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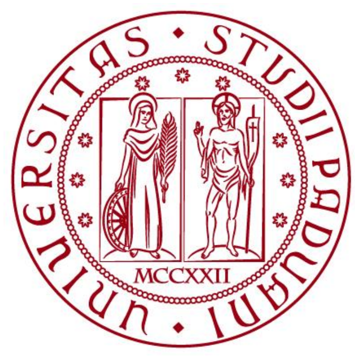
DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biologia



ELABORATO DI LAUREA

Ruolo del canale ionico VRAC nella chemioresistenza del carcinoma testa collo a cellule squamose (HNSCC)

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SOMMARIO

ABBREVIAZIONI 2
ABSTRACT 3

1 - STATO DELL'ARTE
1.1 - Il cancro 4
1.2 - Il tumore della testa e del collo 4
1.3 - I canale ionico VRAC 6
1.4 - VRAC e HNSCC 7

2 - MATERIALI E METODI
2.1 - Analisi dei dati clinici 8
2.2 - Coltura cellulare 8
2.3 - Generazione di un modello resistente al cisplatino 8
2.4 - Sequenziamento RNA 9
2.5 - Vitalità cellulare 9
2.6 - Western blotting 10
2.7 - Microscopia a fluorescenza 10
2.8 - Espressione transitoria di LRRC8A 10

3 - RISULTATI E DISCUSSIONI
3.1 - Identificazione dei fattori clinicamente rilevanti alla resistenza al cisplatino 12
3.2 - Profilazione delle vie di sensibilità al cisplatino e rilevanza di VRAC come determinante critico per la resistenza al cisplatino 13
3.3 - L'espressione di VRAC nelle cellule tumorali HNSCC è fondamentale per la sensibilità e la specificità dei farmaci al platino 15
3.3.1 - Analisi *in vitro* 15
3.3.2 - Analisi in vivo 17
3.4 - Il trasportatore VRAC svolge una funzione determinante nella resistenza al cisplatino nelle cellule tumorali naturali 18
3.5 - Conclusioni 19
3.6 - Analisi critica 20

BIBLIOGRAFIA 21
APPENDICE 23

ABBREVIAZIONI

AVD: *Apoptotic volume decrease*
ATP7B: *ATPase Copper Transporting Beta*
bp: *base pair*
cDNA: *Complementary DNA*
cKRT24: *Cytokeratin2*
CLC: *Chloride channel 4*
crRNA: *RNA CRISPR*
CTR1: *High affinity copper uptake protein 1*
DMSO: *Dimethyl sulphoxide*
dsDNA: *Double strand DNA*
EF1alpha*:* *Elongation factor 1 alpha*
EpCAM: *Epithelial cell adhesion molecule*
gDNA: *Genomic DNA*
GFP: *Green Fluorescent Protein*
HNSCC: *Head and neck squamous cell carcinoma*
KO: *knock-out*
LRRC8A-KO: *LRRC8A knock-out*
mRNA: *Messenger RNA*
MRP1: *Multidrug resistance-associated protein 1*
MRP2: *Multidrug resistance-associated protein 2*
ncRNA: *Non-coding RNA*
NHEJ: *Non-homologous end joining*
OCT1: *Organic cation transporter 1*
PANX: *Pannexins*
PBS: *Phosphate Buffered Saline*
PCR: *Polymerase Chain Reaction*
PFA: *Paraformaldehyde*
PVDF: Polivinilidenfluoruro
ROI: *Region of interset*
RRC: *Leucine-rich repeats containing protein*
RVD: *Regulatory volume decrease*
SOX2: *SRY (sex determining region Y)-box 2*
Taq: *Thermus aquaticus*
TGCA: *The Cancer Genome Atlas Program*
TP53: *Tumor-protein 53*
tracrRNA: *Trans-activating crRNA*
USCS: *Unified Soil Classification System*
VRAC: *Volume-regulated anion channel*
WT: *Wild-type*
γH2AX: *Phosphorylated form of histone H2AX*
2PE: *Two-photon excitation*

ABSTRACT

VRAC (*Volume-Regulated Anion Channel*) è un canale anionico ubiquitario, localizzato principalmente nella membrana plasmatica e nella membrana lisosomiale delle cellule. Il canale svolge un ruolo importante nella regolazione del volume cellulare e partecipa a svariati processi fisiologici. Esistono vari studi che ne dimostrano il coinvolgimento nella proliferazione, migrazione e morte cellulari in vari tipi di cancro. Il suo ruolo nei tumori della testa e collo (HNSCC) rimane tuttora soggetto di studi.

Questa tesi ha l'obiettivo di presentare ed analizzare i risultati e le metodologie utilizzate da Siemer *et a*l. (*Cancers 2021, 13, 4831. https://doi.org/10.3390/ cancers13194831*, vedere appendice), studio che ha dimostrato come VRAC svolge un ruolo importante nella resistenza al cisplatino nell'HNSCC e la sua possibile rilevanza come bersaglio per farmaci antitumorali e come biomarcatore prognostico per la resistenza chemioterapica.

Con lo scopo di verificare sperimentalmente il ruolo critico di VRAC nella resistenza al cisplatino, gli autori dello studio hanno utilizzato linee cellulari *knock-out* (KO) CRISPR/Cas9, ottenendo cellule HNSCC resistenti al cisplatino, nelle quali poteva essere ristabilita l'espressione del VRAC. Il sequenziamento di nuova generazione ha ulteriormente sottolineato l'importanza di VRAC e ha identificato le reti di segnalazione regolate da VRAC, che potenzialmente contribuiscono alla resistenza al cisplatino. Oltre ai modelli bidimensionali di HNSCC, per replicare la situazione in *vivo*, gli autori hanno utilizzato colture tridimensionali di sferoidi tumorali confermando ulteriormente il ruolo di VRAC nella sensibilità al cisplatino.

1-STATO DELL'ARTE

1.1 Il cancro

Il cancro non è una malattia unica, non ha un'unica causa né un unico tipo di trattamento. Esistono oltre 200 tipi diversi di cancro, ognuno con le proprie peculiarità. In generale, i tumori possono essere distinti tra tumori benigni e maligni. I tumori benigni sono forme neoplastiche che non si diffondono in tutto il corpo, hanno una dimensione ben delineata e condividono le stesse caratteristiche del tessuto da cui si sono originate. I tumori maligni, invece, sono forme neoplastiche caratterizzate dalla presenza di una massa cellulare in progressiva replicazione, capace di diffondersi e coinvolgere varie parti dell'organismo. Queste cellule anormali viaggiano attraverso il flusso sanguigno, il sistema circolatorio e il sistema linfatico. Le forme tumorali maligne si possono classificare in diversi modi, in base all'organo nel quale si sviluppano, alla tipologia di cellule che vanno a generare, allo stadio, all'aggressività e alla probabilità dello sviluppo di metastasi.
Per combattere il cancro si hanno a disposizione diversi strumenti come la sorveglianza attiva, la chirurgia, la radioterapia e la farmacoterapia. Tuttavia, ogni tumore richiede un approccio diverso e, spesso, anche tempi di cura diversi.

1.2- Il tumore della testa e del collo

I tumori della testa e del collo HNSCC, chiamati anche tumori testa-collo, includono tumori molto diversi tra loro che hanno origine negli organi e nelle strutture dell'area del collo e della testa, incluse le vie aerodigestive superiori. Globalmente, i tumori della testa e del collo rappresentano circa il 10-12% di tutti i tumori maligni e rappresentano la sesta causa di mortalità per tumore in tutto il mondo (Johnson *et al.,* 2020). Questi tumori rappresentano un problema clinico e sociale importante per la delicatezza delle funzioni degli organi che possono compromettere ed inoltre non esistono terapie mirate realmente efficaci.
L'HNSCC ha origine dalle cellule epiteliali della mucosa che rivestono la cavità orale, la faringe, la laringe e il tratto sinonasale (**Figura 1**).

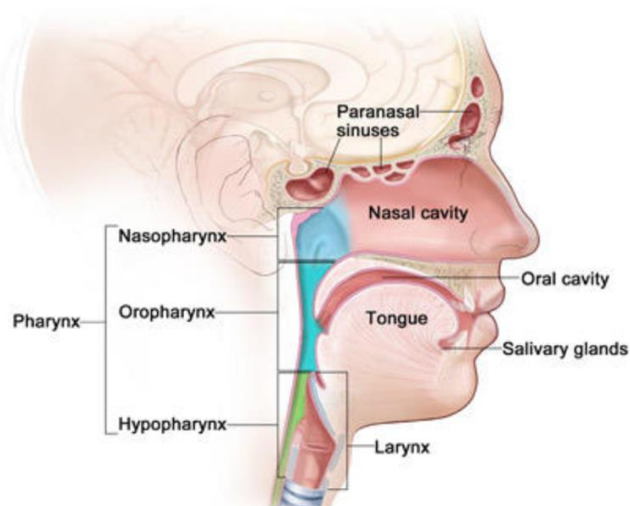

Figura 1: Regioni del cancro della testa e del collo. I tumori noti collettivamente come tumori della testa e del collo di solito iniziano nelle cellule squamose che rivestono le superfici mucose della testa e del collo (per esempio, quelle all'interno della bocca, della gola e della scatola vocale). I tumori della testa e del collo possono avere inizio anche nelle ghiandole salivari, nei seni paranasali, nei muscoli o nei nervi della testa e del collo, ma questi tipi di tumore sono molto meno comuni dei carcinomi a cellule squamose. Immagine modificata da www.cancer.gov

Istologicamente, la progressione verso l'HNSCC invasivo segue una serie ordinata di fasi che inizia con l'iperplasia delle cellule epiteliali, seguita dalla displasia (lieve, moderata e grave), dal carcinoma *in situ* e, infine, dal carcinoma invasivo (Johnson *et al.,* 2020; Califano *et al.,* 1996) (**Figura 2**). È stato riscontrato un arricchimento di eventi genetici specifici in ogni fase della progressione. A differenza della maggior parte dei tumori, in cui le mutazioni oncogeniche guidano tipicamente la tumorigenesi, la formazione dell'HNSCC comporta solitamente l'inattivazione di geni soppressori del tumore, come *CDKN2A* e *TP53* (che codificano rispettivamente p16INK4A e p53) nelle fasi iniziali e *PTEN* (che codifica l'omologo della fosfatasi e della tensina) nelle fasi successive (**Figura 2**).

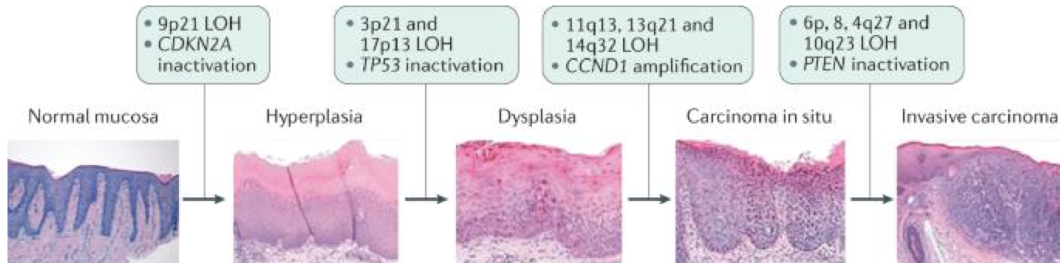

Figura 2: Sito di origine del HNSCC. L'epitelio mucoso che riveste la cavità orale, la faringe, la laringe e il tratto sinonasale è il sito di origine del HNSCC. In un modello di progressione istologica ordinata dell'HNSCC, l'iperplasia delle cellule epiteliali mucosali è seguita dalla displasia e il carcinoma *in situ* precede lo sviluppo del carcinoma invasivo. Gli eventi genetici specifici in ogni fase della progressione sono indicati. Immagine presa da Johnson *et al.*, 2020.

1.3 - Il canale ionico VRAC

I canali ionici sono proteine che controllano il flusso di ioni positivi o negativi attraverso le membrane delle cellule. Esistono molti tipi di canali ionici con proprietà diverse, coinvolti in molte funzioni dell'organismo: principalmente la produzione e lo scambio di segnali elettrici cellulari (es. nel controllo muscolare o nelle funzioni cerebrali) ed il controllo del bilancio ionico.

Il canale anionico regolato dal volume (VRAC) è ampiamente espresso nella maggior parte dei tipi di cellule, dove media le correnti di Cl^- attivate dal rigonfiamento, necessarie per la regolazione del volume cellulare. Essendo permeabile ad una serie di anioni organici e inorganici, VRAC svolge molti altri ruoli importanti nelle cellule. L'ampia gamma di processi cellulari che coinvolgono questo canale ionico suggerisce che VRAC sia un nuovo e potente bersaglio terapeutico per una varietà di malattie umane incluso il cancro.

Nonostante gli intensi sforzi compiuti per oltre tre decenni, i progressi nella caratterizzazione di VRAC e dei suoi ruoli biologici sono stati fortemente limitati dalla mancata identificazione della/e proteina/e principale/i. La svolta è arrivata nel 2014, quando è stato dimostrato che il canale VRAC è codificato da cinque membri della famiglia di geni *LRRC8* (*LRRC8A-E*) (Qiu *et al.,* 2014; Voss *et al.,* 2014) (**Figura 3**). Le proteine LRRC8 originano dalla combinazione di una pannexina (PANX) e di una proteina con dominio di ripetizione ricco di leucina (LRRD) (Abascal *et al*., 2012). Le proteine LRRC8 sono composte da quattro segmenti transmembrana (TM) e da un dominio di ripetizione ricco di leucina contenente il segmento C-terminale che, come il dominio N-terminale, risulta essere citoplasmatico (**Figura 3**).

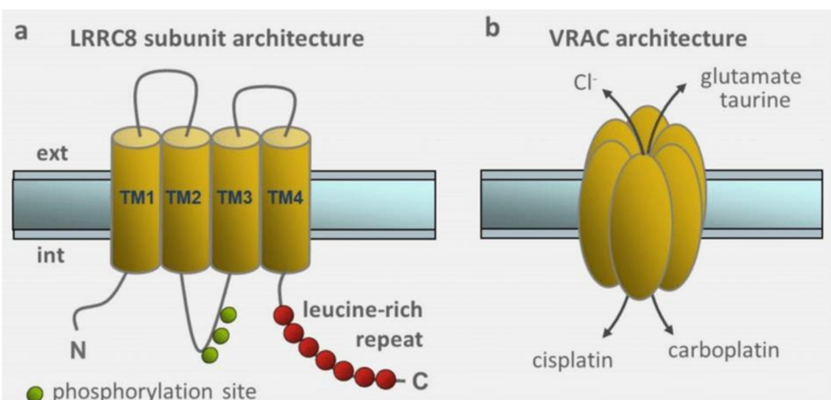

Figura 3: Topologia transmembrana delle subunità LRRC8 e VRAC. (a) Le subunità LRRC8 presentano quattro domini transmembrana (TM1-4) e una terminazione C con fino a 17 ripetizioni ricche di leucina. Le subunità LRRC8 hanno vari siti di fosforilazione (in verde) e di glicosilazione (non indicati). (b) Stechiometria esamerica di VRAC, basata sulla sua parziale omologia con i canali pannexina/connexina. Immagine presa da Caramia *et al.,* 2019.

