Infectious Agents and Cancer

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cIAP-2 protein is upregulated by human papillomavirus in oropharyngeal cancers: role in radioresistance in vitro

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Abstract

Background High-risk human papillomaviruses are the causal agents of a subset of head and neck cancers. A previous transcriptomic analysis showed that cIAP2 protein, involved in cell survival and apoptosis, is upregulated in OKF6 oral cells that express HPV16 E6/E7. In addition, cIAP2 promotes radioresistance, a very important concern in HNC treatment. However, cIAP2 increase has not yet been evaluated in oropharyngeal carcinomas (OPCs), nor has been the role of cIAP2 in HNC radioresistance.

Methods We carried out a descriptive-analytical retrospective study in 49 OPCs from Chilean patients. We determined the expression of cIAP2 at transcript and proteins levels using reverse-transcriptase -polymerase chain reaction and immunohistochemistry, respectively. HPV and p16 expression were previously analyzed in these specimens. In addition, SCC-143 HNC cells ectopically expressing HPV16 E6/E7 were analyzed for cIAP2 expression and after transfection with a siRNA for HPV16 E6/E7 knocking down.

Results We found a statistically significant association between HPV presence and cIAP2 expression ($p=0.0032$ and $p=0.0061$, respectively). An association between p16 and cIAP2 levels was also found ($p=0.038$). When SCC-143 cells were transfected with a construct expressing HPV16 E6/E7, the levels of cIAP2 were significantly increased ($p=0.0383$ and $p=0.0115$, respectively). Conversely, HPV16 E6 and E7 knocking down resulted in a decrease of cIAP2 levels ($p=0.0161$ and $p=0.006$, respectively). Finally, cIAP2 knocking down in HPV16 E6/E7 cells resulted in increased apoptosis after exposure to radiation at 4 and 8 Gy ($p=0.0187$ and $p=0.0061$, respectively).

Conclusion This study demonstrated for the first time a positive relationship between HPV presence and cIAP2 levels in OPCs. Additionally, cIAP2 knocking down sensitizes HNC cells to apoptosis promoted by radiation. Therefore, cIAP2 is a potential therapeutic target for radiation in HPV-driven HNC.

Keywords CIAP-2, Human papillomavirus, Oropharyngeal, Radioresistance, Cancer

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Introduction

Human papillomaviruses (HPVs) are a group of small epitheliotropic viruses that infect the skin and mucous membranes [1]. HPVs cause various diseases, including genital warts and some types of cancer, such as cervical, anogenital, and oropharyngeal cancer (OPC) [2]. Indeed, the so-called high-risk (HR)-HPVs are involved in almost 100% of cervical cancer cases and 30.8–42.7% of OPCs worldwide [3–5]. Specifically, HPV16 is the most prevalent HR-HPV found in more than 60% of anogenital carcinomas and 44% to 91% of OPCs worldwide [6]. The main mechanism by which HR-HPVs contribute to cancer involves the sustained overexpression of viral oncoproteins, including E6 and E7 [7]. These oncoproteins can disrupt normal cell cycle regulation and promote cell growth, potentially leading to cancer development. HR-HPV E7 induces pRb downregulation and HR-HPV E6 promotes p53 degradation thus disrupting the apoptosis pathway [8]. The role of both HR-HPV E6/E7 in cancer initiation and progression depends exclusively on their capacity to interact with cellular proteins [9]. Thus, a plethora of additional functions of HR-HPV E6/E7 have been previously described [10]. These functions involve interactions with cellular partners including c-Myc to induce hTERT promoter activation [11]; signaling pathways activation including MAPK [12] and PI3K/Akt/mTOR [13]; angiogenesis [14] and epithelial mesenchymal induction [15], among other functions.

cIAP-2 protein, encoded by the BIRC3 gene, belongs to IAP family of proteins that includes eight members with importance as potential molecular and treatment targets. cIAP-2 regulates cell survival and programmed cell death, although also is involved in inflammatory signaling and immunity modulation, cell proliferation, invasion, and metastasis [16–18]. Utilizing a transcriptomic approach, we had previously observed that the BIRC3 gene was significantly upregulated in oral epithelial cells ectopically expressing HPV16 E6/E7 oncoproteins [19]. Additionally, it was shown that HR-HPV E6/E7 mediates the expression of multiple genes including cIAP-2 in lung carcinomas, contributing to increased proliferation, angiogenesis, and immortalization [20]. Moreover, cIAP-2 is regulated by HPV16 E6 by EGFR/PI3K/AKT activation in lung cancer cells [21] and this protein increased in HPV-associated Bowenoid papulosis when compared to condyloma acuminatum samples and normal skin [22]. Importantly, cIAP2 protein is overexpressed in different HR-HPV positive cell lines and clinical specimens, conferring apoptosis resistance [21–24]. Interestingly, it has been established that cIAP2 inhibition in HNC improves the response to radiotherapy in vitro and in vivo [21, 24–28]. Accordingly, certain cIAP2 antagonists are in clinical phase 2 of approval for co-adjuvant therapy [29]. The relationship between HPV and cIAP2 in OPC has never been evaluated. In addition, the role of this association in radioresistance has not been determined in HNC cells. Thus, in this study, we investigated the cIAP-2 expression in OPCs from Chilean patients and a potential association with HR-HPV. In addition, we evaluated whether cIAP-2 expression depends on ectopic HPV16 E6/E7 expression in HNC cells and the role of cIAP2 in radioresistance in vitro.

Material and methods

Clinical samples

Formalin-fixed paraffin-embedded tissues were collected from 49 Chilean patients with a diagnosis of OPC. The specimens were obtained from the Service of Pathological Anatomy from Clinical Hospital José Joaquín Aguirre, between 2010 and 2020. Additionally, 31/49 (63.3%) of the patients were men and 18/49 (36.7%) were women, with a mean age of 62.6 years. HPV presence/genotyping and p16 detection by IHC were previously determined in these specimens [30]. This study was approved by the Ethics Committee of the Clinical Hospital of the University of Chile (approval code 47/19). The epidemiological data contained in the anatomical-pathological report were obtained and those missing were looked up in the electronic clinical record. The clinical specimens were labeled with an internal number for their management. Each sample was analyzed by an experienced pathologist who selected the areas of the biopsy specimen in which neoplastic involvement was observed, from which material was obtained both to make tissue microarrays (tissue arrays) and for nucleic acid extraction (2 cuts of 10 microns, each).

