UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

DIEILA GIOMO DE LIMA

Resistência à cisplatina induz células SCC-9 a uma assinatura transcricional e diferenciação morfológica relacionada à transição epitélio-mesênquima

Oral SCC-9 cell line cisplatin resistance induces an epithelial-mesenchymal transition-linked transcriptional signature and morphological differentiation

PIRACICABA
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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Biologia Buco-Dental, na Área de Histologia e Embriologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Buco-Dental Biology, in Histology and Embryology area.

Orientadora: Profª Drª Ana Paula de Souza

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA DIEILA GIOMO DE LIMA E ORIENTADA PELA PROFª DRª ANA PAULA DE SOUZA

PIRACICABA

2024

Ficha catalográfica
Universidade Estadual de Campinas (UNICAMP)
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

Lima, Dieila Giomo de, 1992-
L629r Resistência à cisplatina induz células SCC-9 a uma assinatura transcricional e diferenciação morfológica relacionada à transição epitélio-mesênquima / Dieila Giomo de Lima. – Piracicaba, SP : [s.n.], 2024.

Orientador: Ana Paula de Souza.
Tese (doutorado) – Universidade Estadual de Campinas (UNICAMP), Faculdade de Odontologia de Piracicaba.

1. Transição epitelial-mesenquimal. 2. Cisplatino. 3. Resistência a medicamentos. 4. Neoplasias bucais. 5. Carcinoma de células escamosas. I. de Souza, Ana Paula, 1975-. II. Universidade Estadual de Campinas (UNICAMP). Faculdade de Odontologia de Piracicaba. III. Título.

Informações Complementares

Título em outro idioma: Oral SCC-9 cell line cisplatin resistance induces an epithelial-mesenchymal transition-linked transcriptional signature and morphological differentiation
Palavras-chave em inglês:
Epithelial-mesenchymal transition
Cisplatin
Drug resistance
Mouth neoplasms
Carcinoma, squamous cell
Área de concentração: Histologia e Embriologia
Titulação: Doutora em Biologia Buco-Dental
Banca examinadora:
Ana Paula de Souza [Orientador]
Juliana Lucena Schussel
Mariana Brait Rodrigues de Oliveira
Paula Fontes Asprino
Aline Cristiane Planello
Data de defesa: 16-07-2024
Programa de Pós-Graduação: Biologia Buco-Dental

Identificação e informações acadêmicas do(a) aluno(a)
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- Currículo Lattes do autor: http://lattes.cnpq.br/5762896297146013

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 16 de julho de 2024, considerou a candidata DIEILA GIOMO DE LIMA aprovada.

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

DEDICATÓRIA

Dedico esse trabalho aos meus pais Marino e Clélia que sempre me incentivaram e auxiliaram a persistir no que eu acredito. Bem como a todos que direta ou indiretamente contribuíram nas diferentes e diversas etapas da minha formação.

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Código de Financiamento 001.

O presente trabalho foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), processo nº 140439/2020-0.

À Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) com o Programa de Internacionalização (PrInt), processo nº 88887.573099/2020-00, pelo apoio financeiro durante meu intercâmbio do doutorado nos Estados Unidos.

À Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas (FOP/UNICAMP).

À Coordenação de Pós-Graduação da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas (FOP/UNICAMP) e ao Programa de Pós-Graduação em Biologia Buco Dental.

Gratidão a Deus por cuidar de cada detalhe da minha vida, me dando oportunidades que eu jamais imaginaria ter.

À Profª Drª Ana Paula de Souza, minha querida orientadora do mestrado e do então doutorado, que me deu a oportunidade de entrar no mundo da pesquisa básica (nada básica), que sempre esteve aberta para novas ideias, me dando autonomia e liberdade para desenvolver o pensamento crítico. Obrigada por esses anos de parceria e amizade.

À Drª Mariana Brait, minha amada supervisora durante o intercâmbio do doutorado na Johns Hopkins University. Obrigada por me ensinar coisas que eu nem sabia que precisava aprender, além de toda ajuda, suporte, apoio e risadas. Muito obrigada por cada oportunidade e conselho.

Ao Dr° David Sidranski por me acolher também durante o intercâmbio do doutorado, pelos ensinamentos e oportunidades.

Obrigada aos meus colegas na Johns Hopkins University por toda ajuda e acolhimento, Keerthana, Dr° Pritam, Drª Ravi, Dr° Jonathan Coulter, Drª Celina, Wanda, Francesca, Faith, Drª Kagohara, Drº Zamuner.

Aos amigos que a vida me apresentou nos Estados Unidos. Obrigada pela amizade Michelle, Rina, Olívia, Luccas, Felipe, Gabrielle, Ricardo, Agustin, Luana, Rafael, Leonardo, Camille.

À Profª Drª Aline Cristiane Planello pela amizade, compartilhamento de ideias e disponibilidade em me ajudar, discutir ciência, mesmo à distância.

À minha eterna orientadora Drª Paula Cristina Trevilatto por ser minha inspiração desde a Iniciação Científica e que me fez amar estar no laboratório.

Obrigada às Drªs Juliana Schussel e Paula Asprino pelo aceite em compor a banca titular da minha defesa de doutorado.

Agradeço aos professores Drº Marcelo e Drº Sérgio por serem sempre solícitos e estarem sempre dispostos a ajudar, tirar dúvidas e discutir pontos do mundo acadêmico científico. Mentes muito brilhantes.

Obrigada a cada funcionário da FOP/UNICAMP, Dona Zefa, Maria, Raquel, Magda, Lu que sempre tornaram meus dias mais leves com uma conversa no corredor, na portaria. Mulheres que fazem a diferença na minha vida.

Às minhas chefes Drª Mariana Maschietto e Drª Patrícia Jotta pela compreensão em diversos momentos que precisei de um tempo para me dedicar na conclusão do projeto e pelo auxílio técnico e científico, além de serem referência de pesquisadoras.

Ao meu “paquera” do mestrado que se tornou meu namorado, noivo e então esposo e parceiro de vida/caminhada, mas também pesquisador competente Dr° Gustavo Narvaes Guimarães que me auxilia diariamente, me dá suporte e sempre faz a base para que eu possa me dedicar aos meus objetivos, além de amar e cuidar da nossa Zarinha, minha fiel cãopanheira da vida.

Aos meus amigos da pós-graduação Larissa, Manuel, Camila, Giovanni, Bianca, Gabriell, Douglas por me oportunizar momentos leves e de boas conversas.

Aos meus amigos do Boldrini que se fazem presente diariamente me ensinando e compartilhando ideias, além de sorrisos.

Agradeço imensamente minha colega mais próxima de trabalho que se tornou uma grande amiga. Mayara, obrigada pelo seu carinho, amizade, por

compartilhar a vida como ela é comigo e por me ensinar tanto, além de me dar todo suporte e uns toques de verdade. Exemplo de pessoa e profissional.

Agradeço também ao meu amado amigo Felipe por ser tão gentil, amável além de um pesquisador competente que muito me ensina diariamente. Obrigada por me acolher, auxiliar.

À minha grande amiga Drª Maraysa pelo suporte, apoio incondicional e trocas científico-acadêmicas.

Às minhas amigas Natália e Helena que caminham lado a lado comigo tanto na ciência quanto na vida pessoal. Obrigada pelas conversas e amizade leal.

Obrigada à Força-Tarefa COVID-19 Unicamp, tanto a frente de diagnóstico quanto social, pela oportunidade de contribuir minimamente durante quase dois anos nos diagnósticos e coletas durante a pandemia. Agradecimento especial aos Drºs Alessandro e André pela excelência na gestão do laboratório, além de compartilharem seus conhecimentos e a toda equipe que fez parte desse momento voluntariamente.

Aos meus pais Marino e Clélia, minha eterna gratidão, por estarem sempre comigo sendo colo, abrigo, suporte. Ao meu irmão Dimas que mesmo distante sempre se faz presente e disponível.

Obrigada à minha cunhada Carolina pelo carinho e paciência que sempre teve comigo, bem como à toda família do Gustavo, que hoje é minha família do coração. Um obrigado especial a vó Ivanilde, sempre tão carinhosa comigo.

Minha eterna gratidão a todos os meus professores da vida por transmitir seus conhecimentos e por me apoiarem e acreditarem em mim. Isso com certeza fez toda diferença para que eu seguisse em frente.

RESUMO

O carcinoma espinocelular (CEC) de cavidade oral é o tipo mais incidente de câncer de cabeça e pescoço no Brasil e no mundo, apresentando diagnóstico tardio e prognóstico ruim. A cisplatina (CDDP) é o quimioterápico de primeira linha utilizado para o tratamento de CEC oral. Porém, fatores intrínsecos e extrínsecos envolvidos na resistência à CDDP continuam a ser desafios para eficácia do tratamento de CEC oral. Nos últimos anos, diversos estudos e modelos de resistência a quimioterápicos apontam a mediação da resistência pela programação transição epitélio-mesênquima (TEM), configurando, assim, um perfil mais invasivo e metástatico do tumor. Neste estudo, propomos um modelo *in vitro* de resistência gradual a cisplatina utilizando a linhagem celular SCC-9, correlacionando alterações fenotípicas e assinatura transcricional. Assim, através do ensaio de viabilidade celular, determinamos o IC50 de CDDP nas células SCC-9. Então, tratamos essas células com o valor de IC50 e em seguida com o dobro do valor de IC50 obtido inicialmente. Logo, observamos três estados celulares diferentes: SCC-9P (parental) de morfologia epitelial, SCC-9CPR5 (resistência intermediária) perfil misto de células poligonais e mais fusiformes e SCC-9CPR11 (resistência final) com fenótipo predominantemente mesenquimal. Com ensaio de curva de crescimento, observamos que as células resistentes apresentaram proliferação mais lenta quando comparadas às parentais, confirmando a aquisição da resistência à CDDP. Além disso, com ensaio de migração *scratch*, as células SCC-9CPR5 mostraram perfil mais migratório em grupo correspondente com estágio de resistência parcial. Através de RT-qPCR, avaliamos a expressão de marcadores clássicos associados a TEM como *SNAIL1*, *Vimentina*, *ZEB1*, *ZEB2*, *N-caderina*. Com análise de sequenciamento de RNA (RNA-Seq) verificamos assinaturas transcricionais para TEM, resistência à cisplatina correlacionada com genes pouco expressos na ligação de íons cálcio (Ca^{2+}) entre os termos do *Gene Ontology* (*GO*) e o enriquecimento de vias, utilizando *Kyoto Encyclopedia of Genes and Genomes* (*KEGG),* relacionadas a ambas as condições, como a via de PI3K-Akt. No geral, destacamos diferentes vias e genes alvos que poderão ser considerados em estudos futuros para investigação de inibição da resistência a cisplatina mediado por TEM em câncer oral num modelo de resistência *in vitro*.

Palavras-chave: Transição epitelial-mesenquimal. Cisplatino. Resistência a medicamentos. Neoplasias bucais. Carcinoma de células escamosas.

ABSTRACT

Oral cavity squamous cell carcinoma (OCSCC) is the most common type of head and neck cancer in Brazil and worldwide showing late diagnosis and a poor prognosis. Cisplatin (CDDP) is commonly used as first-line chemotherapy for the treatment of OSCC. However, intrinsic and extrinsic factors involved in CDDP resistance prevent effectiveness of OSCC treatment. Recently, several studies on chemotherapy resistance models have associated epithelial-mesenchymal transition (EMT) programming-mediated resistance and an invasiveness and metastasis profile of the tumor. In this study, we propose a model of gradual resistance to cisplatin in vitro using the SCC-9 cell line, where we correlate phenotypic changes and a gene transcriptional signature. By cell viability assay, we determined the CDDP IC50 in SCC-9 cells. We then treated the cells using the IC50 value and then with the 2-fold IC50 value. Therefore, we obtained three cellular states, SCC-9P (parental) with epithelial morphology, SCC-9CPR5 (intermediate resistance) with a mixed profile of epithelial and more spindle-shaped cells a hallmark of mesenchymal cells, and SCC-9CPR11 (full resistance) showing predominantly mesenchymal phenotype. Resistant cells showed a suppression of proliferation compared to the parental cells configuring the acquisition of resistance to CDDP, highlighted by the growth curve assay. Furthermore, in the scratch migration assay analyses, SCC-9CPR5 cells showed a more migratory profile corresponding to group cell migration partial EMT (pEMT). Through RT-qPCR, we assessed the expression of markers associated with EMT such as *SNAIL1*, *Vimentin*, *ZEB1*, *ZEB2*, *N-cadherin*. RNA sequencing (RNA-Seq) analysis showed transcriptional signatures for EMT among the differentially expressed genes. Cisplatin resistance correlation with genes down-regulated in the binding of calcium ions (Ca^{2+}) among the Gene Ontology (GO) terms, and the enrichment of canonical and non-canonical pathways related to resistance and EMT using Kyoto Encyclopedia of Genes and Genomes (KEGG), such as the PI3K-Akt signalling pathway. Overall, we highlight several signalling pathways and target genes that could be considered in future studies to investigate EMT-mediated inhibition of cisplatin resistance in oral cancer using an in vitro resistance model.

Keywords: Epithelial-mesenchymal transition. Cisplatin. Drug resistance. Mouth neoplasms. Carcinoma, squamous cell.

SUMÁRIO

1 INTRODUÇÃO

Os cânceres de boca, nasofaringe, orofaringe e hipofaringe são tumores malignos incluídos no grande grupo de cânceres de cabeça e pescoço (CCP) (Marur e Forastiere, 2016).

Vários são os termos utilizados para classificar o câncer de boca, conforme documentado por Moore e colaboradores em carta publicada na revista *Oral Diseases* (Moore et al., 2000). Neoplasias definidas como câncer de lábio e cavidade oral, câncer de nasofaringe, orofaringe e hipofaringe devem ser consideradas entidades patológicas distintas em relação ao local, histopatologia, diagnóstico, mecanismos genéticos e epigenéticos, epidemiologia e comportamento tumoral (Chi et al., 2015; Seiwert et al., 2015; Conway et al., 2018).

Anatomicamente, a cavidade oral é posicionada anteriormente em relação à orofaringe e que se comunica com a nasofaringe superiormente e com a hipofaringe inferiormente. Ambas consistem em áreas próximas e representam coletivamente o trato aerodigestivo superior (TADS). A cavidade oral começa na junção mucocutânea dos lábios e inclui os dois terços anteriores da língua, assoalho da boca, gengiva, palato duro e retromolar.

O tipo histológico mais comum de câncer bucal é o carcinoma espinocelular (CEC), que constitui mais de 90% de todos os casos de câncer que surgem na região de cabeça e pescoço, incluindo cavidade oral e orofaringe (Woolgar e Triantafyllou, 2009).

Os locais de CEC de cavidade oral mais acometidos são a língua e o assoalho da boca, representando mais de 50% de todos os casos, seguidos pela gengiva, mucosa palatina, mucosa bucal e mucosa labial (Bagan et al., 2010; Farhood et al., 2019).

Em 2012, o CEC de cavidade oral e de orofaringe ocuparam juntos a posição de sexto tipo de câncer mais comum no mundo (3,1%), com distribuição geográfica variável. No entanto, inquéritos seguintes, de 2018, mostraram que, apesar do aumento da incidência, ambos se tornaram o décimo sexto tipo de câncer mais diagnosticado (2,0% de todos os tumores malignos). Ainda, de 2012 (Ferlay et al., 2015) a 2018 (Bray et al., 2018) a incidência de câncer de lábio e cavidade oral aumentou cerca de 18%, mantendo o número de novos casos duas vezes maior no sexo masculino. O número de óbitos representou cerca de 2% da mortalidade por

câncer em todo o mundo. Enquanto isso, apesar do aumento na contagem de novos casos dos demais cânceres de faringe (orofaringe e hipofaringe, exceto nasofaringe) em 31.495 casos, houve queda na mortalidade de 12% entre os anos analisados. Essa taxa de mortes por neoplasias de orofaringe e hipofaringe é referida na proporção de 5:1 para homens. Neste inquérito dos últimos 6 anos, os tumores de lábio, cavidade oral e outros tumores da faringe continuaram a ser altamente frequentes no Sul da Ásia, bem como nas Ilhas do Pacífico, com um índice de desenvolvimento humano (IDH) médio, acesso limitado à métodos preventivos e hábitos relacionados a fatores de risco para gênese tumoral (Ferlay et al., 2015; Bray et al., 2018; Filho & Warnakulasuriya, 2024).