La caratterizzazione di topi LRRC8A KO ha rivelato l'importante ruolo di VRAC nello sviluppo delle cellule T (Sawada *et al*., 2003), nella secrezione di insulina

stimolata dal glucosio della cellule-β delle isole del Langerhans del pancreas (Kang *et al.*, 2018) e nella funzione metabolica degli adipociti (Zhang *et al.*, 2017). L'incorporazione di complessi proteici purificati contenenti LRRC8A in *bilayer* lipidici è sufficiente a ricostituire le attività dei canali (Osei-Owusu *et al.*, 2014). Tuttavia, la composizione delle subunità LRRC8 determina le proprietà del canale e la selettività del substrato (Osei-Owusu *et al.*, 2014). La capacità di permeare osmoliti e metaboliti organici è una delle caratteristiche principali della VRAC. L'elenco dei substrati della VRAC è destinato a crescere, includendo ora anche alcuni farmaci antitumorali e antibiotici anche in condizioni di non rigonfiamento cellulare. Pertanto, sta emergendo un ruolo critico della VRAC nella resistenza ai farmaci e nella comunicazione cellula-cellula.

1.4 - VRAC e HNSCC

Le opzioni di trattamento dell'HNSCC includono l'asportazione chirurgica con o senza radioterapia (chemio)adiuvante o la radioterapia con chemioterapia concomitante. Sebbene recentemente si siano riscontrati risultati favorevoli grazie all'uso dell'inibitore del *checkpoint* α-PD-1 pembrolizumab (Burtness, *et al.*, 2019), la chemioterapia è principalmente basata sull'utilizzo di farmaci a base di platino, con il cisplatino come opzione principale. Nonostante il cisplatino sia ampiamente utilizzato, la resistenza alla terapia e le conseguenti recidive in tempi più o meno ravvicinati sono ancora comuni (Jou *et al.*, 2017).
I trasportatori che facilitano l'afflusso e l'efflusso attivo dei farmaci a base di platino sembrano essere candidati particolarmente promettenti in questo contesto, in quanto hanno un impatto diretto sulle concentrazioni finali intracellulari dei farmaci. Si è ipotizzato che diversi trasportatori di farmaci, come CTR1, OCT1, VRAC e MRP1, siano coinvolti nel trasporto e nella resistenza dei farmaci a base di platino, mentre la diffusione passiva trans-membrana svolge solo un ruolo secondario. In particolare, recenti studi hanno dimostrato la rilevanza di VRAC per la resistenza al cisplatino ed un ruolo aggiuntivo per le proprietà di assorbimento dei farmaci.

2 - MATERIALI E METODI

2.1 - Analisi dei dati clinici

I *set* di dati riguardanti l'espressione genica e la sopravvivenza dei pazienti con HNSCC sono stati ottenuti dal TCGA. Filtrando i casi con informazioni cliniche dettagliate sono stati identificati 41 casi in cui i pazienti hanno ricevuto cisplatino come chemioterapia di prima linea. I dati sono stati valutati tramite il server USCS Xena (Goldman *et al.*, 2020) e i pazienti sono stati raggruppati in base alle caratteristiche fenotipiche o cliniche.

2.2 - Coltura cellulare

La linea cellulare Pica è stata generata da un carcinoma laringeo a cellule squamose con un nuovo protocollo sviluppato per imitare il tumore in modo più preciso. Per generare la linea cellulare sono stati incisi piccoli espianti da biopsie primarie (Comer *et al.,* 2012). Le cellule tumorali sono state recuperate in modo riproducibile dal terreno di coltura entro uno o tre giorni e utilizzate per test *in vitro*. *In vivo*, le singole cellule hanno formato tumori che imitano perfettamente i tumori umani di origine. Inoltre, dalle cellule espiantate è stato possibile generare linee cellulari che esprimono i marcatori classici delle linee cellulari di carcinoma mediante diluizioni seriali. Le cellule sono state coltivate in condizioni di coltura cellulare standard (37 °C, 5% CO2) in *Dulbecco's Modified Eagle's Medium* e diluite in mezzo fresco ogni 3 giorni. L'assenza di micoplasmi è stata regolarmente controllata con un *kit* di rilevamento *Venor GeM Advance* (*Minverva biolabs*, Berlino, Germania) secondo le istruzioni del produttore. Il numero di cellule è stato determinato con *Casy Cell Counter and Analyzer TT* (*OMNI Life Science GmbH & Co KG*, Brema, Germania).

2.3 - Generazione di un modello resistente al cisplatino

Il sistema CRISPR/Cas9 è molto utilizzato nel campo della regolazione genica e dell'*editing* genomico. Il sistema CRISPR/Cas9 è stato identificato originariamente studiando i batteri, dove la proteina Cas9 svolge la sua funzione di forbice molecolare aiutando questi microrganismi a proteggersi da virus patogeni, svolgendo quindi la funzione di una sorta di sistema immunitario dei batteri. La programmazione del bersaglio di Cas9 avviene attraverso una molecola di RNA, chiamata RNA guida, che può essere facilmente modificata e, una volta associata a Cas9, la ancora alla sequenza di DNA bersaglio scelta. CRISPR fa riferimento a ripetizioni palindromiche brevi regolarmente intercalate, che sono segmenti di DNA procariotico contenenti brevi ripetizioni di sequenze di basi. Ogni ripetizione è seguita da una breve sequenza di "DNA *spacer*" che deriva dal genoma di un virus precedentemente infettato (Wiedenheft *et al.,* 2012). Successive trascrizioni delle unità di ripetizione-separazione CRISPR producono due RNA non codificanti: un RNA CRISPR (crRNA) contenente sequenze guida di nucleasi compilate dagli

spaziatori e un altro RNA non codificante, complementare alla sequenza di ripetizione, noto come crRNA trans-attivante (tracrRNA). Cas9 è un'endonucleasi con due domini enzimatici: un dominio HNH che taglia il filamento complementare di DNA che si accoppia con l'RNA guida e un dominio RuvC1 che taglia il filamento non complementare (Nishimasu *et al.,* 2014). Una volta ottenute le rotture del dsDNA nel gene bersaglio, le cellule attivano le loro vie di riparazione delle estremità non omologhe (NHEJ), soggette a errori, per riparare il danno, dando luogo a mutazioni casuali di inserzione/delezione (indels) delle basi del DNA nel sito di taglio. L'introduzione di indels nella struttura codificante del gene bersaglio porta di conseguenza a cambiamenti nell'espressione del gene bersaglio, compreso il knockdown genetico (Cong *et al.,* 2013).
Il KO di LRRC8A è stato ottenuto mediante la delezione di una regione genomica di LRRC8A usando la tecnologia CRISPR-Cas9. Entrambi gli alleli di loci genetici previsti sono stati bersagliati dalla tecnologia (Trothe *et al.,* 2019). Le cellule sono state trasfettate con i plasmidi (2 μg) utilizzando Lipofectamin 2000 e selezionate mediante trattamento con blasticidina per 9 giorni. Sono state ricavate linee cellulari derivate da singole cellule mediante diluizioni seriali e verificate per un KO corretto e omozigote. I *primers* per le PCR analitiche sono stati progettati in modo da coprire l'area *target* di interruzione per KO. Infine, è stato eseguito il sequenziamento Sanger per garantire il corretto KO. L'esperimento è iniziato dopo una cultura cellulare di sei mesi per permettere alle cellule di riprendere il loro normale metabolismo e quindi una proliferazione cellulare regolare. L'espressione di LRRC8A nelle linee cellulari è stata verificata mediante analisi *Western blot*.

2.4 - Sequenziamento RNA

La trascrittomica mira principalmente: (i) a catalogare tutte le specie di trascritti (compresi mRNA, ncRNA e piccoli RNA), (ii) a determinare la struttura trascrizionale dei geni (in termini di siti di partenza, estremità 5' e 3', schemi di splicing e altre modifiche post-trascrizionali) e (iii) a quantificare i mutevoli livelli di espressione di ogni trascrizione. L'RNA-Seq invece utilizza tecnologie *deep-sequencing* di recente sviluppo. In generale, una popolazione di RNA (totale o frazionato) viene convertita in una libreria di frammenti di cDNA con adattatori collegati a una o entrambe le estremità. Ciascuna molecola viene sequenziata con metodi *high-throughput* per ottenere sequenze brevi da un'estremità (sequenziamento *single-end*) o da entrambe le estremità (sequenziamento dell'estremità della coppia). Le letture sono in genere 30-400 *bp*, a seconda della tecnologia di sequenziamento del DNA utilizzata.

2.5 - Vitalità cellulare

Il metodo più diretto e rapido per misurare l'attività proliferativa di una popolazione cellulare consiste nel contare le cellule presenti ad un certo tempo dalla semina. Le cellule sono state seminate in piastre da 96 pozzetti e trattate a partire da 24 ore dopo la semina. 48 ore dopo il trattamento, i segnali luminescenti

sono stati registrati con un Tecan Spark® (Tecan Group Ltd., Männedorf, Svizzera). I segnali sono stati normalizzati rispetto ai campioni di controllo non trattati. Per i saggi di vitalità cellulare nel caso degli sferoidi, le cellule sono state seminate in piastre di coltura a 96 pozzetti a fondo tondo e a bassissima adesione. Si è attesa la formazione iniziale di sferoidi (3 giorni). Quindi, gli sferoidi sono stati trattati e la vitalità è stata testata dopo 72 ore.

2.6 Western blotting

Il Western blotting è una tecnica che permette di rilevare, analizzare e quantificare le proteine. Il Western blotting prevede la separazione delle proteine mediante elettroforesi su gel seguita dal trasferimento su una membrana di PVDF o nitrocellulosa. Dopo il trasferimento, le proteine possono essere evidenziate mediante immunorivelazione grazie al legame con anticorpi specifici.

2.7 Microscopia a fluorescenza.

Le immagini fluorescenti sono state acquisite con un microscopio a fluorescenza o con il microscopio automatizzato ad alto contenuto di *screening*. Le cellule sono state seminate in piatti da microscopia (35 mm, MatTek) o in piastre a 96 pozzetti a fondo trasparente (Greiner Bio-One GmbH, Frickenhausen, Germania) e fissate con PFA al 4% (20 min, RT). Per la colorazione a immunofluorescenza, sono state ulteriormente permeabilizzate mediante incubazione con Triton-X 100 (0,1%, 10 min, RT). Gli anticorpi sono stati diluiti in 10% FCS/PBS e incubati con i campioni per 1 ora a temperatura ambiente. Dopo un ampio lavaggio (con PBS), gli anticorpi marcati con fluorofori sono stati incubati con i campioni per 1 ora a RT. Infine, i nuclei sono stati colorati con l'aggiunta di *Hoechst* 33342 (50 ng/mL in PBS) per 30 minuti a temperatura ambiente. Per lo *screening* automatico ad alto contenuto, sono state create regioni di interesse utilizzando il segnale del nucleo acquisendo almeno 5000 eventi per campione. Per la microscopia a due fotoni (2PE) degli sferoidi, gli sferoidi sono stati coltivati per 3 giorni e poi fissati mediante incubazione in PFA al 4% a temperatura ambiente (20 min). La permeabilizzazione è stata eseguita in PBSTD (PBS, 0,3% Triton-X 100, 1% DMSO, 1% BSA) e gli sferoidi sono stati incubati, a 4 ◦C, con anticorpi primari diluiti in PBSTD *overnight*. L'incubazione con anticorpi secondari è stata eseguita per 3 ore a RT. Infine, i nuclei sono stati colorati mediante incubazione con *Hoechst* 33342 (50 ng/mL) per 15 ore a temperatura ambiente. La raccolta e il lavaggio degli sferoidi tra le fasi di incubazione sono stati ottenuti mediante centrifugazione delicata (100 g, 3 min). Le immagini sono state acquisite con un sistema Leica TCS SP8 DIVE (Leica Microsystems, Weimar, Germania).

2.8 Espressione transitoria di LRRC8A

Gli autori hanno ristabilito la sensibilità al cisplatino delle cellule Pica-KO riespimendo LRRC8A. Le cellule sono state trasfettate con il plasmide pBix-Ep contenente la sequenza codificante di LRRC8A umano o LRRC8A fusa con un tag

FLAG C-terminale, un promotore costitutivo EF1alfa e il gene di resistenza all'antibiotico puromicina. Per marcare le cellule che esprimono LRRC8A, è stato co-trasfettato il plasmide pC3 che codifica per l'espressione di GFP. Per garantire la veridicità del processo è stata condotta in parallelo una trasfezione di controllo con il plasmide vuoto pC-DNA3 e il plasmide codificante per la GFP. Il terreno è stato cambiato 4 ore dopo le trasfezioni con un normale terreno di coltura cellulare e le cellule sono state trattate con cisplatino per 48 ore a partire da 24 ore dopo la trasfezione. Le cellule sono state fissate con PFA e il numero di cellule verdi è stato quantificato. Il FLAG-tag è stato identificato con anticorpi specifici e le cellule sono state analizzate sia con microscopia convenzionale sia con microscopia automatica ad alto contenuto.

3- RISULTATI E DISCUSSIONE

3.1-Identificazione dei fattori clinicamente rilevanti della resistenza al cisplatino

I processi di assorbimento ed efflusso cellulare dei chemioterapici a base di platino (**Figura 4**) influenzano in maniera decisiva le concentrazioni intracellulari del farmaco e di conseguenza il successo o il fallimento nel trattamento del cancro.

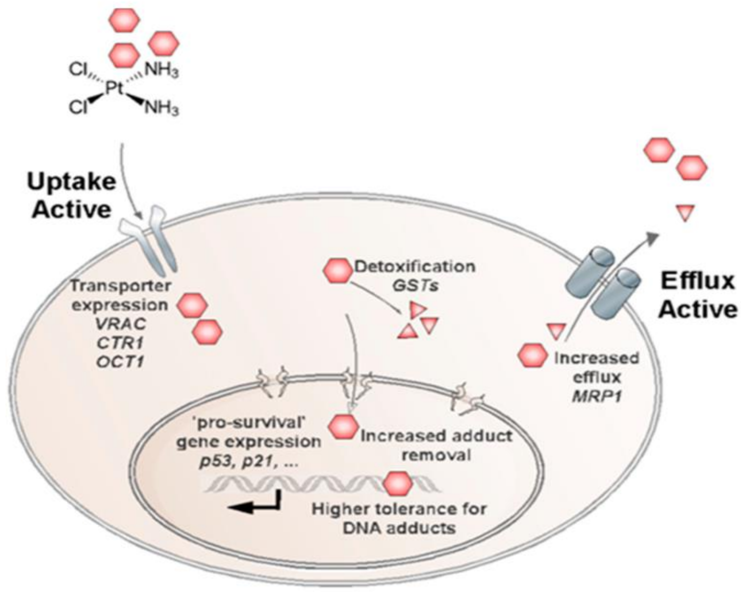

Figura 4. Processi di assorbimento ed efflusso cellulare dei chemioterapici a base di platino. L'immagine illustra i meccanismi potenzialmente coinvolti nella resistenza al cisplatino. La riduzione delle concentrazioni intracellulari di farmaco può essere la conseguenza di un assorbimento ridotto, di un efflusso accelerato o di una detossificazione intracellulare.

Il primo step degli autori è stato quello di analizzare i livelli di espressione delle proteine potenzialmente coinvolte nel trasporto del cisplatino, quali CTR1(gene *SLC31A1*), VRAC (gene *LRRC8A*), OCT1 (gene *SLC22A1*) o MRP1 (gene *ABCC1*). Tali dati sono stati reperiti dal *dataset* TCGA HNSCC, che comprende 565 pazienti oncologici con diversi stati patologici e *background* clinici. In particolare, per capire l'importanza specifica della terapia mediante cisplatino gli autori si sono concentrati solamente sui dati dei pazienti che erano stati sottoposti a chemio-radio con cisplatino come terapia principale. I dati riguardanti l'espressione genica sono stati estratti dal database e con questi sono stati generati *cut-off* per l'alta e bassa espressione. L'analisi eseguita ha mostrato che non vi sono differenze significative nei casi di tumore residuo in base al livello di espressione dei trasportatori CTR1 e OCT1, mentre la bassa espressione del canale VRAC e l'aumento dell'espressione di MRP1 influenzano in modo significativo la permanenza del tumore in seguito alla chemio-radioterapia (rispettivamente $p = 0,042$ e $p = 0,06$) (**Figura 5**).

