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Os receptores de vitamina D e retinoide X alfa em lesões malignas e benignas de pele: Análise Imunohistoquímica e Transcriptômica

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Patologia

Orientadora: Profa. Dra. Mariângela Esther Alencar Marques

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Palavras-chave: Imunohistoquimica; Receptor de vitamina D; Receptor retinoide X; Transcriptomica.

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“Estrelas são os olhos de

Deus vigiando para que corra tudo bem. Para sempre.

E, como se sabe, “sempre” não acaba nunca.”

Como nasceram as estrelas / Doze lendas brasileiras - Página 6, de Clarice

Lispector – Escrito em 1976 – Edição da Editora Nova fronteira - 40 páginas

Resumo

INTRODUÇÃO: A vitamina D se liga a seu receptor (VDR) para exercer suas funções e este se heterodimeriza com um receptor retinoide X (RXRα) e desta forma, em heterodimero, atua em regiões genéticas responsivas a vitamina D (VDRE) ativando ou inibindo transcrições gênicas. A principal fonte de vitamina D é a exposição à radiação ultravioleta, mas a mesma também é a principal causadora de cânceres de pele. Entretanto, diversos estudos, principalmente experimentais demonstraram efeito antiproliferativo, pró-apoptótico, anti-angiogênico e favorável a senescência celular pela vitamina D e seus metabólitos em células de melanoma. Da mesma forma, estudos demonstraram atividade antiproliferativa dos retinoides e seus análogos, levando, inclusive, a seu uso em transplantados renais e em algumas síndromes cutâneas, visando a prevenção de cânceres de pele, especialmente os não melanoma.

OBJETIVO: Analisar a expressão do VDR e do RXRα em lesões de pele não melanocíticas (queratose actínica, carcinoma basocelular e carcinoma espinocelular) e melanocíticas (nevo intradérmico, nevo displásico e melanoma) através de análise imuno-histoquímica e transcriptômica e compará-las a grupo controle.

MÉTODOS: Estudo retrospectivo transversal, com materiais histopatológicos embebidos em parafina provenientes de exéreses realizadas entre 2012 a 2018, no Hospital das Clinicas de Botucatu, da Faculdade de Medicina de Botucatu (UNESP). Foi considerado controle o grupo de peles provenientes de ampliação de margem de melanomas, com ausência de qualquer lesão no material. Após

todos os grupos serem submetidos aos critérios de inclusão e exclusão, foram submetidos à análise imuno-histoquímica para VDR da marca Abcam e RXRα da marca Novus Biologicals. Foram considerados positivos os casos cujo escore, que observava intensidade e porcentagem de positividade das células, fosse maior que 4. Posteriormente, alguns grupos foram selecionados para análise molecular em plataforma para análise múltipla de RNAm (Nanostring®). Para esta etapa foram selecionados outros 20 genes correlacionados a VDR e RXRα e mais 2 genes housekeeping (GAPDH e B-actina) para terem seus respectivos RNAm analisados em conjunto. Os genes selecionados foram: NCOA1, NCOA2, NCOA3, NCOR1, NCOR2, RAR A, RAR B, RAR G, RXR G, MED1, SMAD3, FOXO3, CYP27B1, CYP24A1, AhR, XPA, XPC. TP53, CREBB e EP300. Todos os dados foram submetidos a análise estatística, considerando $p< 0,05$ significativo. O trabalho foi submetido e aprovado no comitê de Ética da instituição.

RESULTADOS E DISCUSSÃO: Nas lesões não melanocíticas, ocorreu perda de RXRα nas queratoses actínicas e perda de ambos os receptores no carcinoma espinocelular. Estes dados favorecem a hipótese de que a vitamina D seria importante no processo de carcinogênese das lesões escamosas. Nas lesões melanocíticas, se observou perda de ambos os receptores em todas as lesões quando comparadas ao grupo controle. Houve correlação estatística da perda dos receptores com a exposição solar, corroborado pela análise do grupo controle em separado. Houve também correlação significativa entre VDR nuclear e regressão histológica, bem como correlação inversa entre RXRα e mitoses. Estas informações favorecem a hipótese de que a exposição solar seria um fator

fundamental na perda dos mecanismos de proteção do VDR e RXRα e que provavelmente essa perda ocorra no início da formação das lesões melanocíticas já que não houve diferença estatística entre as lesões avaliadas, a despeito de sua malignidade ou benignidade. Por fim, a análise transcriptômica comprovou a perda de VDR e RXRα nas lesões melanocíticas (nevo intradérmico e melanoma) e demonstrou perda de VDR e RXRα também em carcinomas basocelulares e espinocelulares. Houve diferença estatística com relação a maioria dos RNAm analisados comparando as lesões com o controle, porém quando comparamos as lesões entre si, carcinoma basocelular se diferenciou de carcinoma espinocelular, mas nevo intradérmico não se diferenciou de melanoma. Houve também diminuição de FOXO3, TP53 e XPC nos melanomas. CONCLUSÃO: A expressão de VDR e RXRα se demonstrou afetada pela exposição solar, mecanismo pelo qual poderia haver diminuição dos efeitos protetivos da vitamina D e RXRα. Os resultados da análise imuno-histoquímica foram corroborados pelos da análise transcriptômica, indicando um importante papel do VDR e RXRα na carcinogênese das lesões escamosas de pele. Com relação às lesões melanocíticas, a perda dos receptores provavelmente não se relaciona ao processo de carcinogênese, haja vista que não houve apresentação diferencial deles nas lesões benignas se comparadas às malignas. A maior compreensão desta via fisiopatogênica abre oportunidade para novos estudos e pesquisas, podendo se tornar alvo de estratégias terapêuticas.