O CEC de cavidade oral permanece um dos subtipos de CCP mais prevalentes por seu perfil heterogêneo, agressivo, invasivo e altamente metastático (Imbesi Bellantoni et al., 2023). No Brasil é o quarto em incidência no sexo masculino na Região Sudeste (13,16 por 100 mil). Estima-se ainda que, de 2023 a 2025, ocorrerão 704 mil novos casos de câncer, sendo que o câncer de cavidade oral representará cerca de 3,2% dos casos em homens (INCA, 2023).

O desenvolvimento de CEC de cavidade oral está relacionado a diversos fatores de risco. Notavelmente, o tabaco continua a ser o principal fator extrínseco que contribui para a gênese de câncer oral (NCI, 1998; IARC, 2007; Hernández-Morales et al., 2023) seguido pelo consumo frequente de álcool (Lubin et al., 2011; Gapstur et al., 2023). O uso combinado de ambos aumenta em cerca de duas ou três vezes a taxa de risco (Hashibe et al., 2009; Gormley et al., 2020). Existem, ainda, outros fatores extrínsecos como a exposição solar prolongada - mais associada ao CEC labial (de Visscher e van der Wall, 1998; Chi, 2009; Kumar et al., 2016) -, má nutrição e má higiene oral contribuindo para a alteração da microbiota tornando um ambiente permissível a infecções crônicas causadas por bactérias, fungos e viroses (Shetty e Vishal, 2019). A infecção pelo vírus do papiloma humano (HPV) se aproxima de ~31% de todos os CEC de orofaringe e uma porcentagem muito pequena de CEC de cavidade oral devido à infecção por HPV (1-10%) (Lingen et al., 2013; Plummer et al., 2016). Dos mais de 200 genótipos, a Agência Internacional de Pesquisa sobre o Câncer (IARC) classificou 12 tipos que apresentam alto risco de gênese tumoral, sendo o HPV16 o mais comum (80%), seguido pelo HPV18 (3%) para CEC de cavidade oral (Kostareli et al., 2012; Castellsagué et al., 2016).

Além destes, temos os fatores intrínsecos que incluem mecanismos genéticos e epigenéticos levando à alterações na atividade de oncogenes e genes supressores tumorais (Baillor e Manjunatha, 2015; Irimie et al., 2018).

A conduta terapêutica e o prognóstico estão intimamente relacionados ao estadio tumoral. O American Joint Committee on Cancer (AJCC) publicou o primeiro manual de sistema de estadiamento TNM em 1977. Este é baseado na avaliação do tamanho do tumor primário (T), envolvimento de linfonodos locorregionais (N) e metástases à distância (M). Em 2017, a 8ª edição foi lançada com algumas modificações como: introdução da profundidade de invasão tumoral (DOI) e extensão extranodal (ENE) para câncer de cavidade oral. Recentemente, novas tecnologias de inteligência artificial e aprendizado de máquina têm sido utilizadas com o objetivo de melhorar a classificação do estadiamento tumoral além de fornecer algoritmos preditivos (Alabi et al., 2019; Bur et al., 2019).

A taxa de sobrevivência em 5 anos do câncer bucal corresponde a menos de 50% dos cânceres com diagnóstico tardio, com detecção em estágios avançados (III ou IV) (Varela-Centelles et al., 2019).

Nas últimas décadas, muitas metodologias para diagnósticos precoces tornaram-se altamente precisas e complementares à exploração e palpação oral. A biópsia tecidual continua sendo o procedimento padrão, embora muitas vezes seja invasivo. Citologia esfoliativa, soro, saliva são técnicas de triagem não invasivas e de baixo custo, mas ainda limitadas em relação à especificidade (Mehrotra et al., 2006; Kakimoto et al., 2008; Sharma et al., 2011). No entanto, a biópsia líquida tem permitido obter um perfil molecular específico para cada paciente com base na detecção de células tumorais circulantes (CTCs), DNA tumoral circulante (ctDNA) e RNA tumoral circulante (ctRNA), proteínas e exossomos (Ma et al., 2015; Siravegna et al., 2017), porém muitos desafios ainda permeiam a aplicação dessas metologiasna realidade clínica. Essas novas abordagens são utilizadas atualmente como auxiliares para prever riscos de transformação maligna, descrevendo alguns biomarcadores obtidos de soro como Ciclina D1 conhecido como um importante regulador de ciclo celular (Ramos-García et al., 2017) e receptor do fator de crescimento epidérmico (EGFR) (Grisanti et al., 2014), diferentes isoformas de CD44 e CD63 (Sharma et al., 2011) de saliva e outros fluidos biomarcadores (Bellairs et al., 2017; Wang et al., 2018), porém baixas concentrações podem não ser detectáveis e sua relevância segue em discussão (Turabi et al., 2024).

A ressecção cirúrgica seguida ou não de radioterapia é o padrão ouro de tratamento para câncer de lábio e cavidade oral (Chi et al., 2015). Contudo, a ressecção cirúrgica está associada à morbidade, desfiguração, comprometimento funcional e consequências fisiológicas ao paciente (Rogers et al., 2009; Pinto et al., 2016). Assim, a escolha do tratamento mais adequado deve depender do tamanho do tumor, localização, estadiamento TNM, características prognósticas derivadas do exame realizado, estado geral de saúde do paciente e dos menores efeitos colaterais possíveis.

A terapia mais indicada para os estágios iniciais (T1 ou T2) ainda é bastante controversa e deve ser discutida em relação à causa e efeito (Gad et al., 2018; Joo et al., 2019; Elicin et al., 2020). Chen e colaboradores (2018) relataram que em estágios mais avançados e com pior prognóstico, adota-se excisão cirúrgica, radioterapia adjuvante e adição de quimioterapia, promovendo maior taxa de sobrevida (Chen et al., 2018).

Atualmente existe uma ampla gama de agentes quimioterápicos utilizados, isoladamente ou em combinação, para o tratamento do câncer oral, principalmente em casos recorrentes ou metástases linfonodais ocultas: cisplatina (CDDP), carboplatina, 5-fluorouracil, paclitaxel, docetaxel e anticorpos (Hartner, 2018).

A cisplatina ou cis-diaminodicloroplatina (II) (CDDP) é um agente anticâncer de primeira geração bem estabelecido, pertencente ao grupo de complexos de platina (carboplatina, oxilaplatina). Cerca de mais de 50% dos tipos tumorais serão tratados inicialmente com CDDP, que possui efetividade tanto a nível curativo quanto paliativo (Ranasinghe et al., 2022). A atividade antitumoral da cisplatina está associada à sua capacidade de interferência na síntese e nos mecanismos de reparo do DNA. Diferentes tipos de interação com o DNA podem ser gerados através da formação de adutos, como monoadutos, ligações intrafitas e entrefitas resultando em danos no DNA. Ao exceder a capacidade da maquinaria de reparo, a cisplatina promoverá a morte celular principalmente por ativação da vida de sinalização apoptótica (Hong et al., 2012). Apesar da alta taxa de sucesso da terapia com CDDP em tumores primários, segundos tumores e metástases, a resistência quimioterápica continua a ser um grande desafio (Liang et al., 2017).

Os mecanismos de resistência a CDDP são intensamente explorados na literatura, podendo ser de origem intrínseca ou adquirida. Estes mecanismos estão correlacionados principalmente à diminuição da captação do medicamento pelas

células, diminuição do influxo ou aumento do efluxo da droga, alteração do alvo do medicamento e pela hiperfunção da maquinaria de reparo de dano ao DNA **(**Brabec e Kasparkova, 2005; Assaraf et al., 2019). No entanto, o mecanismo molecular envolvido na resistência continua limitado. A transição epitélio-mesênquima (TEM) tem emergido como um mecanismo importante no processo de resistência na terapia.

TEM é uma programação celular altamente conservada, mas reversível que promove uma reorganização celular baseada em sua plasticidade como perda da polaridade apico-basal, contato célula-célula, modificação no citoesqueleto, aumento na mobilidade e invasividade. TEM mantém essas alterações associadas à progressão tumoral, metástases e mediadoras de resistência a quimioterápicos **(**Chaffer et al., 2016; Yang et al., 2020). Essa plasticidade celular é regulada positivamente pela perda da expressão de E-caderina no contato célula-célula levando a redução da expressão de outros marcadores epiteliais como citoqueratinas, e aumento da expressão de marcadores msenquimais como N-caderina, Vimentina (Yang et al., 2020). A programação TEM conta com o envolvimento de fatores de transcrição (FT) repressores de E-caderina que inclui as famílias de Snail, Zeb e Twist (Herranz et al., 2008; Sanchez-Tillo et al., 2010; Yang et al., 2010). No entanto, esses marcadores podem ser dependentes do tipo de tumor e apresentar níveis de expressão alterados de acordo com a fase de TEM. Atualmente sabe-se que em um estágio intermediário de TEM, também conhecido como TEM parcial (TEMp) ou híbrido, ocorre uma internalização e consequente co-expressão de E-caderina, potencializando a progressão tumoral e aumentando a invasão celular (Yamashita et al., 2018). Ao se ativar TEM experimentalmente, observou-se a expressão de CD44, correspondente a células-tronco tumorais ou *cancer stem-cells* (CSC). Assim, muitos estudos mostram essa relação da ativação de TEM mediando a conversão de não-CSCs em CSCs que possuem a propriedade de promover metátases que é aumentada em TEMp (Baccelli eTrumpp, 2012; Jolly et al., 2015), bem como são postuladas como sendo uma população resistente aos quimioterápicos e protocolos de radioterapia (Lerner e Harrison, 1990), mas ainda pouco evidenciado.

Portanto, nosso objetivo nesse trabalho é entender como a resistência a cisplatina altera o fenótipo celular e encontrar assinaturas transcricionais envolvidas tanto na resistência quanto na ativação da programação de TEM em um modelo de resistência a CDDP *in vitro* em CEC de língua.

2 ARTIGO: Comparative analysis of differential gene expression in cisplatin-resistant SCC-9

Artigo submetido ao periódico Cellular Signalling (Anexo 1)

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Running Tittle: Cisplatin resistant OSCC induces EMT programing

Key words: EMT, cisplatin, drug-resistance, oral cancer, SCC-9

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Abstract

Therapy resistance remains the major cause of death from cancer. Epithelial-to-mesenchymal transition (EMT) has been widely investigated and significantly associated to resistance in different tumor types. Cisplatin (CDDP) is commonly used as the first-line of treatment agentin oral cancer with increasing cases of intrinsic or extrinsic resistance. However, EMT mediated cisplatin resistance is still poorly understood in oral cancer. Here, using *in vitro* estabilishment of a cisplatin resistance model with SCC-9 cell line, we observed morphological changes associated to high expression of *VIMENTIN*, *SNAIL1*, *ZEB1*, *ZEB2* and *CDH2* according to resistance level. Furthermore, we observed supression of proliferation in resistant cells and a significant migratory profile in partial CDDP resistance level (SCC-9CPR5). On the other hand, SCC-9CPR11, the final resistant cells, did not show a bulk migration profile. Using RNA sequencing (RNA-Seq), we found various differentially expressed genes (DEGs) related to cell adhesion, extracellular matrix organization, binding calcium ion (Ca2+) Gene Ontology (GO) terms. Additionally, using Kyoto Encyclopedia of Genes and Genomes (KEGG), we identified phosphoinositide 3-kinase-related protein kinase (PI3K-Akt) signalling pathway enriched, which plays an important role in CDDP resistance and EMT programing. Together, our study uncovers a new 2D mechanistic model of cisplatin resistance showing important DEGs for further investigation and validation.

Keywords: EMT. Cisplatin. Drug-resistance. Oral cancer. SCC-9.

1. INTRODUCTION

Oral cavity cancer (OCC) remains the most prevalent subtype of head and neck cancer (HNC) and the 11th most frequent carcinoma worldwide due to its heterogeneity, aggressiveness, invasiveness, and highly metastatic profile. OCC accounts globally approximately 390,000 new cases (2% of all cancers) and over 188,000 deaths (1.8% of all cancers) in 2022 [1]. Differentiated oral squamous cell carcinoma (OSCC) represents almost 90% of this cancer [2].

Tobacco use and alcohol consumption are well-established risk factors to OSCC [2,3], but in recent years oral carcinogenesis has increased among non-smokers, suggesting the involvement of other intrinsic and extrinsic factors [4].

Surgery is the gold standard approach for OSCC [5], however the treatment of patients with metastatic OSCC remains one of the greatest clinical challenges [6]. Even with several advances in tumor therapy research, for example, with tools of predictions supported by precision medicine, the 5-year survival rate in OSCC patients remains at 50% [7].

Among the different therapeutic approaches, cisplatin based chemotherapy (cis-diaminedichloroplatinum, CDDP) remains the first line of treatment for OSCC - alone or in combination with pre/post-operative radiotherapy, depending on clinical management and tumor stage [8]. CDDP is an alkylating agent that has a cytotoxic effect mainly on the formation of genomic DNA adducts with intra-chain 1,2 cross-links of purine bases, directly damaging DNA and inhibiting its replication, thus interrupting the cell cycle and eventually leading to death [9,10]. Considering the side effects, researchers have developed some platinum-based analogues, such as carboplatin and oxaliplatin, but despite reducing side effects, they are less effective [11,12,13]. Furthermore, the cost-benefit ratio of CDDP associated with an improvement in the patient's general prognosis, increasing the disease-free survival (DFS) rate, exposes the immediate benefits of its adoption in therapeutic management [14,15]. However, one of the challenges for patients undergoing treatment with chemotherapy is the emerging resistance to both the platinum compound and multiple drugs (MDR). This resistance can be intrinsic or *de novo* and acquired or adaptive, considerably attenuating the efficacy of cisplatin in the treatment of OSCC [16].

Neoplastic cells have high cellular plasticity, resulting in the acquisition of new phenotypic characteristics with distinct cellular states, culminating in tumor heterogeneity. Current evidence suggests that this phenotypic diversity within the tumor is one of the main drivers of the development of resistance to therapy [17,18]. These drug-tolerant cells are often referred to as minimal residual disease (MRD), that is, they play a role in the tumor relapse rate if treatment is interrupted [19].

Epithelial-mesenchymal transition (EMT) is one of the extensively studied processes promoting the invasive and migratory capacity of tumor cells, contributing to the initiation of MRD. EMT is characterized by the loss of epithelial markers, cell-cell and cell-extracellular matrix adhesion, along gaining mesenchymal-like transcriptional programming, display fibroblast-like morphology and cytoarchitecture, as well as increased migratory capacity [20]. The most reported signaling pathways are TGF-β, WNT, and NOTCH, shown to be regulated by transcriptional factors such as SNAIL, ZEB, SLUG and TWIST. In previous reports PI3K-Akt signaling pathway is closely related to EMT programing with phosphorylated Akt inhibiting GSK-3β by decrease E-

cadherin levels in non-small cell lung carcinoma (NSCLC) [21]. Additionally, Vimentin and N-cadherin are classically identified EMT markers associated with cancer stem cells (CSCs), which in turn are indicative of MRD, due to the release of these CSCs by invasive tumors [22]. Some mechanisms that configure the presence of epigenetic modifiers (histone deacetylases, DNA methyltransferases) may also involve changes in chromatin and DNA leading to the CSC state [23].

