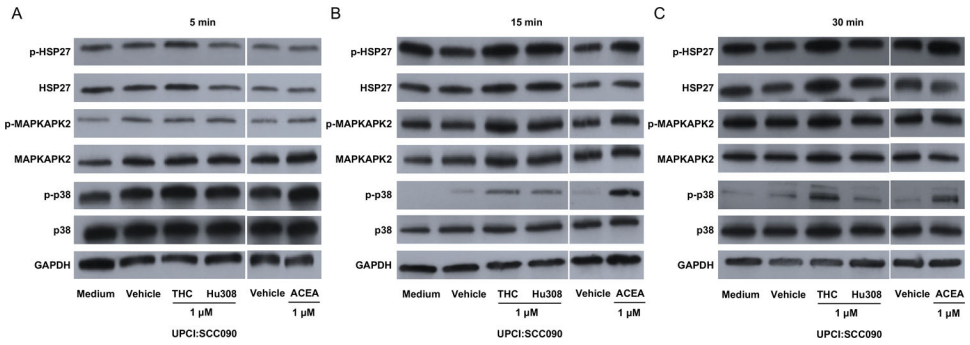

Figure 5. Activation of cannabinoid receptor is associated with p38 MAPK pathway

(A–C) Treatment with 1 μM ACEA, Hu308 and THC for 5 min (A), 15 min (B) and 30 min (C) in UPCI:SCC090 cells, in general, the expression of p-p38, p-MAPKAPK2 and p-HSP27 in p38 MAPK pathway is elevated.

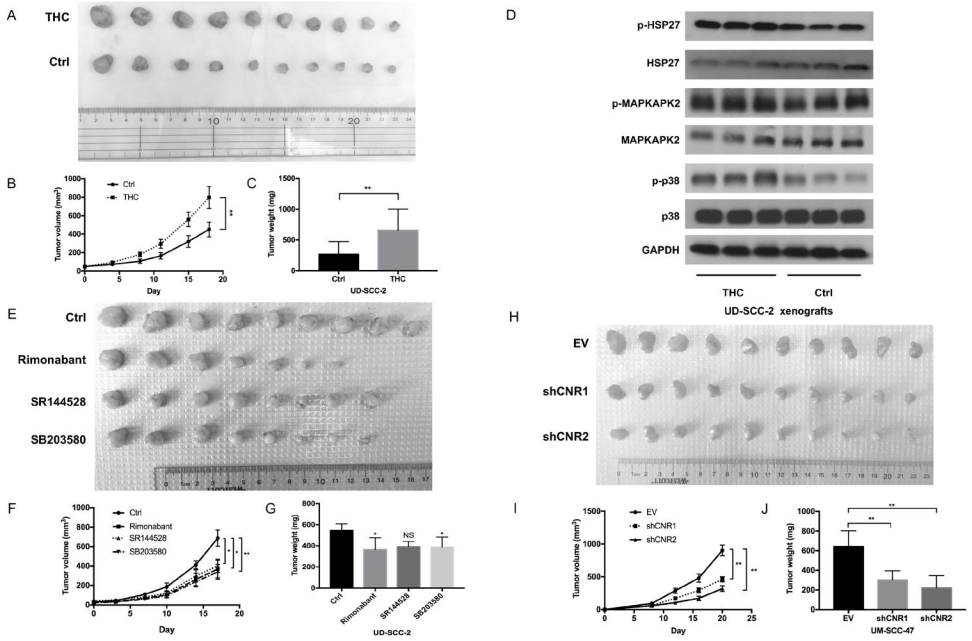

Figure 6. Activation of cannabinoid receptor promotes proliferation of HPV positive HNSCC cells xenografts

(A, B) THC treated tumor xenografts grow substantially faster compared to those in control group. (C) The average weight of the tumors in THC treated group is significantly heavier than those of the control group. (D) Western blotting shows p38 MAPK pathway is activated in THC treated tumor xenografts. (E, F) Tumor xenografts grow substantially slower in Rimonabant, SR144528 and SB203580 treated group compared to those in control group. (G) The average weight of the tumors in Rimonabant and SB203580 treated group is lighter than those of the control group. (H, I) CNR1 or CNR2 silenced tumor xenografts grow substantially slower compared to those in empty vector group. (J) The average weight of the tumors in CNR1 or CNR2 knockdown group is significantly lighter than those of empty vector group. *: $P < 0.05$, **: $P < 0.01$, NS: not significant.

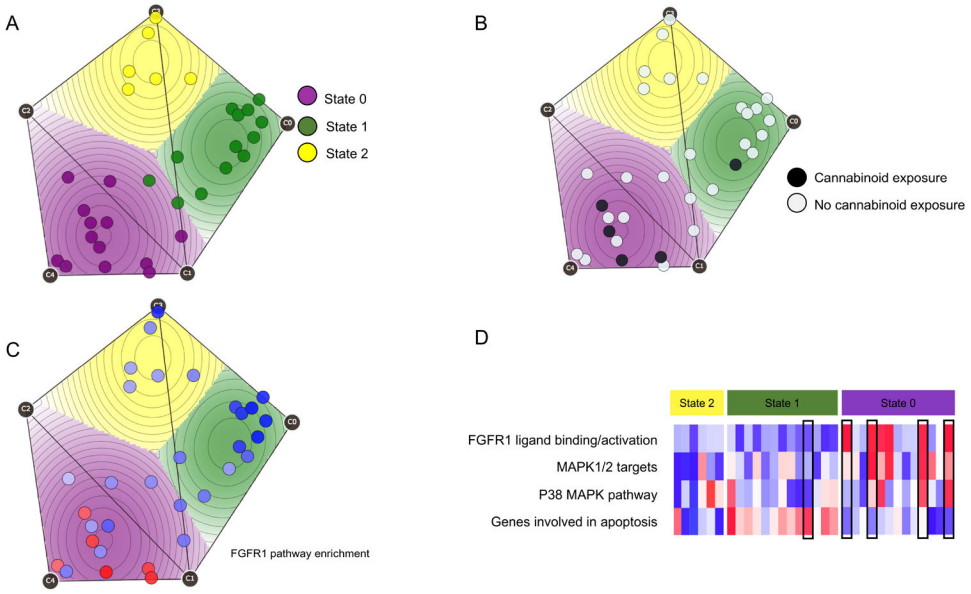

Figure 7. The OncoGenome Positioning System map

(A) Onco-GPS map of cellular states including all 32 HPV positive tumor samples. The tumors aggregate roughly into three cellular, or oncogenic states, each characterized by a unique pattern of pathway expression. (B) Onco-GPS map showing tumor samples derived from patients with cannabinoid exposure (shown in black) and without cannabinoid exposure (shown in grey). Four of the five cannabis positive positive samples aggregate into State 0 (purple). Membership in State 0 includes 13 samples overall. (C) Onco-GPS shows samples colored according to FGFR1 pathway activity. All tumors with higher enrichment in FGFR1 signaling activity are present in State 0, the majority of these samples deriving from cannabinoid positive patients. (D) Heatmap demonstrating ssGSEA enrichment of four selected pathways. FGFR1 signaling (high) and apoptosis (low) represent robust oncogenic characteristics of State 0. Expression patterns corresponding to cannabinoid positive samples are indicated with a black box.