Palavras chave: receptores de calcitriol, receptor x retinoide alfa, carcinoma basocelular, carcinoma de células escamosas, melanoma

Abstract

BACKGROUND: Vitamin D connects to its receptor (VDR) to exert its activities and heterodimerizes with a retinoid X receptor (RXRα), and acts, as a heterodimer, in vitamin D responsive elements (VDRE) activating or inhibiting genic transcriptions. The main source of vitamin D is ultraviolet radiation exposure, but it is the same main cause of skin cancers. Therefore, multiples studies, especially experimental ones showed antiproliferative, proapoptotic, antiangiogenic effects, favorable to cell senescence by vitamin D and its metabolites in melanoma cells. In the same way, studies have demonstrated antiproliferative activity from retinoids and its analogues, leading to their use in kidney transplantation patients and also in some cutaneous syndromes, aiming skin cancer prevention, primary against non-melanoma cancers.

OBJECTIVE: To analyze VDR and RXRα expression in non-melanocytic skin lesions (actinic keratosis, basal cell carcinoma and squamous cell carcinoma) and melanocytic (intradermal nevi, dysplastic nevi and melanoma) through immunohistochemistry and transcriptomic analysis and compare it to the control group.

METHODS: Retrospective transversal study, with formalin fixed and paraffin embebbed material from excision realized between 2012 and 2018 in Botucatu Clinical Hospital, from Botucatu Medicine Faculty (UNESP). The control group consisted of skin provided by margin ampliation of melanomas, when there was not any lesion remained. After all groups were submitted to inclusion and exclusion criteria, they were submitted to immunohistochemistry analysis of VDR (Abcam®) and RXRα (Novus Biologicals®). There were considered positive cases which the score, that observed intensity and percentage of positive cells,

was above 4. Finally, some groups were selected to molecular analysis in multiple mRNA platform analyzer (Nanostring®). In this stage, there were selected other 20 genes correlated to VDR and RXRα and two other housekeeping genes (GAPDH and B-actine)

to have their respective mRNA analyzed together. The genes selected were: NCOA1, NCOA2, NCOA3, NCOR1, NCOR2, RAR A, RAR B, RAR G, RXR G, MED1, SMAD3, FOXO3, CYP27B1, CYP24A1, AhR, XPA, XPC. TP53, CREBB and EP300. All data were submitted to statistical analysis and $p<0,05$ was considered significant. The study underwent institutional Ethics committee analysis and was approved.

RESULTS AND DISCUSSION: In non-melanocytic lesions, there was a loss of RXRα in actinic keratosis and loss of both receptors in squamous cell carcinomas. These data favor the hypothesis that vitamin D would be important in the carcinogenic process in squamous lesions. In melanocytic lesions, we observed loss of both of the receptors in all lesions when comparing to control group. There was statistical correlation of the loss of the receptors and sun exposure, endorsed by the isolated analysis of the control group. There was also statistical correlation between nuclear VDR and histological regression, and also inverse correlation between RXRα and mitosis. These data endorse the hypothesis that sun exposure would be a main factor in the loss of protection mechanisms from VDR and RXRα and probably this loss occurs in the beginning of the development of melanocytic lesions, once there was not statistical difference between the analyzed lesions, despite of their malignance or benignancy. Finally, transcriptomic analysis confirmed the loss of VDR and RXRα in melanocytic lesions (intradermal nevi and melanoma) and demonstrated loss

of them in basal cell and squamous cells carcinomas. There was statistical difference to the most of analyzed mRNA comparing lesions to control,

however, when we compare lesions between each other, basal cell carcinoma differentiated from squamous cell carcinoma but intradermal nevi not differentiated from melanoma. There was also low FOXO3, TP53 and XPC in melanomas.

CONCLUSION: VDR and RXRα expression was affected by sun exposure, and this mechanism could low vitamin D and RXRα protective effects. Immunohistochemistry analysis were endorsed by transcriptomic analysis, indicating an important role of VDR and RXR α in the squamous cell carcinogenesis. Considering melanocytic lesions, the loss of the receptors is not related to carcinogenesis, once there was no different presentation comparing benign and malign lesions. The comprehension of this physio pathogenic pathway opens opportunity to new researches and studies, making possible new insights to therapeutical strategies.

Keywords: Receptors, calcitriol; retinoid X receptor alpha; carcinoma, basal cell; carcinoma, squamous cell; melanoma

VDR – receptor de vitamina D

RXRα – receptor retinoide X, variante alfa

RXR in SK – receptor retinoide X alfa nos queratinócitos adjacentes

CBC – carcinoma basocelular

CEC– carcinoma espinocelular

QA = queratose actínica

NI= nevo intradérmico

ND = nevo displásico

MM = melanoma

SS = extensivo superficial (subtipo de melanoma)

AC = acral (subtipo de melanoma)

LM = lentigo maligno melanoma

ND = nodular (subtipo de melanoma)

UVA = radiação ultravioleta do tipo A

UVB = radiação ultravioleta do tipo B

RNAm = ácido ribonucleico mensageiro

TE = tris EDTA

Sumário

CAPÍTULO I – Revisão da Literatura

A radiação ultravioleta é a principal causa conhecida de lesões pré-neoplásicas e cânceres de pele. Muitos estudos associam a exposição à radiação ultravioleta com melanoma, contudo essa ação não é dose dependente, uma vez que a exposição continua (em trabalhadores ao ar livre) foi associada a menos risco de melanoma do que episódios de queimadura e bronzeamento ao sol. (Field & Newton-Bishop., 2012; Slominski et al., 2017) Hipoteticamente, a queimadura solar leva a imunossupressão e perda dos mecanismos de reparo de dano ao DNA. (Wang et al. 1990; Jeevan et al. 1992; Field & Newton-Bishop., 2012)

A radiação UVA aparentemente é menos carcinogênica, porque gera oxidação de guanina no DNA, ao passo que a UVB gera dímeros de pirimidina e ciclobuteno, que são mais carcinogênicos, porém isso ainda é alvo de discussão. (Podgorska et al, 2021)

Vitamina D e metabolismo

A principal fonte de vitamina D, entretanto, é a exposição a UVB. (Field & Newton-Bishop., 2012; Slominski et al., 2017) Não há consenso sobre o nível ideal de vitamina D, já que este nível depende do fototipo, índice de massa corpórea, ingestão dietética, suplementação oral, latitude e genética. (Field & Newton-Bishop., 2012) Portanto, avaliar a ação da vitamina D através dos seus níveis séricos apresenta muitos vieses. Baixos níveis de vitamina D foram associados com pior prognóstico em melanoma. (Field & Newton-Bishop., 2012; Slominski et al., 2017) Um estudo prospectivo envolvendo 47800 homens com baixos níveis de vitamina D mostrou uma tendência maior de desenvolver

melanoma, entretanto, níveis altos não foram associados com proteção ao desenvolvimento da neoplasia. (Giovannucci et al., 2006)

A radiação ultravioleta do tipo B (UVB) com 260-320 nM de comprimento de onda transforma 7-dehidrocolesterol em lumisterol, taquisterol e secosterol. Eles sofrem hidroxilação sistemicamente, no fígado e rins, resultando na forma ativa da vitamina D: $1,25(OH)_2D_3$. (Slominski et al., 2020). Esta é a via clássica ou canônica, conforme demonstrado na figura 1.