Current studies reveal that partial EMT (pEMT) plays a central role in tumor progression, acquiring mesenchymal functions, but retaining epithelial characteristics. This hybrid state has an increased metastatic potential compared to the initial and final transition states. Therefore, they are classified into different stages of transition and consequently of function according to the identification of distinct genetic markers in addition to unique morphological changes [24].

Despite an extensive literature on EMT and resistance to chemotherapy drugs, the mechanisms driving this resistance at different stages remain unclear in oral cancer. Therefore, the present study aimed to identify the main genetic and morphological changes related to the process of resistance to cisplatin in an immortalized cell line model of tongue cancer subjected to different levels of resistance in vitro.

2. MATERIALS AND METHODS

2.1. Parental cell line maintenance

SCC-9 cells (ATCC® CRL-1629™) were kindly provided by Dr. Edgar Graner (Piracicaba Dental School, University of Campinas, Piracicaba, Brazil). Cells were cultured in DMEM/Ham's F12 medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) (Gibco™, USA) supplemented with 400 ng/mL hydrocortisone (Sigma-Aldrich, USA), 10% fetal bovine serum (FBS) (Gibco™, USA) and an antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomycin) (Gibco™, USA). Cells were growth at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. IC_{50} analysis

The anticancer drug cisplatin (Takara Bio USA, Inc.) was diluted in 0,9% of saline solution. The drug concentration for cisplatin-resistance establishment in the SCC-9 cell line was assessed through of the determination of half-maximal (50%) inhibitory concentration (IC_{50} analysis) (GraphPad Prism v9.1.2 Software Inc., USA). Briefly, cells were seeded in 96-well plates at a density of 4×10^3 cells/well, allowed to adhere for 24 h, and then, exposed to range of cisplatin concentration varying from 0 to 20 µM (n=8). After 72 h, cell viability was determined with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, USA) assay, according to manufacturer's protocol. Cells cultivated with vehicle were considered as control.

2.3. Induction of cisplatin-resistance in SCC-9 cell line

Cisplatin-resistant cell lines (SCC-9CPR) were derived from parental cisplatin-sensitive cell line (SCC-9P) through intermittent exposure to stepwise increasing concentrations of cisplatin (Figure 1). Briefly, exponentially growing cells were exposed to cisplatin IC_{50} concentration for 72 h. Then, the media was changed to fresh medium

and surviving cells were allowed to recover until normal growth restored. At this point the concentration was increased approximately 2-fold and the above process was repeated. Aliquots of cells CDDP-treated cells were cryopreserved at each incremental concentration. This development period was carried out for about one year and two of resistant cells passages were collected and named as SCC-9CPR5 and SCC-9CPR11. Additionally, parental cell line (SCC-9P) was kept in culture during this period as control cell line.

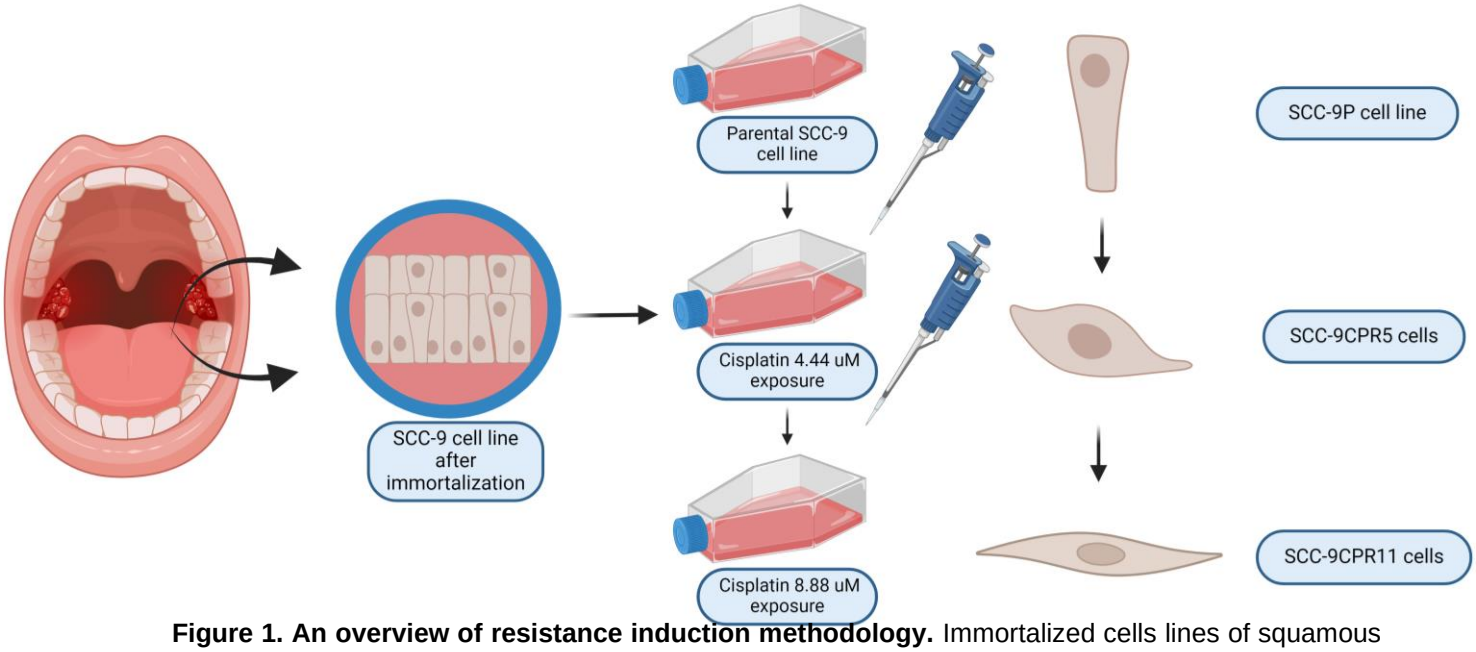

Figure 1. An overview of resistance induction methodology. Immortalized cells lines of squamous cell carcinoma of tongue, HPV-negative (SCC-9) subjected to cycles of intermittent cisplatin applications, generating two new different cells SCC-9CPR5 and SCC-9CPR11, presenting distinct phenotypes at the end of the drug exposure times. Figure made with BioRender.

2.3.1. Measuring the level of drug resistance

Confirmation of cisplatin resistance in established treated cells was caried out using cell viability assay followed of the IC_{50} analysis in both established cell lines, SCC-9CPR5 and SCC-9CPR11. The cells were treated with different concentrations of cisplatin for 72 h and the resistance index (RI) was determined as the ratio of the IC_{50} of the cisplatin resistant cell variant / IC_{50} of parental.

2.3.2. Cell morphological analysis by phase contrast microscopy

The cell phenotype was monitored by an inverted microscope (Zeiss Axiovert 40 CFL Inverted Microscope Fluorescence Phase Contrast, ZEISS, Germany) and photomicrographs were taken in bright field using ZEN 2011, Blue edition (Zeiss Software, Germany).

2.4. Growth curve analysis

SCC-9P and SCC-9CPR cells were seeded at a density of $3,75x10^4$ cells/well into a 24-well plate and cultured in cisplatin-free culture medium for 7 days. In this period, the cells (n=4) were detached by trypsinization (Sigma-Aldrich, Missouri, EUA #cat. T4049) and the number of viable cells was obtained daily in a Neubauer chamber using Trypan Blue Exclusion method. Finally, counting data were plotted on a semi-log scale and culture growth rate was calculated for each cell line. According to the equation, population doubling level (PDL) and population doubling time (PDT) were calculated as follow: PDL = [log10(Nf) - log10(Ni)] / log10(2) and PDT = [log2 x Δt] / [log10(Nf) - log10(Ni)] where, Ni denotes the number of cells seeded, Nf denotes the number of cells harvested at the final of experiment and Δt denotes the time of culture for cell growth from Ni to Nf [25]. The experimental triplicate was statistically compared between the groups by Student's t test, significance level at 5%.

2.5. Scratch assay

To assess cell migration, SCC-9P and SCC-9CPR cells were seeded at high density into 24-well culture plate (n=4) and cultured for 48 h until they reached 100 % of confluency. The confluent monolayer was scratched using a sterile 200-µl pipette tip to create cell-free areas. After washes with PBS (PBS-Gibco BRL, life technologies, Rockville, MD, USA) to remove detached cells and debris, cells were cultured in fresh medium for 24 h without FBS. The scratched area closure by cell migration was assayed taking images at six previously selected different spots every 6 hours for 24 hours, using an inverted microscope (Zeiss Axiovert 40 CFL Inverted Microscope Fluorescence Phase Contrast, ZEISS, Germany). It was determined the scratch area, wound coverage of total area, and average and standard deviation of the scratch width using the ImageJ MRI Wound healing tool [26,27]. The average values of the area relative to the wound were obtained every 6 h, dividing the wound area at each time evaluated by the wound area at time 0. In addition, the wound closure speed was also evaluated, dividing the area occupied by the cells for the time evaluated in hours. Results are shown as mean ± SEM. Statistical analysis was achieved using Student's t test, *$p < 0.1$, **$p < 0.05$, ***$p < 0.001$.

2.6. RNA isolation, cDNA synthesis and RT-qPCR

Analysis of EMT gene expression was performed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Cells were seeded at a density of $18.75x10^4$ cells/well into a 6-well (n=3) plate. After 72 h, the total RNA was purified by Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and the concentration and purity of RNA were determined by spectrophotometric analysis (NanoDrop, Thermo Fisher). Afterward, total purified RNA (1 µg) was treated with DNAse (Invitrogen, Carlsbad, CA, USA) and used for cDNA synthesis carried out using SuperScript III First-Strand Synthesis Systems for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to manufacturer's recommendation. The expression levels of the EMT genes were determined using specific primers (Supplementary Table 1) and SYBR® Green PCR master mix (Applied Biosystems, USA). The PCR reaction solution was carried out in a final volume of 10 µL, containing 1 µL of cDNA diluted 1:10, 5 µL of SYBR Green PCR master mix, 150 nM each primer and nuclease-free H_2O. Quantitative PCR was

conducted in the 7500 Fast Real-Time PCR System (Applied Biosystems™, USA) and the SYBR® Green amplification conditions consisted in an initial denaturation of 5 min at 95 ˚C, followed by 45 cycles of 15 s at 95 ˚C (denaturation), 15 s at 60 ˚C (annealing temperature), and 15 s at 72 ˚C (extension). Target genes expression were normalized by the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Gene ID: 2597) (forward 5'-GTGCTGAGTATGTCGTGGAGT-3'; reverse 5'-TTGTCATATTTCTCGTGGTTCA-3'; amplicon 154-pb, GenBank NM_002046.3) and the relative gene expression was determined using $2^{-\Delta\Delta CT}$ method [28]. Statistical analysis of data was performed by one-way ANOVA followed by Dunnett's post-hoc test.

2.7. RNA-seq and analysis

Total RNA of SCC-9P, SCC-9CPR5 and SCC-9CPR11 cells (n = 2) was isolated and purified using Trizol® reagent protocol (Invitrogen, Carlsbad, CA, USA). RNA was quantified using the Qubit™ RNA BR Assay Kit (#Q10210) with the Qubit Fluorometer (Thermo Fisher Scientific, MA, USA). RNA integrity number (RIN) of each sample ranged between 9.7 and 9.8 (RNA ScreenTape kit, TapeStation 4200, Agilent Tech). A minimum of 400 ng input of total RNA was used to isolate mRNA with an NEBNext® Poly(A) mRNA Magnetic Isolation Module kit (NEB #E7490S/L, New England BioLabs) according to the manufacturer's instructions. The mRNA was eluted from magnetic beads with Tris buffer. cDNA libraries were prepared using VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina (NR605, Vazyme). Briefly, 8 µL (~100 ng) from each sample was fragmented at 85°C for 5 min (fragments of 250 - 450 bp). dUTP buffer (stranded mRNA library) was used to double strand cDNA synthesis. In the ligation step, we used 3 µL of adapter (to ~100 ng of mRNA input) from TRuSeq RNA Single Indexes – Set A kit (Illumina). Purified libraries were amplified for 12 cycles and final libraries were quantified using quality checked using Agilent Tapestation and were then pooled in equimolar solutions. Sequencing was performed using an Illumina NextSeq 2000 system (Central Laboratory of High-Performance Technologies in Life Sciences from State University of Campinas - LaCTAD-UNICAMP) operated under a 150-base paired-end read configuration. Image analysis, base calling and quality check were performed with the Illumina data analysis and Bcl2fastq version 2.20. Clean reads were aligned to the human genome assembly (GRCh38.p13) by using STAR version 2.7.11b (https://github.com/alexdobin/STAR) [29] with two-pass mode.

2.7.1. Differential expression (DE) analysis and functional enrichment analysis

DE analysis for bulk RNA-seq data were conducted by using DEseq2 packages in R. $|\log2FC| \leq -1$ and adjusted p-value ≤ 0.05 were considered as downregulated genes and $|\log2FC| \geq 1$ and adjusted p-value ≤ 0.05 were considered as upregulated genes, both were used as the threshold for differentially expressed genes (DEGs) [30]. For matched samples of P (SCC-9P), CPR5 (SCC-9CPR5) and CPR11 (SCC-9CPR11), genes with top 25% expression were adopted for principal component analysis (PCA) and heatmap plots indicating variance between CPR5, CPR11 and P samples. The geom_mark_ellipse function (ggforce) was implemented for area annotation in PCA plots. Venn diagrams and UpSet plot were utilized to visualize common and exclusive genes across comparisons. The Database for Annotation,

Visualization and Integrated Discovery (DAVID) was used to identify the functional enrichment of Gene Ontology (GO) terms across Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) on lists of differentially expressed genes (CPR5 or CPR11 vs P) ranked by fold-change. To explore the potential functional implications of these differentially expressed genes (DEGs), we performed pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The KEGG pathway enrichment analysis on lists of DEGs was conducted in the R envirorment using the clusterProfiler [31] and enrichplot [32] packages.

2.8. Statistical analysis

Statistical analysis for growth, migration and gene expression by RT-qPCR was conducted using the R environment (R version 4.3.1). The Shapiro-Wilk test was used to assess normality, and the F-test examined the homogeneity of variances. If the data were normally distributed with homogeneous variances, a t-test was performed. For non-normally distributed data or those with unequal variances, the Mann-Whitney U test was used. All tests were conducted at a significance level of 0.05, and results are reported with their respective p-values.

3. RESULTS

3.1. OSCC showed phenotypic alterations during establishment of chemo-resistant cell lines

Before the cisplatin resistance induction, the SCC-9P cells, with their typical polygonal cell morphology (Figure 2d) presented IC_{50} value for cisplatin of 4.44 μM (Figure 2a). After four intermittent rounds of treatment with 4.44 μM cisplatin (~115 days), it was possible to observe the generation of a heterogeneous cell population, displaying both polygonal and fusiform cells (SCC-9CPR5), which exhibited an IC50 value of 8.67 μM (Figure 2b,e). Following cisplatin resistance induction, with further four intermittent rounds of treatment with cisplatin 8.88 μM (2-fold higher to the initial IC50 value) generating a new cell population, SCC-9CPR11. That new cell population exhibited a fibroblast-like or mesenchymal morphology (Figure 2f), suggesting a complete epithelial-mesenchymal transition. Furthermore, SCC-9CPR11 cells presented a IC50 value for cisplatin of 11.13 μM (Figure 2c), indicating the establishment of cisplatin chemo-resistant cells. The RI of SCC-9CPR11 (2.5-fold), indicates cells with a high level of cisplatin-resistance, when compared to RI of SCC-9CPR5 of 1.95-fold [33].