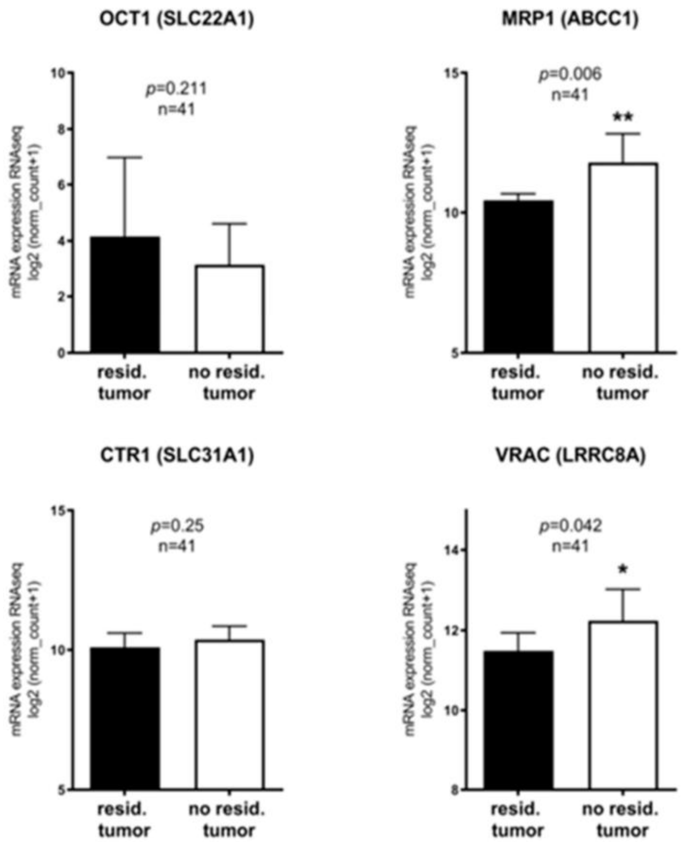

Figura 5: Relazione tra la permanenza residua del tumore e l'espressione dei trasportatori cellulari. Identificazione bioinformatica di potenziali trasportatori di farmaci clinicamente rilevanti. Si prevede che una bassa espressione dei canali di importazione dei farmaci, ovvero una ridotta captazione del cisplatino, favorisca la sopravvivenza delle cellule tumorali e quindi le recidive. Abbiamo riscontrato una tendenza inaspettata alla diminuzione dell'espressione di OCT1, mentre i livelli di espressione del trasportatore di assorbimento del farmaco CTR1 sono rimasti simili. Tuttavia, una maggiore espressione del trasportatore di esportazione dei farmaci MRP1 e una maggiore espressione del trasportatore di assorbimento dei farmaci VRAC sono significativamente correlate a una riduzione delle recidive tumorali.

La correlazione tra un'elevata espressione del trasportatore di esportazione dei farmaci MRP1 ed un miglioramento della terapia è meccanicamente difficile da comprendere e dimostrare. Al contrario, la correlazione osservata tra il successo della terapia con cisplatino e l'alta espressione di VRAC/LRRC8A, che abbassa le concentrazioni intracellulari di cisplatino e quindi promuove la sopravvivenza delle cellule tumorali, è sembrata più rilevante e adatta a ulteriori indagini sperimentali.

3.2-Profilazione delle vie di sensibilità al cisplatino e rilevanza di VRAC come determinante critico per la resistenza al cisplatino

Per approfondire il ruolo di VRAC nella risposta al cisplatino e per verificare sperimentalmente l'ipotesi che esista una correlazione diretta tra la risposta alla terapia e l'espressione di VRAC, gli autori hanno creato un modello di coltura cellulare *in vitro*. È stato utilizzato il metodo CRISPR/Cas9 per generare specificamente un KO completo di LRRC8A nelle cellule HNSCC Pica, senza indurre ulteriori alterazioni genetiche. Le cellule LRRC8A-KO non sono in grado di produrre canali VRAC funzionali (Voss *et al*., 2014).

Per assicurare la massima omogeneità genetica gli autori hanno generato diversi cloni monocellulari dal pool LRRC8A-KO (PicaKO36) e dalla linea cellulare WT (PicaWT04). Le analisi genetiche a livello di gDNA e cDNA e l'analisi Western blot (**Figura 6**) hanno confermato l'assenza di espressione della proteina LRRC8A nella linea cellulare PicaKO36.

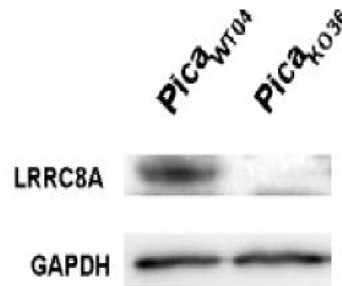

Figura 6: Risultati Western Blot. Western blot per confermare l'assenza di espressione della proteina LRRC8A nelle cellule PicaKO36. L'antibiotico contro la proteina GAPDH viene usato per confermare il corretto funzionamento del Western Blot.

Macroscopicamente, le cellule generate non hanno mostrato differenze apparenti rispetto alla popolazione cellulare iniziale. Tuttavia, esaminando la risposta al cisplatino delle linee cellulari, gli autori hanno osservato che le cellule PicaKO36 erano significativamente più resistenti al cisplatino rispetto alle cellule PicaWT04 (**Figura 7**), confermando così un ruolo diretto dell'espressione di VRAC nella risposta al cisplatino.

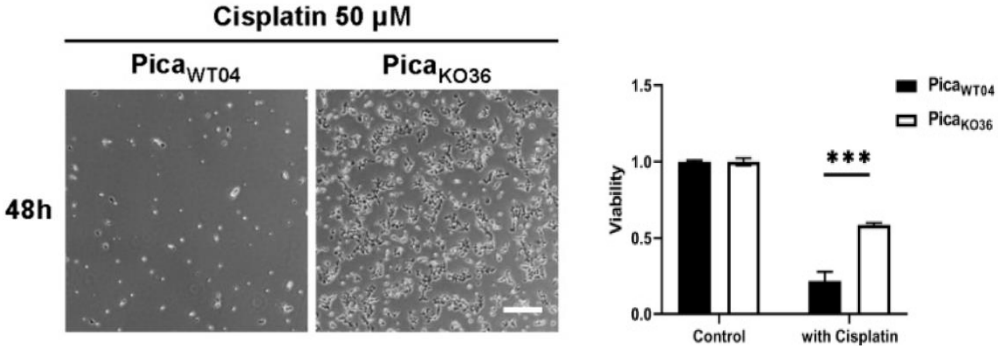

Figura 7: Resistenza differenziata al cisplatino. Il pannello di destra mostra come PicaKO36 sia in grado di sopravvivere al trattamento con alte concentrazioni di cisplatino. Il pannello di sinistra mostra come la linea PicaKO36 sia significativamente resistente al cisplatino.

Per identificare ulteriori *network* di resistenza potenziale, gli autori del lavoro hanno eseguito anche un'analisi trascrittomica (RNA-Seq) di nuova generazione su tre campioni per linea cellulare. La successiva analisi bioinformatica dei geni differenzialmente espressi ha rivelato un elenco di candidati con un potenziale impatto sulla resistenza al cisplatino (**Figura 8**). Tra questi fattori, TP53 risulta essere *down*-regolato nelle cellule KO. Il gene *TP53* codifica una proteina soppressore del tumore contenente domini di attivazione trascrizionale, di legame al DNA e di oligomerizzazione. Fino all'80% di tutti i tumori presenta aberrazioni

dell'espressione di p53, il che la rende una delle proteine più decisive per lo sviluppo del cancro. Contemporaneamente, hanno osservato differenze nei livelli di espressione di SOX2, un fattore di trascrizione oncogeno, coinvolto nello sviluppo di carcinomi a cellule squamose. È importante notare che altri potenziali candidati canali di trasporto del cisplatino, come CTR1, OCT1 o MRP1, non sono stati né *up*-regolati né *down*-regolati (**Figura 8**), sottolineando ulteriormente la rilevanza di VRAC per la resistenza ai farmaci.

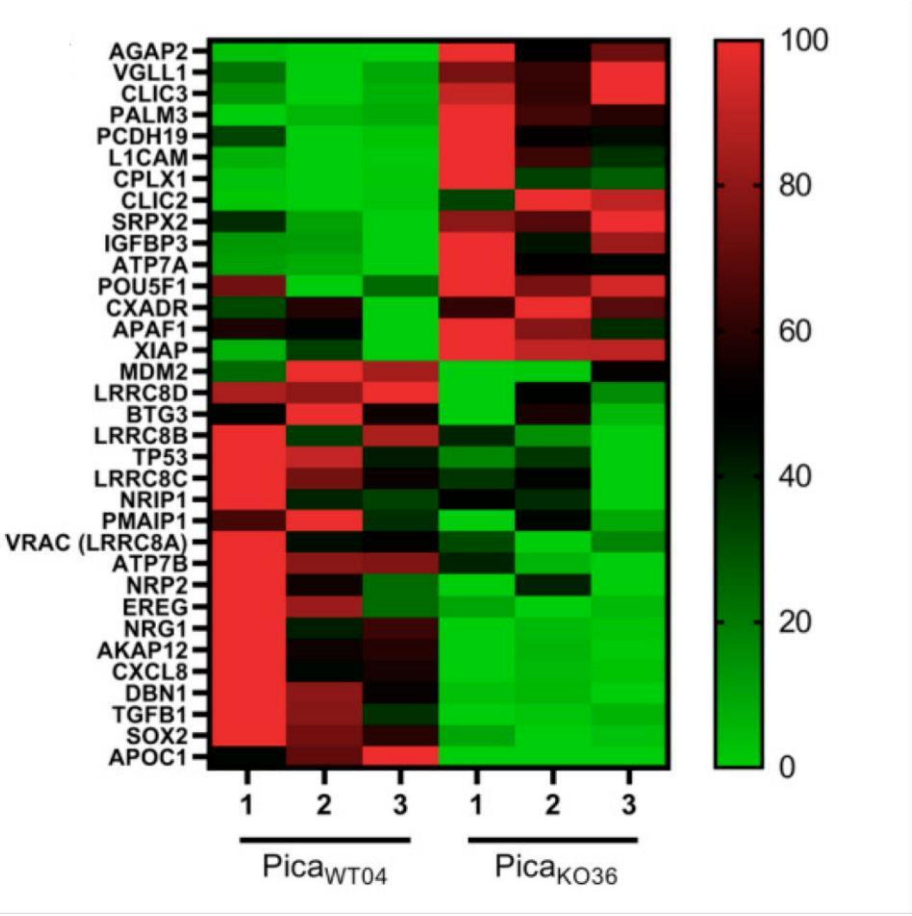

Figura 8: Possibili geni che influiscono sulla resistenza al cisplatino. L' *Heatmap* mostra i livelli di espressione dei geni potenzialmente associati alla resistenza al cisplatino, i quali sono espressi in maniera diversa nelle cellule PicaKO36 rispetto che nelle cellule PicaWT04 (verde: *down*-regolati, rosso: *up*-regolati).

3.3- L'espressione di VRAC nelle cellule tumorali HNSCC è fondamentale per la sensibilità e la specificità dei farmaci al platino

3.3.1-Analisi *in vitro*

Gli autori, dopo aver dimostrato come VRAC sia effettivamente un forte candidato per il trasporto e la resistenza al cisplatino, hanno ulteriormente analizzato le conseguenze del LRRC8A-KO in relazione alla tossicità del cisplatino. Le cellule PicaKO36 sono state trattate con il cisplatino in dosi sempre più alte e ciò ha rivelato che queste cellule presentavano un significativo aumento nella resistenza

al cisplatino anche per concentrazioni maggiori rispetto a quelle che vengono normalmente somministrate nelle terapie cliniche (**Figura 9a**).
Escludendo gli errori correlati ai metodi utilizzati, il numero di rotture del doppio filamento di DNA indotte per cellule risulta direttamente correlato alla concentrazione intracellulare di cisplatino. Nello specifico le cellule KO hanno mostrato un numero significativamente inferiore di danni al DNA rispetto alle corrispondenti cellule WT sia a livello di singola cellula che a livello complessivo (**Figura 9b**). Le cellule PicaKO36 hanno mostrato un numero inferiore di eventi di danno al DNA indotti dal cisplatino (valutati come foci di danno γH2AX) per cellula. In particolare, gli autori hanno sviluppato un protocollo che automaticamente quantifica i segnali di fluorescenza delle immagini al microscopio di singole cellule colorate utilizzando il metodo dell'immunofluorescenza sfruttando il marcatore di danno al DNA yH2AX. A tale scopo è stato utilizzato un microscopio di *screening* ad alto contenuto e anticorpi specifici per i foci γH2AX (**Figura 9c**).Gli autori hanno voluto indagare ulteriormente e quindi hanno deciso di rendere nuovamente sensibili al cisplatino le cellule PicaKO36 attraverso la trasfezione del plasmide di espressione di LRRRC8A permettendo loro di ricostituire la funzione del canale VRAC. Così facendo le cellule hanno riacquistato anche la sensibilità alla morte cellulare mediata dal cisplatino confermando ancora una volta e in maniera indipendente come l'azione di VRAC sia fondamentale per mediare la sensibilità al cisplatino.

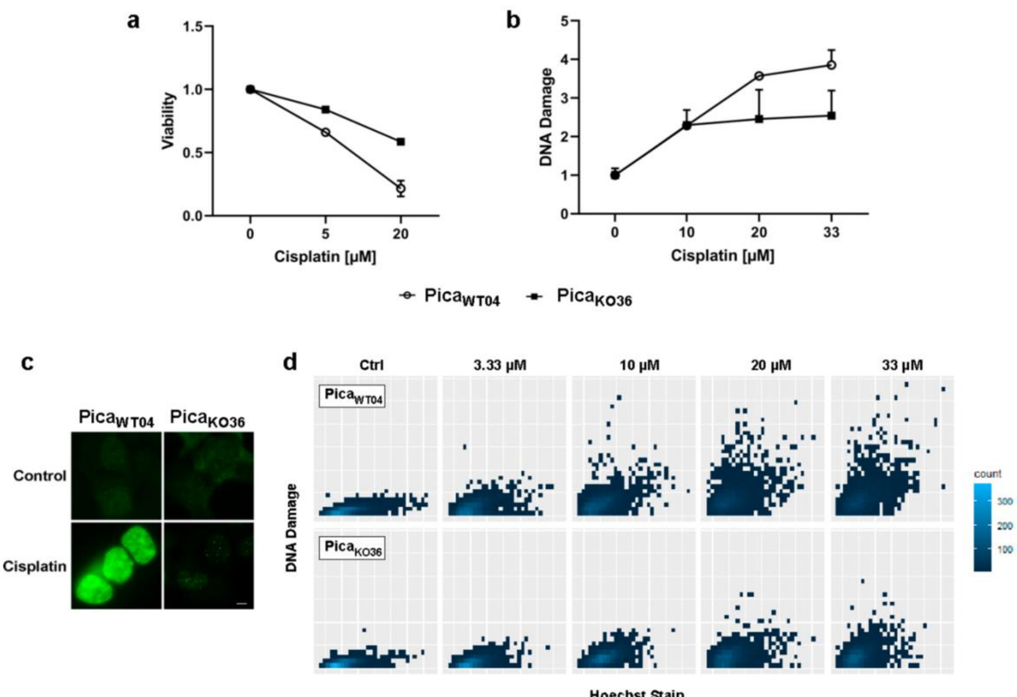

Figura 9: L'espressione di VRAC nelle cellule tumorali HNSCC è fondamentale per la sensibilità e la specificità dei farmaci al platino. Le cellule PicaKO36 sono resistenti in modo dose-dipendente al cisplatino (a). Le cellule PicaKO36 mostrano un numero inferiore di eventi di danno al DNA indotti dal cisplatino (valutati come foci di danno γH2AX) per cellula (b). Microscopia a fluorescenza per visualizzare il danno al DNA. Danno al DNA (foci γH2AX) colorato da specifici anticorpi fluorescenti (verde) (c). I foci γH2AX di una singola cellula sono stati

quantificati tramite il microscopio automatico ad alto contenuto di screening Array Scan VTI e tracciati tramite ggplot2/R (d).