Há uma outra via, alternativa, com formação de outros derivados da vitamina D: 20,23(OH)2D3 e 20(OH)D3. Estes produtos são detectados in vivo no plasma, epiderme e em adrenal. Eles inibem a proliferação de queratinócitos epidérmicos, melanócitos e fibroblastos dérmicos, e promovem a diferenciação de queratinócitos (Slominski et al., 2018) Eles também inibem as atividades fibróticas dos fibroblastos e têm propriedades imunomoduladoras. Diferentemente do 1,25(OH)2D3, eles não promovem aumento de cálcio em doses farmacológicas. (Slominski et al, 2018)

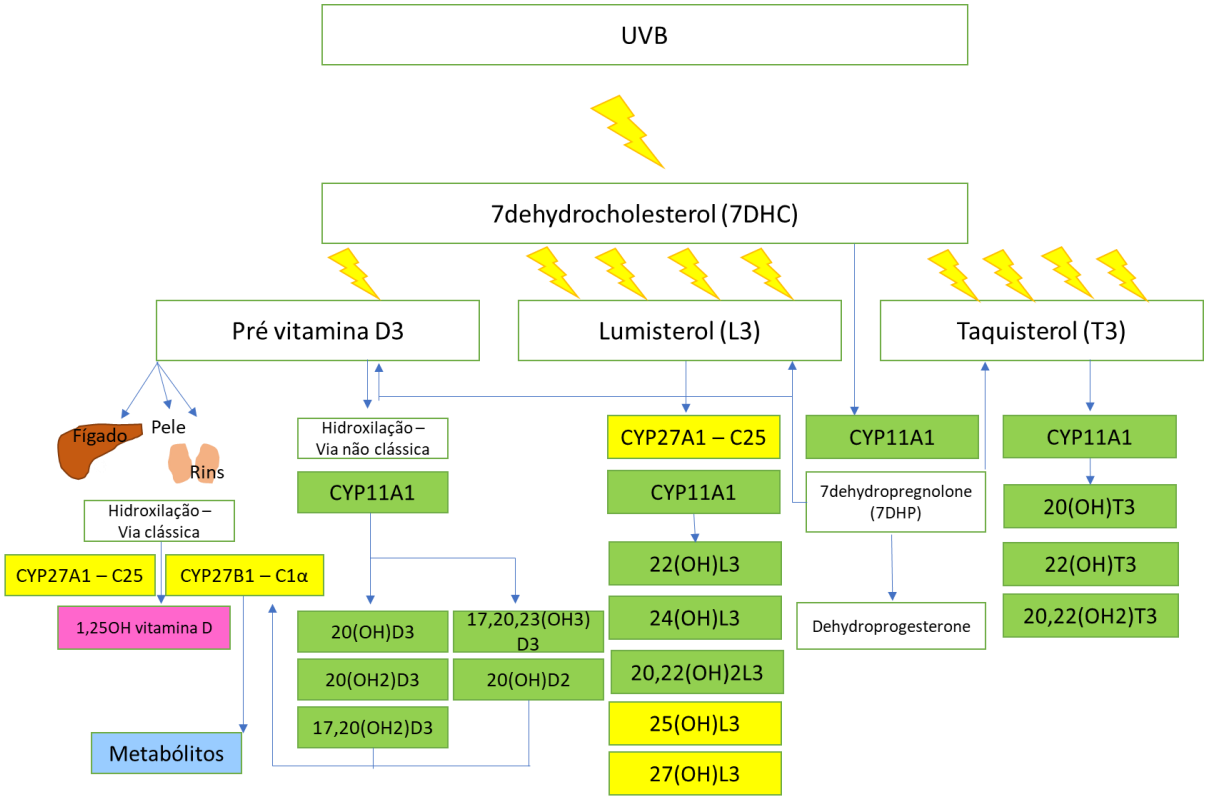

Figura 1 - Desenho esquemático das vias clássica (amarelo) e não clássica (verde) de vitamina D, segundo Slominski et al, 2020.

A 1,25(OH)2D3 inicia sua resposta biológica quando se liga ao seu receptor, VDR, um receptor nuclear que se heterodimeriza preferencialmente ao receptor retinoide RXRα (figura 2), outro receptor nuclear. Este heterodímero reconhece os elementos responsivos a vitamina D (VDREs) presentes nos genes, e corregula a transcrição ou supressão deles. (Haussler et al., 2011)

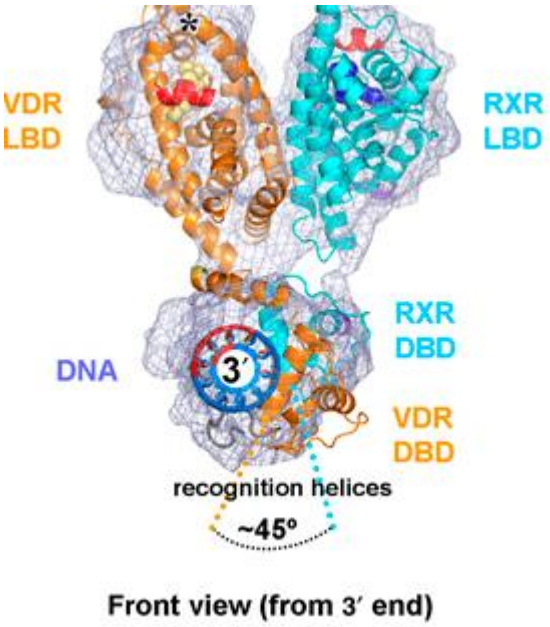

Figura 2. Nesta figura, demonstração do heterodímero de VDR e RXR (Orlov et al, 2012).