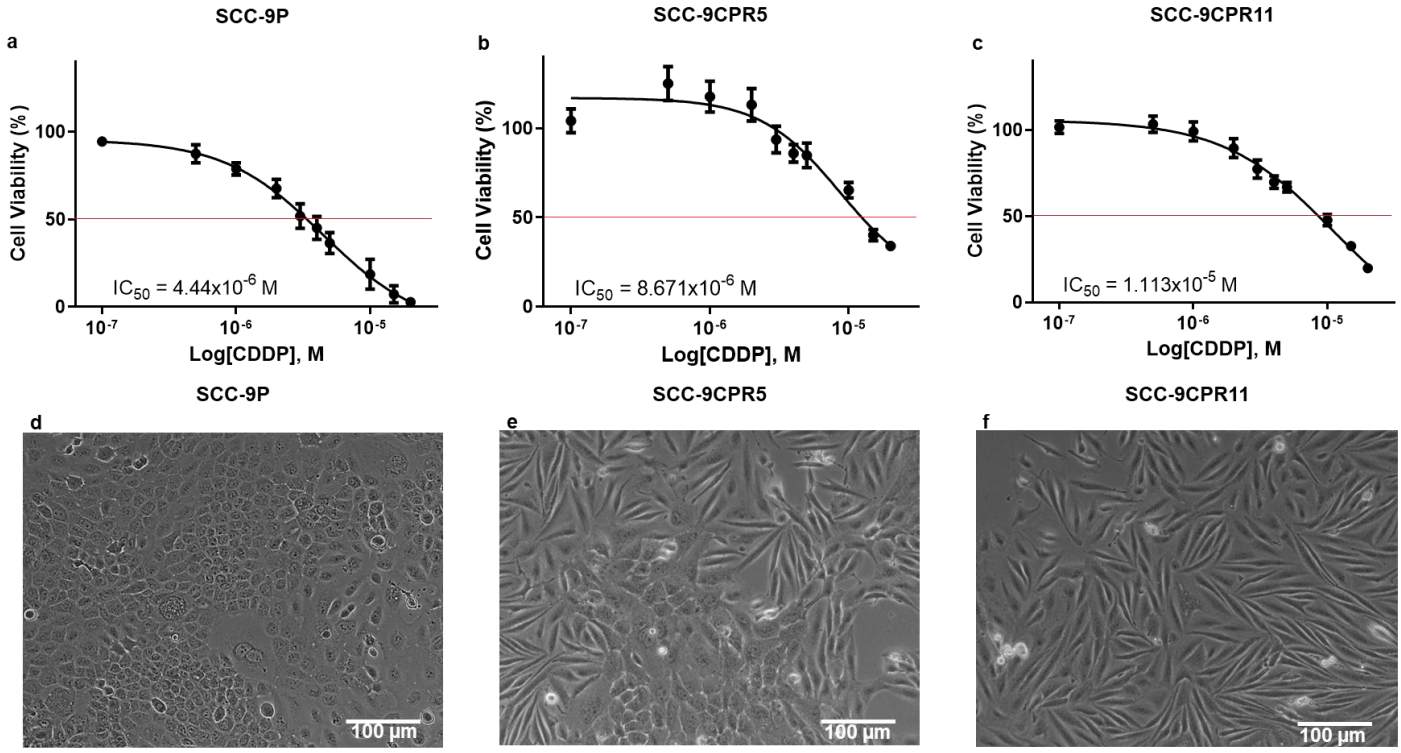

Figure 2. Morphological alterations and IC_{50} analysis after cisplatin resistance induction in OSSC cells (a-c) IC_{50} curve (log concentration) based on cell viability assay (%) express in molar (M) after 72 h of cisplatin exposure, showing higher IC_{50} values for cisplatin on the SCC-9CPR11. **(d-f)** Representative photomicrographs exhibiting the morphological changes occurred on the SCC-9P after cisplatin resistance induction in the SCC-9CPR5 and SCC-9CPR11. (The photomicrographs were taken at 10x magnification). Scale bars = 100 µm.

3.2. SCC-9CPR cells showed reduced cell growth

Besides exhibiting a different morphology in relation to SCC-9P cells, SCC-9CPR11 cells also showed a different growth pattern (Figure 3, Table 1). According to analysis of PDT, the SCC-9P cells took approximately 1.52 days to double its population, while SCC-9CPR11 presented a PDT of approximately 3.41 days. These results reveal that cell growth rate was almost 2-fold decreased in cisplatin resistant cells.

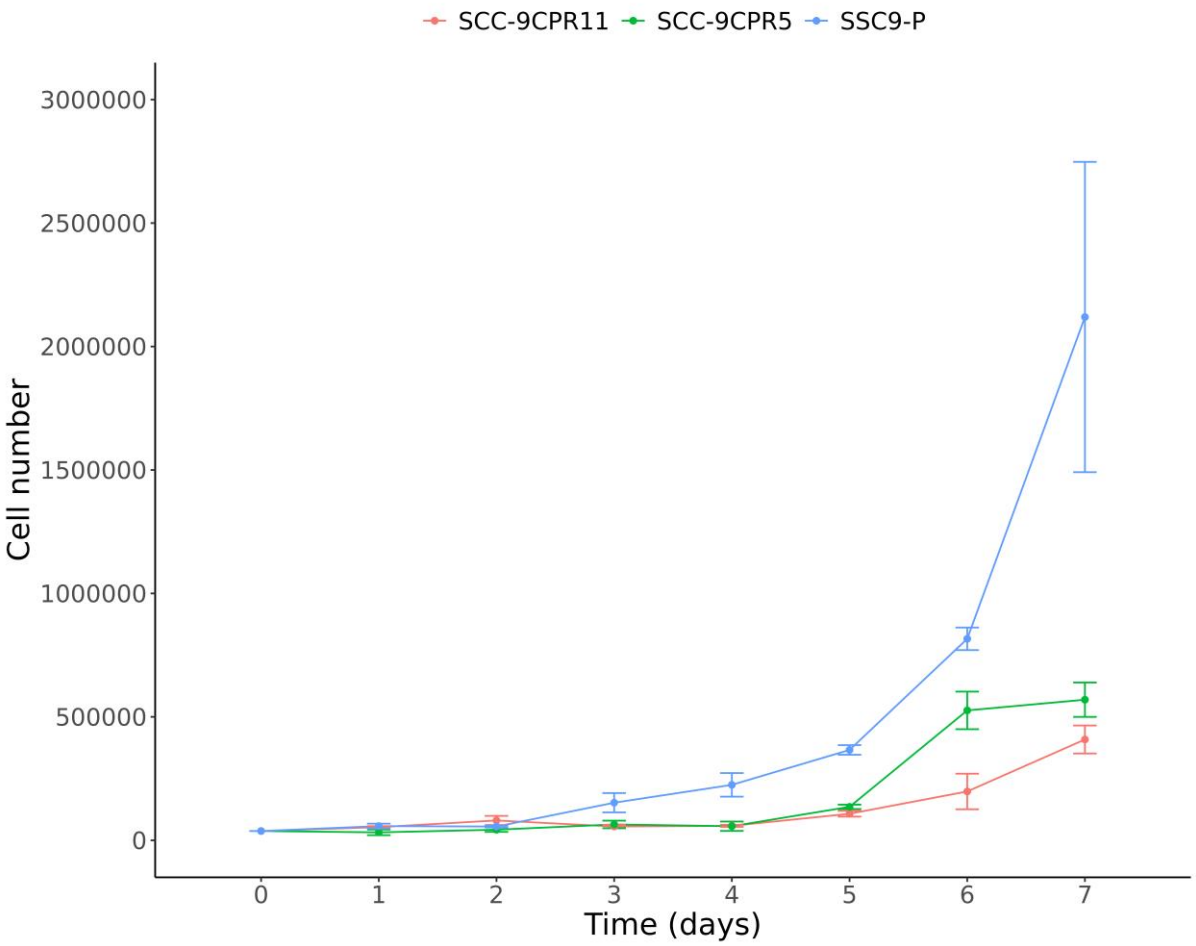

Figure 3. Comparison of the growth curves of cisplatin-resistant cell lines (SCC-9CPR5 and SCC9-CPR11) and parental cell line (SCC-9P) over 7 days in cisplatin-free culture medium.

Table 1. Growth characteristics of SCC-9P cell line and the established cisplatin resistant SCC-9CPR5 and SCC-9CPR11

Cell line	Population Doubling (PD)	Population Doubling time (PDT) (day)
SCC-9P	5.25	1.52
SCC-9CPR5	3.73	2.15
SCC-9CPR11	2.35	3.41

3.3. SCC-9CPR5 cells present increase in migratory profile

To further support the association of EMT characteristics with cisplatin resistance, the cell migration was evaluated by scratch assay. None of the cells were able to close completely the scratched area in 24 hours (Figure 4a). While the SCC-9-CPR5 cells exhibited a more accentuated migratory profile, both in terms of occupancy of the wound area (SCC-9P: 30%; SCC-9CPR5: 55%; p=0.000379, Figure 4b) and wound closure speed (SCC-9P: 9 µm2 /h; SCC-9CPR5 15 µm2 /h; p=0.04389, Figure 4c), the SCC-9CPR11 cells showed a stationary profile. However, the cells bordering the scratch displayed long processes and spindle like structures oriented towards the

scar, demonstrating a typical feature of migrating cells. Taken together, these findings suggest that the cisplatin-resistant SCC-9CPR5 cell line exhibits a migration pattern similar to metastatic cells.

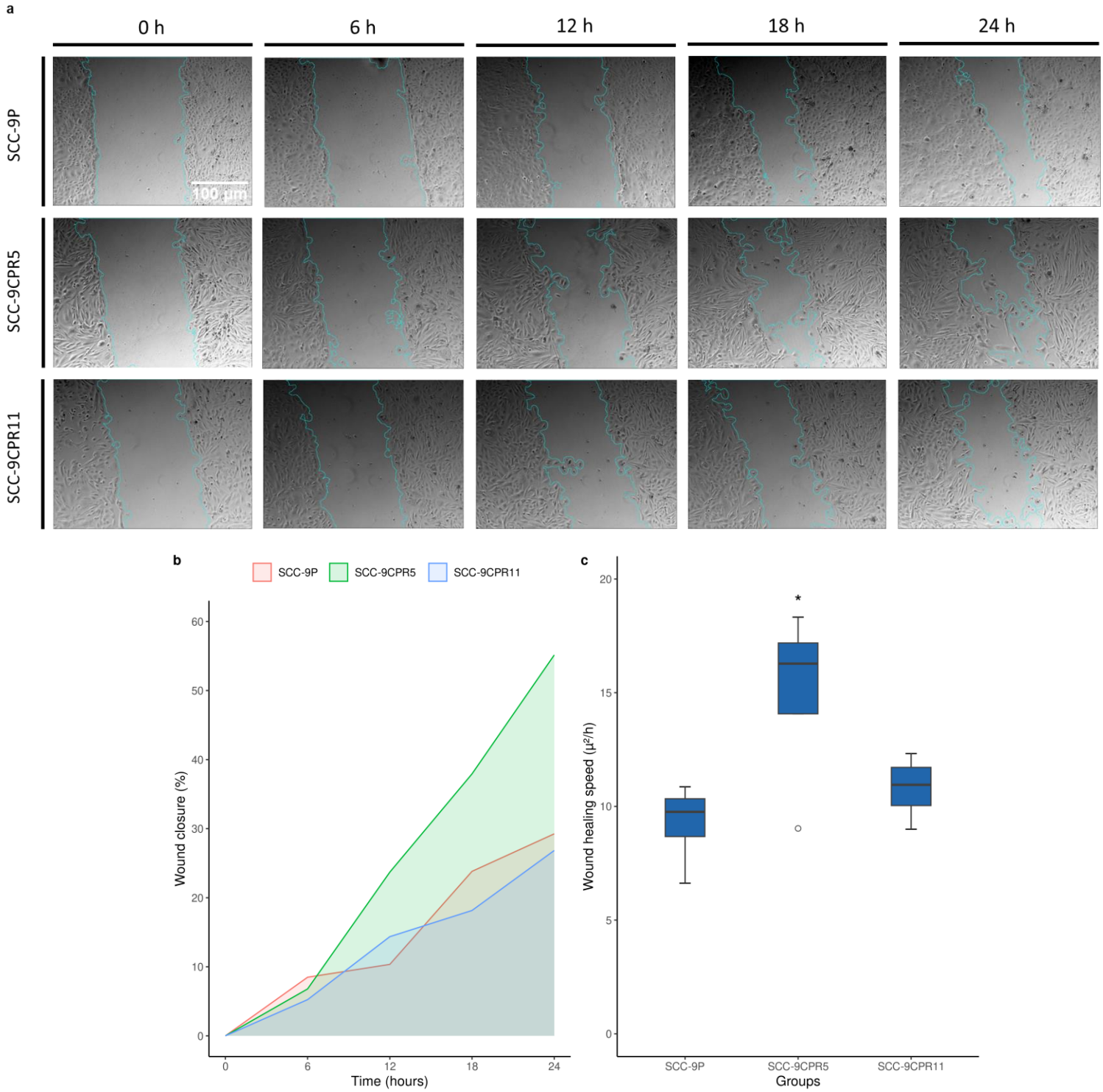

Figure 4. Cell migratory profile in scratch assay for 24 hours. (a) Images show scratched areas at 0 h and wound closure by different cell lines every 6 hours for 24 hours of culture. **(b)** Plot shown represent the mean of percentage of the cell-free area covered by cells after every 6 hours (n = 4). **(c)** Boxplot of migration speed to closing scratched area after 24 h (*p<0.05). Scale bar = 100 µM.

3.4. SCC-9-CPR11 cells overexpress mesenchymal markers

Some markers have been shown to induce EMT, in which epithelial cells undergo morphological alterations acquiring mesenchymal cell phenotype and other properties. Based on that, we analyzed the EMT-markers gene expression in the SCC-9P and SCC-9CPR (CPR5 and CPR11) cells (Figure 5). The results showed an overexpression of ZEB1, ZEB2, SNAIL1 and Vimentin (VIM) in both cisplatin-resistant cells compared to SCC-9P cells. Specifically, in CPR5 cells, ZEB1 expression was increased in 3.85-fold ($p < 0,001$), ZEB2 in 28.33-fold ($p < 0,01$) SNAIL1 in 3.33-fold ($p < 0,01$) and VIM in 17.89-fold ($p < 0,001$). Meanwhile, CPR11 cells exhibited an increase of ZEB1, ZEB2, SNAIL1 and VIM gene expression in 6.85-fold ($p < 0.001$), 35.91-fold ($p < 0.001$), 2.57-fold ($p < 0.05$) and 49.15-fold ($p < 0.001$), respectively, in relation to SCC-9P cells (Figure 5). In addition, the mRNA levels of other mesenchymal markers such as TWIST2 and N-CAD (CDH2, N-cadherin) were increased in CPR11 cells (Figure 5). Interestingly, SNAIL2 gene was found downregulated in CPR11 in relation to parental cell line ($p < 0,01$). We could observe the clearly the inversion gene expression in relation to CDH1 (E-CAD, E-cadherin) (downregulated) and CDH2 (upregulated) in CPR11.

