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Cisplatin overcomes radiotherapy resistance in OCT4-expressing head and neck squamous cell carcinoma

Johannes Routila [a,b], Xi Qiao [a], Jere Weltner [c], Juha K Rantala [d], Timo Carpén [e], Jaana Hagström [f], Antti Mäkitie [e], Ilmo Leivo [f,g], Miia Ruuskanen [b], Jenni Söderlund [i], Marjut Rintala [i], Sakari Hietanen [i,j], Heikki Irjala [b], Heikki Minn [j,k], Jukka Westermarck [a,h,j], Sami Ventelä [a,b,j,*]

[a] *Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, Finland*
[b] *Department for Otorhinolaryngology – Head and Neck Surgery, University of Turku and Turku University Hospital, Kiinamyllynkatu 4-8, 20521 Turku, Finland*
[c] *Department of Clinical Science, Intervention and Technology, Karolinska Institutet, SE-14186 Stockholm, Sweden and Division of Obstetrics and Gynecology, Karolinska Universitetssjukhuset, SE-14186 Stockholm, Sweden*
[d] *MISVIK Biology Ltd, Karjakatu 35 B, 20520 Turku, Finland*
[e] *Department for Otorhinolaryngology – Head and Neck Surgery, Helsinki University Hospital and University of Helsinki, P.O.Box 263, FI-00029 HUS Helsinki, Finland*
[f] *Department of Oral Pathology and Radiology, University of Turku, Turku, Finland*
[g] *Institute of Biomedicine, Pathology, University of Turku, Kiinamyllynkatu 10 D, 20520 Turku, Finland*
[h] *Biomedical Institute, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland*
[i] *Department of Obstetrics and Gynecology, Turku University Hospital and University of Turku, Turku, Finland*
[j] *FICAN West Cancer Centre, Turku, Finland*
[k] *Department of Oncology and Radiotherapy, University of Turku and Turku University Hospital, Turku, Finland*

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ABSTRACT

Objectives: Cisplatin is combined with radiotherapy for advanced head and neck squamous cell carcinoma (HNSCC). While providing a beneficial effect on survival, it also causes side effects and thus is an important target when considering treatment de-escalation. Currently, there are no biomarkers to predict its patient-selective therapeutic utility. In this study, we examined the role of the stem cell factor OCT4 as a potential biomarker to help clinicians stratify HNSCC patients between radiotherapy and chemoradiotherapy.
Materials and methods: OCT4 immunohistochemical staining of a population-validated tissue microarray (PV-TMA) (n = 166) representative of a standard HNSCC patients was carried out, and 5-year survival was analyzed. The results were validated using *ex vivo* drug sensitivity analysis of HNSCC tumor samples, and further cross-validated in independent oropharyngeal (n = 118), nasopharyngeal (n = 170), and vulvar carcinoma (n = 95) clinical datasets. *In vitro,* genetically modified, patient-derived HNSCC cells were used.
Results: OCT4 expression in HNSCC tumors was associated with radioresistance. However, combination therapy with cisplatin was found to overcome this radioresistance in OCT4-expressing HNSCC tumors. The results were validated by using several independent patient cohorts. Furthermore, CRISPRa-based OCT4 overexpression in the HNSCC cell line resulted in apoptosis resistance, and cisplatin was found to downregulate OCT4 protein expression *in vitro*. *Ex vivo* drug sensitivity analysis of HNSCC tumors confirmed the association between OCT4 expression and cisplatin sensitivity.
Conclusion: This study introduces OCT4 immunohistochemistry as a simple and cost-effective diagnostic approach for clinical practice to identify HNSCC patients benefitting from radiosensitization by cisplatin using either full or reduced dosing.

* Corresponding author at: Department of Otorhinolaryngology – Head and Neck Surgery, Turku University Hospital, FI-20521 Turku, Finland.
E-mail address: satuve@utu.fi (S. Ventelä).

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Introduction

Head and neck cancers have an increasing frequency, accounting for up to a total of 890 000 new cases worldwide [1,2]. Whereas recent advances in both surgical and oncological treatments have contributed to increases in the quality of life of patients with HNSCC, they have resulted in only negligible improvements in patient survival [1]. Recent randomized trials investigating targeted therapy options have failed to provide significant survival benefits to patients [3–7]. Regardless of extensive genomic characterization of the disease, no therapy-stratifying biomarkers exist in routine clinical use, and the only selection criteria for therapy remain to be based on the patient's overall state of health, tumor site and extent [8,9].

Since radiotherapy is used in the treatment of more than half of HNSCC patients, the radioresistance of individual tumors has a tremendous impact on the overall success of therapy [10]. Cisplatin has been used in the radiosensitization of HNSCC since 1970 as well as in combination chemotherapy of advanced disease. The absolute patient benefit from cisplatin-based radiosensitization remains uncertain [11,12]. This is reflected in the lack of biomarkers predicting sensitivity to cisplatin and concerns about renal toxicity and hearing loss, which limit the use and enthusiasm for cisplatin radiosensitization, especially in elderly patients with comorbidities [11,13]. A clinically unmet need for a biomarker selecting patients who would benefit from cisplatin would help spare the other patients from cisplatin-elicited side effects.

In the present study, we evaluated the predictive potential of OCT4 immunochemistry in stratifying patients for radiotherapy versus cisplatin-based chemoradiotherapy. The primary patient cohort of the study was a population-validated cohort of 288 HNSCC patients treated with radiotherapy or chemoradiotherapy [14]. Primary findings were investigated in independent patient cohorts of oropharyngeal carcinoma (OPSCC) [15], nasopharyngeal carcinoma (NPC) [16], or vulvar squamous cell carcinoma (VSCC), as well as *in vitro* and prospective *ex vivo* study settings using HNSCC cell lines and primary patient-derived tissue samples from surgically treated HNSCC patients.

Materials and methods

Primary HNSCC patient cohort

The primary HNSCC patient cohort was formed by identifying all patients treated for new HNSCC at the Turku University Hospital (TUH) region in 2005–2010 [14]. In this patient population, patient age, high T class, nodal positivity and alcohol use made up a powerful prognostic panel [14], which was used in all multivariable survival analyses. The usage of human tissue samples was approved by the Finnish National Supervisory Authority for Welfare and Health (V/39706/2019), regional ethics committee of University of Turku (51/1803/2017) and Auria Biobank scientific board (AB19–6863). Formalin-fixed, paraffin-embedded (FFPE) tissue samples were acquired from pathology archives through Auria Biobank. TMA blocks with duplicate 0.6 mm core biopsies were made using TMA Grand Master (3D Histech).

Validation datasets

A previously reported OPSCC patient cohort from the Helsinki University Hospital (Helsinki, Finland) was acquired through collaboration [15]. The Finnish nationwide NPC cohort of patients treated in 1990–2009 was used as an independent validation cohort and was collected as previously reported [16].

The VSCC patient material included clinical and follow-up data of 95 patients treated in 2000–2013 at the TUH. Primary FFPE samples were included in TMA (biobank scientific board approval AB15–9293 and local hospital research permit T100/2018).

Immunohistochemistry (IHC)

FFPE blocks were cut into 6 μm sections. OCT4 IHC was performed as previously described with anti-OCT4 antibody sc-5279 (1:200 mouse monoclonal, Santa Cruz Biotechnology) and with MRQ-10 (1:200 mouse monoclonal, Sigma, 309 M−14) [17]. Stainings were analyzed by two authors (JR, SV) independently, and differences were discussed until consensus was reached. Nuclear OCT4 positivity was scored as described previously [14].