Infine, gli autori hanno voluto dimostrare come la mancata espressione del canale VRAC conferisca alla linea cellulare PicaKO36 anche la resistenza al carboplatino, un chemioterapico alternativo sempre a base di platino (**Figura 10**). Allo stesso tempo hanno dimostrato come non esista una resistenza incrociata ai chemioterapici alternativi doxorubicina e paclitaxel (**Figura 10**). Tutto ciò certifica una rilevanza specifica per il cisplatino del meccanismo molecolare VRAC-dipendente identificato.

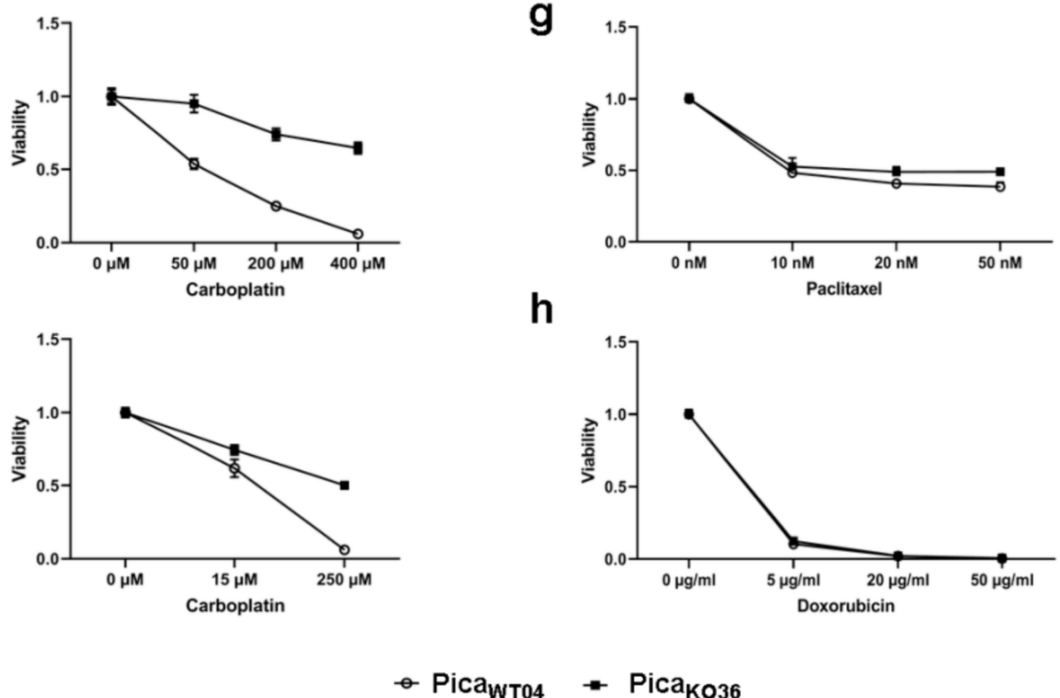

Figura 10: Resistenza al carboplatino. Le cellule PicaKO36 2D e gli sferoidi tumorali 3D con deficit di VRAC sono resistenti al trattamento con carboplatino. La carenza di VRAC influisce sulla resistenza al cisplatino ma non sulla risposta ad altri farmaci che danneggiano le cellule quali il paclitaxel o la doxorubicina.

3.3.2- Analisi *in vivo*

Per avvicinarsi maggiormente alla situazione tumorale in vivo, gli autori hanno sviluppato un modello di sferoidi 3D che imita la microarchitettura tumorale dei pazienti. Per confermare oggettivamente la crescita riproducibile degli sferoidi tumorali è stata sfruttata la microscopia automatizzata ad alto contenuto. La **Figura 11** illustra l'affidabilità del metodo e mostra sferoidi rappresentativi durante il periodo di coltura (**Figura 11b**). In particolare, la microscopia 3D a due fotoni è stata utilizzata per visualizzare la microarchitettura degli sferoidi e confermare l'espressione del marcatore della superficie epiteliale EpCAM (**Figura 11d**). È interessante notare che gli sferoidi con deficit di VRAC, PicaKO36, sono rimasti intatti anche dopo un trattamento prolungato con alte concentrazioni di cisplatino, mentre gli sferoidi WT si sono disassemblati nelle stesse condizioni di trattamento (**Figura 11e**).

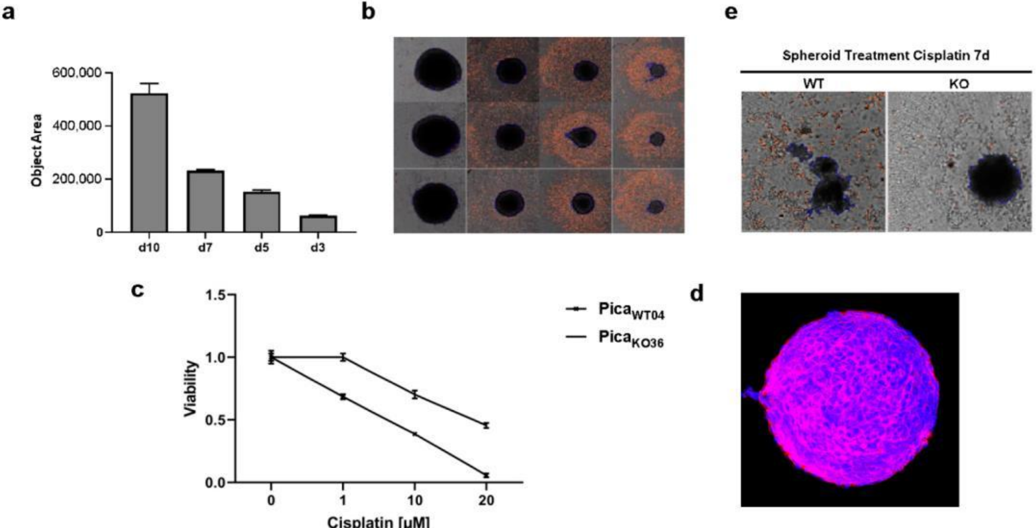

Figura 11: L'assenza di espressione di VRAC protegge gli sferoidi tumorali 3D dalla morte delle cellule tumorali indotta dal cisplatino. Microscopio ad alto contenuto Array Scan VTI usato per visualizzare la crescita degli sferoidi e determinare automaticamente le dimensioni medie degli sferoidi (a). Visualizzazione delle regioni di interesse (ROI) durante periodo di crescita (b). Grafico che mostra la rilevanza di VRAC nella resistenza al cisplatino anche in colture 3D. (c) Microarchitettura degli sferoidi visualizzata mediante microscopia a due fotoni a eccitazione profonda (2PE). EpCAM è stata rilevata con anticorpi fluorescenti specifici (rosso), mentre i nuclei sono stati colorati con colorante Hoechst (blu) (d). La microscopia dimostra come gli sferoidi PicaWT04 abbiano subito maggiormente gli effetti tossici del cisplatino rispetto agli sferoidi PicaKO36 (e).

3.4 - Il trasportatore VRAC svolge una funzione determinante nella resistenza al cisplatino nelle cellule tumorali naturali

In modo da verificare in maniera indipendente le intuizioni ottenute attraverso l'utilizzo dei modelli ingegnerizzati gli autori hanno stabilito una linea cellulare Pica che è naturalmente resistente al cisplatino (PicaC) trattandola con concentrazioni subtossiche di cisplatino (3-5 μM) per sei mesi. Gli autori hanno atteso che le cellule recuperassero il loro normale metabolismo e quindi una normale proliferazione cellulare in modo da garantire una popolazione cellulare stabile e omogenea prima di iniziare con gli esperimenti. Si è osservato come PicaC mostri livelli di resistenza simili a quelli della linea cellulare PicaKO36, sia nei saggi 2D che in quelli 3D, si può inoltre constatare anche con un numero ridotto di danni al DNA (**Figura 12**).

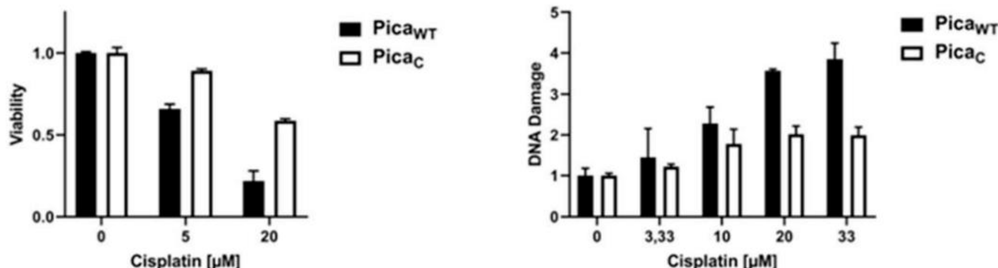

Figura 12: Cellule Pica naturalmente resistenti al cisplatino. Gli sferoidi PicaC trattati con il farmaco mostrano una maggiore vitalità rispetto agli sferoidi PicaWT trattati con la stessa dose di farmaco (a destra). Le cellule PicaC mostrano un numero inferiore di eventi di danno al DNA indotti dal cisplatino (foci γH2AX) per cellula rispetto alle cellule PicaWT, quantificati automaticamente mediante microscopia ad alta risoluzione (a sinistra).

I livelli di espressione di VRAC sono stati ridotti di circa il 25% rispetto ai livelli riscontrati normalmente nelle PicaWT04, sottolineando ancora una volta il ruolo centrale e la rilevanza generale di VRAC per la sensibilità alla chemioterapia con cisplatino nelle cellule HNSCC (**Figura 13**).

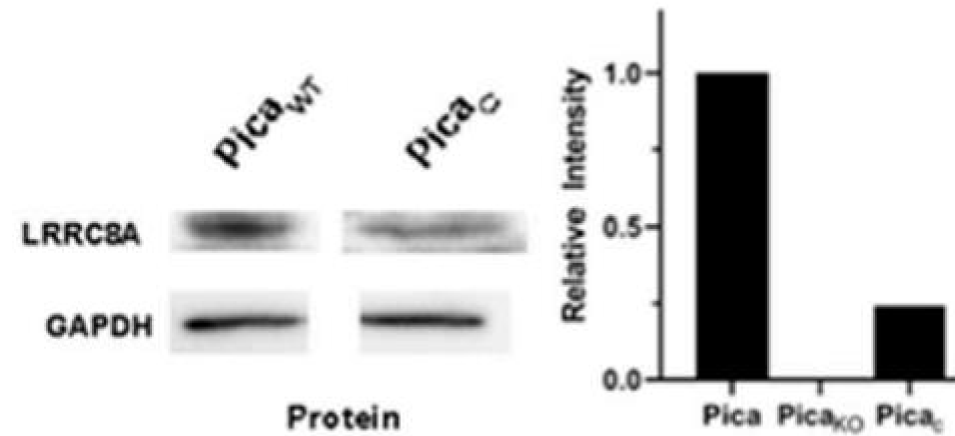

Figura 13: Riduzione espressione VRAC. L'analisi immunoblot conferma la *downregulation* dei livelli proteici di VRAC nelle cellule PicaC. L'anticorpo contro la proteina GAPDH è stato usato come controllo di caricamento del western blot.

3.5 - Conclusioni

I tumori della testa e del collo (HNSCC) vengono solitamente trattati con terapia a base di cisplatino, spesso però il successo del trattamento è ostacolato dalle recidive del tumore dovute alla resistenza alla terapia. Gli autori dell'articolo scelto si sono impegnati ad indagare i potenziali attori molecolari coinvolti in modo critico nella resistenza al cisplatino. Hanno seguito un processo a più tappe partendo dalla formulazione di ipotesi basate su analisi bioinformatiche, fino alla conferma delle ipotesi *in vitro* e poi *in vivo*. Hanno concluso come il canale anionico VRAC è fondamentale per la resistenza al cisplatino nelle cellule del tumore HNSCC. Gli autori sono giunti a questa conclusione basandosi sui risultati ottenuti grazie agli esperimenti e le analisi svolte. Hanno confermato la rilevanza di VRAC per la resistenza e la specificità del cisplatino grazie a cellule *knockout* CRISPR/Cas9 per

LRRC8A, subunità principale di VRACC, le quali dimostrano una maggiore resistenza al cisplatino. Per confermare ulteriormente questo dato hanno ristabilito la sensibilità delle cellule al cisplatino. Grazie a cellule tumorali naturalmente resistenti al cisplatino hanno dimostrato che una bassa espressione di VRAC è correlata alla resistenza al cisplatino. Nel complesso gli autori propongono VRAC come un promettente biomarcatore per la resistenza al cisplatino nell'HNSCC e suggeriscono come possa costituire la base per studi clinici futuri per la prevenzione e cura del cancro.

3.6 - Analisi critica

Dal mio punto di vista, l'articolo oggetto di questo elaborato presenta alcuni limiti. Gli autori si sono basati sul lavoro di Voss *et al.* del 2014 nel quale si dimostra che la proteina LRRC8A e i suoi quattro omologhi (LRRC8B-E) formano canali VRAC eteromerici (composti da subunità non uguali). Nello stesso studio si è osservato come la formazione di diversi eteromeri LRRC8 indica che esistono diversi VRAC, che possono variare nelle proprietà e nella distribuzione tissutale. Ad esempio, la composizione delle subunità degli eteromeri LRRC8 potrebbe specificare la preferenza del canale per il cloruro rispetto alla taurina o viceversa (Voss *et al.*, 2014). Basandosi su queste evidenze hanno deciso di svolgere un KO sulla subunità LRRC8A portando allo sviluppo di cellule in cui il canale VRAC non si costituisce. Però come si è visto da uno *screening* genomico imparziale (Planells-Cases *et al.*, 2015) la perdita di una delle due subunità VRAC, LRRC8A o LRRC8D, aumenta la resistenza al carboplatino e al cisplatino. Il trasporto dei farmaci richiede come abbiamo già detto la subunità obbligatoria del canale LRRC8A però dipende anche da LRRC8D, una subunità che aumenta fortemente la permeabilità di VRAC al cisplatino/carboplatino. Inoltre, LRRC8D aumenta la permeabilità di VRAC alla taurina e le cellule prive di questa subunità hanno mostrato una ridotta regolazione del volume cellulare (Planells-Cases *et al.*, 2015.) Tenendo in considerazione tutto questo gli autori avrebbero potuto svolgere un KO su un'altra tra le subunità LRRC8 (B,C,E) per vedere se anche queste influenzano in qualche modo il trasporto e la resistenza al cisplatino e capire se la loro *up-* o *down-* regolazione possa influire sulla cura del cancro.
Un ulteriore aspetto poco chiaro è la rappresentazione dei dati nei grafici e nelle figure. Ad esempio, nella figura 10 non è ben chiaro nel grafico il confronto tra la vitalità delle cellule PicaWT04 e le cellule PicaKO36 in seguito al trattamento con doxorubicina. Mentre nella figura 13 il grafico non mostra in maniera chiara l'espressione relativa del VRAC nelle tre linee cellulari diverse.
Nonostante le criticità sopra esposte l'articolo risulta molto chiaro e di facile comprensione anche dal punto di vista linguistico. Si riesce a seguire il processo degli autori e le motivazioni che li hanno spinti a svolgere gli esperimenti e le analisi che hanno svolto. Le conclusioni a cui sono giunti sono coerenti e propongono molti spunti per proseguire la ricerca riguardo il cancro e le sue possibili terapie.