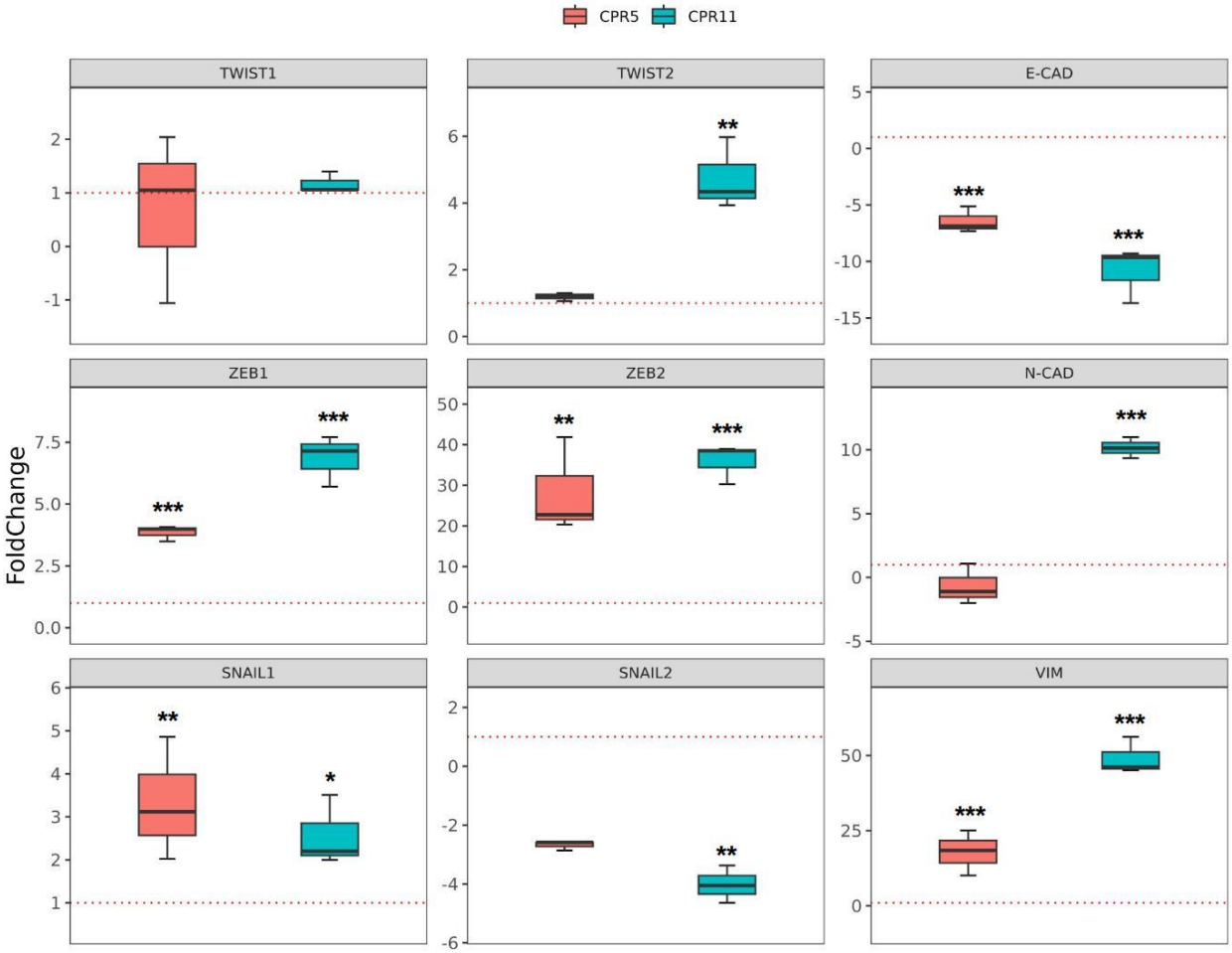

Figure 5. The overexpression of Snail and Vimentin are correlate to epithelial-mesenchymal transition (EMT). RT-qPCR was performed to determine the relative mRNA expression levels of Zeb1, Zeb2, CDH2 (N-CAD, N-cadherin), CDH1 (E-CAD, E-cadherin), VIM (Vimentin), Snail1, Snail2, Twist1 and Twist2, with *GAPDH* as housekeeping gene. The boxplot data are shown at significance level *p<0.05 **p<0.01 ***p<0.001.

3.5. Resistance level displays clusters of different heterogeneity

Using RNA-Seq data to establish determinants intracellular and intercellular transcriptomic diversity, we performed independent principal component analyses (PCAs) within the major CDDP resistant SCC-9 groups revealing that samples were aligned along resistance levels (Figure 6a) after pre filtering and normalization of data (Supplementary Figure 1a,b) . Next, we selected the top 1000 highly variable genes based on fold change and padj and visualized their expression profiles on a heatmap with the rows (genes) and columns (samples replicates) clustered across resistance levels (Figure 6b). The heatmap was partitioned into the three top branches identified by hierarchical clustering. Importantly, while CPR11 showed an opposite pattern from P, the CPR5 samples displayed some similar features with CPR11. Therefore, we also could identify two small clusters representing opposite identities among the CPR sample groups at the same region. From this heatmap emerged genes related to transcriptional signatures in EMT and resistance drug.

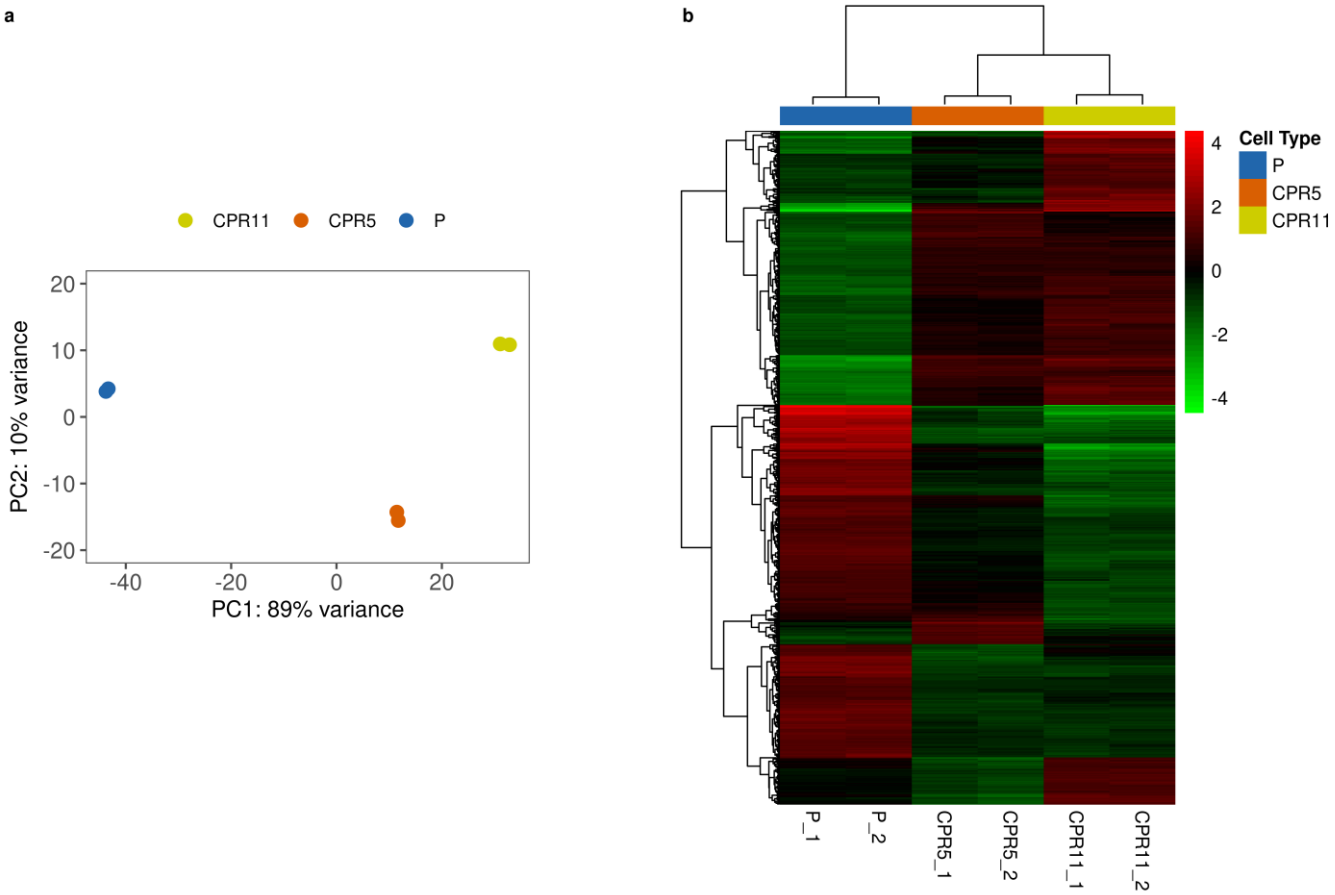

Figure 6. Transcriptional heterogeneity among different groups CDDP resistant levels (a) Scatter plot of the PCs PC1 (89%) and PC2 (10%) of RNA-seq samples based on resistance group levels from P, CPR5 and CPR11 (n = 2). **(b)** Heatmap of top 1000 highly varying genes, with rows (genes) and columns (samples) sorted by hierarchical clustering. The columns are split into three groups as determined by a 3-way cut of the dendrogram. P blue, CPR5 red and CPR11 green.

3.6. Identification of DEGs between resistant and non-resistant samples indicates a pattern between resistance level and the number of genes regulated unmasking EMT genes

There were 2930 DEGs between CPR5 and P (1476 down-regulated and 1454 up-regulated), 3697 DEGs between CPR11 and P (1936 down-regulated and 1761 up-regulated) and 1672 DEGs between CPR11 and CPR5 (848 down-regulated and 824 up-regulated). Volcano plot (Figure 7a) indicated all the differentially expressed mRNAs that were statistically significant among the groups. Furthermore, to identify distinct and shared transcriptional signatures between resistant cells, we performed a comparison among the groups. Of the total DEGs found between CPR5 and P (2930) and between CPR11 and P (3697), 2137 genes were shared between CPR5 and CPR11, while 1560 genes were differentially expressed only in CPR11 (relative to P) and 793 genes were differentially expressed only in CPR5 (relative to P) (Figure 7b). Interestingly, we could identify 54 genes that shown down-regulated in CPR5 and up-regulated in CPR11, and 16 genes that shown up-regulated in CPR5, but down-regulated in CPR11 (Figure 7c, Supplementary Figure 2a,b). Besides that, the major DEGs to EMT could be identified such as *CDH1*, *CDH2*, *ZEB1*, *ZEB2*, *SNAIL1*, *SNAIL2*, *TWIST1*, *TWIST2* and *VIM*. In both resistant groups, CPR5 and CPR11, *VIM*, *ZEB1*, *ZEB2*, *TWIST1* were found up-regulated, otherwise *CDH1* was down-regulated. While *SNAIL1* was the fourth highly expressed at CPR5 in relation to P, *SNAIL2* kept unchanged not only in CPR5, but also in CPR11. Some genes showed no expression differences such as *TWIST2* and *CDH2* in CPR5 – but up-regulated in CPR11 -, and *SNAIL1* in CPR11. Taken together, these results showed that although many genes were differentially expressed, the EMT markers can be variated between CDDP resistance levels groups.

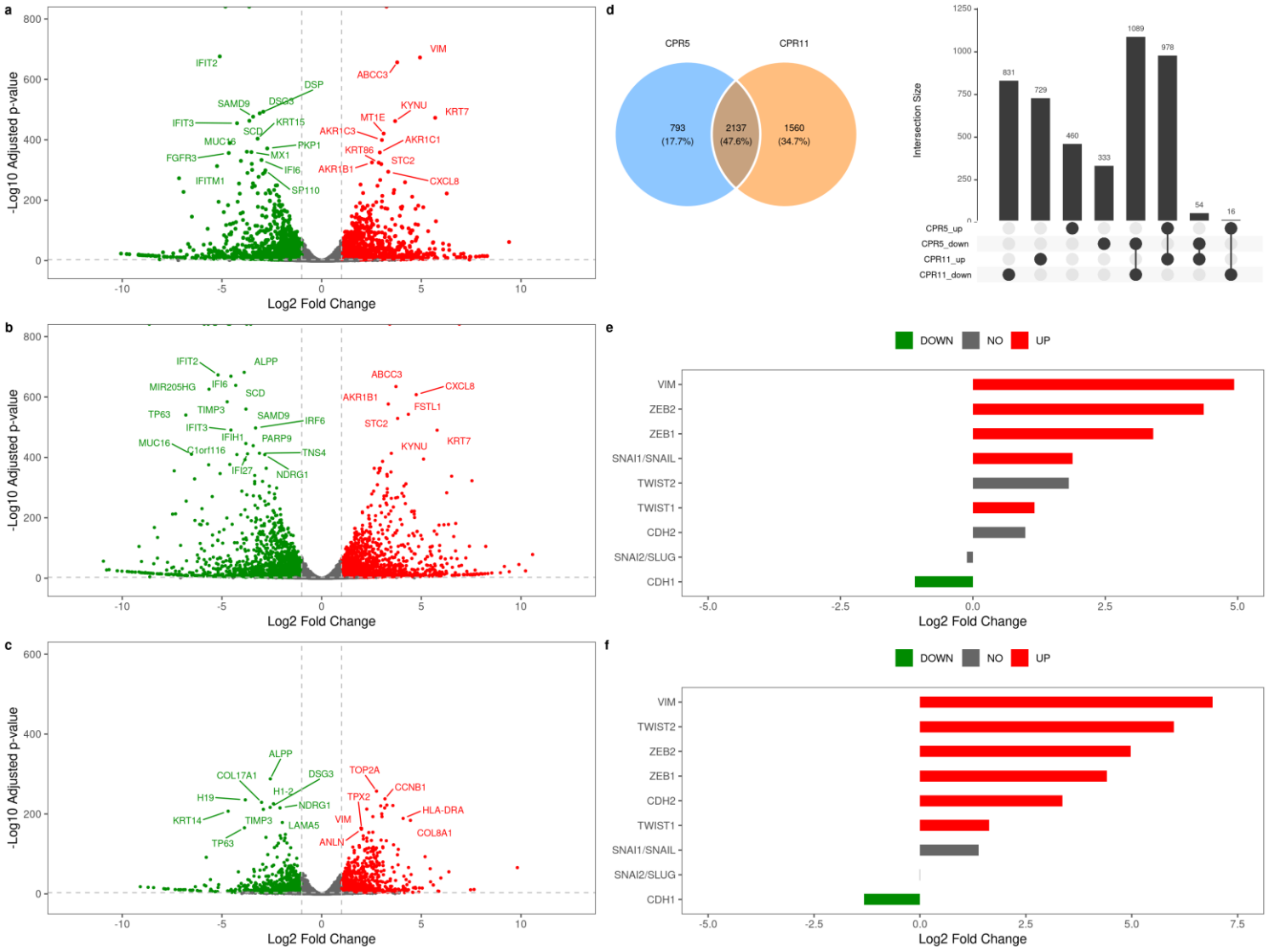

Figure 7. Transcriptional signatures based on DEGs isolated and shared between resistant groups relative to P group and identification of driver genes profiling EMT (a) Volcano plot showing all DEGs in each comparison. Genes with Log2FC ≤ -1 and padj ≤ 0.05 were considered down-regulated (green), while those with Log2FC ≥ 1 and padj ≤ 0.05 were considered up-regulated (red). **(b)** Venn diagram showing DEGs common (intersection point) and unique between the groups. Genes highlighted in light blue and orange are showed altered expression in CPR5 or CPR11 relative to P, respectively; and genes highlighted in dark blue are observed to be altered in both CPR5 and CPR11. **(c)** Upset plot displays DEGs in combinations of down and up-regulated (connected bullet points) between groups. Numbers of exclusive DEGs are indicated in each bar according to expression status. **(d)** Bar graphs showing the most frequent EMT DEGs down-regulated in green, up-regulated in red and without changes in grey in both groups CPR5 and CPR11**.**

3.7. Shared enriched GO-terms and genes could be identified between CPR5 and CPR11

In order to identify potential biological roles of DEGs in these groups, we performed Gene Ontology term enrichment analysis. The plotted graphs represent the top 10 terms based on the adjusted p-value (Fisher's exact test corrected by Benjamini-Hochberg). We found the following biological process enriched in both resistant groups: cell adhesion, angiogenesis, extracellular matrix organization, epidermal

development, inflammatory response and cell-cell adhesion, with cell adhesion (GO:0007155, p = 5.992337e-22 for CPR5, p = 2.702383e-24 for CPR11) and angiogenesis (GO:0001525, P = 6.030375e-12 for CPR5, p = 2.44496e-15 for CPR11) the GO-terms most significantly enriched. Besides that, extracellular exosome and calcium ion binding were also GO-terms enriched in relation to molecular function (Figure 8a,b, Supplementary Figure 3a-d). Further enrichment chord analysis (Figure 8c,d) showed important genes shared between more than one biological process and both levels of resistance. The differential genes involved in the cell and cell-cell adhesion were downregulated such as: *CDH8*, *DSC3* and *NECTIN1,* otherwise *ITGA4* was found upregulated, however in CPR5 it is involved in an additional biological process, extracellular matrix organization. Importantly, *MMP2* gene showed highly expressed in both CPR5 and CPR11 composing angiogenesis process, but also positive regulation of cell migration for only CPR11 and extracellular matrix organization in CPR5.