Cell line experiments and CRISPRa cell line derivation

For creation of the Oct4-CRISPRa cell line,[18] cultured cells were transfected with designated plasmids. Cells were detached as single cells from the culture plates with TrypLE Select (Gibco) and washed with PBS. Cells were electroporated using the Neon transfection system (Invitrogen). A total of 1 million cells and 3.5 μg of plasmid mixture, containing 1 μg of PB-tight-DDdCas9VP192-GFP-IRES-Neo activator plasmid (Addgene plasmid # 102889), 1 μg of PB-CAG-rtTAM2-IN plasmid (Addgene plasmid # 60612), 1 μg of PB-GG-OCT4-1–5 PGK-Puro (Addgene plasmid # 102893) and 0.5 μg of PiggyBac transposase plasmid, were electroporated in a 100 μl tip with 1100 V, 20 ms, and 2 × pulse settings. Electroporated cells were plated on 100 mm diameter cell culture plates in fibroblast medium (Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% fetal bovine serum (FBS; Life Technologies), 2 mM GlutaMAX (Life Technologies), and 100 μg/ml penicillin–streptomycin (Life Technologies). Three days after electroporation selection was started with 1 μg/ml Puromycin (Sigma) and 250 μg/ml G418 (Life Technologies) for four days, after which Puromycin concentration was halved, and selection continued for an additional week.

Cell cultures were continued for 48 h after treatment with the indicated cisplatin doses or irradiation. For drug treatment, the cells were cultured 48 h after treatment with indicated cisplatin (S1166, selleckchem) doses. The radiation was done by using Faxitron Multirad 350.

Western blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 0.5 % DOC, 0.1 % SDS, 1% NP-40, and 150 mM NaCl) with protease and phosphatase inhibitors (4693159001 and 4906837001, Roche). The lysate was sonicated, added with 6X SDS loading buffer, boiled and resolved by 4–20% precast protein gels (456–1093 and 456–1096, Biorad). Proteins were transferred to PVDF membranes (1704156, Biorad). Membranes were blocked in 5% Milk-TBS-Tween 20 for 30 min under RT, and then incubated with primary antibodies overnight at 4 °C. Secondary antibodies was incubated in 5% Milk-TBS-Tween 20 for 1 h under RT, and developed by ECL western blotting substrate (32106, Pierce). The following are antibodies used for western blot: Oct-3/4 (sc- 5279, Santa Cruz), Vinculin (sc-25336, Santa Cruz), cleaved PARP (ab32064, Abcam) and GAPDH (5G4-6C5, HyTest Ltd). Secondary antibodies are from Dako (P0447 and P0399).

Immunocytochemistry

To induce dCas9 activator expression, cells were treated with doxycycline (DOX, 2 μg/ml; Sigma) and trimethoprim (TMP, 1 μM; Sigma) for three days. Cells were fixed with 4% PFA, permeabilized using 0.5% Triton X-100, and treated with Ultra Vision block (ThermoFisher). Primary antibodies for OCT4 (1:1000, sc-8628, Santa Cruz; 1:500, sc-9081, Santa Cruz) were diluted in 0.1% Tween-20 PBS and incubated for 2 days in 6 °C. Secondary antibody incubations were done in room temperature for 45 min in the presence of Hoechst33342 to stain the nuclei. Secondary antibodies used were: AlexaFluor 594: donkey anti-goat (1:500, 11058; Invitrogen) and donkey anti-rabbit (1:500, A21207; Invitrogen).

Ex vivo drug screening analysis

Five surgical biopsy samples were collected for *ex vivo* drug screening after patient informed consent and in accordance with the local research ethics council permit (Dnro 166/1801/2015). Drug screens were performed as previously described [19]. Briefly, the therapeutic compound collection used in the drug screening consisted of 163 anti-cancer agents, purchased from commercial chemical vendors (Selleck biochemical, Santa Cruz Biotechnology). Each compound was tested in four different adjusted concentrations with 2-fold dilutions. The single-cell suspension of freshly isolated tumor tissue derived cells (45 μl per well; 1,000 cells per well) was transferred to each well using a peristaltic MultiDrop Combi dispenser (ThermoScientific). The 384-well plates were incubated for 96 h in standard cell culture conditions; 37 °C, 5% CO_2. Analysis of cell viability was performed with CellTiter-GLO (Promega) luminescence cell viability assay according to manufacturer instructions and a Labrox luminescence plate reader. Growth rate normalized viability data was used for calculation of IC_{50} estimates in GraphPad Prism software (V8, GraphPad Software).

Statistical analysis

Clinical patient data for each cohort, staining results, and quantified *in vitro* results were entered into SPSS 25 software (SPSS, IBM). For all patient cohorts, tumors were staged according to TNM criteria applicable at the time of diagnosis and treatment protocols were decided in multidisciplinary Tumor Boards for head and neck cancer. OS was defined from end-of-treatment to end-of-follow-up or death.

TMA inclusion bias was analyzed using logistic regression analysis, reporting odds ratios with 95 % confidence intervals and p values. For Cox proportional hazards models, the proportionality of hazards was testing using log-minus-log plotting and plotting Schoenfeld residuals against survival time, when appropriate. For all stepwise multivariable analysis, backward likelihood ratio method was applied, if not otherwise indicated, with p value limit for exclusion set at 0.10. Hazard ratios with 95 % confidence intervals and p values were calculated. Survival was plotted using the Kaplan-Meier method, using sticks to indicate censoring.

For analysis of immunochemistry results, OCT4 staining data was combined with patient data. For multivariable models, prognostic models based on clinical variables were established and survival analyzed. For quantitative western blot analysis, General Linear Model statistics were applied, reporting estimated marginal means with 95 % confidence intervals determined by bootstrapping and p values.