Tissue arrays and immunohistochemistry (IHC)

Four tissue microarrays were manufactured, in which 13 5 mm samples of oropharynx, a control of non-neoplastic spleen tissue, and a control of neoplastic breast carcinoma tissue were included. The Benchmark Ultra equipment (Ventana, Medical Systems Inc., Tucson, AZ, USA) was used in which the tissue microarrays were introduced into it and Tris–Borate-EDTA (TBE) pH 8.0 buffer was added for 36 min to antigen recovery. Subsequently, nonspecific proteins were blocked by incubation with 3% albumin for 1 h. For cIAP2 IHC, the microarrays were incubated at 4 °C in a humid chamber overnight with the cIAP2 monoclonal antibody (E40 clone; 1:500 dilution). The p16 IHC data was obtained from a previous study by us [30]. Briefly, for p16 IHC, antigenic recovery was carried out in TBE buffer pH 8.0 for 92 min, incubation with the Cintec p16 antibody clone E6H4 (Ventana, Medical Systems Inc., Tucson, AZ, EE. USA) for 1 h at 37°. For cIAP2 and p16,

revealing was performed using the UltraView Dab Kit (Ventana, Medical Systems Inc., Tucson, AZ, USA). For cIAP2 analysis, samples were classified as negative when the staining was negative or very slight, and positive when the staining was moderate to intense, which was evaluated by an experienced pathologist. The results for p16 IHC were expressed as negative and positive according to the standard of the Pathological Anatomy Service according to the indications provided by the American College of Pathologists (positive with ≥ 70% nuclear and cytoplasmic staining) (de C Ferreira et al., 2021).

Cell cultures and transfections

SCC-143 cells (squamous cell carcinoma of the floor of the mouth) were obtained from the University of Pittsburgh (White et al., 2007). Cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Fremont, CA, USA) and antibiotics (100 U/mL penicillin and 100 g/mL streptomycin) and were maintained at 37ºC in a 5% CO2 atmosphere incubator. For subculture, cells were incubated with trypsin for 3–5 min and maintained with fresh medium containing Fetal Bovine Serum (FBS) (Hyclone, Fremont, CA, USA). The cells were tested against mycoplasma contamination. The pLXSN and pLXSNVPH16E6/E7 plasmids were donated by Dr. Massimo Tommasino†, from the International Agency for Research on Cancer (IARC), Lyon, France. Retroviral transduction was performed with GP + envAM-12 packaging cells previously transfected with the pLXSN or pLXSNHPV16E6/E7 plasmids with lipofectamine 2000 and maintained for 24 h at 37 °C under conditions of 5% CO2 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. SCC-143 cells were stably transduced with retroviruses and then selected with 0.3 mg/mL geneticin (GIBCO, Carlsbad, CA, USA). Knocking down of HPV16 E6, HPV16 E7 and cIAP2 was performed in SCC-143 E6/E7 cells using small interfering RNA (siRNA) (SC-156008 for E6, SC-270423 for E7, SC-29850, Santa Cruz). A scramble sequence was used as the negative control (SC-37007, Santa Cruz). Transfection of SCC-143 cells with the siRNAs was performed using FuGENE® 6 (Promega, Madison, WI, USA), according to the manufacturer's instructions.

RNA extraction, reverse-transcriptase PCR, and reverse-transcriptase quantitative PCR

The RNA from clinical specimens was purified using the High Pure RNA Paraffin Kit (Roche, Pleasanton, CA, USA), following the manufacturer's instructions. The RNA from SCC-143 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After chloroform purification and isopropanol precipitation, the RNA was suspended in diethylpyrocarbonate (DEPC)-treated water and stored at − 80 °C. Next, the RNA was incubated with RQ1 RNase-free DNAse (Promega, Madison, WI, USA) at 37 °C for 60 min and then treated with RQ1 DNAse Stop Solution for 10 min. cDNA was prepared using a 20 µL-reaction volume containing DNAse treated RNA (1 µg), 1 U RNAse inhibitor (Promega, Madison, WI, USA), 0.04 µg/µL random primers (Promega, Madison, WI, USA), 2 mM dNTPs (Promega, Madison, WI, USA) and 10 U Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was incubated for 1 h at 37 °C. The obtained cDNA was stored at − 20 °C until use. For cIAP2 transcript detection in clinical specimens, a semiquantitative RT-PCR was carried out, using the following primers: cIAP2 F 5′-AGCTACCTCTCAGCC TACTTT-3′ and cIAP2 R 5′-CCACTGTTTTCTGTA CCCGGA3′. The conditions of amplification were: 94 °C for 5 min, followed by 33 cycles that included 95 °C for 45 s, 56 °C for 40 s and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The PCR products were characterized by 2.5% agarose gel electrophoresis and stained with SafeView PlusTM (abm, Vancouver, BC, Canada) and visualized by UV exposure in a transilluminator (Vilber-Lourmat, Marne La Vallée, France). The ImageJ software version 1.52a was used for semiquantitative analysis. For experiments in the SCC-143 cell line, RT-qPCR was performed on the AriaMx Real-Time machine (Agilent, Santa Clara, CA, USA) in a final volume of 25 µL. The reaction components were as follows: 12.5 µL of 2X SYBR Green Mastermix (Bioline, London, United Kingdom), 7.5 µL of nuclease-free water, and 1 µL of cDNA. The amplification conditions were as follows: initial denaturation at 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 20 s, for a total of 40 cycles. The relative copy number of each sample was calculated using the $2-\Delta\Delta Ct$ method. A melting curve was carried to determine the specific melting temperature of each amplicon. Endogenous β-actin mRNA levels were used for the normalization of RNA expression. All reactions were performed in triplicate.