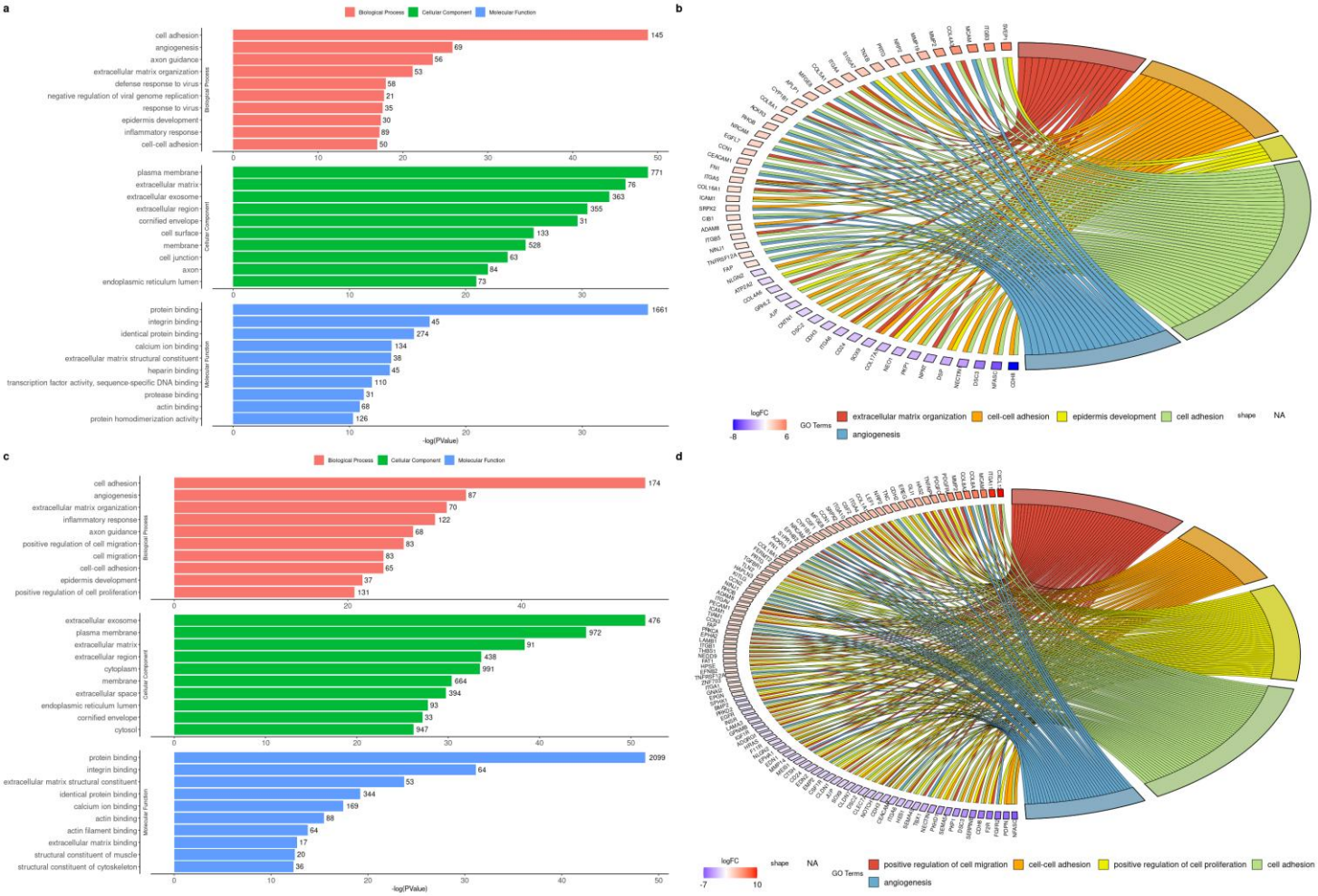

Figure 8. GO-terms and genes enriched in groups with different levels of cisplatin resistance. Bar graphs representing the top 10 Gene Ontology terms. Differential expression enriched GO-terms between **(a)** CPR5 and P **(b)** CPR11 and P (Biological Process in red, Cellular Component in green and Molecular Function in blue). The number of DEGs in each term is represented in front of each bar. **(c,d)** Chord plot with genes involved/shared in five biological process GO-terms enriched, being to CPR5 (c): extracellular matrix organization, cell-cell adhesion, epidermis development, cell adhesion, angiogenesis; CPR11 (d): positive regulation of cell migration, cell-cell adhesion, positive regulation of cell proliferation, cell adhesion and angiogenesis.

3.8. Hierarchical clustering of enriched pathways highlighting the PI3K pathway with genes related to cisplatin resistance

The lists of genes differentially expressed between the resistant groups, together with the Log2FC data, were subjected to pathway enrichment analysis in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Of all the mapped pathways, the top 20 pathways, based on Benjamini-Hochberg (BH)-corrected p-values, were plotted in bubble plots. Some pathways are enriched in both groups, such as proteoglycans in cancer, cytoskeleton in muscle cells, PI3k-Akt signaling and HPV (Human Papilloma Virus) infection (Figure 9a,b). According to SCC-9 cell line origin, the HPV infection pathway enrichment was pointed out in downregulated genes both in CPR5 and CPR11 which is independent of the level of resistance to cisplatin (Supplementary Figure 3e-h). As described above, the differential signatures were tested for enrichment with pathways between CDDP resistance levels groups in relation to P group. We then used the hierarchical clustering of enriched pathways analysis based on ontology as input identifying a significant node to PI3K-Akt signaling pathway (padj≤0.01) in both CPR5 (80 genes) and CPR11 (100 genes) (Figure 9c,d). However, while CPR5 up-regulated showed a relationship with MAPK signaling pathway (30 genes) and proteoglycans in cancer (20 genes) (padj≤0.01), besides PI3K-Akt signaling, CPR11 up-regulated was significantly connected to focal adhesion (30 genes) (padj≤0.01) with around 30 genes (Supplementary Figure 4b,d). Otherwise, looking about down-regulated genes, in both CPR5 and CPR11, the connection was significantly established with HPV infection and proteoglycans in cancer (padj≤0.01) (Supplementary Figure 4a,c). In order to investigate more about genes in PI3K-Akt signaling pathway, we used PATHVIEW (data not showed) to visualize enriched KEGG pathways. Based on that, we could identify an overexpression of Akt3 while PIK3 is downregulated in both groups. Interestingly, the CPR11 group showed an upstream Hsp90 upregulation at PI3K-Akt signaling pathway.

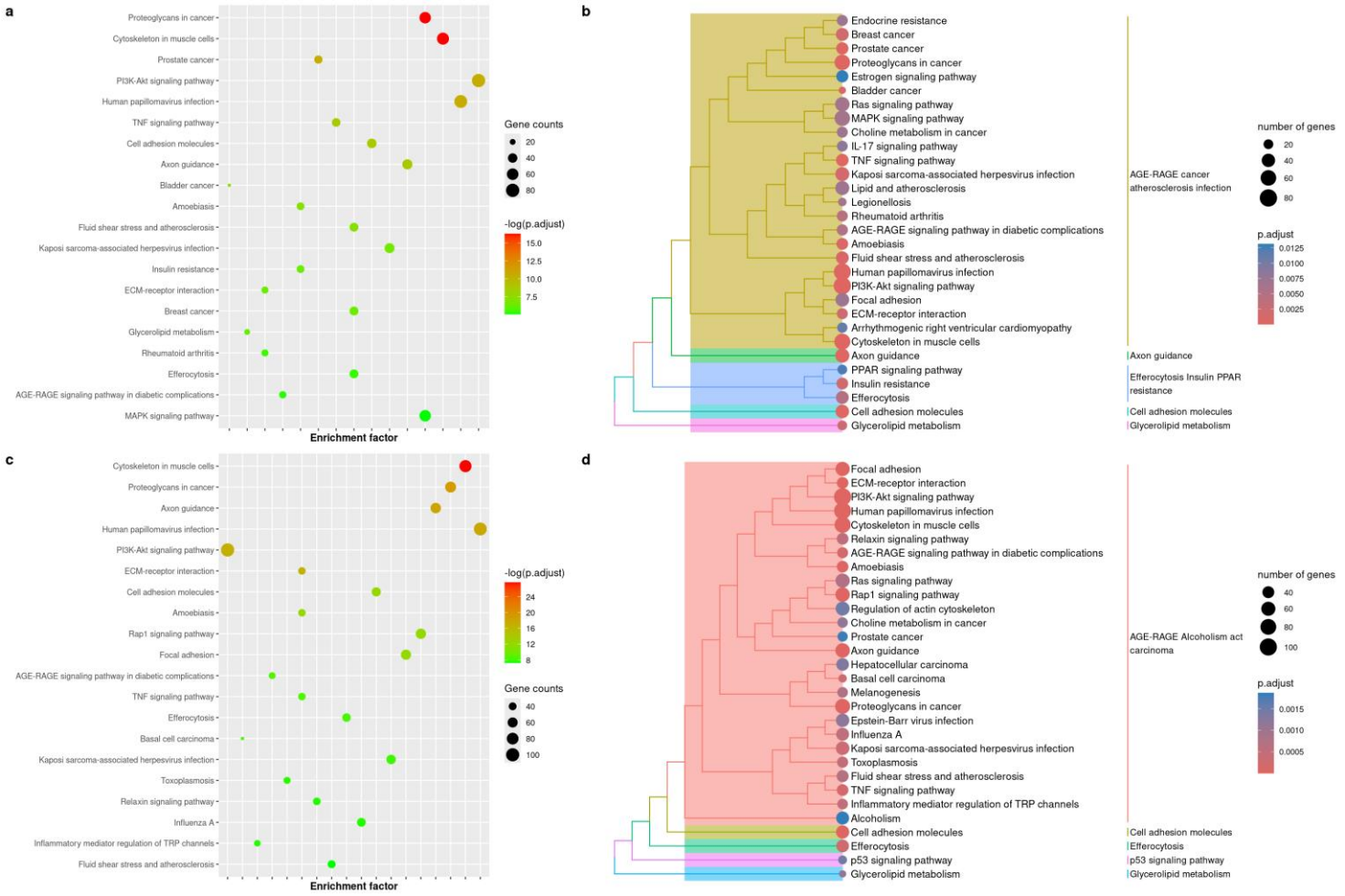

Figure 9. KEGG pathways enriched in groups based on DEGs. Bubble plots showing the top 20 KEGG pathways, which the size of the bubbles in the plot represents the number of enriched DEGs in each pathway, color of the bubbles represents the p.adjust value, the Y-axis shows the relevant pathways and X-axis label represents enrichment factor (enrichment factor = amount of differentially expressed genes enriched in the pathway/amount of all genes in the background gene set) **(a)** CPR5 and **(b)** CPR11, in comparison to P. **(c)** Hierarchical clustering tree plot of enriched pathways in CPR5 and **(d)** CPR11. Pathway ranking was based on p.adjust (BH) values (node color), with size representing the number of genes found to be differentially expressed in each pathway.

4. DISCUSSION

In the present study, we established an in vitro cisplatin resistance model in the tongue squamous cell carcinoma cell line (SCC-9), considered one of the most aggressive oral cavity tumor cell lines. Although this kind of mechanistic study is well evidenced in the literature, with different models of resistance to chemotherapy drugs in vitro, we set out to develop a new resistance protocol. This allowed us to access three different levels of drug sensitivity and characteristics related to their transcriptional signature and phenotypes. Thus, when evaluating the growth and migration profile of the resistant cells considered as final, SCC-9CPR11, we were able to observe a reduction in cell proliferation associated with the more fusiform morphological profile like fibroblasts. On the other hand, its migratory potential was reduced, especially when compared to SCC-9CPR5, identified with intermediate resistance level to cisplatin. These data agree with the segregation of groups according

to the cellular state, and while a totally mesenchymal state remains controversial in relation to metastasis and resistance to treatment, many studies confirm the presence of cells in the hybrid state has a relevant association with these conditions as well as poor prognosis and outcomes for patients [34].

Furthermore, Pastushenko et al., in a study with single-cell RNA (scRNA) sequencing and Assay for Transposase-Accessible Chromatin (ATAC-seq), showed different stages of EMT associated with chromatin remodeling, identifying the hybrid state associated with a greater metastatic potential in mammary tumors in mice [35].

Although some genes are classically correlated with EMT, following an expression pattern for different tumor types, this profile can vary according to the level of resistance. Thus, we can see, for example, that the process of gene regulation during resistance to CDDP and phenotypic change is cancer-dependent, since, for some tumor types such as ovarian cancer, SNAIL2 is up-regulated and is strongly correlated with EMT and cisplatin resistance [36]. However, intriguingly we found SNAIL2 down-regulated in the CPR5 and CPR11 groups, suggesting that SNAIL2 may be supressed by SNAIL1 in these resistant cell lines [37]. As what characterizes EMT is mainly the loss of cell-cell adhesion and extracellular matrix [20], when we identified the enrichment of genes involved in cell adhesion by GO-PB, we were able to verify that these were terms enriched in both resistant groups. Interestingly, Desmocollin 3 (DSC3), a desmosomal cadherin required for maintaining cell adhesion in the epidermis, was found down-regulated in cell adhesion/cell-cell adhesion GO-terms in both CPR5 and CPR11 groups. Previously, a study reported DSC3 down-regulation is correlated with dedifferentiation and more aggressive tumor in OSCC [38]. Still in order to elucidate the relationship between resistance and morphological changes in GO, we observed a term enriched in CPR5 down-regulated genes related to the binding of calcium ions (Ca2+). Increasing evidence shows that in several tumor types such as breast, ovary, bladder, the increase in Ca2+ concentration with the use of cisplatin leads to apoptosis [39]. Therefore, low levels of calcium influx are characteristic of cells resistant to cisplatin, in addition to microcalcifications being correlated with EMT markers in an unclear manner [40].