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APPENDICE

Si allega a seguire il lavoro oggetto di questo elaborato.

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Article

Profiling Cisplatin Resistance in Head and Neck Cancer: A Critical Role of the VRAC Ion Channel for Chemoresistance

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Simple Summary: Treatment success of head and neck cancers (HNSCC) is often hindered by chemoresistance. In this study, next-generation sequencing transcriptomics and CRISPR/Cas9 knockout strategies were used to identify cisplatin resistance mechanisms and potential (personalized) biomarkers. Moreover, employing a tiered experimental pipeline, the cisplatin uptake transporter VRAC was found to be critical for cisplatin sensitivity and specificity in 2D/3D-HNSCC cell culture models as well as for tumor relapses in the clinical setting. Our study suggests exploiting VRAC as a potential drug target as well as a personalized prognostic biomarker to improve the treatment of HNSCC patients in the future.

Abstract: Treatment success of head and neck cancers (HNSCC) is often hindered by tumor relapses due to therapy resistances. This study aimed at profiling cisplatin resistance mechanisms and identifying biomarkers potentially suitable as drug targets and for patient stratification. Bioinformatic analyses of suggested resistance factors in a cohort of 565 HNSCC patients identified the VRAC ion channel as a clinically relevant indicator for recurrent diseases following radiochemotherapy ($p = 0.042$). Other drug import/export transporters, such as CTR1, OCT1, or MRP1, were found to be less relevant. To experimentally verify VRAC's critical role for cisplatin resistance, we used CRISPR/Cas9 knockout resulting in cisplatin-resistant HNSCC cells, which could be resensitized by VRAC expression. Next-generation sequencing further underlined VRAC's importance and identified VRAC-regulated signaling networks, potentially also contributing to cisplatin resistance. CTR1, OCT1, or MRP1 did not contribute to increased cisplatin resistance. In addition to two-dimensional HNSCC models, three-dimensional tumor spheroid cultures confirmed VRAC's unique role for cisplatin sensitivity. Here, resistance correlated with DNA damage and downstream apoptosis. The cisplatin specificity of the identified VRAC pathway was verified by testing paclitaxel and doxorubicin. Our results were independently confirmed in naturally occurring, cisplatin-resistant HNSCC cancer cell models. Collectively, we here demonstrate VRAC's role for cisplatin resistance in HNSCC and its relevance as a potential drug target and/or prognostic biomarker for chemotherapy resistance.

Keywords: chemotherapy resistance; HNSCC; tumor therapy; drug transporter; personalized medicine

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1. Introduction

Cancers of the head and neck range among the ten most frequent malignant diseases, and head and neck squamous cell carcinomas (HNSCCs) account for roughly 90% among

them. Despite extensive and radical primary treatment options, the median 5-year overall survival averages around 65% with a range of 30–85%, depending on tumor stage. HNSCC treatment options with intention to cure mainly include either surgical removal with or without adjuvant (chemo)radiation or radiotherapy with concurrent chemotherapy. There is increasing evidence indicating that the expression of molecular markers, primarily p16 as a surrogate for human papilloma virus (HPV)-associated carcinogenesis, could be decisive when choosing the right first-line treatment regimen [1]. However, highly heterogeneous cancer cell populations [2] in HNSCC did prevent extensive efforts to establish other predictive markers on the road to personalized therapy.

First-line chemotherapy of HNSCCs is predominantly platinum-based with cisplatin being the primary option. While recently, the field could see promising results by using α-PD-1 checkpoint inhibitor pembrolizumab [3] as platinum alternative in a subset of cases, platinum remains the most effective and economic therapy for the majority of HNSCC patients. On the other hand, therapy resistance and associated subsequent near or distant relapses are still common and are associated with high patient morbidity and a median survival of only 10 months [4,5]. The development of resistance mechanisms toward (platinum-based) chemotherapeutics is clearly a complex multilevel process, potentially including alterations in drug import and export, apoptosis initiation/inhibition, improved drug detoxification, as well as DNA repair [6–10] (Figure 1a). The impact of pre-existing resistance factors due to high mutational loads of HNSCCs compared to dynamic adaption on transcriptome level remains unclear. Thus, resistance to chemotherapeutics is manifold, complex, and not yet fully understood. Previous studies aimed to identify gene signatures for HNSCC, allowing predicting patients' therapy responses, pathobiology, and survival. However, the suggested signatures still failed to be used in the clinical routine to guide (targeted therapy) treatments for all patients [11,12]. Alongside methodological complications, the fact that HNSCC is characterized by highly heterogeneous tumors and anatomical localizations makes the selection of relevant patient subpopulations difficult and prevents rapid progress. Investigating crucial cellular components involved in the transport of platinum-based drugs could lead to further understanding of platinum resistance here. Transporters facilitating active influx and efflux of platinum drugs seem to be especially promising candidates in this context, as they have a direct impact on final intracellular drug concentrations (Figure 1a). A variety of drug transporters such as CTR1, OCT1, VRAC, and MRP1 were suggested to be involved in the transport and resistance of platinum-based drugs, with passive trans-membrane diffusion playing only a subordinate role. Particularly, the pioneering work by the Jentsch group not only demonstrated VRACS's relevance for cisplatin resistance but also showed that the subunit composition of VRAC plays additional roles for drug uptake properties [13–19]. Taking into consideration these complexities, we argued that it is necessary to employ a tiered experimental pipeline from in silico to analytical and in vitro to understand and potentially overcome cisplatin resistance in HNSCC.

In our study, we first used TCGA data analyses of genomic and transcriptomic alterations in drug transport channels to correlate their relevance with clinical phenotypes. Subsequently, we experimentally identified the ion channel VRAC as a critical component for cisplatin-specific uptake and resistance and confirmed its clinical relevance. Our results strongly suggest exploiting VRAC as a potential drug target as well as a personalized prognostic biomarker to improve the treatment of HNSCC patients in the future.

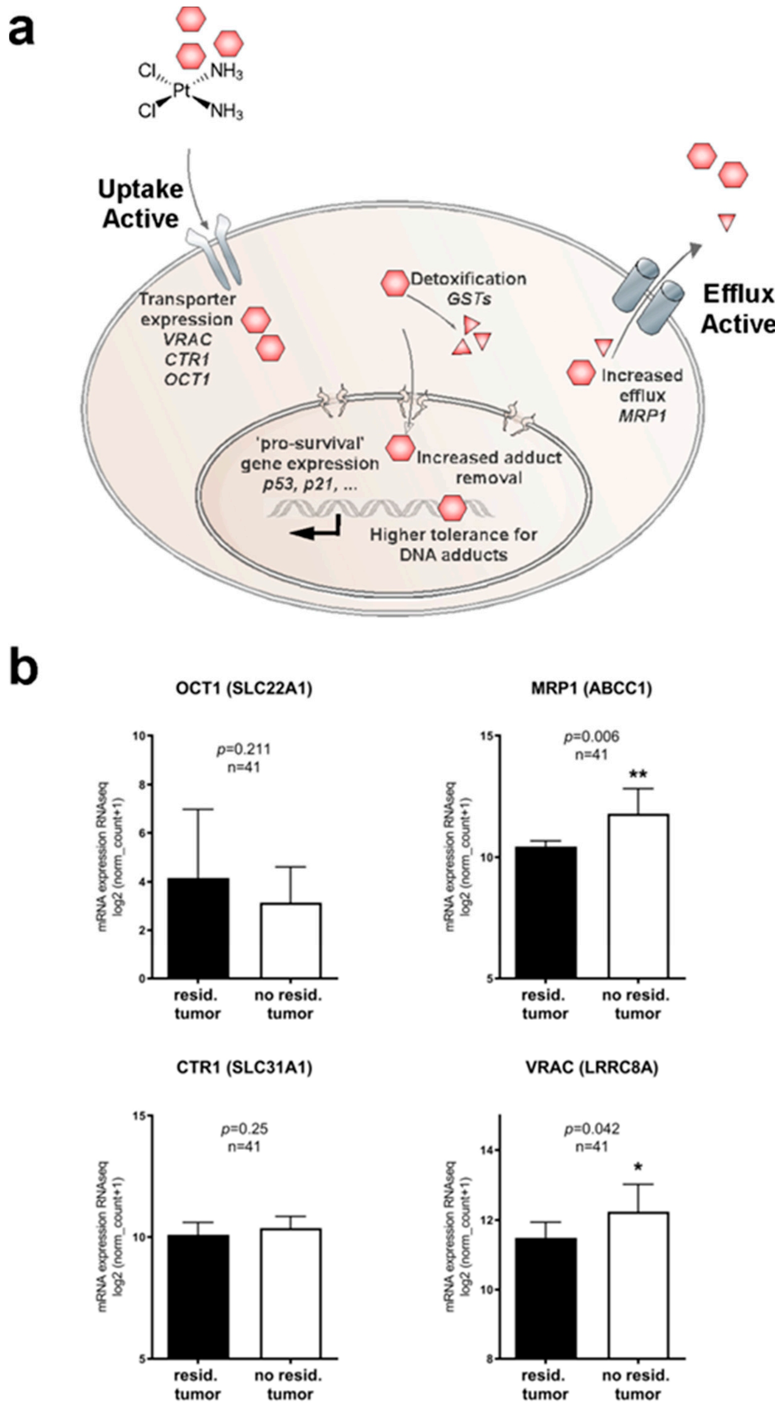

Figure 1. Molecular pathways and potential clinically relevant players contributing to cisplatin resistance in HNSCC. (**a**) Cartoon depicting mechanisms potentially involved in cisplatin resistance. Reduced intracellular drug concentrations can be a consequence of reduced uptake, accelerated efflux, or intracellular detoxification. Additionally, improved DNA repair and various (indirect) pro-survival pathways may improve cancer cells' ability to cope with cisplatin toxicity. (**b**) Bioinformatic identification of potential clinically relevant drug transporters in the transcriptomics dataset of HNSCC patients from *The Cancer Genome Atlas* (TCGA) ($n = 565$). Correlation of drug transporter transcription levels with residual tumors after first-line chemoradiotherapy and full clinical documentation ($n = 41$) were assessed. Low expression of drug import channels, i.e., reduced uptake of cisplatin, is expected to favor cancer cell survival and thus, tumor recurrences. We found an unexpected trend of decreased expression of OCT1, and expression levels of the drug uptake transporter CTR1 remain similar. However, enhanced expression of the drug export transporter MRP1 and increased expression of the drug uptake transporter VRAC significantly correlated with lower tumor recurrences. *, $p < 0.05$; **, $p < 0.01$.

2. Results and Discussion

2.1. Identifying Clinically Relevant Players of Cisplatin Resistance

Cellular uptake and efflux processes of chemotherapeutics are certainly decisive for intracellular drug concentrations and ultimately cancer treatment success or failure (Figure 1a). Therefore, we analyzed the expression levels of transport channels suggested to be involved in cisplatin transport in the publicly available TCGA HNSCC dataset, comprising 565 cancer patients of various disease states and clinical backgrounds. Gene expression data of such candidates, e.g., CTR1 (gene *SLC31A1*), VRAC (gene *LRRC8A*), OCT1 (gene *SLC22A1*), or MRP1 (gene *ABCC1*) were extracted from the database and cut-offs for high and low expression were generated. To identify cisplatin therapy-specific relevance, we first focused on patients that underwent cisplatin-based chemoradiation as a first-line therapy. Limited by the availability of exact information regarding clinical treatments and remission status information, we were able to identify 41 cases. While there were no significant differences in residual tumor cases depending on expression level for transporters CTR1 and OCT1, low expression of the drug import channel VRAC and increased expression of the drug export transporter MRP1 significantly correlated with residual disease after chemoradiation in curative intent ($p = 0.042$ and $p = 0.06$ respectively; Figure 1b). However, the correlation of high expression of the drug efflux transporter MRP1 with improved chemoradiation is mechanistically hard to understand. In contrast, the observed correlation of cisplatin therapy success with high VRAC/LRRC8A expression, lowering intracellular cisplatin concentrations and thus promoting cancer cell survival, seems more relevant and suitable for further experimental investigations. Notably, when we analyzed overall survival for all patients irrespective of treatments, these trends were lost, showing various correlations of expression levels with disease prognosis (Figure S1). As VRAC is suspected to additionally support tumor cell survival pathways next to drug uptake, such heterogeneity can be expected in the absence of cisplatin selection pressure.

2.2. Profiling Cisplatin Sensitivity Pathways and Relevance of VRAC as a Critical Determinant for Cisplatin Resistance

Clearly, bioinformatic results are helpful for hypothesis building but need to be carefully confirmed experimentally employing HNSCC models and molecular mechanistic approaches. Thus, to further investigate VRAC's unique role for cisplatin response on the cellular level and to test our hypothesis of a direct correlation of therapy response with VRAC expression, we next established an in vitro cell culture model. Here, CRISPR/Cas9 was used to specifically generate a complete LRRC8A knockout (KO) in HNSCC Pica cells, without inducing additional genetic alterations. LRRC8A is the only constituting subunit of the heteromeric transporter VRAC, leaving LRRC8A-KO cells unable to build functional VRAC channels [15,20] (Figure 2a). The Pica cell line was generated from a laryngeal squamous cell carcinoma with a novel protocol developed to mimic the tumor more precisely [21]. For maximal comparability and genetic homogeneity, different single cell clones were then generated from the LRRC8A-KO pool ($\text{Pica}_{\text{KO36}}$) as well as from the wild-type (WT) cell line ($\text{Pica}_{\text{WT04}}$). Genetic analysis on gDNA as well as cDNA level as well as Western blot analysis confirmed successful homozygous gene KO and absence of LRRC8A protein expression in cell line $\text{Pica}_{\text{KO36}}$ (cells did not show an apparent difference to the initial cell population (Figure 2b,e). Probing cisplatin response of the cell lines, $\text{Pica}_{\text{KO36}}$ cells could be shown to be significantly more resistant than WT cells, thus confirming a direct role of VRAC expression for cisplatin Figure 2c–d, Figure S3). Additionally, sequencing verified successful silencing of the respective genetic locus due to an insertion of a frameshift after amino acid 10, resulting in an additional stop codon after amino acid 66 of the LRRC8A protein (Figure S4, Tables S2 and S3). Macroscopically, the generated cells did not show an apparent difference to the initial cell population (Figure 2c). Probing cisplatin response of the cell lines, $\text{Pica}_{\text{KO36}}$ cells could be shown to be significantly more resistant than WT cells, thus confirming a direct role of VRAC expression for cisplatin response (Figure 2d).