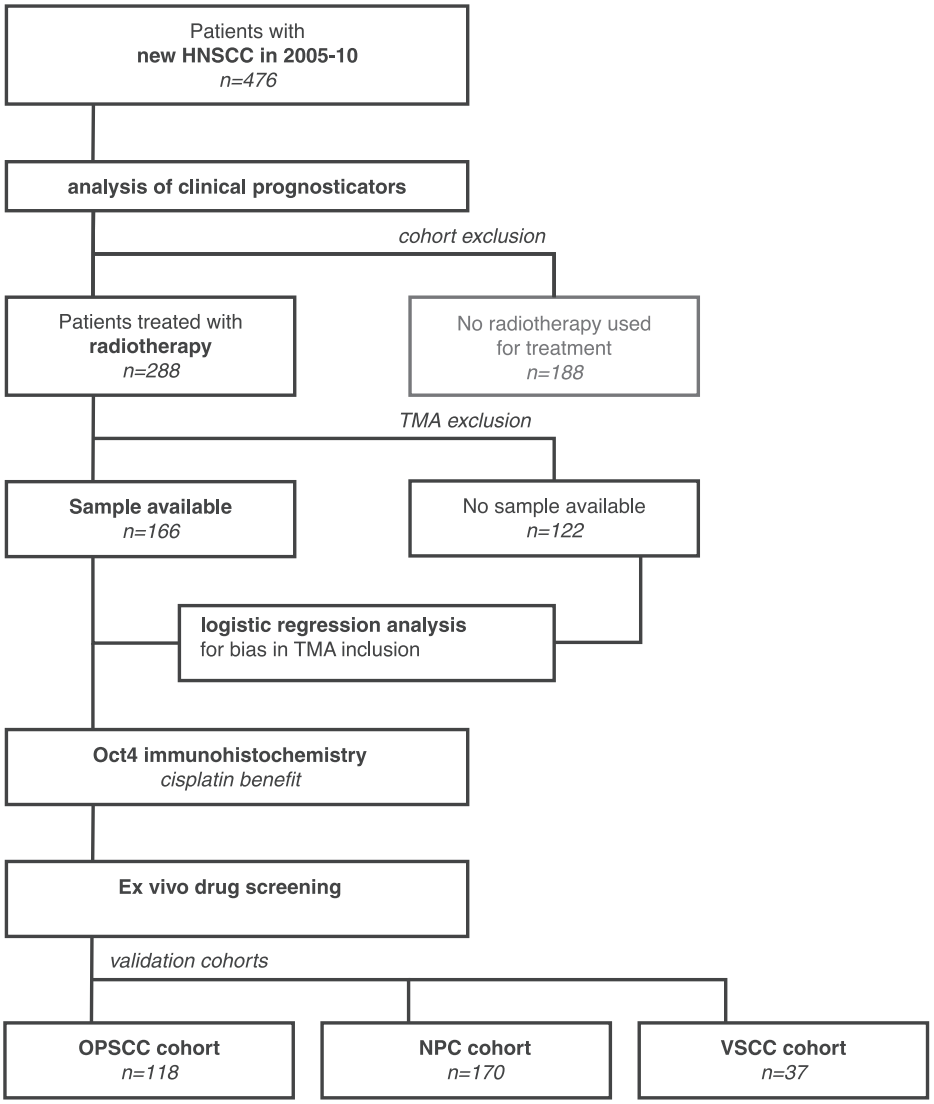


Fig. 1. A) Flowchart representation of the study protocol. Clinical prognostic factors were established, and the representativeness of the population-validated tissue microarray (PV-TMA) was analyzed using logistic regression analysis. After representativeness was confirmed, OCT4 immunohistochemistry staining was analyzed to assess the possible survival benefit from cisplatin addition. Further analysis of *ex vivo* drug screenings was carried out. For validation, independent oropharyngeal carcinoma (OPSCC), nasopharyngeal carcinoma (NPC), and vulvar carcinoma (VSCC) patient cohorts were investigated.

Results

OCT4 expression in HNSCC tumors predicts impaired survival in the radiotherapy-only cohort but not in patients treated with concurrent cisplatin plus radiotherapy

The stemness characteristics of cancer cells have been linked to clinical radioresistance [20]. Based on the association between the stem cell marker OCT4 and HNSCC radiotherapy resistance [17], we investigated whether OCT4-based patient stratification would prove useful in larger patient cohorts. To study the role of OCT4 in HNSCC, we used previously published PV-TMA [14] consisting of samples from 166 patients treated with radiotherapy or chemoradiotherapy with or without surgery (Fig. 1A). The resulting PV-TMA population was highly representative and comparable to the clinical characteristics of the patients diagnosed with a new HNSCC at our institute during 2005–2010 (Table 1).

OCT4 antibody specificity was confirmed by Western blot and immunofluorescence analyses of HNSCC cells that do not endogenously express OCT4 (Supplemental Figure 1) but in which OCT4 promoter activity was activated by CRISPRa [18] (Fig. 2A-B, Supplemental Figure 2). In HNSCC tissues, nuclear OCT4 positivity or negativity was easily recognized based on a simple IHC staining procedure (Fig. 2C-D), indicating potential in clinical translation. In OCT4-negative patients, there was no OS difference between patients treated using radiotherapy or chemoradiotherapy (Fig. 2E). In OCT4-positive patients, however, treatment with radiotherapy only was associated with significantly impaired prognosis (Fig. 2F). In fact, the OS of OCT4-positive patients treated with the radiosensitization protocol was indistinguishable from that of OCT4-negative patients treated with radiotherapy alone (Fig. 2G).

Importantly, OCT4-positive patients, whose cisplatin course was interrupted due to adverse side effects, achieved a significant survival benefit in comparison to patients treated with radiotherapy alone (Fig. 2H). However, OCT4 positivity was associated with poor survival when radiotherapy was combined with cetuximab or taxane (Supplemental Figure 3). The inclusion of neck dissection in the treatment algorithm did not affect the survival of either OCT4-positive or OCT4-negative patients (data not shown). In multivariable analysis, OCT4 was associated laryngeal site only and not with other clinical characteristics (OR 3.55; 95% CI, 1.29 to 9.80, p = 0.015) (Supplemental Table 1), demonstrating an independent predictive effect of OCT4 positivity.

Table 1
Clinicopathological variables of the primary HNSCC patient population (left columns) and the population-validated TMA (right columns). TMA inclusion was influenced by primary tumor site and local operation but not by other clinical variables.

		Total	TMA patients			TMA inclusion		Multivariable	
		n	%	n	%	OR (95% CI)	p	OR (95% CI)	p
Gender									
	male	210	73 %	112	67 %	1	–	NS	–
	female	78	27 %	54	33 %	1.97 (1.13–3.42)	0.016	–	–
Age at diagnosis									
	<65	180	63 %	104	63 %	0.99 (0.97–1.01) / yr	0.35	not included	–
	>65	108	38 %	62	37 %	–	–	–	–
Smoking status									
	current smoker	91	32 %	64	39 %	0.51 (0.29–0.88)	0.017	NS	–
	former smoker	47	16 %	20	12 %	0.31 (0.15–0.65)	0.002	–	–
	non-smoker	150	52 %	82	49 %	1	–	–	–
Alcohol consumption									
	no	176	61 %	105	63 %	1	–	not included	–
	yes	112	39 %	61	37 %	0.81 (0.50–1.31)	0.39	–	–
Primary tumor site									
	oral cavity	87	30 %	63	38 %	1	–	1	–
	oropharynx	82	28 %	59	36 %	0.98 (0.50–1.92)	0.95	1.65 (0.74–3.66)	0.22
	larynx	79	27 %	23	14 %	0.16 (0.080–0.31)	<0.001	0.29 (0.13–0.66)	0.003
	hypopharynx	15	5 %	9	5 %	0.57 (0.18–1.78)	0.33	1.20 (0.34–4.22)	0.78
	other	25	9 %	12	7 %	0.35 (0.14–0.88)	0.025	0.61 (0.22–1.71)	0.35
T class									
	T0-2	153	53 %	90	54 %	1	–	not included	–
	T3-4	135	47 %	76	46 %	0.90 (0.57–1.44)	0.67	–	–
N class									
	N0	137	48 %	67	40 %	1	–	NS	–
	N+	151	52 %	99	60 %	1.99 (1.24–3.20)	0.004	–	–
Stage									
	I-II	79	27 %	39	23 %	1	–	NS	–
	III-IV	209	73 %	127	77 %	1.59 (0.94–2.68)	0.082	–	–
Recurrence in 5 yrs									
	yes	85	30 %	53	32 %	1.36 (0.80–2.32)	0.25	not included	–
	no	175	61 %	96	58 %	1	–	–	–
	no curative treatment	28	10 %	17	10 %	1.27 (0.56–2.87)	0.56	–	–
Living at 5 yrs									
	yes	142	49 %	78	47 %	0.74 (0.36–1.55)	0.42	not included	–
	no, died of HNSCC	106	37 %	66	40 %	1	–	–	–
	no, died of other cause	40	14 %	22	13 %	0.74 (0.44–1.23)	0.25	–	–
Surgical treatment									
	No surgery	126	44 %	50	30 %	–	–	–	–
	Local operation	110	38 %	82	49 %	3.28 (1.95–5.51)	<0.001	2.47 (1.24–4.92)	0.010
	Neck dissection	132	46 %	96	58 %	3.28 (2.00–5.38)	<0.001	NS	–
Treatment type									
	RT only	51	18 %	20	12 %	1	–	NS	–
	CRT only	75	26 %	30	18 %	1.03 (0.50–2.14)	0.93	–	–
	RT + surgery	46	16 %	34	20 %	4.39 (1.85–10.44)	0.001	–	–
	CRT + surgery	116	40 %	82	49 %	3.74 (1.88–7.45)	<0.001	–	–