Several pathways have already been identified related to EMT and cisplatin resistance, as well as the description of many driver genes. The TGF-β pathway has been one of the most studied in relation to EMT markers, including in oral cavity cancer, in which it appears enriched [41]. However, TGF-β signaling is involved in a complex intracellular signaling network with several other pathways, one of which is the non-canonical PI3K-Akt pathway [42]. Here we show an enrichment of the PI3K-Akt pathway, in which Akt is found up-regulated, which can be correlated with resistance to cisplatin and CSCs as already reported in the study by Kouba et al., highlighting authors who demonstrated this associative relationship in their study [39]. Additionally, we could identify up-regulation of TGF-β1I1 and TGF-β2 in both comparisons performed. Interestingly, the HPV pathway was found enriched in both resistant groups but correlated with down-regulated genes (Supplementary Figure 3a,c). This is possibly due to the fact that these cells are originally HPV negative. We were also able to observe poorly described genes for oral cavity cancer DKK1, NEK2 [43], CMIP, DGLAP5 (Supplementary Figure 2a,b), which DKK1 (Dickkopf-related protein 1) highly up-regulated in CPR11, but down-regulated in CPR5 and its mechanistic role remains unclear in OSCC.

Although we could identify different phenotype and a molecular signature to EMT programing in SCC-9 resistant induced with cisplatin, the protocol was only applied to one type of oral cancer cell line and in monolayer model, what configures limitations of our study. Finally, our transcriptome analysis approach not utilized the public database to better estimate the correlation with primary oral tumors.

In summary, the development of an in vitro model of cisplatin resistant tongue SCC encompassed important changes during the EMT process, as well as by identifying different cell migration and growth profiles, in addition to showing variation in genes differentially expressed between resistant cells and between resistant and parental cells, where we were able to observe enrichment of canonical and non-canonical pathways revealing new genes involved in this process. Therefore, our study may contribute in the future to more complex data mining associated with other factors such as the involvement of epigenetic mechanisms that may be regulating some of the complexes already described so far.

AUTHOR CONTRIBUTIONS

Dieila Giomo de Lima: Formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **Gabriell Bonifácio Borgato:** Formal Analysis; methodology; visualization; writing – review and editing; **André S Vieira:** Resources; visualization; writing – review and editing; **Alessandro Santos Farias:** Resources; visualization; writing – review and editing; **Gustavo Narvaes Guimarães:** Formal analysis; data curation; methodology; writing – review and editing. **Ana Paula de Souza:** Conceptualization; project administration; resources; supervision; funding acquisition; writing – review and editing. The work reported in the article has been performed by the authors, unless clearly specified in the text.

FUNDING INFORMATION

This research project was partially supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (Proc. 140439/2020-0). The authors thank Life Sciences Core Facility (LaCTAD), University of Campinas-UNICAMP

CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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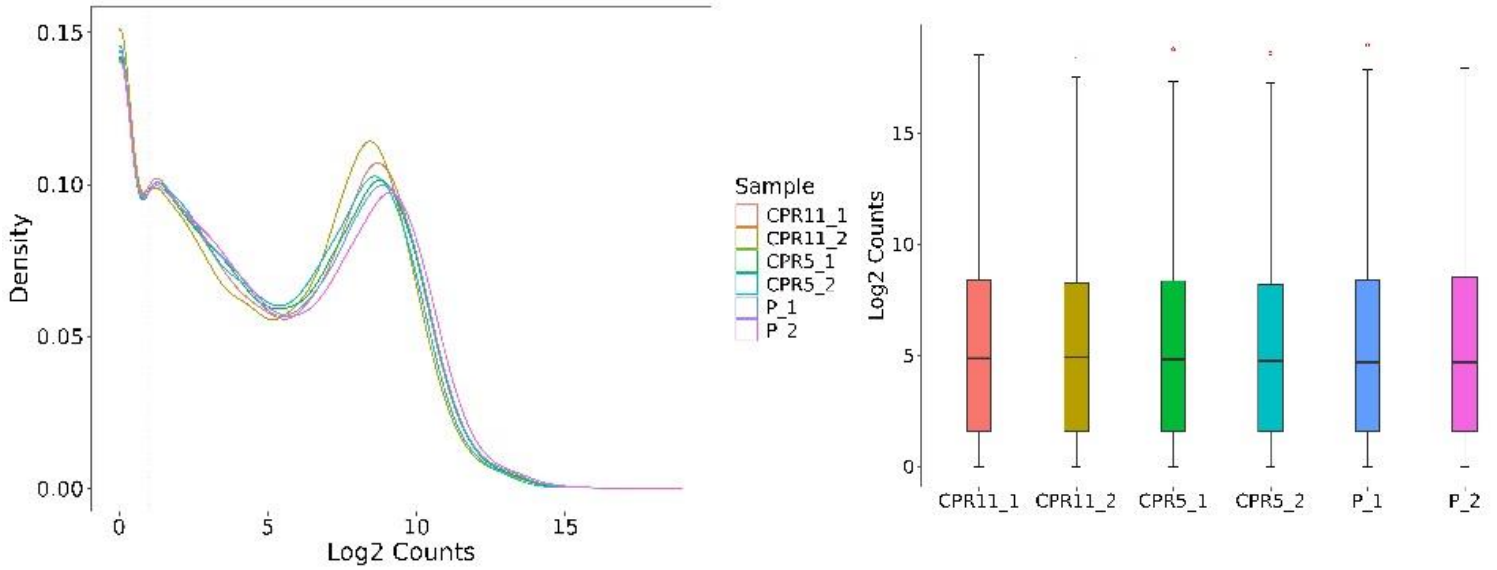
Supplementary Table 1. Primer sequences for RT-qPCR

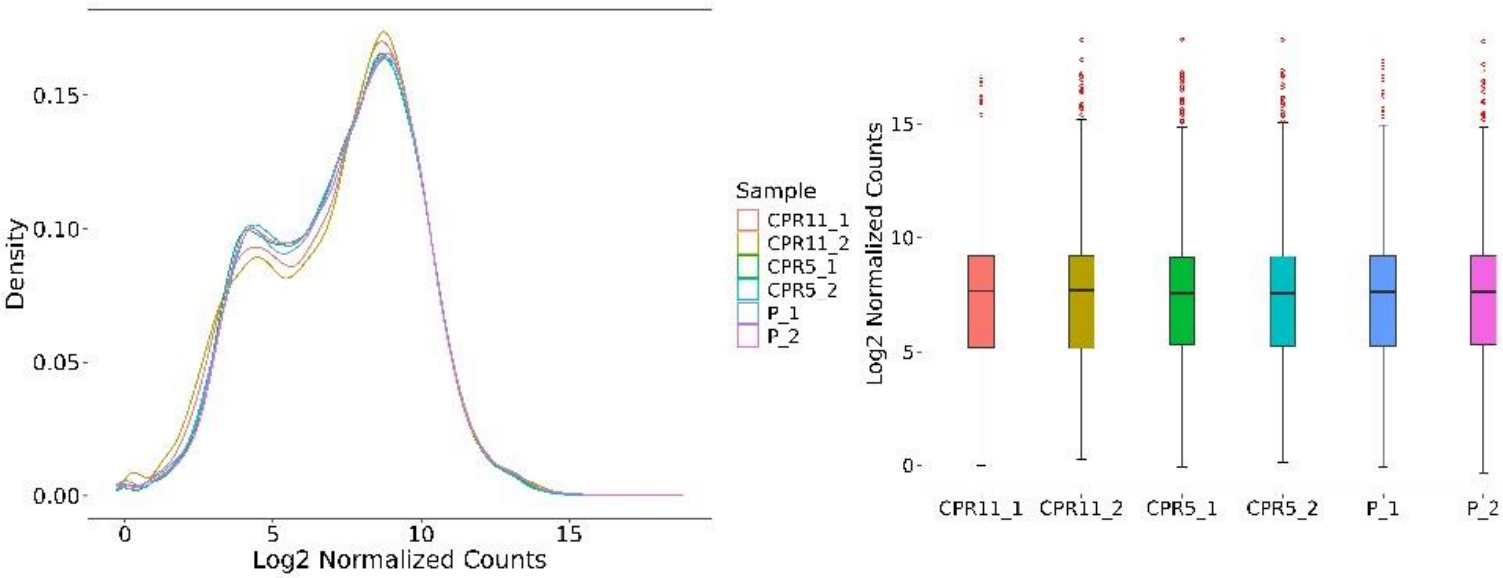
Gene name	Forward 5’ – 3’	Reverse 5’ – 3’	Product (bp)	Tm (˚C)
ZEB1	GCTTTTCCCATTCTGGCTCCTA	TCTTGGTCGCCCATTCACA	81	60
ZEB2	CACACACATACACAGAAAGGA	ATAACAGGAGGCATAGCATT	157	60
N-cadherin	GCGTCTGTAGAGGCTTCTGG	GCCACTTGCCACTTTTCCTG	293	60
E-cadherin	ACAGCCCCGCCTTATGATT	TCGGAACCGCTTCCTTCA	56	60
Vimentin	GACGCCATCAACACCGAGTT	CTTTGTCGTTCGTTAGCTGGT	248	60
Snail2	GGAGCATACAGCCCCATCA	TGGGTAGCTGGGCGTGGAA	61	60
Snail1	GCGTGTGCTCGGACCTTCT	ATCCTGAGCAGCCGGACTCT	67	60
Twist1	AAGCTGAGCAAGATTCAGACC	CGTGAGCCACATAGCTGC	118	60
Twist2	GCAAGAAGTCGAGCGAAGAT	GCTCTGCAGCTCCTCGAA	92	60

All of the primers were synthesized by Exxtend (Exxtend, São Paulo, Brazil).

a

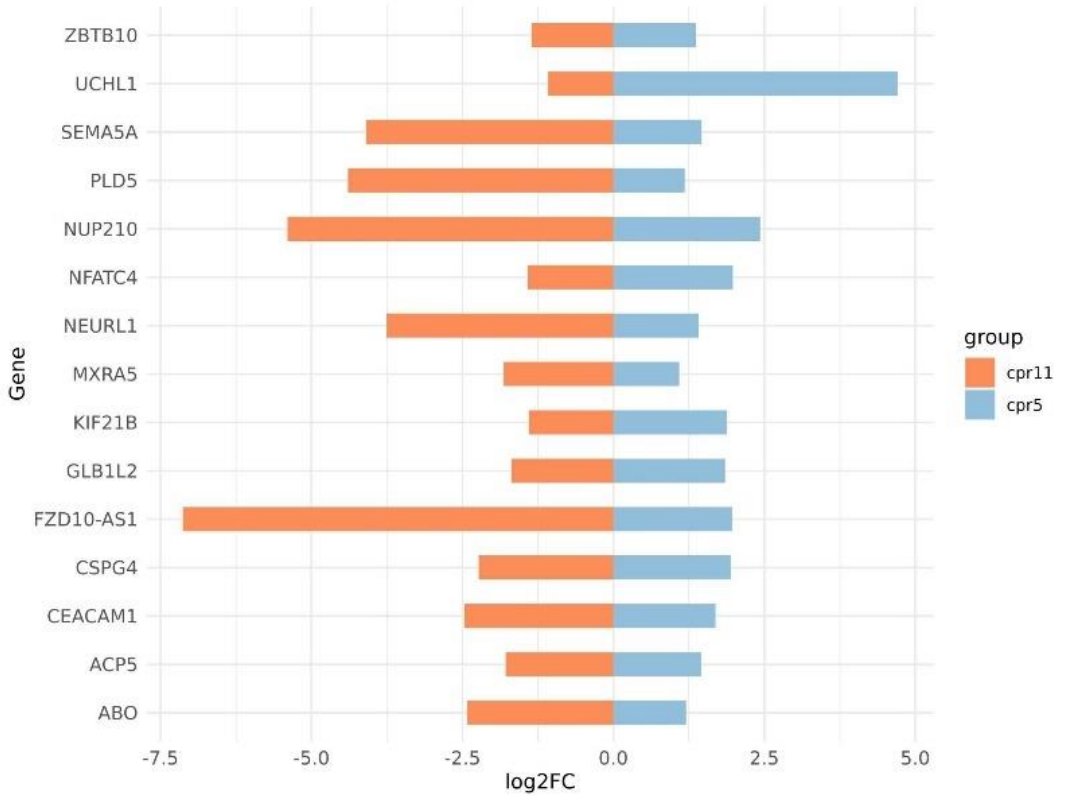


b

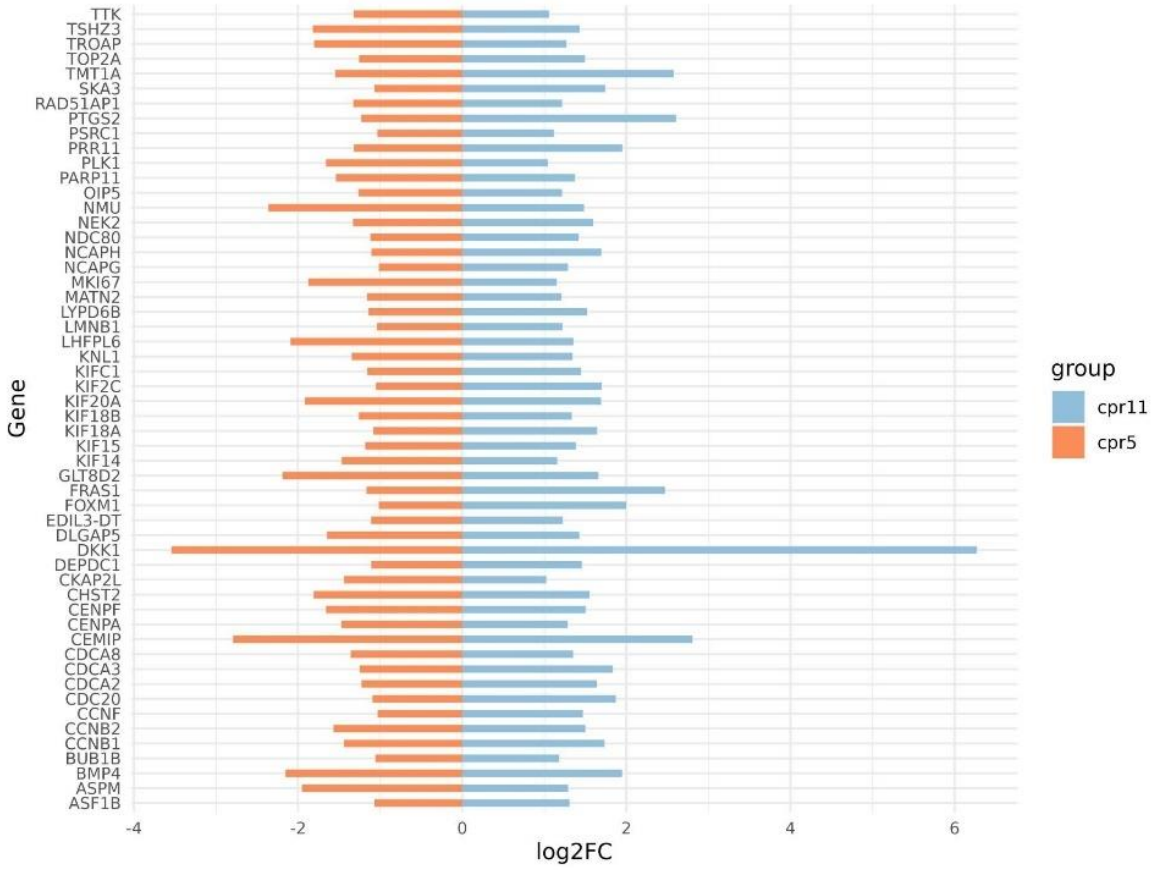


Supplementary Figure 1. Initial parameters from DESeq2 (a) Density graph and boxplot of raw counts (log2) per sample. **(b)** Density graph and boxplot of counts (log2) filtered and normalized per sample.