In order to further identify additional potential resistance networks, we also performed next-generation RNA sequencing transcriptomics on three samples per cell line. Subsequent bioinformatic analysis of differentially expressed genes revealed a list of candidates with potential impact on cisplatin resistance (Figure 2f, for raw data of significantly differentially expressed genes, see Table S4). Among these factors *TP53*, coding for the tumor suppressor protein p53 was downregulated in the knockout cells. Up to 80% of all cancers show aberrations of p53 expression, making it one of the most decisive proteins for cancer development. However, bearing in mind its multiple functions for DNA damage repair and correlation with tumor disease prognosis across several indications [22–24], this observation suggests a rather indirect mediator role of *TP53*. Likewise, we found differences in SOX2 expression levels. SOX2 has been reported also as an oncogenic transcription factor that is involved in the development of squamous cell carcinomas, and its increased expression was reported to correlate with drug resistance against tamoxifen in breast cancers [25–27]. Critically, changes in transcription factor expression as exemplified by these two candidates are able to further impact different downstream pathways and networks potentially resulting in vast changes of the cellular machinery. In contrast, growth factor receptors, e.g., EGFR, survival proteins such as survivin [28,29] or protease networks, e.g., the HNSCC-relevant protease taspase1 [30,31], were not affected (see Table S4). Importantly, other potential cisplatin transport channel candidates such as CTR1, OCT1, or MRP1 were neither up- or downregulated (Figure 2g), further underlining VRAC's relevance for drug resistance.

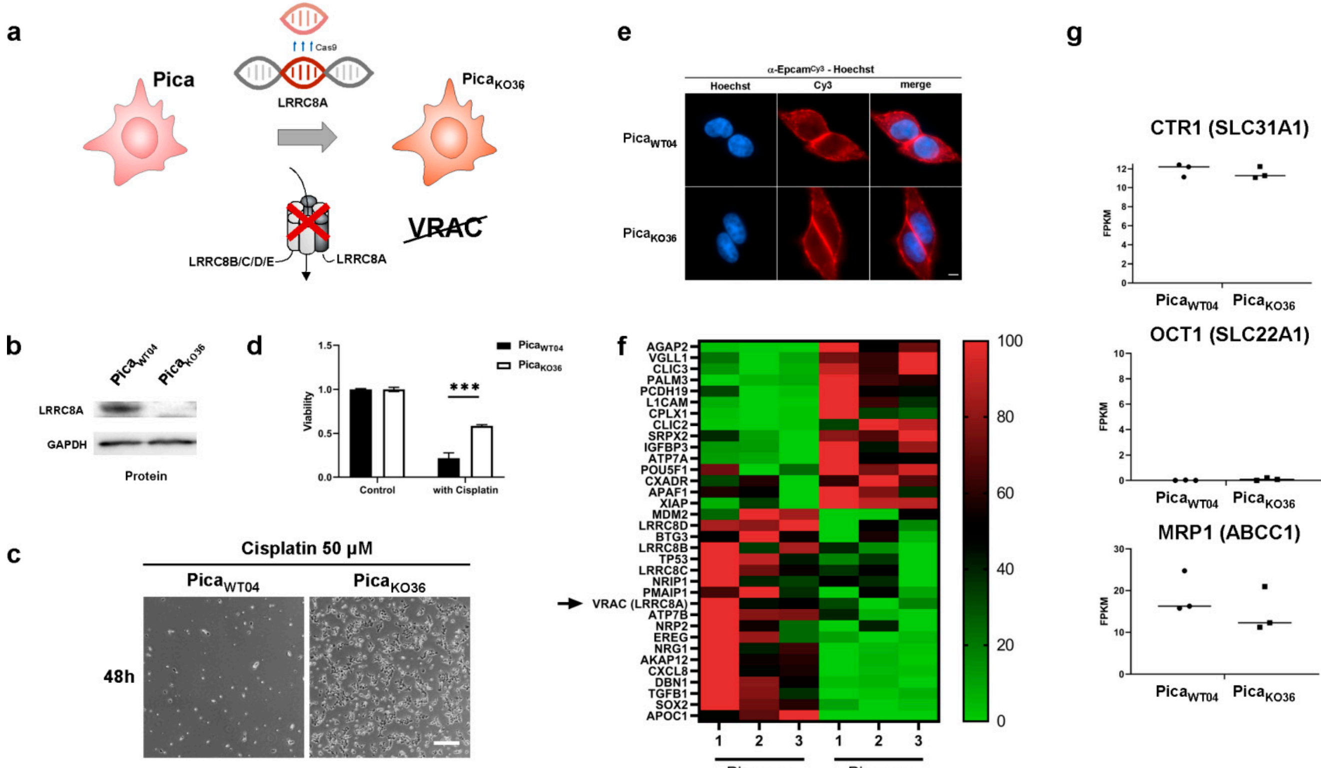

Figure 2. Profiling cisplatin sensitivity pathways underlines the relevance of VRAC as critical for cisplatin resistance. (**a**) Illustration of CRISPR/Cas9 technology to establish the VRAC-deficient, cisplatin-resistant, knockout cell line ($Pica_{KO36}$). Scheme of the VRAC channel, consisting of six heteromeric subunits, indicated (**b**) Western blot to confirm the absence of LRRC8A protein expression in $Pica_{KO36}$ cells. GAPDH served as loading control. (**c**) In contrast to WT cells, the VRAC-deficient cell line $Pica_{KO36}$ is able to survive treatment with high cisplatin concentrations. Cells were treated with cisplatin (50 µM) for 48 h and imaged by live cell microscopy. Scale bar, 200 µm. (**d**) In contrast to WT cells, the VRAC-deficient cell line, $Pica_{KO36}$, is significantly cisplatin resistant. Cells were exposed to cisplatin (20 µM) for 48 h and viability was normalized to untreated controls. ***, $p < 0.005$ (**e**) $Pica_{WT04}$ and $Pica_{KO36}$ cells show similar morphology. Fluorescence

microscopy visualized EpCAM expression (stained with specific antibodies (red)), nuclei were stained with Hoechst (blue). Scale bar, 5 μm. (**f**) Applying RNASeq transcriptomics to identify cisplatin resistance players. Heatmap visualizing expression levels of potential cisplatin resistance-associated genes, which are differentially expressed in KO ($Pica_{KO36}$) vs. cisplatin sensitive WT ($Pica_{WT04}$) cells (green: downregulated, red: upregulated; full list of differentially expressed genes in Table S4). Constituting subunit of VRAC (LRRC8A) indicated. (**g**) Expression levels of previously described cisplatin-resistance channel proteins (CTR1, OCT1, MRP1; see Figure 1b) are unaffected in the HNSCC knockout cell line and thus are less relevant for HNSCC. RNA intensities as FPKM values are displayed. For uncropped blots, refer to Supplementary Materials.

2.3. VRAC Expression in HNSCC Cancer Cells Is Key for Platinum Drug Sensitivity and Specificity

As a strong candidate for cisplatin uptake and resistance identified in our dataset, we further analyzed the consequence of LRRC8A-KO on cisplatin toxicity. Cells were treated and revealed a significantly increased resistance toward cisplatin beyond clinically applied concentrations (<20 μM vs. ≈2 μM) (Figure 3a). Excluding methodical artefacts, the amount of induced DNA double-strand breaks per cell were additionally quantified as a second readout directly correlated to intracellular cisplatin levels. To this means, a protocol enabling the automated quantification of fluorescence signals in microscopy images of single cells immunofluorescently stained for DNA damage marker γH2AX was established using high content screening microscope Array Scan VTI. As shown in Figure 3, the KO cells showed significantly less double-strand breaks when compared to the respective WT cells both on the single-cell level (Figure 3d) as well as overall (Figure 3b), excluding methodical artefacts and confirming cellular resistance. Fluorescent microscopy confirmed the absence of background staining (Figure 3c). Notably, the transfection of LRRC8A expression plasmid reconstituted VRAC channel function and significantly resensitized the resistant LRRC8A-knockout $Pica_{KO36}$ cells to cisplatin-mediated cell death (see Figure S5). These data provide independent strong evidence that mainly VRAC function is key for mediating cisplatin sensitivity.

Additionally, the absence of VRAC channel expression could be shown to confer resistance against carboplatin, which is an alternative platinum-based chemotherapeutic to cell line $Pica_{KO36}$ (Figure 3e,f). In contrast, no cross-resistance was detectable for alternative chemotherapeutics doxorubicin and paclitaxel (Figure 3g,h), demonstrating a cisplatin-specific relevance of the identified VRAC-dependent molecular mechanism.

In order to more closely approach the tumor situation in vivo, a 3D spheroid model and viability protocol was established. Cisplatin resistance was confirmed not only in 2D conventional cell cultures but also in 3D tumor spheroids, mimicking more closely the tumor microarchitecture in patients. Here, automated high content microscopy allowed to objectively confirm the reproducible growth of tumor spheroids. Figure 4a illustrates the reliability of our method (n = 8) and showed representative spheroids over the cultivation period (Figure 4b). Notably, two-photon 3D microscopy was used to visualize the spheroids' microarchitecture and confirm expression of the epithelial surface marker EpCAM (Figure 4d, Supplementary Video S1). The resistance of the VRAC-deficient cells against cisplatin as well as carboplatin is shown in Figures 3f and 4c. Interestingly, VRAC-deficient, $Pica_{KO36}$, spheroids stayed intact even after prolonged treatment with high concentrations of cisplatin, while the WT spheroids disassembled under the same treatment conditions (Figure 4e).

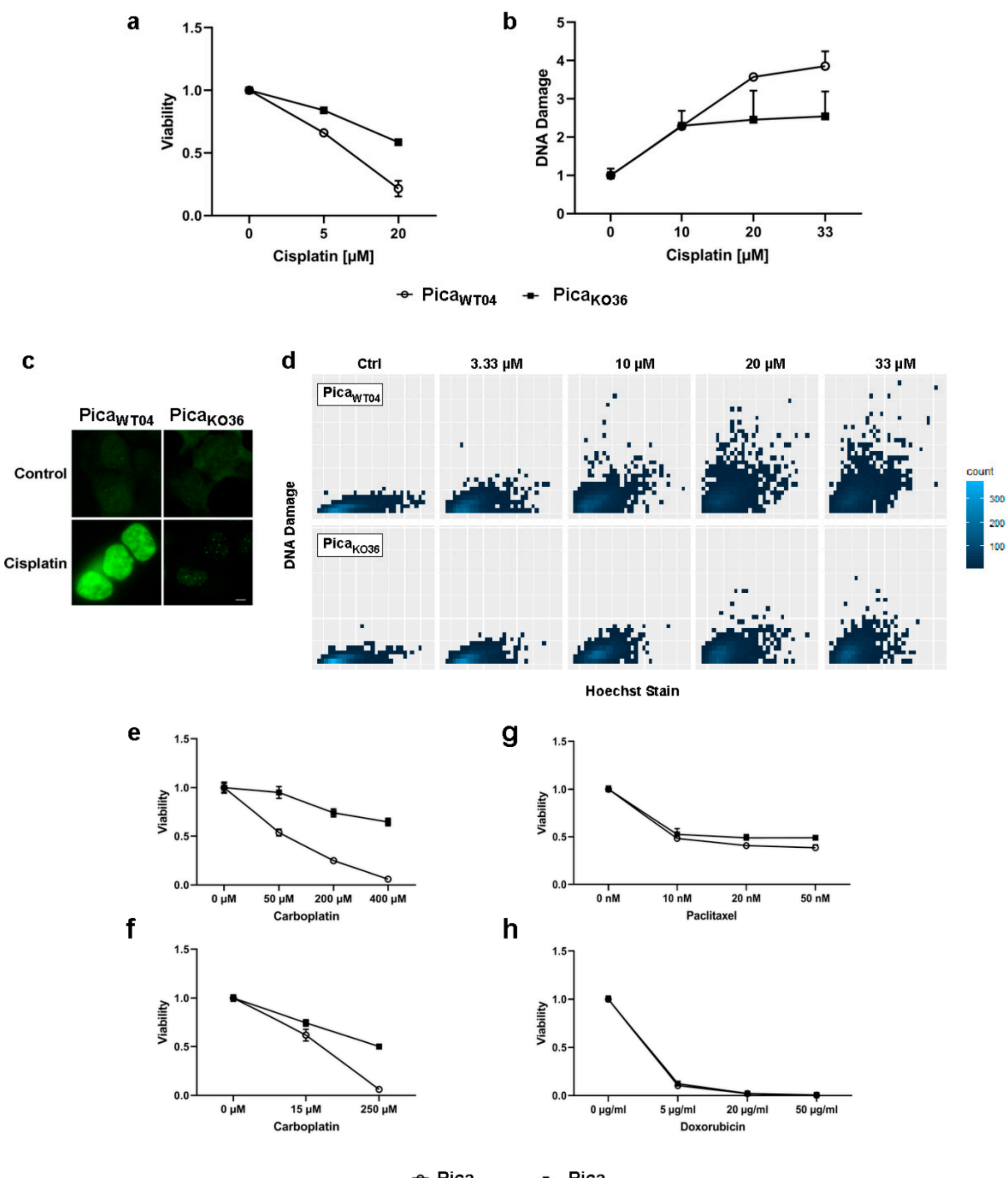

Figure 3. VRAC expression in HNSCC cancer cells is critical for platinum drug sensitivity and specificity. (**a**) VRAC-deficient $Pica_{KO36}$ cells are dose-dependent resistant against cisplatin. Cells were treated for 48 h, and viability was normalized to untreated controls. (**b**–**d**) Resistant VRAC-deficient cells show lower number of cisplatin-induced DNA damage events (assayed as γH_2AX damage foci) per cell. Cells were treated for 24 h and γH_2AX foci detected by specific antibodies. (**b**) DNA damage automatically quantified by high-throughput microscopy and normalized to untreated controls. (**c**) Fluorescence microscopy to visualize DNA damage. DNA damage (γH_2AX foci) stained by specific fluorescent antibodies (green). Scale bar, 5 µm. (**d**) Single-cell γH_2AX foci were quantified via automatic high content screening microscope Array Scan VTI and plotted via ggplot2/R [32]. (**e**,**f**), VRAC-deficient $Pica_{KO36}$ 2D cells (**e**) and 3D-tumor spheroids (**f**) are resistant against carboplatin treatment. Cells/spheroids were treated for 48/72 h and viability was normalized to untreated controls. (**g**,**h**) VRAC deficiency mediates cisplatin resistance but does not affect response to other cell-damaging drugs, such as paclitaxel or doxorubicin. Cells were treated for 48 h and viability was normalized to untreated controls.