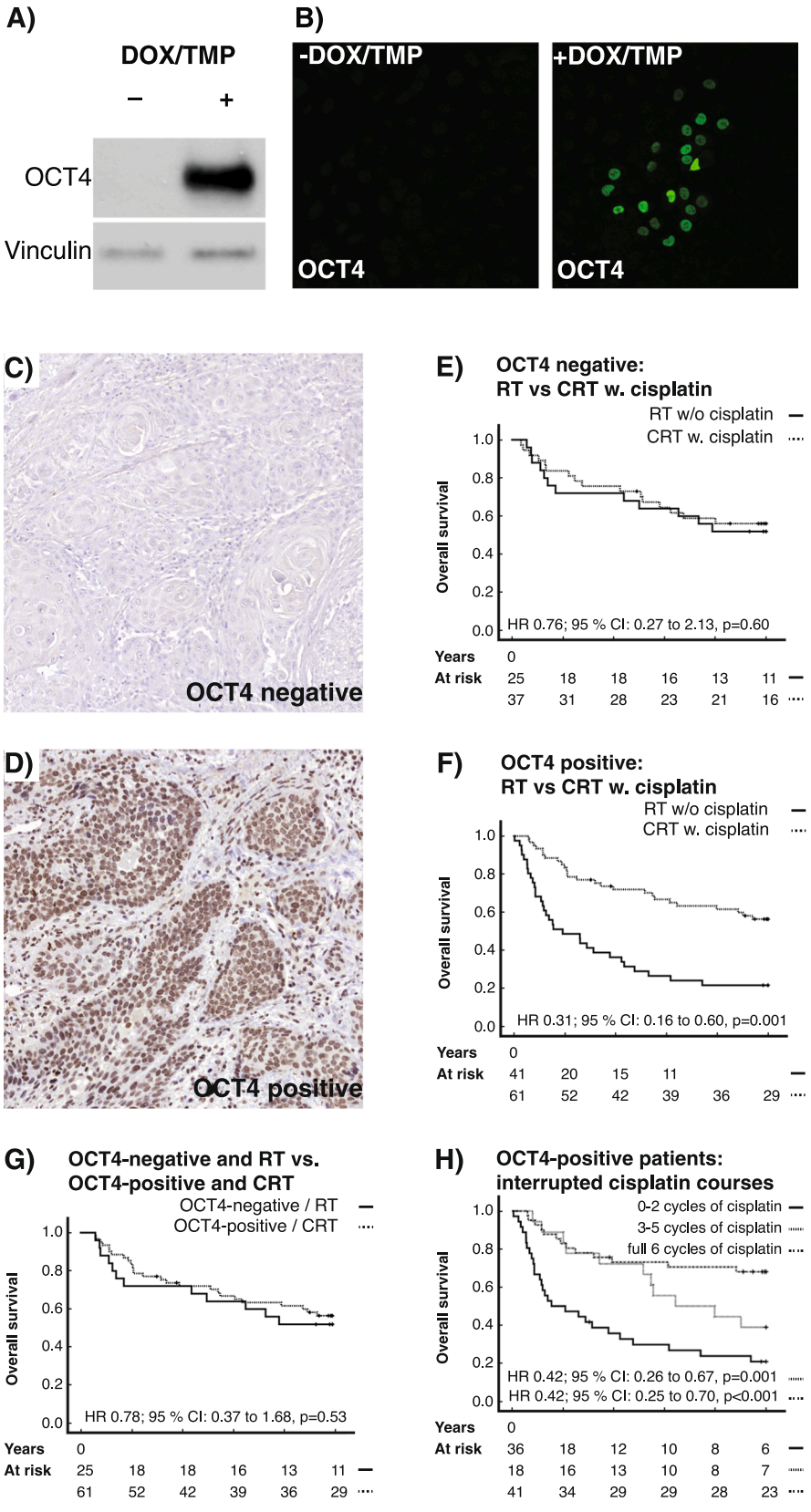


Fig. 2. Analysis of OCT4 in HNSCC. The specificity and reliability of the OCT4 antibody was demonstrated in a constructed UT-SCC-36-CRISPRa-OCT4 cell line, which overexpresses OCT4 after administration of doxycycline and trimethoprime (DOX-TMP), using A) Western blot (the full Western blot gel is included as Supplemental Fig. 1) and B) immunofluorescence. Immunohistochemical analysis of HNSCC tumors divided tumors dichotomously C) OCT4-negative and D) OCT4-positive cancers. E) There was no survival difference after radiotherapy (RT) alone versus cisplatin-based chemoradiotherapy (CRT) in OCT4-negative patients. However, F) in OCT4-positive patients, survival was severely impaired in patients treated with radiotherapy alone. Importantly, G) there was no survival difference between OCT4-negative patients treated with radiotherapy alone and OCT4-positive patients treated with cisplatin-based chemoradiotherapy. Interestingly, H) an interrupted or failed cisplatin-chemosensitization protocol was associated with a similar improvement in prognosis compared to a full cisplatin dose in multivariable survival analysis.