Western blotting

Protein lysate was obtained from SCC-143 cells transduced with either an empty vector or HPV16 E6/E7 vector, using 1X RIPA lysis buffer (Abcam, Cambridge, United Kingdom) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). The suspensions were centrifuged at 14,000 rpm for 15 min at 4 °C. Protein concentration was determined using the PierceTM BCA

Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Next, 15 µg of total protein was loaded per well and separated by SDS-PAGE on 12% gels. Proteins were then transferred by semidry electrotransfer to Hybond-P ECL membranes (Amersham, Piscataway, NJ, USA), using a Tris–glycine transfer buffer pH 8.3 (20 mM Tris, 150 mM glycine and 20% methanol) and the Trans-Blot SD® kit (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5% bovine serum albumin/0.5% Tween-20 in Tris-buffered saline pH 7.6 (TBS) for 1 h at room temperature and then incubated overnight at 4 ∘C with primary antibodies against cIAP2 (ab32059), p53 (BD554294) (BD PharmigenTM, San Diego, CA, USA), pRb (ab24) or β-actin (SC47778) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted 1:1000 in TBS/Tween 20 (TBS–T20). After three washes in TBS-T20, the membranes were incubated with Anti-Mouse IgG (BD Pharmingen; BD Biosciences, Heidelberg, Germany) or Anti-Rabbit IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). USA) conjugated with HRP, diluted 1:1000 in 5% BSA blocking buffer for 1 h at room temperature. Membranes were washed three times for 15 min and revealed with ClarityTM Western ECL detection reagent (Bio-Rad, Hercules, CA, USA) in a ChemiDoc™ Imaging System (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

Radiation exposure and apoptosis analysis by flow cytometry

The cellular radiation assays were performed at the National Cancer Institute (INC) under the supervision of a radiation oncologist and a medical physicist. After 24 h post-transfection, SCC143 E6/E7 control and SCC143 E6/E7 si-cIAP2 cell cultures were irradiated in 6-well plates (200,000 cells per well), using the Trilogy clinical electron linear accelerator (Varian) at doses of 0, 2, 4, 6 and 8 Gy. SCC143 E6/E7 and SCC143 E6/E7 si-cIAP2 cells were trypsinized and harvested 8 h post-irradiation. The flow cytometry assay for the evaluation of apoptosis was performed using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher, Waltham, MA, USA) according to the manufacturer's instructions. The tests were carried out in triplicate.

In silico analysis

The UCSC Xena web source (https://xenabrowser.net) allowed us to explore functional genomic datasets and correlational genomic and phenotypic variables. We selected 612 HNSSC samples from GDC TCGA Head and Neck Cancer Database—first, BIRC3 gene expression concerning the HPV status (ISH information). Next, only 33 HNSSCs met the following criteria: (1) HPV status information, (2) BIRC3 expression, and (4) tumor primary site (tonsils and base of tongue cancers).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 (Version 9.3.1) and Stata 17.0 (Stata Corp, Texas, USA) softwares. The results were considered statistically significant when p was less than or equal to 0.05.

Results

cIAP2 is overexpressed in high-risk HPV positive OPCs

Firstly, we evaluated BIRC3 (cIAP2) gene expression at transcript levels in HPV positive and negative patients with head and neck carcinomas from the TCGA database. A statistically significant increase in BIRC3 transcripts is observed in HPV positive patients, characterized by ISH testing ($p = 0.0112$, Fig. 1A). Next, we focused exclusively on OPCs from the TCGA database. 34 cases were found, 11 of which were HPV negative, and 23 of which were HPV positive. We observed cIAP2 transcript

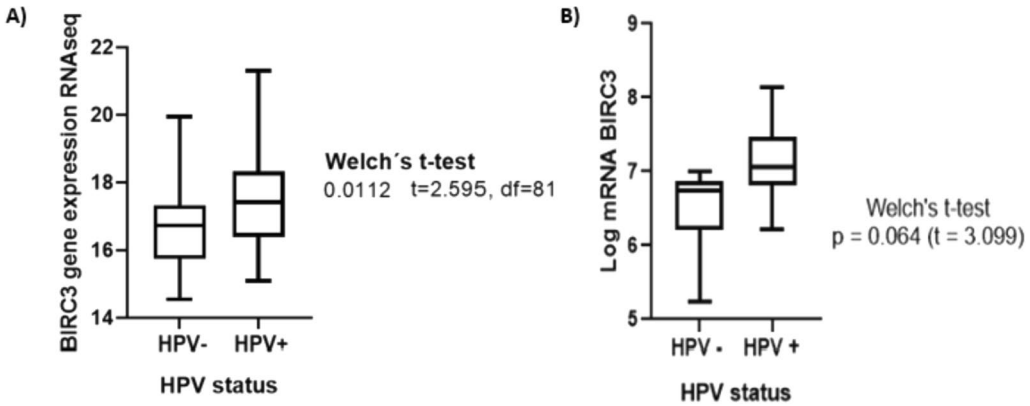


Fig. 1 BIRC3 (cIAP2) expression in HNCs from TCGA database (extracted from Xena Browser). **A** HNCs ($p = 0.0112$, Welch´s t-test). **B** OPCs (tonsils and base of tongue cancers ($p = 0.064$, Welch´s t-test)

increase in HPV positive cases when compared with the HPV negative cases, although this difference was not statistically significant ($p=0.064$, Fig. 1B). After that, we evaluated the relationship between cIAP2 expression and HPV in OPCs from Chilean patients. For this purpose, 49 oropharyngeal tumors were collected and analyzed for p16 (a surrogate HPV biomarker) and cIAP2 IHC. The anatomopathological features of patients are shown in Table 1.