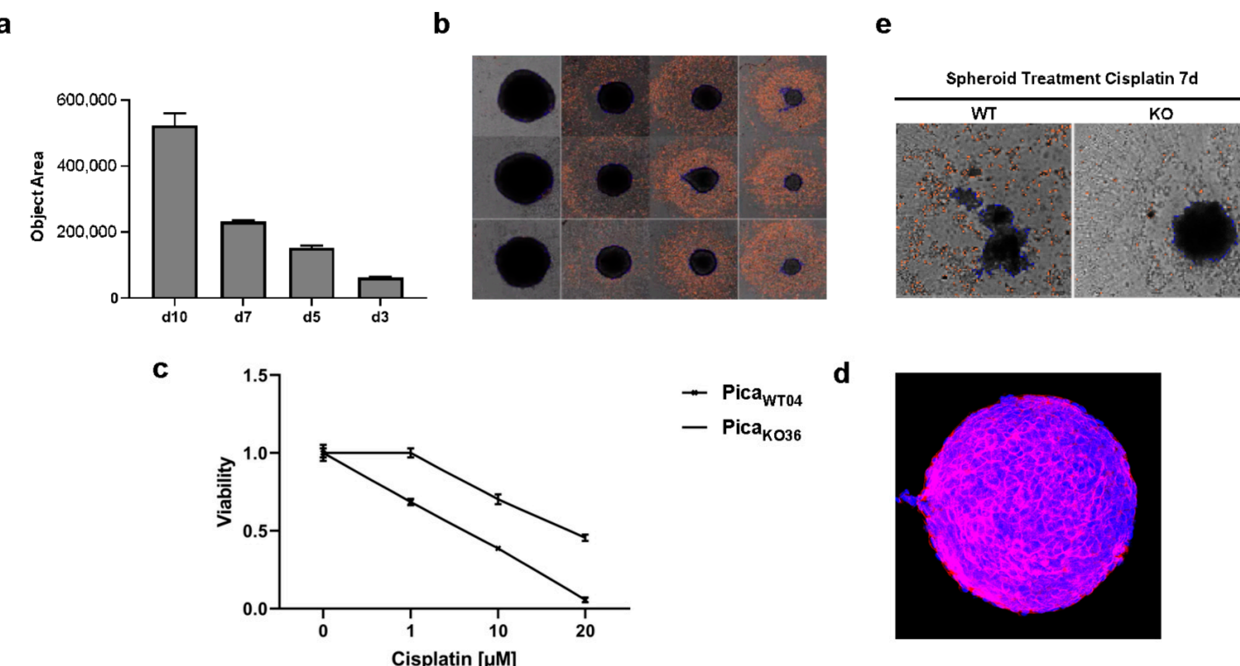

Figure 4. Absence of VRAC expression protects 3D tumor spheroids against cisplatin-induced cancer cell death. (**a**,**b**) Automated high content microscopy to visualize tumor spheroid growth. (**a**) Mean object sizes of spheroids (n = 8) automatically determined by high-content screening microscope Array Scan VTI. (**b**) Exemplary images of automatically detected regions of interest (ROI). Magnification, 5-fold. (**c**) Application of spheroids to confirm VRAC's relevance for cisplatin resistance even in tumor mimicking 3D cultures. Spheroids were treated for 72 h, and viability was normalized to untreated controls. (**d**) Microarchitecture of spheroids visualized by deep-tissue two photon excitation (2PE) microscopy. EpCAM was detected by specific fluorescent antibodies (red), nuclei were stained with Hoechst dye (blue). Magnification, 20-fold. (**e**) Microscopy demonstrates that $Pica_{WT04}$ (WT) spheroids were killed and disassemble under cisplatin treatment compared to $Pica_{KO36}$ (KO) spheroids. Magnification, 5-fold.

2.4. The Drug Uptake Transporter VRAC Is a Critical Determinant for Cisplatin Resistance in Naturally Occurring Cancer Cells

To independently verify our insights obtained in our engineered cell models, we additionally established a naturally occurring cisplatin-resistant Pica cell line ($Pica_C$) by treatment with subtoxic concentrations of cisplatin (3–5 µM) for six months. Experiments were conducted after regular proliferation was regained to ensure a homogenous, stable cell population (Figure 5a). Notably, $Pica_C$ showed similar levels of resistance compared to our VRAC KO cell line, both in 2D as well as 3D assays, also correlating with reduced numbers of DNA damage events (Figure 5b–d). VRAC expression levels were also reduced to about 25% compared to sensitive WT baseline levels, underlining again the central role and general relevance of VRAC for cisplatin chemotherapy sensitivity in HNSCC cells (Figure 5e).

Of note, the generation of cisplatin-resistant cell culture models is not trivial as expected for an effective anticancer drug, and we did not succeed in selecting cisplatin-resistant Hunkel or Deuser HNSCC cells, as used in previous studies by us and others [33].

However, we successfully established a cisplatin-resistant cell line, $Fadu_{CisR}$, by selecting HNSCC Fadu cells with subtoxic concentrations of cisplatin (3–5 µM) for six months (Figure S6a). Similar to the results obtained in the Pica cell models, immunoblot analysis confirmed decreased VRAC expression in the cisplatin-resistant $Fadu_{CisR}$ cells (Figure S6b), thereby confirming our results by an additional independent cell model.

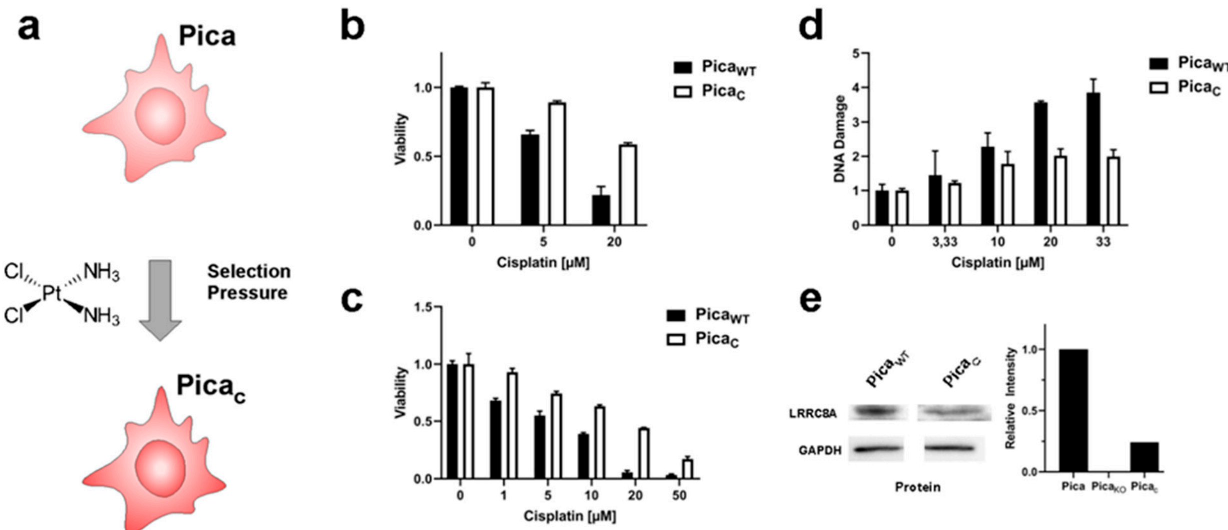

Figure 5. VRAC levels affect cisplatin sensitivity also in naturally occurring cisplatin-selected cells. (**a**) Scheme to illustrate the establishment of cisplatin-resistant Pica$_C$ cells by chronic exposure to cisplatin. (**b**,**c**) 2D/3D Pica$_C$ cell models to demonstrate cisplatin resistance. Cells (**b**) or spheroids (**c**) were drug treated (cells—48 h; spheroids—72 h), and viability was normalized to untreated controls. (**d**) Resistant Pica$_C$ cells show a lower number of cisplatin-induced DNA damage events (γH_2AX foci) per cell as automatically quantified by high-throughput microscopy. γH_2AX foci stained by specific fluorescent antibodies. (**e**) Immunoblot analysis confers downregulation of VRAC (LRRC8A) protein levels in resistant Pica$_C$ cells. GAPDH served as loading control. Data from one representative experiment of three independent experiments shown. For immunoblot analysis of Pica$_{KO36}$ cells, refer to Figure 2b. For uncropped blots, refer to Supplementary Materials.

3. Discussion

This study aimed at profiling cisplatin resistance mechanisms and identifying biomarkers potentially suitable as drug targets or patient stratification. The development of resistance mechanisms is clearly a complex process. We hypothesized that particularly transporters facilitating active influx or efflux of platinum drugs are the most relevant candidates, as they have a direct impact on the intracellular drug concentrations causing tumor cell death (see also Figure 1a). Several drug transporters such as CTR1, OCT1, VRAC, or MRP1 were suggested to be involved in resistance to various chemotherapeutics, although the underlying mechanisms are not fully understood [13,14,16–19,34]. In line with previous reports pioneered by the Jentsch group, we argue that our in silico and in vitro data justify our decision to focus on VRAC rather than other transporters, such as MRP1, as the most relevant factor for cisplatin resistance in HNSCC [13–15,20]. For one, to guide the selection of clinically most relevant candidates, we first used the TCGA HNSCC datasets to correlate drug transporter expression levels with resistances in cancer patients, focusing on patients that underwent cisplatin-based chemoradiation as a first-line therapy. While we found no significant differences in therapy response depending on the expression levels for the transporters CTR1 or OCT1, low expression of the drug import channel VRAC and increased expression of the drug export transporter MRP1 significantly correlated with residual disease (Figure 1b). However, as increased expression of the cisplatin efflux transporter MRP1 is expected to result in cancer cell resistance, and its correlation with improved clinical response to cisplatin treatment cannot be explained by the current knowledge of MRP1's pathobiological functions. One may speculate that additional, so far unknown functions of MRP1, independent of cisplatin efflux, may ultimately contribute to improved therapy response observed in patients. In contrast, low expression of the drug import channel VRAC is expected to reduced uptake of cisplatin and thus favor cancer cell survival and tumor recurrences, as we found in patients. Second, when we analyzed resistant vs. cisplatin-sensitive cell lines, the potential cisplatin transport channel candidates CTR1, OCT1, or MRP1 were neither up- nor downregulated (Figure 2f)

in contrast to the downregulation of VRAC further underlining VRAC's relevance for drug resistance in HNSCC. However, we agree that it might be interesting for the field to further explore so far unknown functions of MRP1 contributing to improved therapy response.

We could strongly confirm in vitro the clinical observation that the response to platinum treatment is dependent on VRAC-mediated drug uptake. We generated an LRRC8A-KO cell line lacking VRAC channel expression, which is significantly less susceptible to treatment with the platinum-based drugs cisplatin and carboplatin. Notably, rescued expression of LRRC8A and thus reconstitution of VRAC channel function significantly resensitized the resistant cells to cisplatin (Figure S5). These data provide independent strong evidence that mainly VRAC function is key for mediating cisplatin sensitivity.

Collectively, the mechanistic insights from our in vitro data help to understand our clinical observation that patients with low VRAC expression in their tumors develop resistances, respond less to chemotherapy, and thus, suffer from a higher residual tumor burden when compared to patients with high VRAC expression levels (Figure S2a,b).

Our findings could further be strengthened by appropriate animal models. However, transgenic LRRC8A knockout mice show multiple physiological impairments and defects [35] and thus seem not to be appropriate models to further investigate cisplatin resistance. Hence, we established and applied our 3D cancer spheroid model, mimicking not only more closely the tumor microarchitecture in patients but also following requests to reduce animal experiments as far as possible following the 3R principles (Replace, Reduce, Refine).

VRAC is a heteromer constituted of six subunits and can be of differing composition between the subunits LRRC8A/B/C/D and E [15,20], of which subunit LRRC8A is the only constituting member, and channels formed from subunits LRRC8A and LRRC8D are mainly involved in the transport of platinum drugs [14,15]. In addition to its involvement in platinum drug transport, VRAC is known to be crucial for active volume regulation of cells, which seems to also affect cell migration, cell cycle processes, and also each cell's highly regulated apoptosis cascade [13]. Therefore, genetic alterations affecting VRAC components may not only lead to decreased cisplatin uptake but also changes in apoptosis induction also in other tumor entities [13]. The dual role of cell volume regulation and active transport of platinum-based drugs may be important for the cancer cells to adapt to the tumor microenvironment and fine tune cell cycle, migration, and metastasis, particularly in the absence of the cisplatin drug selection pressure [13,36–38].

Analyzing the suggested molecular networks of potential interaction partners of LRRC8A, previously known to be involved in the development of cancer (Figure S7) and cisplatin resistance (Figure S8), the Ingenuity Pathway Analysis software indicates that all subunits LRRC8B-E are suggested to be associated with cancer. Interaction partners include different proteins in various cellular localizations, including nuclear proteins such as HDAC4. In addition to facilitating the deacetylation of histones and thereby regulating genetic transcription and epigenetic repression, histone deacetylase HDAC4 could be shown to promote cancer proliferation and invasion and to be associated with poor patient outcome in esophageal cancer [39,40]. LRRC8A networks of cisplatin resistance are suggested to also include NOTCH and SNAI1, which are two transcription factors with high cancer relevance. While NOTCH signaling is centrally involved in cell proliferation, differentiation, and survival [41,42], SNAI1 is involved in the induction of EMT processes, which are critical for the shedding of circulating tumor cells and metastasis [43,44]. In this context, an involvement of VRAC expression in the promotion of metastatic growth in the absence of cisplatin therapy was suggested [38,45].

VRAC expression is also discussed to be (in)directly involved in EMT processes [46]. When we examined marker proteins for both the epithelial as well as the mesenchymal phenotype, the KO cells tend to show slightly enhanced expression of molecular markers favoring an epithelial phenotype, such as EpCAM and reduced N-cadherin expression. Moreover, the KO cells are characterized by a reduced migratory potential (Figure S9) in wound assays and high VRAC expression levels seems associated with increased perineural

sheath infiltration in the TCGA HNSCC patient cohort ($p = 0.02$). Taken together, these results signal a potential role of high VRAC expression to promote the migratory potential of HNSCC tumor cells, leading to metastasis. However, although these pathways may influence overall patient survival, it is mechanistically not resolved how these signaling cascades and their individual players do directly contribute to cisplatin resistances, which may be considered a limitation of our study and of the field. Clearly, a still unresolved limitation of bioinformatical interaction partner prediction software is the need to confirm the results by independent experimental assessment. In addition, a limitation of most large 'omics' studies, including ours, is the fact that corresponding non-malignant tissue, primary tumor, as well as metastases are not always available from the same patient, and treatment information often is not available. For example, in the HNSCC cohort of *The Cancer Genome Atlas* (TCGA), 565 tumors and only 50 normal adjacent tissues (i.e., 10%) were analyzed, which may preclude the identification of unknown signaling pathways and/or biomarkers. However, there is agreement in the field that besides these limitations, the high number of samples analyzed seem to outweigh these potential limitations, and the TCGA data sets formed the basis for numerous follow-up studies. We are unaware of large data sets in which corresponding non-malignant tissue, primary tumor as well as metastases have been analyzed from the same patient for all included patients, which can be considered a potential general limitation for the whole field. Notably, not only VRAC's importance for cisplatin sensitivity seems to be masked when regarding solely overall survival profiles of very diverse patient collectives with various disease (progression) and therapy backgrounds. It is understood that these limitations apply to other previously suggested resistance proteins, such as MRP1. Only the selection of a relevant subset of data is able to reveal correlations of molecular profiles with clinical properties such as cisplatin sensitivity, as shown by our study. In addition, it would be interesting to further define the type of molecular, chemical, or physical signals (e.g., toxic agents in tobacco, HPV, DNA damage, treatments, signaling cascades, oncogenes, etc.) which cause VRAC downregulation. Concerning the relevance of the observed correlations for HNSCC, LRRC8A expression is particularly high compared to other cancer subtypes ($n = 565$, Figure S2d) and seems to also depend on the tumors' anatomical localization (Figure S2c). Interestingly, we found that VRAC expression was significantly lower in HPV-positive (p16-positive) tumors (Figure S10a). In addition, these patients seem to respond better to chemoradiation (Figure S10b) and showed a better overall survival (Figure S10c). However, so far, there are neither data nor hypotheses explaining how the HPV status could be linked to VRAC expression and tumor pathobiology. Hence, among various potential mechanisms, a cross-talk of p16 signaling and VRAC function or the impact of the VRAC transporter during HPV infection need to be investigated in detailed follow-up studies.

Aiming at increasing patient treatment success, personalized treatment approaches are currently considered one of the most promising strategies. However, bioinformatically harnessing the full potential of powerful data sets such as TCGA is only possible by a combination of clinical hypothesis formation, educated data analysis, and in vitro confirmation of the hypothesis, as exemplified here for VRAC channel expression in HNSCC. Prospectively, personalized treatment approaches will certainly benefit from improved understanding of such personal tumor predispositions, which might signal already early the need for alternative treatment strategies (Figure 6). Such strategies might involve other drugs in clinical use, such as paclitaxel or doxorubicin, which did not show any change in effectiveness upon differential VRAC expression in our study. Alternative strategies may include nano-formulated cisplatin, which is expected to enter tumor cells independent of the VRAC transporter to cause cancer cell death. As such formulations are currently already under (pre)clinical investigation, the findings of our study may aid in selecting clinical study populations based on VRAC expression [47–51]. However, despite the current enthusiasm on nanomedicals, their clinical applicability and superiority compared to 'standard' drug formulations needs to be based on a mechanistic understanding of their advantages.