O receptor de Vitamina D (VDR)

O VDR é receptor nuclear com estrutura de 14 hélices, mas comumente referido como 13 hélices já que a estrutura E420 da figura trata-se de resíduo (figura 3).

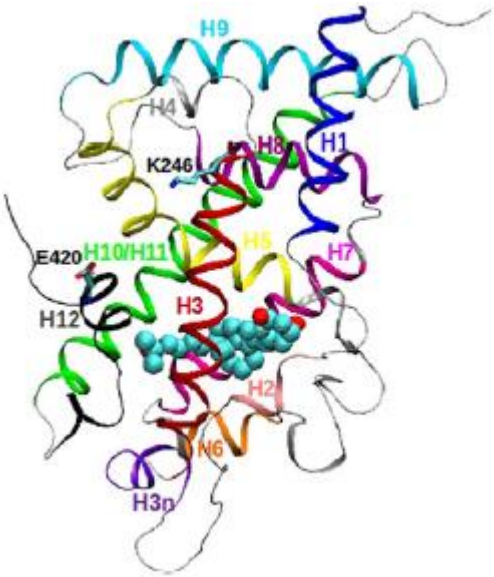

Figura 3: Imagem do VDR na sua forma ativa definido por Raio X, ligado à

vitamina D (representada na figura pelas esferas azuis – que são átomos de carbono e esferas vermelhas – átomos de oxigênio) (Yaghmaei et al, 2013).

O VDR é geralmente positivo na pele normal, mais robustamente expresso em células basais e na bainha externa do folículo piloso do que nas células mais superficiais. (Reichrath et al., 1999) Ele foi estudado nas lesões benignas e malignas de pele, mama, ossos, próstata e trato gastrointestinal. (Gallagher et al., 2012; Wen et al., 2015; Paolino et al.,2017; Zheng et al., 2017; Campolina-Silva et al., 2018)

Carcinomas basocelulares (CBCs) expressam VDR no núcleo mais fortemente do que nos queratinócitos adjacentes, de acordo com estudos em imuno-histoquímica e reação em cadeia da polimerase (PCR). (Mitschele et al., 2004; Haussler et al., 2011) Outro estudo reportou VDRs menos intensos nos carcinomas espinocelulares (CECs) do que pele normal e CBCs. (Slominski et al., 2020)

Os melanomas apresentaram uma redução dos VDRs citoplasmáticos e aumento dos receptores nucleares, comparados com pele normal. (Reichrath et al., 1999) Essa associação variou com o subtipo, a espessura tumoral e a exposição solar, sendo mais positivo em áreas cobertas do que expostas. (Reichrath et al., 1999)

Muitos estudos experimentais demonstraram efeitos da vitamina D em células de melanoma *in vitro*. No seu estudo clássico, Reichrath et al. relataram que nas linhagens celulares de melanoma MeWo, SkMel-28 e SM, a vitamina D e seus análogos tiveram efeito antiproliferativo. (Reichrath et al., 2007) Aparentemente, os efeitos dependem da linhagem celular, já que as linhagens

SkMel-5, SkMel-25, IGR e MelJuso não responderam à vitamina D. (Reichrath et al., 2007) Outros estudos experimentais demonstraram este potencial efeito antiproliferativo da vitamina D no melanoma (Podgorska et al., 2021; Gorman et al., 2007; Mason et al., 2010), outros demonstraram efeito de diferenciação e senescência celular, (Slominski et al., 2011; Slominski et al., 2012) apoptose, (Fleet et al., 2012; Feldman et al., 2014) inibição de angiogênese, (Mantell et al., 2000) interferência na inflamação (Swami et al., 2007; Fleet et al., 2012) através de mecanismo dependente do receptor VDR.

Após muitos estudos conflitantes, uma metanálise envolvendo 14 estudos considerou os polimorfismos gênicos do VDR responsáveis por diferença no risco de desenvolver melanoma. (Birke et al., 2020) O alelo dominante Fok1 aumentou o risco de melanoma em 22%, o alelo dominante Apa-1 aumentou o risco em 20%, enquanto BsM-1 diminuiu o risco em 15%. Outras variantes como A1012G, Cdx2, Bg-III e Taq-1 não tiveram associação significativa com o risco de melanoma. Distribuição diferente dos polimorfismos nas populações ou vieses de seleção podem explicar os resultados conflitantes dos estudos prévios.

O receptor retinoide X

Os receptores retinoides são uma superfamília de receptores nucleares. O receptor preferencial que se liga ao VDR é o RXR, com suas variantes α, β, and γ. (Jusu et al., 2018; Rambow et al., 2018) RXR α é mais frequente na pele do que nos outros tecidos (vide figura 4), enquanto a β ocorre em órgãos internos, a γ é frequentemente relacionada a desordens neurológicas.