We previously established the presence and genotyping of HPV in these specimens. Briefly, HPV was detected in 61.2% of OPCs, with HPV16 being the most prevalent genotype [30]. Additionally, p16 was positive by IHC in 58.3% of the cases [30]. In this study, we evaluated cIAP2 at transcript and protein levels in the previously collected OPCs from Chilean patients. Representative tissue array results for p16 and cIAP2 protein detection are shown in Fig. 2. cIAP2 IHC was positive in 27 out of 49 (55.1%) OPCs with non-statistically significant difference among anatomical locations ($p=0.09$). Interestingly, a statistically significant association between p16 and cIAP2 transcript levels was found ($p=0.038$, Fig. 3 left). Furthermore, when we compared cIAP2 IHC and cIAP2 transcripts with HPV DNA presence, a significant association was found ($p=0.0027$, Table 2 and $p=0.007$, respectively, Fig. 3 right). As expected, p16 was positively associated with the HPV presence ($p=0.0004$, Table 2). Taken together, these findings suggest a positive association between HPV DNA presence and cIAP2 at transcript and protein levels in OPCs from Chilean patients.

HPV16 E6/E7 are involved in increasing cIAP2 levels in head and neck *cancer* cells

We evaluated whether HPV16 E6/E7 oncoproteins were involved in explaining the increase in cIAP2 levels. For this purpose, gain and loss of function experiments were designed using SCC-143 cancer cells as a model. These HNC cells were stably transduced with a retroviral construct for HPV16 E6/E7 expression and characterized, according to a previously published protocol [31]. Firstly, we confirmed the E6/E7 expression in these cells by RT-qPCR (Suppl. Figure 1). We confirmed that cIAP2 expression levels were significantly increased in SCC143 E6/E7 cells when compared to control cells, at both the transcript and protein levels (Fig. 4, $p=0.0383$ and $p=0.0115$, respectively). In addition, E6 and E7

Table 1 Clinicopathological features, p16 and cIAP-2 expression in OPCs from Chilean patients

	Anatomical site				
	Tonsils N (%)	**Base of Tongue N (%)**	**Palate N (%)**	**Total**	***p* value***
Total	26	14	9	49	
Gender					*0.85*
Female	9 (50)	6 (33.33)	3 (16.66)	18	
Male	17 (54.38)	8 (25.8)	6 (19.35)	31	
Age					*0.3035*
≤65 years	18 (62.07)	7 (24.24)	4 (13.79)	29	
>65 years	8 (40)	7 (35)	5 (25)	20	
Keratinization					*0.2676*
Keratinized	15 (45.45)	10 (30.3)	8 (24.24)	33	
Non-keratinized	10 (66.66)	4 (26.66)	1 (6.66)	15	
Non-informed	1	0	0	1	
Differentiation					*0.0414*
Poor	16 (69.56)	5 (21.73)	2 (8.26)	23	
Moderate	8 (47)	4 (23.52)	5 (29.41)	17	
Well differentiated	1 (12.5)	5 (62.5)	2 (25)	8	
Non-informed	1	0	0	1	
p16					*<0.0001*
Positive	22 (78.57)	5 (17.85)	1 (3.57)	28	
Negative	3 (15)	9 (45)	8 (40)	20	
cIAP2					*0.09*
Positive	18 (66.66)	5 (18.51)	4 (14.81)	27	
Negative	8 (36.36)	9 (40.9)	5 (22.72)	22	

* Fisher´s exact test

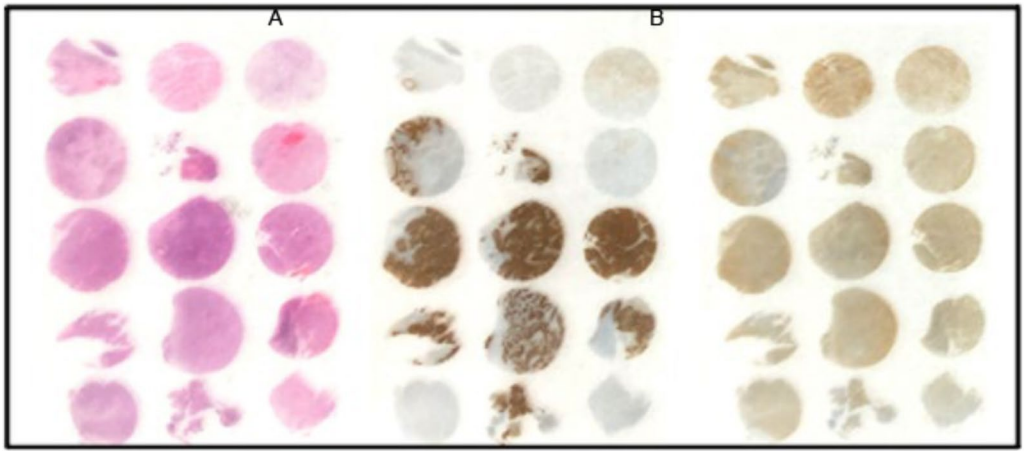


Fig. 2 Tissue array of OPCs from Chilean patients. **A** Hematoxylin–Eosin, **B** IHQ for p16 and **C** IHQ for cIAP2

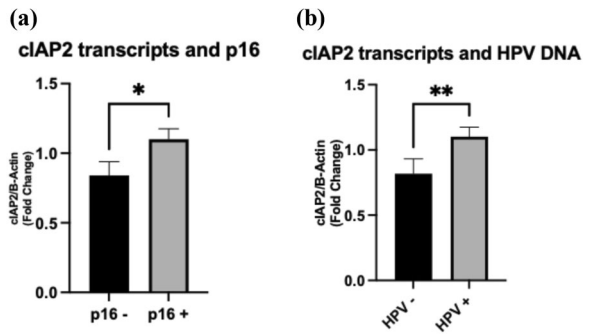


Fig. 3 cIAP2 mRNA levels correlate with p16 and HPV presence. cIAP2 expression at transcript levels were evaluated in 49 OPCs from Chilean patients and the samples were stratified considering p16 IHC positivity ($p=0.038$) **a** and HPV DNA presence ($p=0.0027$) **b**. β-actin was used as a housekeeping gene for normalization (*$p \leq 0.05$; **: $p \leq 0.01$; *Fisher´s exact test)*

Table 2 Relationship between p16 and cIAP2 status (IHC) with HPV DNA presence in OPCs from Chilean patients

	HPV presence			
	HPV negative	HPV positive	Total	*p* value[#]
Total	19	29	48*	
p16				0.0004
Positive	5 (17.85)	23 (82.14)	28	
Negative	14 (70)	6 (30)	20	
cIAP2				0.0027
Positive	5 (18.51)	22 (81.48)	27	
Negative	14 (63.63)	8 (36.36)	22	

[*] One clinical specimen was exhausted; [#]t-student test

oncoproteins were functional because they were able to promote p53 and pRb loss, respectively (Fig. 4b).