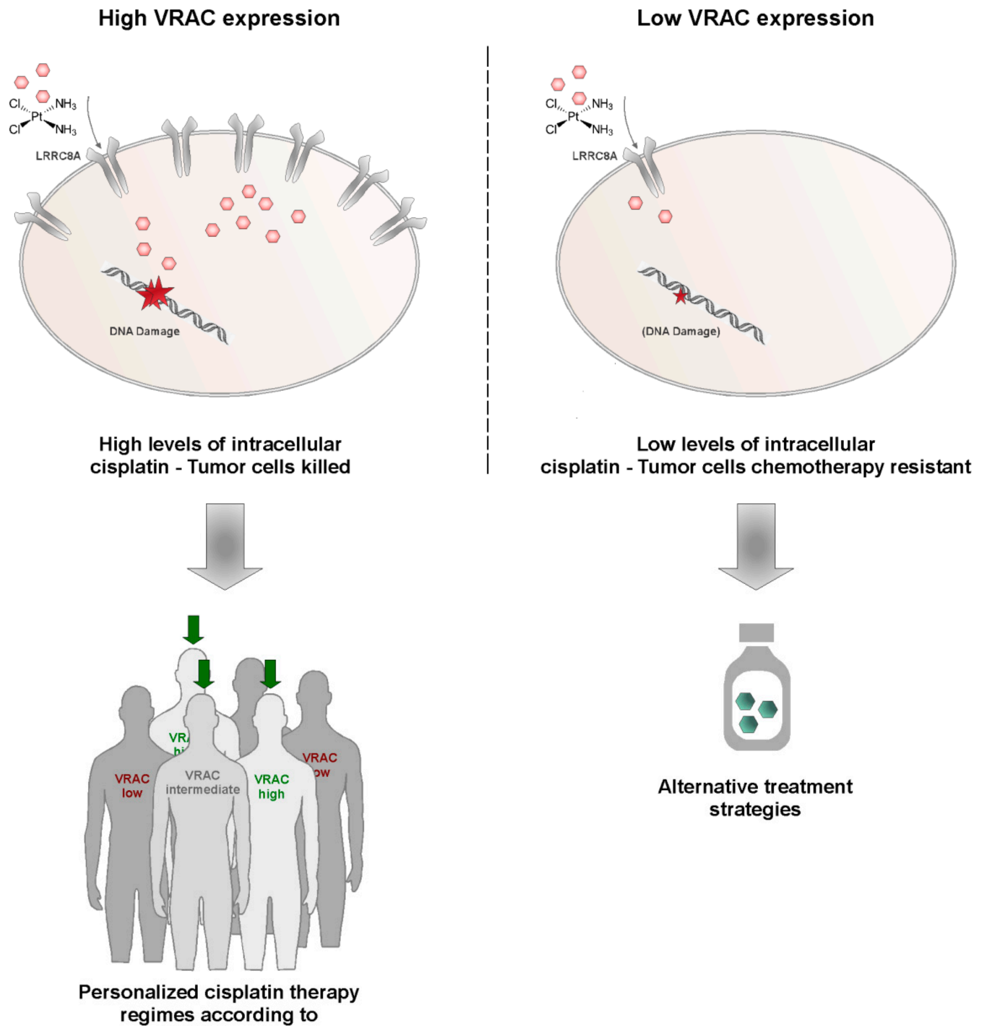

Figure 6. Exploiting VRAC expression levels as a strategy for personalized medicine in HNSCC. While tumors expressing high VRAC levels (green) are likely to be killed by cisplatin therapy, low VRAC levels may signal the development of resistance and thus may indicate the need for alternative treatment strategies. VRAC-based patient stratification could improve success rates in first-line chemotherapy.

Clearly, VRAC's pathobiological and clinical relevance concerning disease progression and therapy responses in HNSCC and other malignancies needs to be examined in detail in large prospective studies. In addition, we do not wish to postulate that VRAC is 'the' unique biomarker predicting (cisplatin) drug resistance for HNSCC but needs to be considered in the context of other potential biomarkers reported so far. Despite these limitations, we feel that our results strongly suggest to exploit VRAC as a potential drug target as well as a personalized prognostic biomarker to improve current and future treatments of HNSCC patients.

4. Materials and Methods

4.1. Chemicals and Reagents

Unless stated otherwise, chemicals were purchased from Sigma Aldrich/Merck (Darmstadt, Germany). Cell culture media and reagents were sourced from Gibco/Thermo Fisher Scientific (Dreieich, Germany). Disposables were purchased from Greiner Bio-One (Frickenhausen, Germany). Kits and antibodies were sourced and used as described in the further methods section und in Table S1.

4.2. Clinical Data Analysis

Publicly available gene expression and survival datasets were obtained from the Cancer Genome Atlas (TCGA) filtering for patients with HNSCCs (TCGA HNSC). Thus, a total of $n = 565$ patients were included. Filtering for cases with detailed clinical information available who have received cisplatin as first-line chemotherapy, $n = 41$ cases could be included where indicated. Data were assessed via the USCS Xena server [52] and patients grouped according to indicated phenotypic or clinical characteristics. Final visualizations were performed with graphpad Prism.

4.3. Cell Culture

Pica cell line was established from laryngeal squamous cell carcinoma as described by Mack et al. [21]. Cells were cultured under standard cell culture conditions (37 °C, 5% CO_2) in Dulbecco's Modified Eagle's Medium and subcultured every 3 d. Absence of mycoplasms was regularly checked via a Venor GeM Advance detection kit (Minverva biolabs, Berlin, Germany) according to the manufacturer's instructions. Cell numbers were determined using Casy Cell Counter and Analyzer TT (OMNI Life Science GmbH & Co KG, Bremen, Germany).

4.4. Generation of Cisplatin Resistant Model

LRRC8A-KO cells were prepared using CRISPR/Cas9 tools, as has been described [53]; however, plasmids were used for expression cassette delivery. Cells were transfected with plasmids (2 µg) using Lipofectamin 2000 according to the manufacturer's instructions and potentially edited cells selected via Blasticidin treatment (Fisher Scientific GmbH, Schwerte, Germany, 2 µg/µL) for 9 d [53]. For primer sequences, see Supplementary Table S2. Then, single cell-derived cell lines were derived from the surviving gene pool via serial dilutions and checked for correct and homozygous knockout. Genomic DNA (gDNA) was isolated using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), while RNA was prepared with the help of an RNeasy Mini Kit (Qiagen) and transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim Germany). Primers for analytical PCRs were designed in order to span the target area of disruption for KO and PCR performed using Taq polymerase according to standard procedures. For primer sequences and expected band sized, see Supplementary Tables S2 and S3. Finally, Sanger sequencing was performed to ensure correct KO. Additionally, constantly selected cell lines were generated by treatment with doses of cisplatin corresponding to IC90 (5 µM) and then constant treatment (3 µM). First experiments were started after constant exposure for 6 months and reestablishment of regular proliferation. LRRC8A expression of cell lines was probed via Western blot analysis. Whole cell lysates were prepared in RIPA buffer and samples separated on 12% SDS gels as has been described at length [54–56]. Blotting onto activated PVDF membranes was achieved with a Trans-Blot Turbo (bio-rad, Feldkirchen, Germany) and blocking as well as antibody incubations (1 h/RT or 16 h/ 4 °C depending on antibody) was performed in 5% milk powder/PBS. Detection of luminescence signal of HRP-coupled secondary antibodies after addition of Clarity Western ECL Substrate was performed with the help of a ChemiDoc^{TM} (bio-rad). For primer sequences and details on plasmids as well as antibodies, see Supplementary Information. Original blots can be found at Figure S11.

4.5. Sequencing of DNA and RNA

Next-generation Sanger sequencing of DNA samples was commercially performed by starseq (Mainz, Germany), and sample analysis was performed with the help of benchling software. The absence of contamination of RNA samples with DNA was ensured by performing cDNA transcription steps without the addition of reverse transcriptase and then checking the amplification of the housekeeping gene actin in a PCR reaction. Then, RNA sequencing was performed as described in [53] and visualizations achieved with the

help of graphpad Prism and Ingenuity Pathway Analysis (Qiagen, Hilden, Germany). For primer sequences, see Supplementary Information.

4.6. Probing Cell Viability

To probe cell viability, cells were seeded in 96-well plates (5000 cells/well) and treated with indicated substances and concentrations (n = 3) starting 24 h after seeding. Then, 48 h after treatment, commercial assay CellTiter-Glo® 2.0 (Promega, Walldorf, Germany) was performed according to manufacturer's instructions and luminescent signals recorded using a Tecan Spark® (Tecan Group Ltd., Männedorf, Switzerland). Signals were normalized to untreated control samples. For cell viability assays using spheroids, cells were seeded in 96-well round bottom, ultra-low adhesion cell culture plates (1000 cells/well, Corning GmbH, Wiesbaden, Germany) and initial spheroid formation was allowed (3 d). Then, spheroids were treated (n = 4) and viability was probed after 72 h using a commercial assay CellTiter Glo® 3D (Promega, Walldorf, Germany) according to the manufacturer's instructions. Luminescent signals were recorded using a Tecan Spark® (Tecan Group Ltd., Männedorf, Switzerland) and normalized to untreated controls.

4.7. Fluorescence Microscopy

Fluorescence images were acquired on an Axiovert 200 M fluorescence microscope (Zeiss, Oberkochen, Germany) or automated high content screening microscope Array Scan VTI (Fisher Scientific, Schwerte, Germany). Cells were seeded in microscopy dishes (35 mm, MatTek) or clear-bottom 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and fixed with 4% PFA (20 min, RT). For immunofluorescence staining, they were additionally permeabilized via incubation with Triton-X 100 (0.1%, 10 min, RT). Antibodies were diluted in 10% FCS/PBS and incubated with samples for 1 h at RT. After extensive washing (PBS), fluorophore-labeled antibodies were incubated with samples for 1 h at RT. Finally, nuclei were stained by the addition of Hoechst 33342 (50 ng/mL in PBS) for 30 min at RT. For automated high content screening, regions of interests were created using the nucleus signal, and each sample was acquired in triplicates, imaging at least 5000 events per sample. For two photon-excitation (2PE) microscopy of spheroids, spheroids were cultured for 3 d and then fixed via incubation in 4% PFA at RT (20 min). Permeabilization was performed in PBSTD (PBS, 0.3% Triton-X 100, 1% DMSO, 1% BSA) and spheroids then incubated with primary antibodies diluted in PBSTD overnight (4 °C). Secondary antibody incubation was performed for 3 h at RT. Finally, nuclei were stained via incubation with Hoechst 33342 (50 ng/mL) for 15 in at RT. Spheroid collection and washing in between incubation steps was achieved via gentle centrifugation (100 g, 3 min). Images were acquired on a Leica TCS SP8 DIVE System (Leica Microsystems, Weimar, Germany). For details on antibodies and dilutions, see Supplementary Information.

4.8. Cell Migration Assay

To measure cell migration, cells were seeded into the two wells of ibidi two-well dishes (70 µL, 5 × 105 cells each, Ibidi GmbH, Gräfelfing, Germany) and incubated for 24 h until cells reached confluency. Then, the silicon barrier was removed with the help of sterile forceps, and the medium including any detached cells was changed. Consistent areas were documented periodically over 48 h via microscopy.

4.9. Transient LRRC8A Expression

In order to reconstitute LRRC8A expression in a KO cell line $Pica_{KO36}$, cells were transfected with plasmid pBix-Ep containing the coding sequence of human LRRC8A or LRRC8A fused to a C-terminal FLAG tag, a constitutive EF1alpha promoter, and the puromycin antibiotic resistance gene. Plasmid and Lipofectamine 3000 (Fisher Scientific, Schwerte, Germany) were mixed according to the manufacturer's instructions and added to the cells, which were cultured in OptiMEM medium as described [33]. To mark LRRC8A-expressing cells, plasmid pC3 coding for GFP expression was co-transfected. To exclude

artefacts, a control transfection of empty plasmid pC-DNA3 and the GFP-coding plasmid was conducted in parallel. Medium was changed 4 h post-transfections to normal cell culture medium and cells were treated with cisplatin for 48 h starting 24 h post-transfection. Cells were fixed with PFA, and the number of green cells was quantified. FLAG-tag was stained by specific antibodies, and cells were analyzed both by conventional as well as by automated high content microscopy as described [57]. To determine changes in viability, CellTiter-Glo® assay was performed as described.

5. Conclusions

Treatment success of head and neck cancers (HNC) is often hindered by tumor relapses due to therapy resistances. This study aimed at profiling potential molecular players critically involved in cisplatin resistance. Following a tiered pipeline from clinical hypothesis formation over educated bioinformatic analysis to in vitro hypothesis confirmation, we conclude that the volume-regulated anion channel VRAC is critical for cisplatin resistance in head and neck squamous cell carcinoma tumor cells and cancer patients. Our conclusion is based on the following findings: For one, low expression of the drug import channel VRAC correlates with reduced response to cisplatin-based chemoradiation and tumor recurrences in the TCGA HNSCC patient cohort. Second, VRAC's key relevance for cisplatin resistance and specificity was confirmed by CRISPR/Cas9 knockout cells, which could be rescued by VRAC expression. Third, low VRAC expression correlated with cisplatin resistance in independent, naturally occurring, cisplatin-resistant HNSCC cancer cell models. Collectively, we suggest that VRAC should be considered a promising biomarker for cisplatin resistance in HNSCC and form the basis for prospective clinical trials to investigate its predictive power.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13194831/s1, Figure S1: Overall survival of HNSCC patients correlating with potential cisplatin resistance candidates, Figure S2: Significance of VRAC's expression for clinical data depends on case stratification, Figure S3: Genetic Analysis of LRRC8A-Knockout clone $Pica_{KO36}$, Figure S4: Next-generation sequencing confirms LRRC8A-knockout in clone $Pica_{KO}$, Figure S5: Ectopic expression of LRRC8A reconstituted VRAC channel function and significantly resensitized the resistant LRRC8A-knockout cell line $Pica_{KO36}$ to cisplatin mediated cell death, Figure S6: Permanent cisplatin selection leads to reduced levels of LRRC8A expression in Fadu cells, Figure S7: Molecular network of LRRC8A interaction partners involved in cancer diseases, Figure S8: Molecular network of LRRC8A interaction partners involved in cisplatin resistance, Figure S9: VRAC expression impacts cellular phenotype and migratory potential, Figure S10: Correlation of LRRC8A expression and clinical characteristics depending on the HNSCC patients' HPV status, Figure S11: Original blots. Table S1: Antibodies, Table S2: Primer Sequences, Table S3: Expected Band Sizes Genomic Analysis, Table S4: RNA Sequencing Results, Video S1: Cancer Cell Spheroid.

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Institutional Review Board Statement: Publicly available gene expression data were obtained from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/ (accessed on 19 August 2021)). The TCGA Research Network included patients in accordance with the guidelines of the Declaration of Helsinki of 1975. Data of n = 50 normal adjacent tissue (NAT) and n = 528 HNSCC tissue samples were included.

Informed Consent Statement: All patients have been recruited by the TCGA research network. The TCGA informed consent guidelines are publically available from https://www.cancer.gov/tcga (accessed on 19 August 2021).

Data Availability Statement: The cell line raw data required to reproduce these findings is available upon request.The clinical results shown here are based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga (accessed on 19 August 2021).

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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