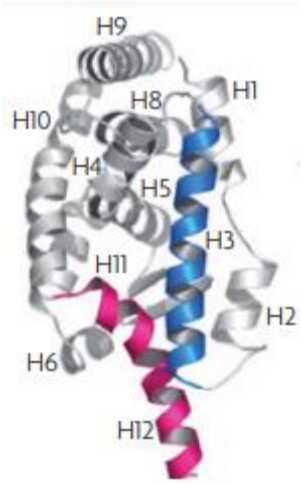

Figura 4: Imagem do RXRα na sua forma inativa. O RXRα é receptor nuclear com estrutura de 12 hélices. (de Lera et al. 2007)

Linfomas cutâneos de células T tratados com agonistas RXR demonstraram melhor resposta ao tratamento com indução de apoptose e diminuição da proliferação celular. (Wang et al., 2017) Agonistas de RXR inibem a proliferação celular nos CECs (Zacheis et al., 1999) A hipótese sobre o mecanismo pelo qual o RXR diminuiria o crescimento celular é através da via MAPKinase. (Crowe et al., 2003) O RXR é progressivamente diminuído em lesões de queratose actínica e CEC. (Xu et al., 2001)

Alguns estudos sugerem o uso de isotretinoina e acitretina para a quimioprevenção de cânceres de pele não melanoma, embora as agências governamentais não endossem o seu uso. Um estudo demonstrou a redução de 63% de cânceres de pele não melanoma em pacientes com xeroderma pigmentoso (Kraemer et al., 1988), e outros relatam que, com o uso de retinoides, houve uma proteção significativa aos pacientes após transplante renal. (de Sévaux et al., 2003; De Graaf et al., 2004)

Alguns estudos demonstraram que a perda de RXRα foi mais

significante no melanoma que nas células de lesões melanocíticas benignas. (Chakravarti et al., 2007) Análogos de retinoide inibiram a proliferação celular em melanomas (Siegrist et al., 1998; Desai et al., 2000; Klopper et al., 2010; Li et al., 2019) Um estudo experimental em ratos utilizando adapaleno (um retinoide tópico) em células de melanoma A375 demonstrou um efeito citotóxico dose dependente, parada na fase S do ciclo celular e indução de apoptose. (Li et al., 2019)

Ratos com genes $RXR\alpha^{-}/NRAS^{+}/CDK4^{+}$ expostos a uma única dose de UVB neonatal demonstraram, no melanoma induzido, alta proliferação celular, com maior expressão de marcadores tumorais, maior vascularização e invasão linfática do que ratos sem a alteração gênica. (Chagani et al., 2017) Como o RXRα foi menos expresso em células de melanoma do que os queratinócitos, os autores especularam que o RXRα nos queratinócitos adjacentes pode ter efeito parácrino na melanogênese, porque a deleção aumentou a proliferação melanocítica. (Chagani et al., 2017)

Análise múltipla de RNAm na plataforma Nanostring®

Amostras em parafina fixadas em formol tem sido a fonte de muitos estudos, porém por um longo tempo, exames moleculares não foram possíveis de serem realizados com este tipo de material. A plataforma Nanostring NCounter® é tecnologia validada para avaliar diferentes e múltiplos RNA mensageiros numa mesma análise em material parafinado. (Veldman-Jones et al., 2015; Tsang et al., 2017; Godoy et al., 2019)

As vantagens de se utilizar esta técnica são muitas: abordagens não rotineiras das lesões não são necessárias, uma vez que a fixação em formol faz parte da rotina diagnóstica dos tumores, longa duração do material fixado antes

de realizar o exame molecular e também a possibilidade de examinar pequenas quantidades de material genético, muito comum nas exéreses de pele, sem necessidade de amplificação gênica. Entretanto, ainda há alguma restrição quanto a quantidade mínima de células para detecção, e é uma limitação a necessidade de microdissecção de material para obtenção de células alteradas em lesões diminutas para separá-las da pele normal (como numa queratose actinica e no nevo displásico, por exemplo).

Estudos anteriores utilizando-se da plataforma Nanostring® analisaram a presença de mutações prévias no CBC com relação à resposta ao Vismodegib (Sternfeld et al., 2020), mas não foi encontrado nenhum estudo envolvendo CBC sobre VDR ou RXRα na literatura. Ainda, considerando o CEC, há alguns estudos sobre resposta ao Pembrolizumab (Stevenson et al., 2017), e estudos fisiopatogênicos em lesões orais. (Yu et al., 2016) Um estudo com 9 CECs e 7 controles gerou uma base de dados de 105 fatores de transcrição que demonstraram níveis alterados nos CECs, incluindo reguladores do desenvolvimento cutâneo (MYC, RELA, ETS1, TP63). (Das Mahapatra et al., 2020) Outro estudo mostrou 81 genes potenciais envolvidos na sinalização MAPKinase induzida por radiação UV, através da análise de micro RNA dos CECs. (Chen et al., 2020) A via de VDR/RXRα, entretanto, ainda permanece não estudada por este método.

Finalmente, considerando estudos em melanoma com o uso da plataforma, alguns foram realizados sobre a resposta à imunoterapia com Pembrolizumab (Awada et al., 2020), e outros tentaram estabelecer novos genes alvo possíveis de terapia alvo, como CSAG2, MAGEA3, MAGEC2, IL13RA2, PRAME, CSPG4 e SOX10. (Beard et al., 2013) Um estudo comparou 12 lesões

benignas e malignas melanocíticas, e após 1000 genes analisados, propuseram S100A8 como um marcador de melanoma expresso pelos queratinócitos ao redor das células de melanoma. (Kiuru et al., 2021) Novamente, não houveram estudos para a via VDR/RXRα com relação ao melanoma.

O presente estudo traz dessa forma, um caráter inovador, à medida que não há, na literatura, estudos realizados com metodologia semelhante sobre o tema.

RNAm avaliados

Além do RNAm dos VDR e RXRα, foram avaliados os RNAm referentes aos genes a seguir:

- NCOA1, NCOA2 e NCOA3 é uma família de coativadores nucleares, através do qual o VDR fecha sua hélice 12 e 4, garantindo estabilidade ao VDR na sua conformação ativa. (Yaghmaei et al., 2013)
- NCOR1 e NCOR2 são moléculas correpressoras nucleares. De uma forma simplificada, moléculas repressoras inibiriam a transcrição e atividade gênicas. Porém estudos demonstraram que o RXRα, de forma dependente do VDR, causa liberação dos NCOR, inibindo a repressão sobre o VDR. (Sánchez-Martínez et al., 2008; Meyer & Pike., 2013)
- RARα, RAR β e RAR γ são integrantes da família dos receptores de ácido retinóico. Os RARs também podem se heterodimerizar com VDR, porém a interação VDR/RXRα é preferencial. (Schräder et al., 1994)