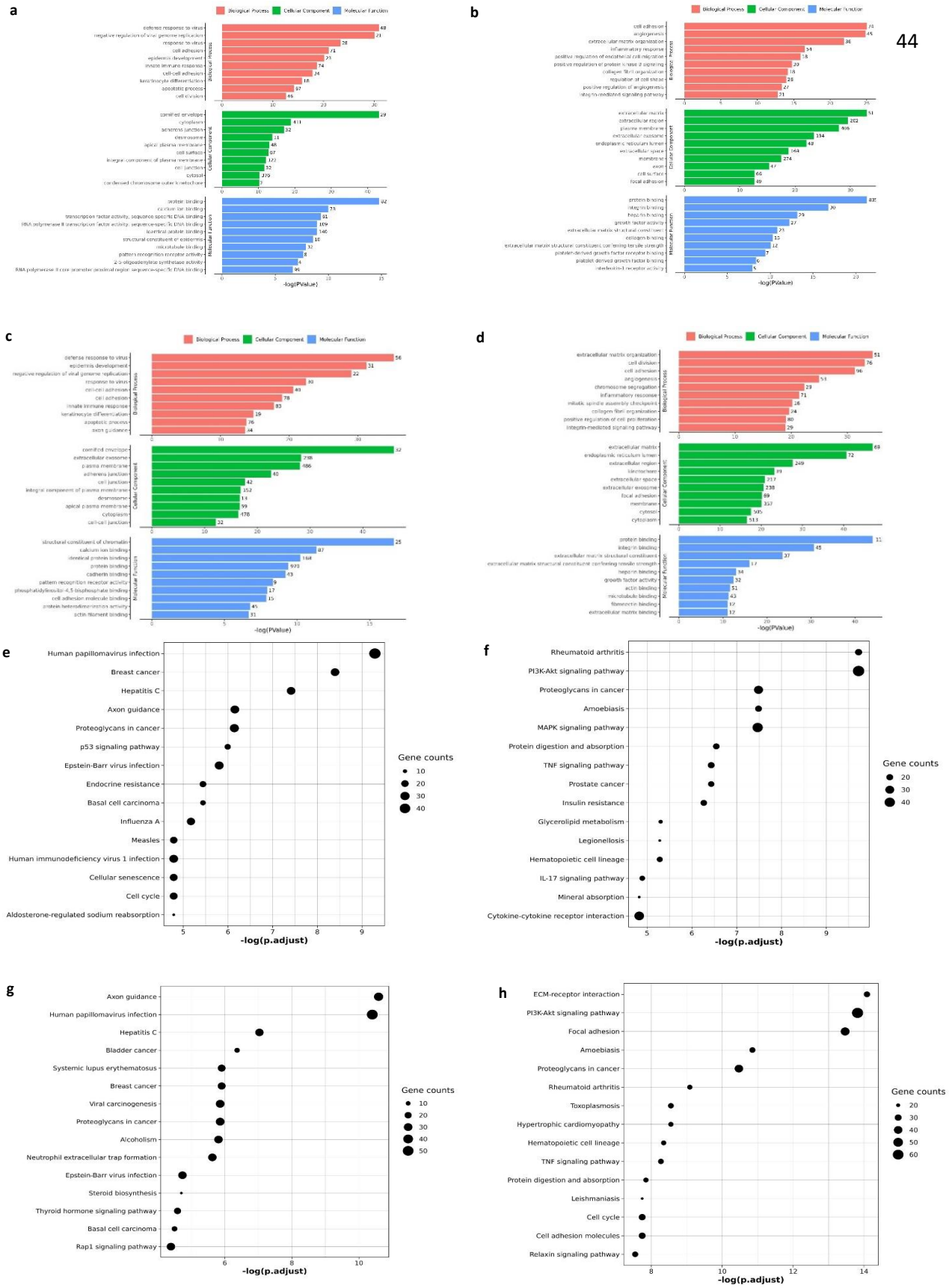
a



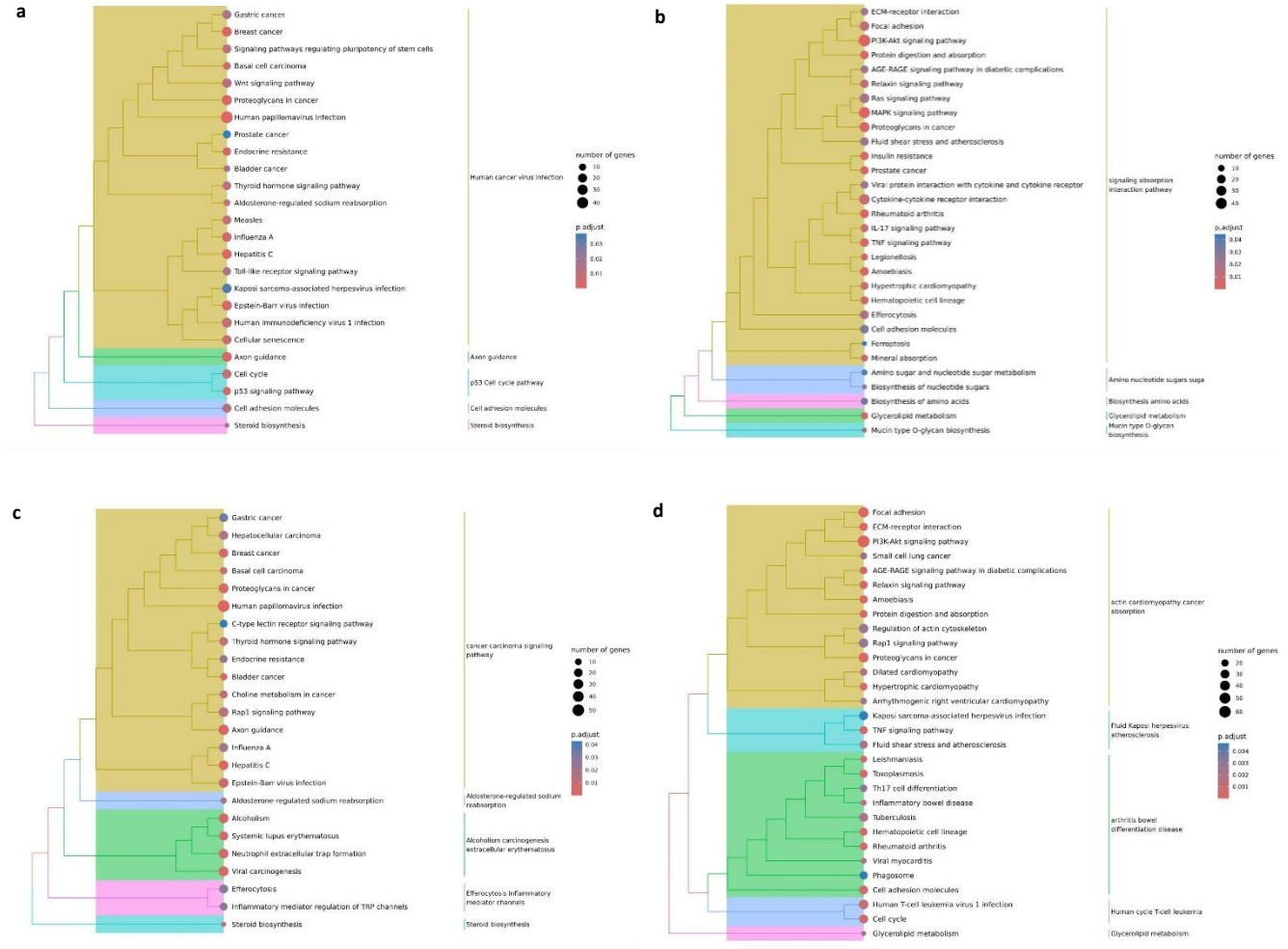
b



Supplementary Figure 2. DEGs comparison between CPR5 and CPR11 in relation to P (a) Genes down-regulated in CPR11 (orange bars) and up-regulated in CPR5 (blue bars) (in relation to P) showing the values to Log2FC on the X-axis. **(b)** Genes up-regulated in CPR11 and down-regulated in CPR5 (in relation to P) showing the values to Log2FC on the X-axis.



Supplementary Figure 3. Top 10 enriched GO-terms showing biological process, cellular component, molecular function and KEGG pathways according to expression status of CPR5 and CPR11 samples (a) GO-terms enriched down-regulated CPR5 genes **(b)** GO-terms enriched up-regulated CPR5 genes **(c)** GO-terms enriched down-regulated CPR11 genes **(d)** GO-terms enriched up-regulated CPR11 genes.**(e)** Bubble plot to enriched KEGG pathway down-regulated CPR5 genes **(f)** Bubble plot to enriched KEGG pathway up-regulated CPR5 genes **(g)** Bubble plot to enriched KEGG pathway down-regulated CPR11 genes **(h)** Bubble plot to enriched KEGG pathway up-regulated CPR11 genes.



Supplementary Figure 4. Treeplot hierarchical clustering of enriched pathways based on GO-terms enrichment (a) showing down-regulated CPR5 **(b)** up-regulated CPR5 **(c)** down-regulated CPR11 **(d)** up-regulated CPR11.

3 CONCLUSÃO

Em resumo, nosso trabalho mostrou um novo modelo *in vitro* de resistência a cisplatina desenvolvido em linhagem imortalizada de CEC de língua, SCC-9, que englobou alterações importantes durante o processo de TEM, bem como de resistência a cisplatina ao identificar diferentes perfis de migração e crescimento celular, além de mostrar variação de genes diferencialmente expressos entre as células resistentes, de acordo com o nível de resistência adquirido, e também em relação as células parentais, onde pudemos observar enriquecimento de vias canônicas e não-canônicas revelando novos genes envolvidos nesse processo. O trabalho possui ainda algumas limitações como o fato de ser um modelo 2D e termos utilizado apenas um tipo celular. No entanto, nosso estudo poderá contribuir futuramente para direcionar outros modelos de resistência e a utilização desses dados em comparação com dados públicos relacionados a tumores primários de câncer de cabeça e pescoço seja em relação à expressão diferencial de genes, enriquecimento de vias até mesmo relacionado ao envolvimento de mecanismos epigenéticos que possivelmente estejam regulando, seja silenciando ou ativando, genes alvos no processo de mediação de TEM e resistência a cisplatina.

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APÊNDICE 1

Projeto 1 – Saliva PAX5 methylation can stratify tobacco-induced oral cancer

O objetivo do estudo é verificar o potencial uso da saliva como fonte para detecção de metilação de marcadores relacionados ao uso do tabaco que podem ser indutores para o câncer oral.

Projeto 2 – Salivary glands shedding RNA into saliva: challenges and opportunities

O estudo tem como objetivo testar diferentes protocolos de coleta e processamento de saliva para identificar possíveis marcadores de câncer de cabeça e pescoço baseado em RNA. Assim, poderemos ter uma otimização para aumento de especificidade, qualidade de amostra e sensibilidade de teste utilizando cinco diferentes protocolos de coleta de saliva, cinco metodologias de proteção das amostras preservando RNA e, posteriormente, teste de quatro protocolos de extração de RNA.

Projeto 3 – HPV status and epigenetic profiling of saliva from a cancer-free population

O estudo visa acessar o perfil de metilação de genes relacionados ao câncer em amostras de saliva da população brasileira livre de câncer e associar com fatores de riscos conhecidos, além de identificar a presença de tipos de HPV menos comuns de outras fontes comparando com a presença de metilação na saliva.

Projeto 4 – A review about DNA methylation studies in HPV-related head and neck cancer

Essa revisão objetiva compilar estudos publicados revisados por pares identificado alterações na metilação do DNA relacionadas ao status de HPV em carcinoma espinocelular de orofaringe e cavidade oral que possam direcionar estudos translacionais futuros.

Projeto 5 – Saliva T cell receptor signature in head and neck cancer patients

Para esse estudo o objetivo é explorar o repertório, diversidade e clonalidade de receptores de linfócitos T que possam ser compartilhados entre tecido tumoral, linfócitos e saliva de pacientes com câncer de cabeça e pescoço.

Projeto 6 e 7 – A precision DNA methylation test to triage HPV positive women before referral to colposcopy-driven biopsies or ablative treatment in cervical cancer screening clinics worldwide / CervicalMethDx: A precision DNA methylation test to identify advanced disease risk in cervical cancer screening algorithms

O objetivo desse estudo é validar e determinar o poder do novo teste CervicalMethDx em distinguir Neoplasia Intraepitelial Cervical (NIC) 2/3 de mulheres

HPV-positivas, identificar a progressão do tecido normal para estágios pré-cancerosos utilizando amostras de citologia líquida.

Projeto 8 – Direct cell testing of methylation markers in liquid cytology samples

Este estudo objetiva a otimização do uso de kits de conversão de bissulfito de maneira direta em amostras de citologia líquida comparando, portanto, quatro kits comerciais avaliando genes já previamente descritos como metilados em amostras de citologia líquida de pacientes com câncer cervical.

Projeto 9 – Nucleosome remodeling complex subunits as adaptors for p53 signaling

O estudo é desenhado para testar as hipóteses de que subunidades chaves do compleco BAF agem como adaptadores que modulam a atividade de p53, bem como genes alvos de p53.

Projeto 10 – New ribosome-targeting therapy compound modulates protooncogenes

O objetivo do estudo é testar novos agentes moduladores de ribossomos em câncer de pulmão de células pequenas a fim de identificar potencial atividade nesses tipos tumorais se induzidos por MYC, uma vez que esses agentes podem inibir seletivamente a tradução de proteínas.

ANEXO 1 – Comprovante submissão artigo

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	CS-D-24-02304	Comparative analysis of differential gene expression in cisplatin-resistant SCC-9 cells	Aug 29 2024 11:52PM	Aug 29 2024 11:52PM	With Editor

Cellular Signalling

Comparative analysis of differential gene expression in cisplatin-resistant SCC-9 cells

--Manuscript Draft--

Manuscript Number:	
Article Type:	Full Length Article
Keywords:	EMT. Cisplatin. Drug-resistance. Oral cancer. SCC-9.
Corresponding Author:	Dieila Giomo de Lima, DDS, MSc, PhD State University of Campinas Piracicaba, São Paulo BRAZIL
First Author:	Dieila Giomo de Lima, DDS, MSc, PhD
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	Gabriell Bonifácio Borgato, DDS, MSc, PhD
	André Schwambach Vieira, PhD
	Alessandro Santos Farias, MSc, PhD
	Gustavo Narvaes Guimarães, MSc, PhD
	Ana Paula de Souza, DDS, MSc, PhD
Abstract:	Therapy resistance remains the major cause of death from cancer. Epithelial-to-mesenchymal transition (EMT) has been widely investigated and significantly associated to resistance in different tumor types. Cisplatin (CDDP) is commonly used as the first-line of treatment agent in oral cancer with increasing cases of intrinsic or extrinsic resistance. However, EMT mediated cisplatin resistance still poorly understood in oral cancer. Here, using in vitro estabilishment of a cisplatin resistance model with SCC-9 cell line, we observed morphological changes associated to high expression of VIMENTIN, SNAIL1, ZEB1, ZEB2 and CDH2 according to resistance level. Furthermore, we observed supression of proliferation in resistant cells and a significant migratory profile in partial CDDP resistance level (SCC-9CPR5). On the other hand, SCC-9CPR11, the final resistant cells, did not show a bulk migration profile. Using RNA sequencing (RNA-Seq), we found various differentially expressed genes (DEGs) related to cell adhesion, extracellular matrix organization, binding calcium ion (Ca2+) Gene Ontology (GO) terms. Additionally, using Kyoto Encyclopedia of Genes and Genomes (KEGG), we identified phosphoinositide 3-kinase-related protein kinase (PI3K-Akt) signalling pathway enriched, which plays an important role in CDDP resistance and EMT programing. Together, our study uncovers a new 2D mechanistic model of cisplatin resistance showing important DEGs for further investigation and validation.
Suggested Reviewers:	
Opposed Reviewers:	

ANEXO 2 - Verificação de originalidade e prevenção de plágio

Resistência à cisplatina induz células SCC-9 a uma assinatura transcricional e diferenciação morfológica relacionada à transição epitélio-mesênquima

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