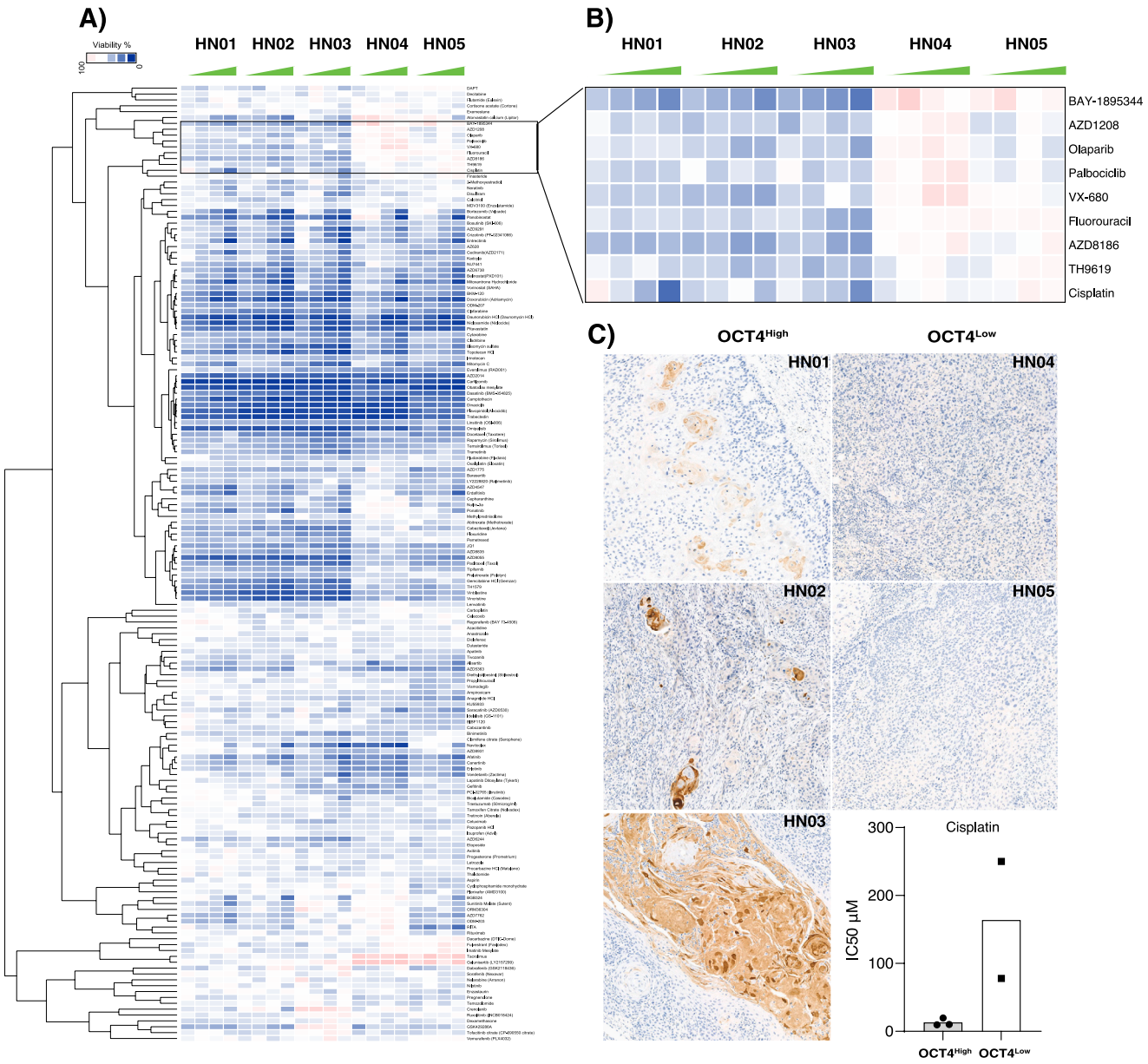
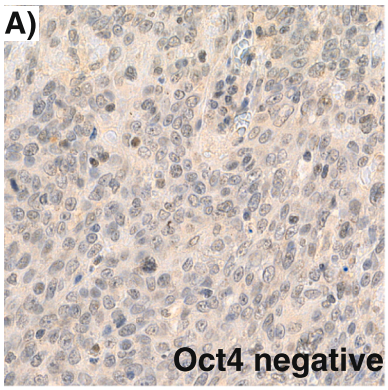
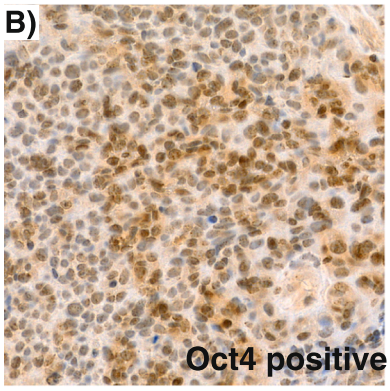


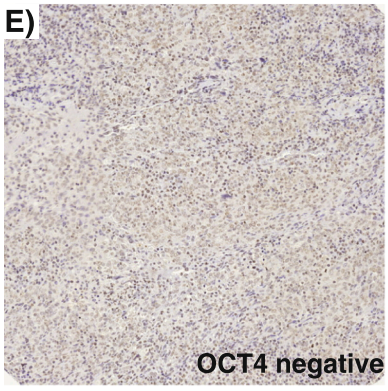
Fig. 3. *Ex vivo* drug screening analysis of five HNSCC tumors (HN01-HN05). A) Heatmap showing unsupervised hierarchical clustering of the *ex vivo* drug screening results. Viability % shown as indicated in the color key. Drugs with the strongest differential efficacy are shown in the magnified heatmap. B) OCT4 immunohistochemical analysis of the samples used for drug sensitivity experiments. C) Bar graph showing the cisplatin IC_{50} (µM) in OCT4-positive (High) and OCT4-negative (Low) patient tumor-derived cell samples.

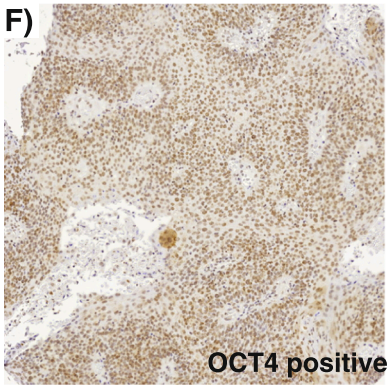
Ex vivo drug screening of HNSCC tumor samples demonstrates an intrinsic role for OCT4 positivity as a biomarker of benefit from DNA damaging chemotherapies

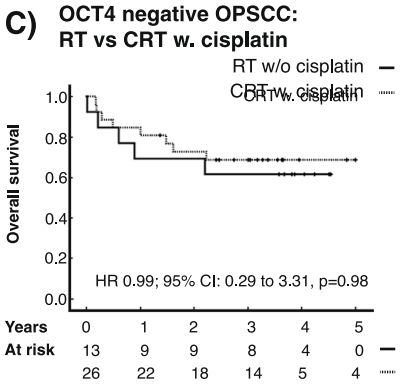
The results above from highly representative retrospective HNSCC material clearly indicate that OCT4 immunohistochemistry analysis could provide a diagnostic approach to identify HNSCC patients with cisplatin-sensitive tumors. To recapitulate such a diagnostic scenario in a prospective setting and to examine whether OCT4 could serve as a biomarker for the sensitivity to other therapies, *ex vivo* drug screening with vital patient-derived tumor cells from freshly isolated HNSCC surgical tumor samples was performed. The drug sensitivity of the cells was assessed after four days of exposure to 164 anticancer therapeutics (Fig. 3A-B). In parallel, the OCT4 status of the tumors from which the cells were derived was studied by OCT4 IHC (Fig. 3C). Interestingly, while the great majority of the tested therapeutics did not display differential effects when comparing OCT4-negative and OCT4-positive tumor samples, OCT4-negative samples did show decreased sensitivity to several drugs associated with the DNA damage response (DDR) (Fig. 3A-B). Among these were the ATR inhibitor BAY-1895344, 5-fluorouracil, the MTHFD2 inhibitor TH9619 and cisplatin (Fig. 3B). OCT4 negativity was also associated with decreased sensitivity to the PI3K inhibitor AZD8186, the calcineurin inhibitor tacrolimus and the TGF-beta receptor I inhibitor galunisertib (Fig. 3A-B).