- MED1 é uma subunidade do complexo mediador, que exerce função coativadora dos genes nucleares. (Belorusova et al., 2020) Um estudo demonstrou que a ablação de MED1 interfere de forma importante no ciclo do folículo capilar, além de causar hiperproliferação de queratinócitos epidérmicos interfoliculares. (Oda et al., 2011) Outro estudo também observou que o bloqueio de MED1/MED21 causou hiperproliferação de queratinócitos, sugerindo que o mediador regula as funções do VDR de controle de proliferação e diferenciação celular nos queratinócitos. (Oda et al., 2010)
- SMAD3 são proteínas coativadoras que podem mediar a interação entre VDR e TGFβ, atuando no controle de proliferação e diferenciação celular. (Yanagisawa et al., 1999)
- FOXO3 são fatores de transcrição que controlam proliferação celular e sobrevida. Funcionam como supressores tumorais e estão associados a longevidade. Estudos demonstram que regulam genes similares aos regulados por VDR. (An et al., 2010)
- CREBBP e EP300 são proteínas que se ligam ao RXR ou RAR e medeiam transcrições genicas nucleares. (Chakravarti et al., 1996) Regulam uma série de eventos do ciclo celular, como senescência e apoptose. (Wang et al., 2018) A mutação de CREBBP/EP300 leva a rara síndrome Rubinstein-Taybi, que tem predisposição a uma série de neoplasias hematológicas, do trato gênito-urinário e lesões benignas de pele, como

pilomatrixomas. (Boot et al., 2018)

- CYP27B1 e CYP24A1 – são genes que codificam as enzimas responsáveis pela ativação da vitamina D (1,25(OH)2D3) pela via clássica ou canônica. Um estudo defende que, enquanto os ratos com deleção VDR tem altos índices de tumores induzidos por radiação ultravioleta, os ratos com deleção CYP27B1 seriam resistentes, indicando uma possível atuação preventiva para o VDR independente de 1,25(OH)2D3. (Dowd & MacDonald et al., 2010) No entanto, há vários estudos demonstrando associação de deleção CYP27B1 e cânceres colorretal, próstata, mama, pulmão, fígado e pancreático. (Latacz et al., 2020) Um estudo demonstrou correlação entre CYP27B1 dos queratinócitos e melanoma, sendo menos positivo na fase de crescimento vertical e metástases, além de sua menor positividade em lesões de pior prognóstico. (Brożyna et al., 2013)

  O aumento da proteína CYP24A1 foi 50 vezes maior em células de adenocarcinoma pulmonar. (Nemazannikova et al., 2019) Este estudo também observou esta associação em queratose actínica, carcinoma basocelular e espinocelular. Esta enzima está associada a degradação de vitamina D da via canônica, o que justificaria essa associação inversa à da CYP27B1.
- AhR – Aryl Hydrocarbon receptor – é um receptor de vitamina D da via não clássica (20,23(OH)2D3), dependente da enzima CYP11A1. (Slominski et al., 2018)
- TP53 – é um gene supressor tumoral com papel na apoptose,

reparo ao DNA e controle de ciclo celular, quando apresenta mutação germinativa dá origem a síndrome de Li Fraumeni, que aumenta a susceptibilidade a diversos tumores, incluindo melanoma. (Sandru et al., 2022) Correlaciona-se a importância do p53 como um alvo para ação ultravioleta acelerando as mutações BRAF de alguns melanomas. (Choi & Fisher et al., 2014) Um estudo populacional não observou correlação entre polimorfismos de TP53 e câncer de pele não melanoma. (Almquist et al., 2010)

- XPC e XPA – genes relacionados ao Xeroderma Pigmentoso, condição que tem aumento de risco de cânceres de pele UV induzidos. Houve um estudo que associou diminuição de risco ao melanoma na presença de um haplótipo de XPC quando comparado ao gene referência, (Paszkowska-Szczur et al., 2013) porém outros estudos relatam associação quando presentes XPA e XPD. A associação de XPC e melanoma é mais documentada. (Paszkowska-Szczur et al., 2013)

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3.1 Objetivo geral

Avaliar a expressão imuno-histoquímica de VDR e RXRα e realizar análise transcriptômica de RNAm relativos a genes relacionados à vitamina D em lesões epiteliais e melanocíticas, benignas e malignas de pele, e comparar esta expressão com grupo controle.

3.2 Objetivos específicos

- Descrever os casos de queratoses actínicas, CBCs, CECs, nevos intradérmicos, nevos displásicos, melanomas e controles através da análise de prontuário com relação a dados demográficos (sexo, idade e exposição solar) e histopatológicos.

- Avaliar a presença de positividade de VDR e RXRα por marcadores imuno-histoquímicos nos queratinócitos e melanócitos; correlacionar com possíveis variáveis epidemiológicas e histopatológicas.

- Realizar exame molecular em plataforma Nanostring® com avaliação de RNA mensageiro de VDR, RXRα e outros 20 RNAm relacionados, nas lesões de CBCs, CECs, nevos intradérmicos e melanomas e comparar com pool de controles. Correlacionar o exame molecular com as variáveis observadas na análise de prontuário e comparar também estes resultados aos da análise imuno-histoquímica.

4.1. Descrição da amostra e Tipo de estudo

4.2. Seleção dos sujeitos

Estudo retrospectivo transversal, em que foram selecionados materiais provenientes de exérese cirúrgica de lesões cutâneas realizadas entre 2012 a 2018, armazenados em parafina, procedentes do Hospital das Clinicas de Botucatu, da Faculdade de Medicina de Botucatu (UNESP).

4.2.1 Critérios de inclusão

Lesões menos frequentes de serem excisadas cirurgicamente, como queratose actínica, determinaram o valor mínimo amostral por grupo. O valor máximo amostral foi de 100 casos, e ocorreu de forma arbitrária. A seleção para os grupos com maior número de casos ocorreu de forma aleatória, seguindo a ordem da data de excisão das lesões.

Foram incluídos como controles, amostras provenientes de ressecção cirúrgica de ampliação de margem de melanomas que não tinham lesão residual.