To confirm the positive association between HPV16 E6 and E7 oncoproteins with cIAP2 levels, we evaluated E6 and E7 knockdown in SCC-143 E6/E7 cells using siRNAs. A significant decrease in the levels of both viral oncoproteins was observed (Fig. 5).

We found that cIAP2 levels decreased significantly after E6 and E7 knocking down ($p=0.0161$ and $p=0.006$, respectively) (Fig. 6a, c). As a control, the interference of E6 and E7 was verified indirectly by measuring the recovery of the p53 and pRb protein levels, respectively (Fig. 6b).

cIAP2 knocking down sensitizes SCC143 cells expressing HPV16 E6/E7 to apoptosis induced by radiation exposure

First, we evaluated the usefulness of a siRNA for cIAP2 knocking down in SCC143 E6/E7 cells. As shown in Fig. 7, a maximum interference efficiency was found at 24 h post-transfection($p \leq 0.01$). Following cIAP2 knockdown, SCC143 E6/E7 cells were irradiated by the Trilogy Clinical Electron Linear Accelerator at 2, 4, 6, and 8 Gy. Eight hours later, early apoptosis was evaluated by flow cytometry. Figure 8a shows representative graphs for each condition. As can be observed in Fig. 8b, at a higher dose of radiation there was a higher percentage of apoptosis in both SCC143 E6/E7 scramble and SCC143 E6/E7 si- cIAP2 cells. However, from 4 Gy of irradiation, cells transfected with siRNA for cIAP2 knockdown presented a higher percentage of apoptosis when compared to the control, with this difference being significant at 4 and 8 Gy ($p=0.0187$ and $p=0.0061$, respectively).

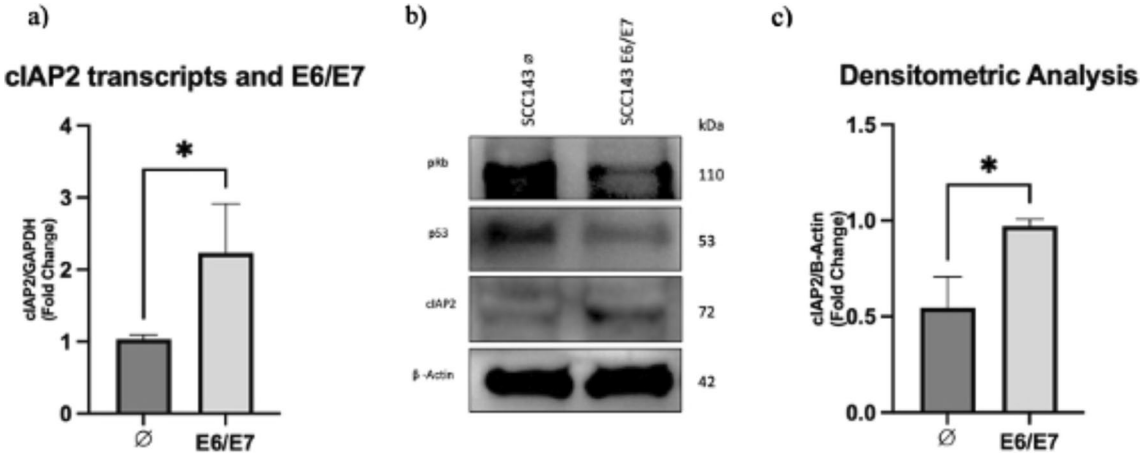


Fig. 4 HPV16 E6/E7 oncoprotein expression correlates with cIAP2 expression in squamous cell carcinoma cells. cIAP2 expression in HPV16 E6/E7 positive SCC-143 cells was measured by **a** RT-qPCR normalized against GAPDH transcripts ($p = 0.0383$) and **b** Western Blotting for pRb, p53 and cIAP2 protein levels in SCC-143 transfected with an empty (Ø) or HPV16 E6/E7 vector. β-actin levels were used as loading control. **c** The bar graph represents the densitometric analysis of three independent experiments ($p = 0.0115$, t student) (*$p \leq 0.05$; *t*-student test)

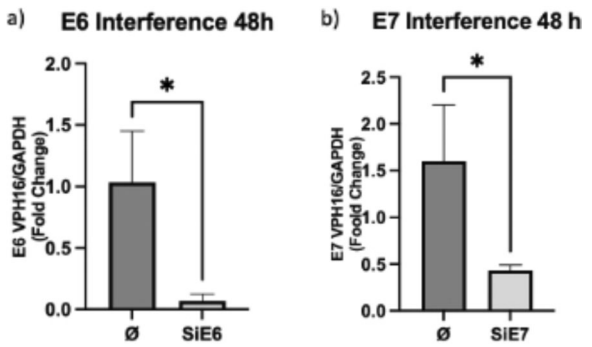


Fig. 5 HPV16 E6 (**a**) and E7 (**b**) knockdown by specific siRNAs as measured by qPCR performed for HPV16 E6 and E7 transcripts normalized against GAPDH housekeeping transcripts ($p = 0.0164$ and $p = 0.0285$, respectively) (*$p \leq 0.01$, *t-student test*)

Discussion

The incidence of OPC is increasing in many countries, including US, probably due to HPV infection [32]. In addition, there are no studies that provide the possibility of OPC reduction after vaccination programs [33, 34]. Interestingly, HR-HPV presence has been considered an independent positive predictor of survival and treatment response in patients with OPC [35, 36]. In this study, we established a significant relationship between HR-HPV presence and cIAP2 or p16 detection (an indirect biomarker of oncogenic viral infection), and cIAP2 transcripts with the presence of HR-HPV by RT-PCR and IHQ. cIAP2 expression was also found to be associated with p16 [37]. Furthermore, using a HNC cell line, we confirmed the results obtained in clinical specimens. After transducing SCC-143 cells with a retroviral vector for HPV16 E6/E7 oncoprotein expression, we observed an increase in the expression of cIAP2 at transcript and at protein levels. In turn, after HPV16 E6/E7 knocking down with a siRNA, we observed a decrease in cIAP2 levels when compared to the control. These results are in accordance with studies carried out in different cell lines, which demonstrates a positive association between cIAP2 and HPV [21, 24, 25].