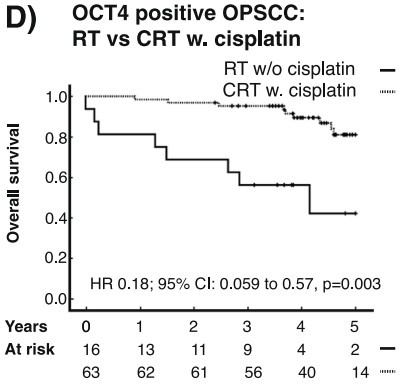


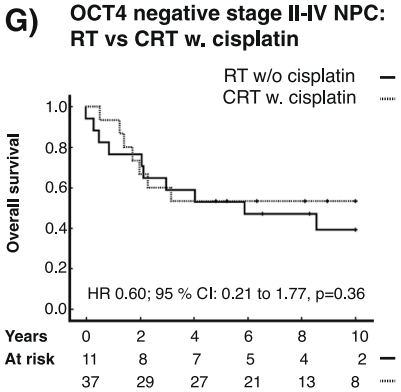












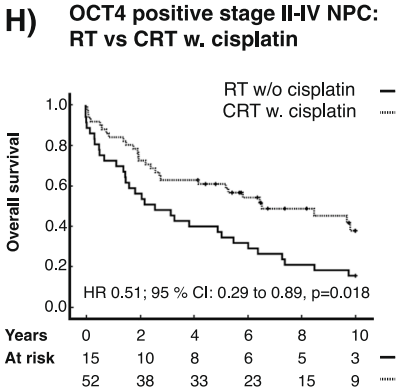


Fig. 4. Validation of the role of OCT4 in an independent dataset of OPSCC and NPC. Immunohistochemical analysis divided OPSCC tumors dichotomously into A) OCT4-negative tumors and B) OCT4-positive tumors. In OCT4-negative patients, there was C) no survival difference between patients treated with radiotherapy (RT) alone and patients treated with cisplatin-based chemoradiotherapy (CRT). In OCT4-positive patients, there was D) a significant improvement in the prognosis of patients treated with cisplatin-based chemoradiotherapy as compared to patients treated with radiotherapy alone. Similarly, in stage II-IV NPC, divided into E) OCT4-negative patients and F) OCT4-positive patients, there was G) no prognostic difference between radiotherapy alone and cisplatin-based chemoradiotherapy, while H) OCT4-positive patients had a significantly impaired 10-year survival when radiotherapy alone was used.

Validation in an independent cohort of oropharyngeal cancer

For validation of PV-TMA results in independent HNSCC clinical material, 118 oropharyngeal carcinoma (OPSCC) patient samples were processed into TMAs (Supplemental Table 2). Patients were treated with radiotherapy alone (n = 29) or with cisplatin-based chemoradiotherapy (n = 89). Similar to HNSCC samples, nuclear OCT4 status was clearly distinguishable by IHC analysis of OCT4-negative and OCT4-positive

OPSCC samples (Fig. 4A-B). OCT4-negative patients had similar prognoses irrespective of treatment type (Fig. 4C), while OCT4 positivity was strongly associated with an improved prognosis in cisplatin-treated patients (Fig. 4D).

Validation in an independent cohort of nasopharyngeal carcinoma

For further validation, the OCT4 status of the tumor samples of 170 patients from the Finnish nationwide TMA cohort of NPC patients treated with radiotherapy with or without cisplatin was investigated (Supplemental Table 3, Fig. 4E-F). Consistent with the aforementioned results, OCT4 positivity was associated with a trend for poor OS after radiotherapy but not after cisplatin-based chemoradiotherapy in NPC (Supplemental Figure 4A-B), reaching significance in stage II-IV NPC (Fig. 4G-H).

OCT4 positivity predicts cisplatin benefit in an independent vulvar carcinoma patient cohort

To test whether our observations were restricted to HNSCC tumors only, OCT4 status was studied in a cohort of 95 primary vulvar squamous cell carcinomas (VSCCs) (Supplemental Table 4, Supplemental Figure 5A-B). In surgically treated patients, OCT4 positivity was associated with a favorable prognosis (Supplemental Figure 5C). In OCT4-