Foram incluídos carcinomas basocelulares do tipo nodular, carcinomas espinocelulares bem ou moderadamente diferenciados, queratoses actinicas, nevos intradérmicos, nevos displásicos e melanomas.

4.2.2 Critérios de exclusão

Foram excluídos nos grupos, casos em que a lesão estudada não foi confirmada em revisão anatomopatológica.

Foram excluídos do grupo nevo os que não eram do subtipo intradérmico ou displásico.

Foram excluídos do grupo carcinoma basocelular os que não eram do subtipo nodular.

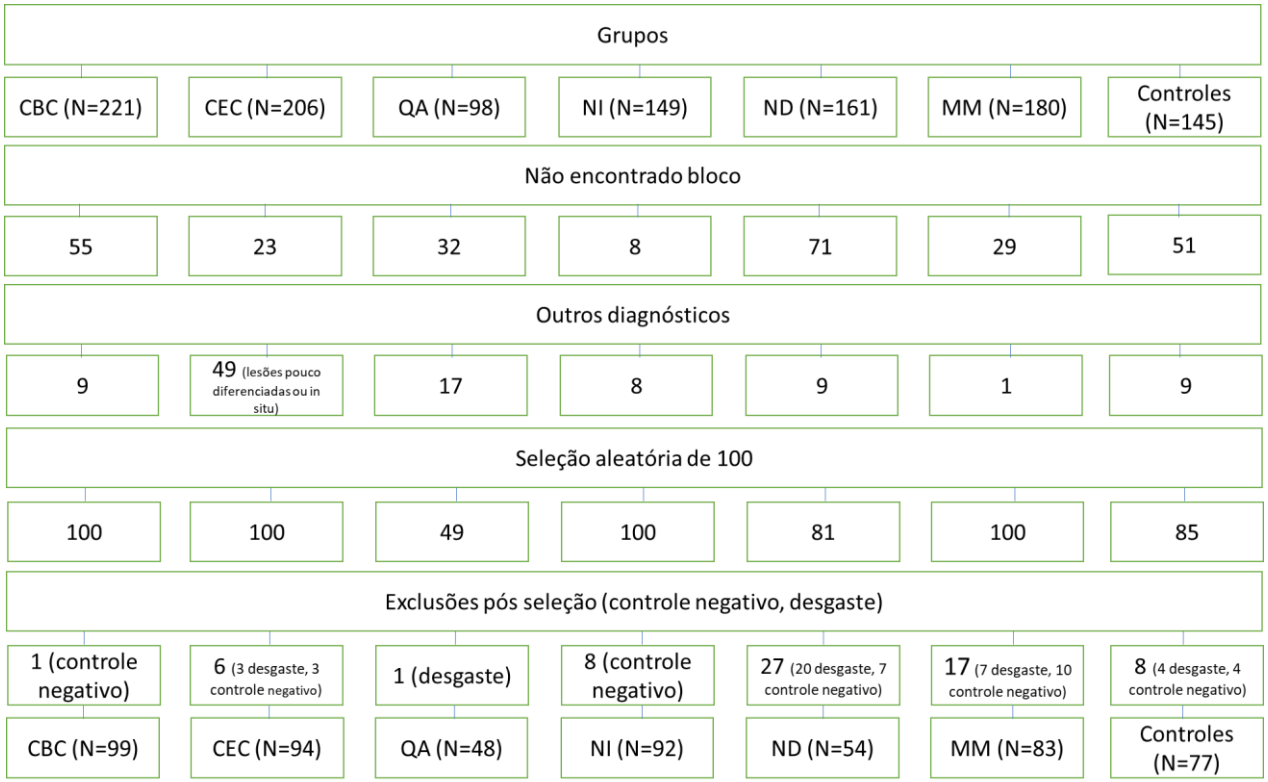
Foram excluídos do grupo carcinoma espinocelular os carcinomas *in situ* e pouco diferenciados (graus 3 e 4 da classificação de Broders - *em Broders AC. Squamous cell cancer of the lip: a study of five hundred and thirty-seven cases. JAMA. 1920;74:656-664*)

Foram excluídos todos os casos em que o material parafinado não apresentou condições técnicas mantidas para sua utilização.

No exame imuno-histoquímico, foram excluídos os casos em que a pele adjacente – normal – era negativa aos receptores.

No exame molecular, foram excluídas lesões menores, em que o desgaste de todo o bloco não resultaria em material genético suficiente após extração.

Organograma 1. Detalhamento de obtenção da amostra após aplicação dos critérios de eligibilidade



CBC = carcinoma basocelular; CEC = carcinoma espinocelular; QA = queratose actinica; NI = nevo intradérmico, ND = nevo displásico; MM = melanoma

4.3.1. Variáveis clínicas

Foram obtidas a partir de laudo anatomopatológico, e consistem de sexo, idade e localização. A lesão foi considerada exposta ou não exposta ao sol, conforme a figura 5 demonstra.

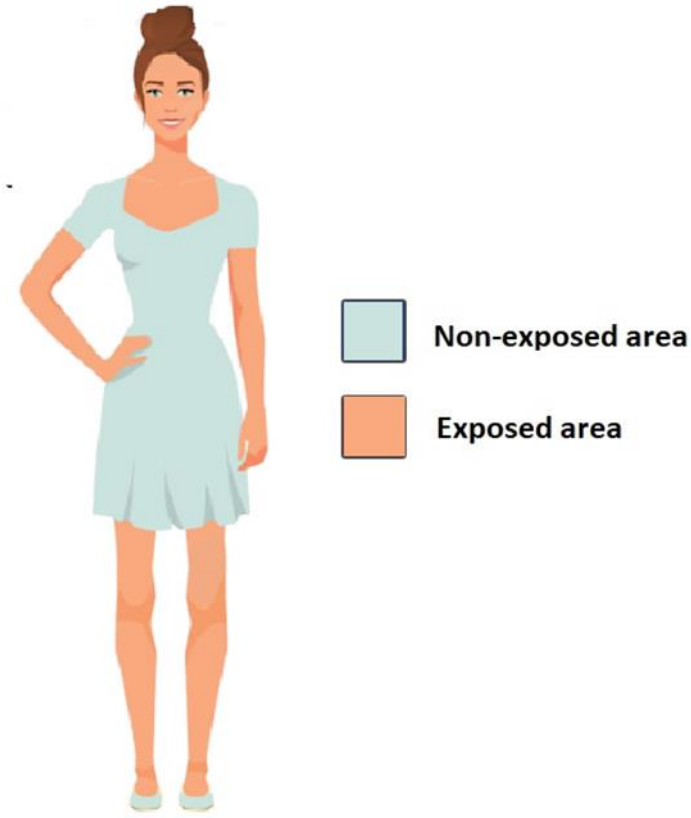


Figura 5: Distribuição dos locais considerados de área exposta (exposed areas) e não exposta (non-exposed areas). Fonte: próprio autor.