Although the levels of the cIAP2 protein decreased when both oncoproteins were knocked down, this was only statistically significant for E6. In fact, previous studies have proposed that E6 oncoprotein plays a more relevant role in promoting cIAP2 increase [21, 25]. Various studies have suggested that HR-HPV E6 and E7 oncoproteins are involved in Nuclear Factor Kappa B (NF-κB) activation by modulating the expression of the genes responding to this signaling pathway [38–41]. However, the mechanism by which both E6 and E7 modulate NF-κB activation is not completely understood. Under hypoxic conditions, HR-HPV E6 stimulates the ubiquitination and proteasomal degradation of cylindromatosis lysine deubiquitinase (CYLD), a negative regulator of the NF-κB pathway that blocks IKK recruitment and activation mediated by TRAF [42]. CYLD deubiquitinates TRAF2, TRAF6 and NEMO, and inactivation of CYLD increases NF-κB signaling in a sustained manner, both in vitro and in vivo [43]. In addition, Xu et al. reported that E6-mediated NF-κB activation could also be mediated by inactivation of the nuclear transcription factor X-box binding 1 (NFX1), an inhibitor of NF-κB [44]. NFX1 increases the expression of p105, which acts to inhibit NF-κB before it is cleaved to generate active p50. Since E6 expression is associated

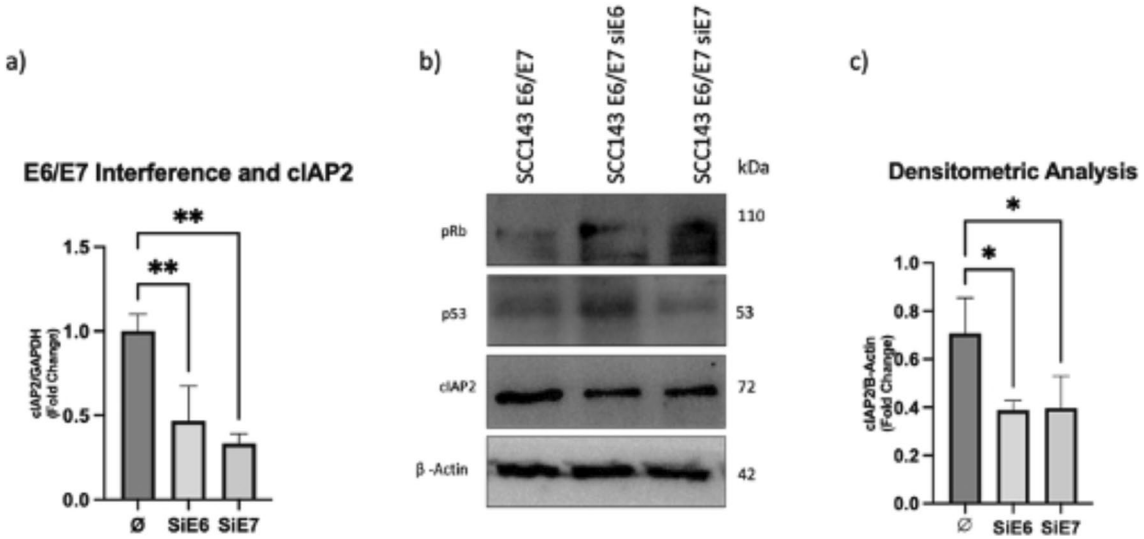


Fig. 6 cIAP2 transcript levels are decreased after HPV16 E6/E7 knocking down. **a** RT-qPCR for cIAP2 transcripts normalized against GAPDH ($p=0.0161$ and $p=0.006$ for E6 and E7, respectively); **b** Western blotting for pRb, p53 and cIAP2 protein levels in SCC143 E6/E7 cells transfected with scramble (Ø), siRNA for E6 and siRNA for E7 knocking down. β-actin levels were used as loading control. **c** Densitometric analysis of three independent western blotting experiments. siRNA for E6 vs control ($p=0.03$) and siRNA for E7 vs control (*NS*). (*$p \le 0.05$; **$p \le 0.01$; *NS* non significant, *t*-student test)