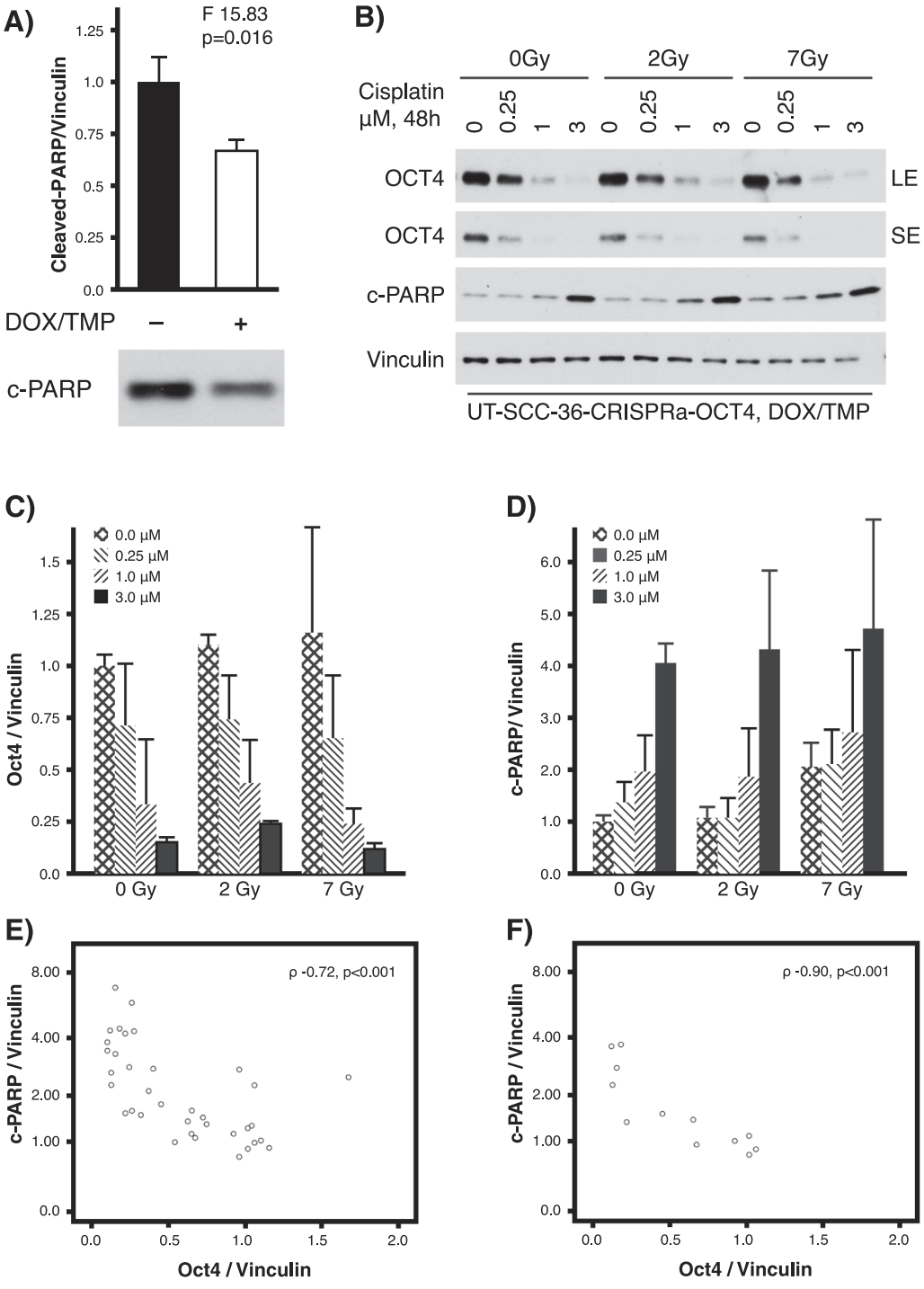


Fig. 5. *In vitro* analysis of OCT4-related cisplatin and radioresistance using the UT-SCC-36-CRISPRa cell line. A) Confirming the functional activity of the induced OCT4 in UT-SCC-36-CRISPRa-OCT4 cells, the expression of apoptosis indicator cleaved-PARP decreased after OCT4 induction. Quantitative analysis (estimated marginal means; error bars indicate the upper limit of 95% CI) of triplicate experiments demonstrated a significant reduction in apoptosis after OCT4 induction (F 15.83, $p = 0.016$). B) Western blot analysis of OCT4 and apoptosis marker cleaved-PARP in UT-SCC-36-CRISPRa-OCT4 cells treated with cisplatin alone (0 Gy) or with concurrent irradiation at the indicated doses. Cells were first treated with cisplatin and, after 24 h of incubation, irradiated with the indicated doses. (Full, uncut gel is included as Supplemental Fig. 6.) C) In quantitation analysis (estimated marginal means; error bars indicate upper limit of 95% CI) of triplicate experiments, OCT4 expression in OCT4-induced cells was insensitive to irradiation, demonstrating a nonsignificant trend toward increased OCT4 (F 0.59, $p = 0.56$). However, OCT4 was dramatically downregulated when increasing concentrations of cisplatin were used (F 31.40, $p < 0.001$). No interaction effect was noted. D) Cleaved-PARP expression in OCT4-induced cells was significantly dependent on cisplatin concentration (F 19.66, $p < 0.001$) but not irradiation (F 3.01, $p = 0.068$). E) A significant correlation between OCT4 and c-PARP levels was detected (rho −0.72; 95% CI, −0.51 to −0.85, $p < 0.001$). F) Importantly, OCT4 depletion and c-PARP induction correlated throughout cisplatin monotherapy experiments (rho −0.90; 95% CI, −0.63 to −0.96, $p < 0.001$) but not in radiotherapy monotherapy experiments (no Fig. shown, rho 0.033; 95% CI, −0.89 to 0.73, $p = 0.93$).

positive but not OCT4-negative patients, the addition of adjuvant cisplatin offered a significant survival benefit compared to radiotherapy alone (Supplemental Figure 5D), confirming the association between OCT4 positivity and a favorable cisplatin-based chemoradiotherapy response in cancers other than HNSCC.

OCT4 is associated with radioresistance but indicates cisplatin sensitivity in CRISPRa-OCT4 HNSCC cells

To establish the causal link between OCT4 and HNSCC chemo-radiosensitivity, we used the UT-SCC-36-CRISPRa cell line introduced above, in which OCT4 protein expression can be induced with doxycycline/trimethoprim treatment (Fig. 2A-B). Consistent with previous reports indicating an association of OCT4 expression with apoptosis resistance [21,22], OCT4 induction in UT-SCC-36-CRISPRa-OCT4 cells inhibited basal apoptotic activity, as indicated by reduced PARP cleavage (Fig. 5A). To study the relationship between OCT4 and radio- or cisplatin sensitivity, UT-SCC-36-CRISPRa-OCT4 cells were exposed to irradiation and cisplatin (Fig. 5B, Supplemental Figure 6). OCT4 protein levels were insensitive to radiotherapy (Fig. 5C-D, Supplemental Figure 7A). However, dramatic and dose-dependent OCT4 protein down-regulation was observed upon the addition of cisplatin (Fig. 5C-D, Supplemental Figure 7A). Importantly, the levels of OCT4 and cleaved-PARP correlated significantly, especially when no irradiation was used (Fig. 5E-F). The OCT4-related reduction in apoptosis levels could be restored using 1 μM cisplatin (Supplemental Figure 7B), after which there was no difference in the apoptotic response of the cells to radiotherapy (Supplemental Figure 7C).

Discussion

The cornerstones of HNSCC primary treatment with curative intent are surgery and radiotherapy with chemotherapy used mainly for radiosensitization of locally advanced cases with a high tumor burden. Thus, this study addresses one of the most crucial questions of HNSCC primary therapy with curative intent (i.e., the lack of biomarkers for the prediction of clinical benefit from cisplatin-based radiosensitization). Our results indicate that OCT4 immunostaining could potentially be used for clinical HNSCC patient therapy stratification between radiotherapy and chemoradiotherapy with cisplatin (*cf.* Fig. 6). In particular, our results exert a significant impact on recent discussion concerning treatment de-escalation [23].

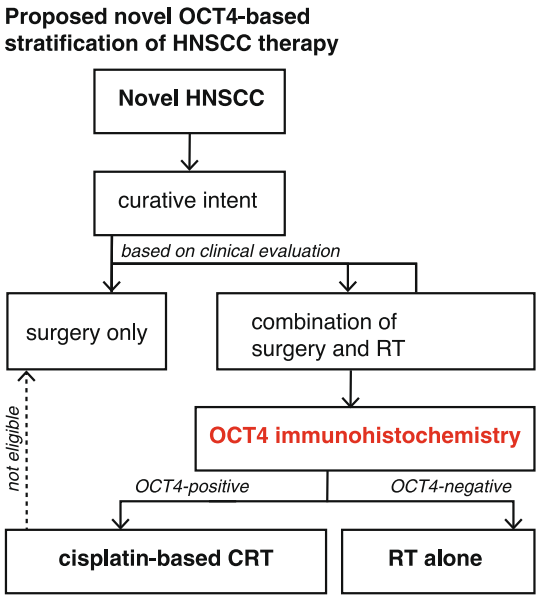


Fig. 6. Proposed OCT4-based stratification for novel HNSCC. When indicated by clinical evaluation, including TNM staging and current guidelines, curative treatment for HNSCC should consist of surgery, radiotherapy (RT), or a combination thereof. However, based on the results presented in this paper, when radiotherapy is used, the decision for chemosensitization should be based on OCT4 immunohistochemical evaluation. While OCT4-negative tumors could be treated without cisplatin radiosensitization, OCT4-positive tumors should be treated with cisplatin-based chemoradiotherapy (CRT). If the patient is not eligible for even low-dose cisplatin treatment, surgical treatment should be preferred.

Worldwide, the decision of optimal oncological treatment is made annually for over 400 000 patients with HNSCC [1]. Thus, the identification of patients who would benefit from already existing therapies is the foundation for clinical HNSCC research investigating novel therapeutic approaches. The application of combined treatment with cisplatin concurrent with radiotherapy in HNSCC has been well established since the 1970s. Despite landmark observations of platinum-based radiosensitization, comprehensive genomic characterization and multiple biomarker-based trials have failed to predict which patients will benefit from combined chemo- and radiotherapy in HNSCC. Furthermore, since cisplatin may be associated with significant renal and ototoxicity and contributes to the incidence of late radiation-induced toxicities, unselected use of cisplatin-based radiosensitization has been questioned, especially in frail and elderly patients [11–13].