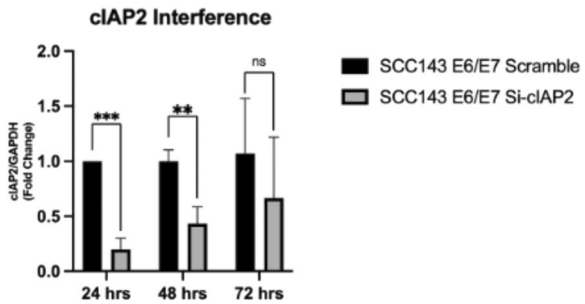
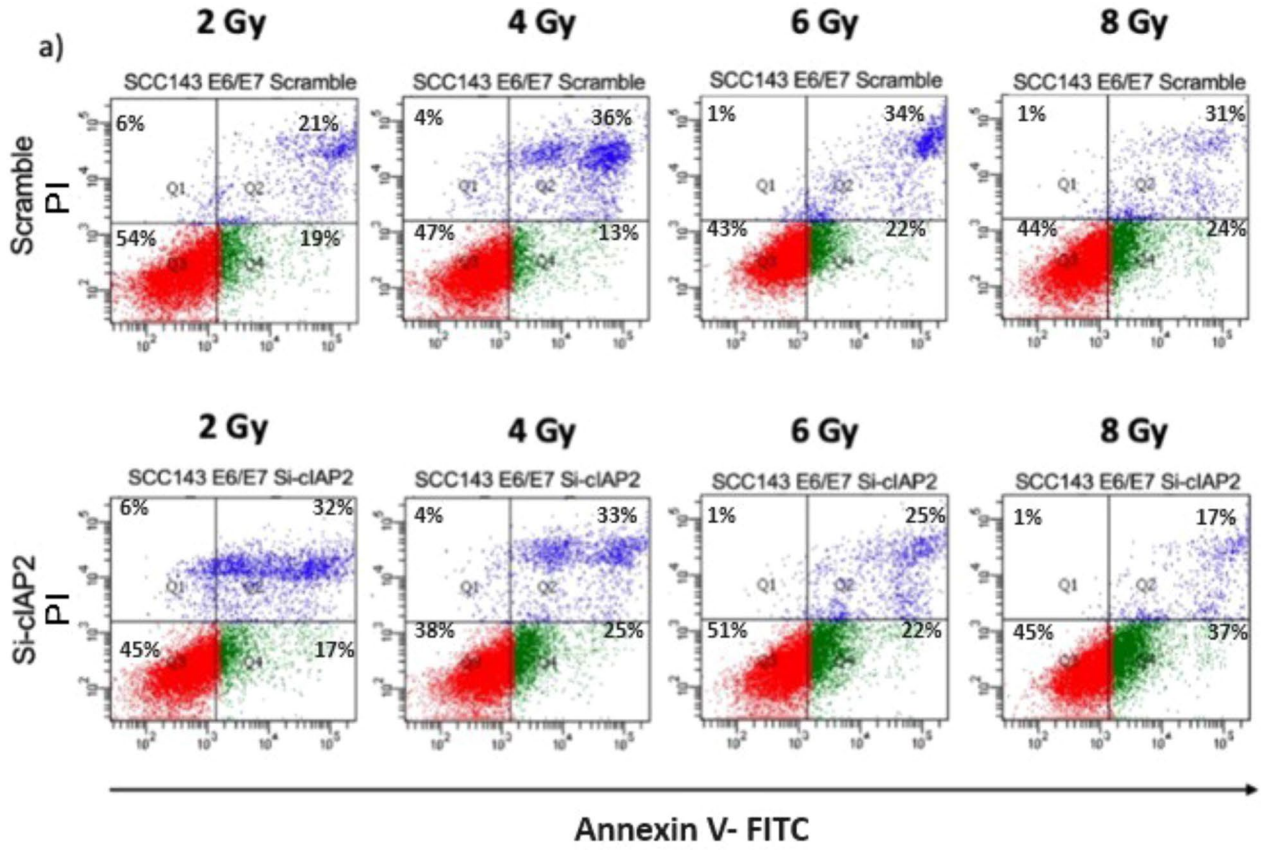


Fig. 7 cIAP2 transcripts are significantly decreased after transfection of SCC143 cells with a siRNA for cIAP2 knocking down. cIAP2 transcript levels were evaluated by RT-qPCR at 24-, 48- and 72 h post-transfection with a scrambled RNA sequence or a siRNA for cIAP2 knocking down. GAPDH transcripts were used as a load normalizer ($p=0.002$, $p=0.0058$ and $p=0.04$, respectively). (**$p \le 0.01$; ***$p \le 0.001$; *t*-student test)

with decreased p105 levels and NF-κB activation, it is suggested that this occurs through NFX1-mediated inhibition of p105 expression [44]. Even though a greater participation of E6 on NF-κB has been reported, studies show that E7 would play a synergistic role by mechanisms that have not yet been clarified [25] (Fig. 9). Finally, cIAP2 is one of the genes that are induced by NF-κB activation, which is consistent with the association between this signaling pathway and aggressive tumor biological behavior and radio-chemoresistance [45, 46].

In this study, we have evaluated the response to different doses of irradiation in SCC143 E6/E7 cells treated with siRNA for cIAP2 knockdown. Using flow cytometry for Annexin V/propidium iodide we found that cIAP2 knocking down results in a higher percentage of apoptosis that increased in a dose-dependent manner, being statistically significant at doses of 4 and 8 Gy ($p=0.0187$ and $p=0.0061$, respectively). cIAP2 regulates the extrinsic apoptotic pathway since it promotes the ubiquitination of RIP1, preventing it from forming a cytosolic complex with the adapter molecule FADD and caspase 8 [47]. Therefore, it inhibits apoptosis by an indirect regulation of caspase activity through its E3 ligase activity [48]. Because cIAPs block the induction of cell death, contributing to radioresistance, it has been proposed that the inhibition of these proteins is a promising approach to enhance the effectiveness of radiotherapy in HNC treatment [26–28]. AZD5582, an inhibitor of cIAPs, induces apoptosis in HNSCC cell lines, though this effect is synergistic with combined therapy with irradiation [28]. In combination with radiotherapy, another dual cIAP/XIAP inhibitor, ASTX660, significantly delayed the growth of HPV (−) and HPV (+) tumor xenografts. Furthermore, in HPV-positive cell lines, the induction of apoptosis was observed and was related to the recovery of p53 [49]. Recently, also combined radiotherapy, the use of a dual cIAP/WEE1 antagonist, AZD1775,, decreased cell proliferation and survival in HNSCC cells [50]