Currently, the only biomarker for HNSCC in clinical use is p16 for OPSCC as a surrogate marker of HPV infection. However, de-escalation strategies using cetuximab or reduced radiotherapy or reduced cisplatin doses are still not recommended outside clinical trials, as exemplified by conflicting results obtained in recent studies [7,24,25]. The possibility of avoiding treatment toxicity by TORS (Trans Oral Robotic Surgery) remains an interesting prospect [26,27]. Challenges and disappointments in HNSCC clinical trials using targeted therapies indicate that our knowledge of the molecular heterogenicity of HNSCC remains insufficient and further demonstrates an unmet need for validated biomarkers to predict the outcome of individual oncological treatments [3,4,28,29].

Previous and recent publications have demonstrated the important role of the stemness-associated transcription factor OCT4 in many cancer-critical functions, such as inhibition of cancer cell apoptosis, induction of cell viability and tumor invasion [30–32]. Furthermore, the potential of OCT4 as a biomarker for radioresistance is well recognized in the cancer stem cell hypothesis [33]. However, the role of OCT4 in radiosensitization and chemoresistance is not well understood, and the conclusions of recent publications are conflicting [34]. As the outcome and the goal of OCT4 stratification in cancer patients has remained unclear, OCT4 detection has not reached clinical practice. OCT4, as an indicator of cisplatin sensitivity, has best been studied in testicular cancers (TCs), which are mostly OCT4-positive [35] and highly sensitive to cisplatin [36,37]. In TCs, loss of OCT4 is known to induce cisplatin resistance [33], whereas in studies of cancer types other than TC OCT4 has been associated with cancer aggressiveness, decreased survival [17,38,39] and radioresistance [40–42]. Our data and a recent study in which OCT4 expression was evaluated in specimens from a series of population-based HNSCCs clearly demonstrate limitations of OCT4-based prognostication [14]. However, our results in this study overall support the conclusion that OCT4 detection could be used in the identification of radioresistant and cisplatin-sensitive tumors, which is in line with the involvement of OCT4 in the regulation of the DNA damage response demonstrated in our prospective drug screening study as well as in previous reports [43–45]. In a recent publication, OCT4 was mechanistically reported to regulate the cell cycle checkpoint kinases CHK1 and WEE1 and the homologous recombination (HR) repair genes PSMC3IP and RAD54L in HNSCC, which lead to HR-mediated deficiency in DNA repair mechanisms [45]. Stem cell-associated, OCT4-linked DNA repair mechanisms are putatively exploited by cancers in the context of radiotherapy-induced DNA damage [46]. Our *in vitro* and prospective HNSCC drug screening findings are well in line with these previous findings, since OCT4 induction reduced apoptotic cell death and,

furthermore, OCT4-positive tumors were sensitive to DNA repair inhibitory substances, such as cisplatin. Thus, we suggest that cisplatin targets OCT4-related DNA repair mechanisms. Whether OCT4 expression plays a role in tumor immune evasion, would be an intriguing focus for future studies.

As a main strength of our study, high-quality clinical cohorts were employed in the investigation of the role of OCT4 in patient stratification. OCT4 immunohistochemistry identified patients benefitting from cisplatin radiosensitization in four cohorts of nonselected HNSCC, OPSCC, NPC and VSCC. Importantly, since OCT4 positivity was not associated with other patient-related clinical characteristics in any of the investigated cancers, it would contribute to the decision-making process by identifying patients likely to benefit from cisplatin-based chemoradiotherapy. In support of OCT4-based stratification, OCT4 immunohistochemical analysis is cost-effective, relatively straightforward to analyze and reliable in terms of staining heterogenicity. OCT4 positivity was associated with significant improvement in OS even when the targeted cisplatin dose was not achieved, suggesting that OCT4 positivity would be beneficial even with low-dose radiosensitization strategies. Importantly, the prospect for avoiding harms associated with adjuvant radiotherapy after TORS in OCT4-positive HPV-related OPSCC is especially intriguing, since no OCT4-related survival impact was found in surgically treated patients with HNSCC [14]. Interestingly, OCT4 positivity was more common in laryngeal cancer, where curative radiotherapy alone is often used in early-stage disease. Further evaluation in larger patient cohorts would allow for verification of our observation, while prospective trials would be needed for the evaluation of benefit from adding cisplatin in early stage laryngeal cancer with respect to OCT4 status of these tumors. Whether OCT4 immunohistochemistry could be analyzed in other than dichotomous way, has to be carefully addressed during the follow-up studies using whole-section samples.

The obvious weakness of this study was the challenge to demonstrate the *in vitro* OCT4-related mechanisms. We used a panel of patient-derived HNSCC cell lines established earlier [47]. Surprisingly, all tested cancer cell lines were negative for OCT4 protein expression, although clear OCT4 positivity was observed in testicular cancer cell lysates. Thus, cultured HNSCC cells or xenograft experiments are not suitable for assessing the clinical role of OCT4 in HNSCC therapy responses and might explain why its role in mediating sensitivity to chemoradiotherapy has not been revealed before. The lack of OCT4 expression in HNSCC cell lines reveals that standard *in vitro* conditions do not support OCT4 expression, which may have influenced earlier HNSCC functional and biomarker studies. The hypothesis that OCT4-associated mechanisms do not easily yield *in vitro* studies is also supported by our demonstration that lentivirus-induced OCT4 expression was not successful. Moreover, during longer *in vitro* culture, OCT4 protein expression was lost even in conditional CRISPRa-OCT4-expressing HNSCC cell lines. However, despite these challenges in OCT4 *in vitro* modeling, we were still able to demonstrate that OCT4 promotes apoptosis resistance and is downregulated by cisplatin treatment. Furthermore, inhibition of apoptosis-inhibiting OCT4 and OCT4-related DNA repair by cisplatin provides a feasible molecular biological explanation for the significantly better clinical outcomes of OCT4-positive patients treated with cisplatin-based chemoradiotherapy compared to patients treated with radiotherapy alone.

Conclusions

In conclusion, we demonstrate that tumor OCT4 positivity could be used in HNSCC as a stratification marker to select patients who should not preferably be treated with radiotherapy without platinum-based sensitization. Importantly, OCT4-negative tumors may be candidates for de-escalation strategies in future prospective trials. However, such trials should carefully evaluate tumor characteristics and behavior to ensure the oncological safety of de-escalation. Finally, corroboration of our findings by independent investigators and in a prospective, multicenter clinical trials would prove useful for the inclusion of OCT4 IHC as a predictive biomarker in HNSCC therapy stratification.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper: [JKR is the founder and CEO of Misvik Biology Ltd. The authors declare no potential conflicts of interest.].

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Finnish National Supervisory Authority for Welfare and Health (V/39706/2019), regional ethics committee of University of Turku (51/1803/2017 and 166/1801/2015) and Auria Biobank scientific board (AB19–6863 and AB15–9293).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the *ex vivo* drug sensitivity screening. For patients involved in the retrospective datasets, patient consent was waived due to retrospective nature of the data in accordance with approval from Finnish National Supervisory Authority for Welfare and Health and regional ethics committee of University of Turku.

Data availability statement

The data sets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2022.105772.

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