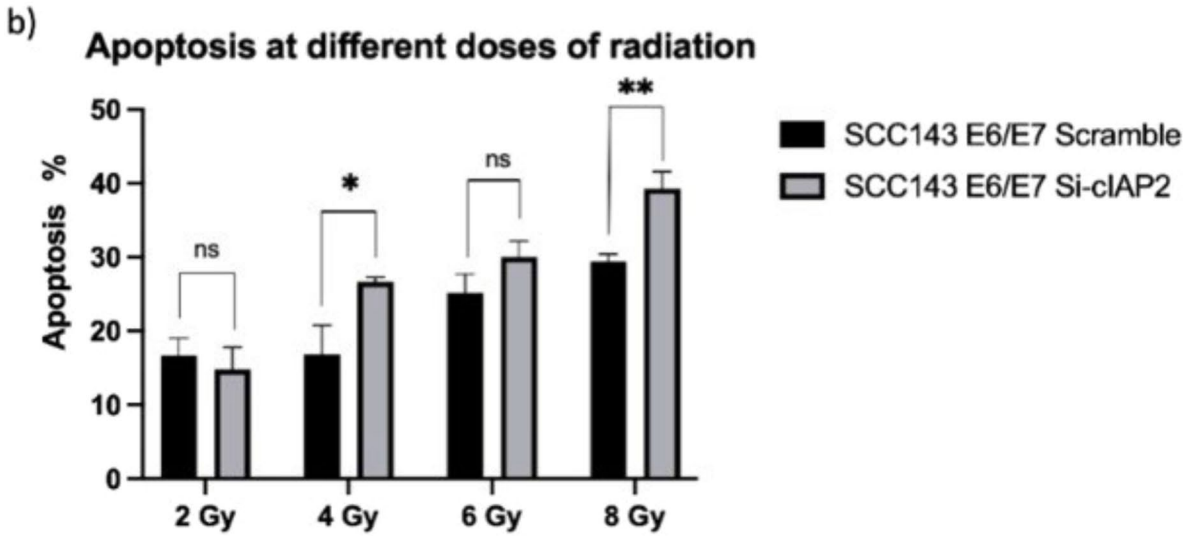


Fig. 8 Radiation increases apoptosis in cIAP2 knocked down SCC-143 cells. **a** Annexin V and Propidium Iodide staining in SCC143 E6/E7 scramble and SCC143 E6/E7 si-cIAP2 cells, exposed to different radiation doses (2, 4, 6 and 8 Gy). **b** Percentage of apoptotic cells at different doses of radiation, comparing SCC143 E6/E7 si-cIAP2 versus scramble, being significant for 4 and 8 Gy ($p = 0.0187$ and $p = 0.0061$, respectively; *t*-student test)

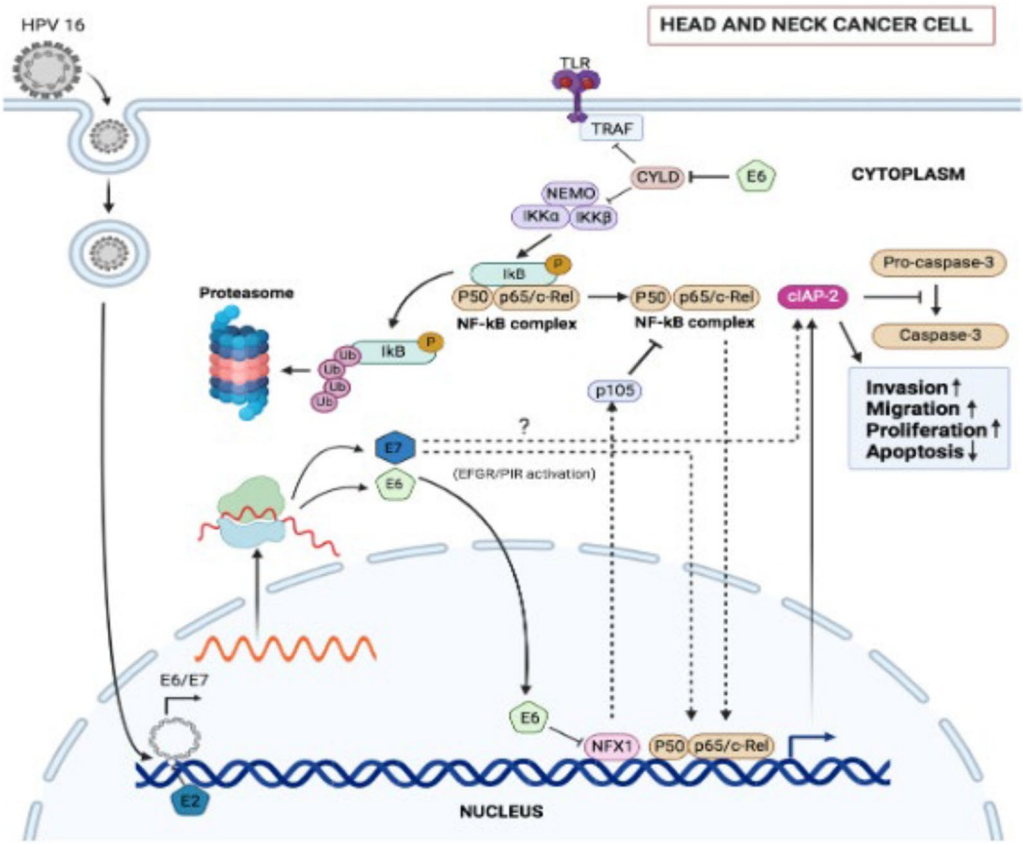


Fig. 9 Representative scheme of the proposed mechanism for the association between HR-HPV E6 and E7, NF-κB and cIAP2

Additionally, it was shown that a decrease in cIAP2 expression caused by the combination of poly(I:C) and cisplatin induces high radiosensitivity in HNSCC [26]. On the other hand, HPV-positive OPCs have an epidemiological, molecular and clinical profile different from those caused by known risk factors such as smoking and/or alcohol consumption, with p16 positivity being an independent positive predictor of overall survival and response to treatment [36]. This background has led to the development of clinical trials of de-escalation treatment to reduce acute and chronic toxicity [51].

The present study has some limitations, for instance, a reduced number of specimens from patients was included, which were collected from a specific public health institution in Santiago, Chile. In addition, clinical information such as tobacco smoking, was not available for this study. In conclusion, we have established that cIAP2 is upregulated in HPV positive OPCs with HR-HPV E6/E7 being involved in increasing the levels of this protein in HNC cells. Additionally, cIAP2 downregulation sensitizes HNC cells to apoptosis promoted by radiation, suggesting that cIAP2 is a potential therapeutic target for HPV positive HNC treatment.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13027-024-00609-z.

Additional file 1.

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Author contributions
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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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