4.3.2 Variáveis histopatológicas

Foram extraídas a partir de revisão das lâminas coradas por Hematoxilina e Eosina (HE), para o grupo melanoma. São elas: subtipo, presença de ulceração e mitose, Breslow (maior espessura da lesão) em milímetros e regressão histológica.

4.4 Coleta dos dados

As informações referentes a idade, sexo e localização das lesões foi obtida através dos laudos anatomopatológicos, obtidos a partir da pesquisa pelos termos diagnósticos. Foram selecionados, 77 controles, 48 QAs, 99 CBCs, 94

CECs, 92 NIs, 54 NDs e 83 MM, totalizando 470 casos e 77 controles (Organograma 1).

4.5 Processamento de dados

Os dados foram armazenados em planilha Excel e analisados posteriormente com auxílio de Estatístico.

4.5.1. Critérios histopatológicos

Os critérios histopatológicos utilizados no diagnóstico das lesões de pele foram os mesmos descritos no livro de tumores de pele *"Classification of Skin Tumours". 4th ed. IARC: Lyon; 2018. da Organização Mundial de Saúde*.

4.5.2. Técnica de Imuno-histoquímica

A técnica de realização de imuno-histoquímica seguiu protocolo específico para cada receptor de acordo com a orientação da empresa que o produziu, e será pormenorizado a seguir.

Preparação das amostras:

1) As amostras incluídas em parafina foram seccionadas com navalhas de aço inox na espessura de 3 micra em micrótomo rotativo.

2) Os cortes das amostras foram estendidos em lâminas histológicas de vidro, foscas de bordas esmerilhadas, próprias para imuno-histoquímica da marca Agilent

3) Foram mantidas as lâminas por 1 hora em estufa a 60o C

Recuperação Antigênica:

Recuperação antigênica pelo calor em equipamento PT-LINK 200 (Dako), solução de pH alto (pH= 9), para o anticorpo VDR, por 20 minutos após atingir a temperatura de 95oC e em solução de pH baixo (pH = 6,1) para o anticorpo RXRα.

Reação de imuno-histoquímica automatizada realizada em equipamento Autostainer Link 48 (Dako).

1) Bloqueio da peroxidase endógena através da incubação das lâminas em solução de peróxido de hidrogênio a 3% por 5 minutos em temperatura ambiente.

2) Lavagem em solução Salina Tamponada com tampão Tris com Tween 20, pH= 7,6.

3) Incubação dos anticorpos VDR (Coelho - Abcam – Reino Unido) na diluição de 1:1500 e RXRα (Anticorpo NR2B1, Rato, Novus Biologicals – CO, EUA) na diluição de 1:800 por 30 minutos. Os anticorpos eram recomendados por manual dos laboratórios em diluição 1/2000 a 1/4000 (VDR) e 1/500 a 1/3000 (RXRα) respectivamente. Foram realizados testes prévios para determinar a diluição melhor para cada anticorpo.

4) Lavagem em solução Salina Tamponada.

5) Incubação do anticorpo conjugado (secundário e terciário) Labelled Polymer Flex/HRP por 10 minutos em temperatura ambiente.

6) Lavagem em solução Salina Tamponada.

7) Revelação pelo cromógeno 3,3-diaminobenzidine (DAB – DAKO) por 5 minutos.

8) Lavagem em solução Salina Tamponada.

9) Contra Coloração em Hematoxilina (Dako) por 10 minutos nas lesões não-melanociticas

9.1) Nas lesões melanocíticas, o anticorpo VDR foi contra corado em Hematoxilina e o anticorpo RXRα em Melan A (Dako) e Magenta (Dako) - dupla contra coloração.

10) Lavagem em água destilada.

11) Desidratação em etanol absoluto e xilol.

12) Montagem com lamínulas em resina sintética.

4.5.4. Análise da imuno-histoquímica

Foi utilizado um score que classifica a intensidade de positividade do marcador de 0 a 3 (0= negativo, 1 = fraco, 2 = moderado, 3 = forte) e outro score que classifica o número das células positivas de 0 a 4 (0 = abaixo de 5% das células, 1 = de 6 a 25%, 2 = de 26 a 50%, 3 = de 51 a 75%, 4 de 76 a 100%). Um score final de 0 a 12 foi obtido pela multiplicação desses fatores. A marcação foi considerada positiva quando o score final era maior que 4. Muitos scores tem sido utilizados, porém este que divide os grupos em quartis iguais foi preferido. (Wen et al., 2015)

O marcador VDR teve dois scores diferentes de acordo com o local: nuclear e citoplasmático. Dessa forma era classificado em 4 categorias: negativo, nuclear, citoplasmático ou ambos (quando não era possível determinar o local predominante)

O marcador RXRα é exclusivamente nuclear. Foi também avaliada a positividade de RXRα nos queratinócitos ao redor das lesões melanocíticas (RXRα in SK).

4.6. – Seleção dos RNAm no site Genecards.org

O site Genecards (www.genecards.org) trata-se de banco de dados online, no qual através de ferramentas de bioinformática há cruzamento de dados de forma atualizada, e através da digitação de um gene de interesse conseguimos estabelecer uma malha de interações gênicas mais frequente nos estudos, podendo se organizar através dessas ligações possíveis vias de atuação. Abaixo, temos a rede de interações geradas a partir do termo VDR (vide figura 6).

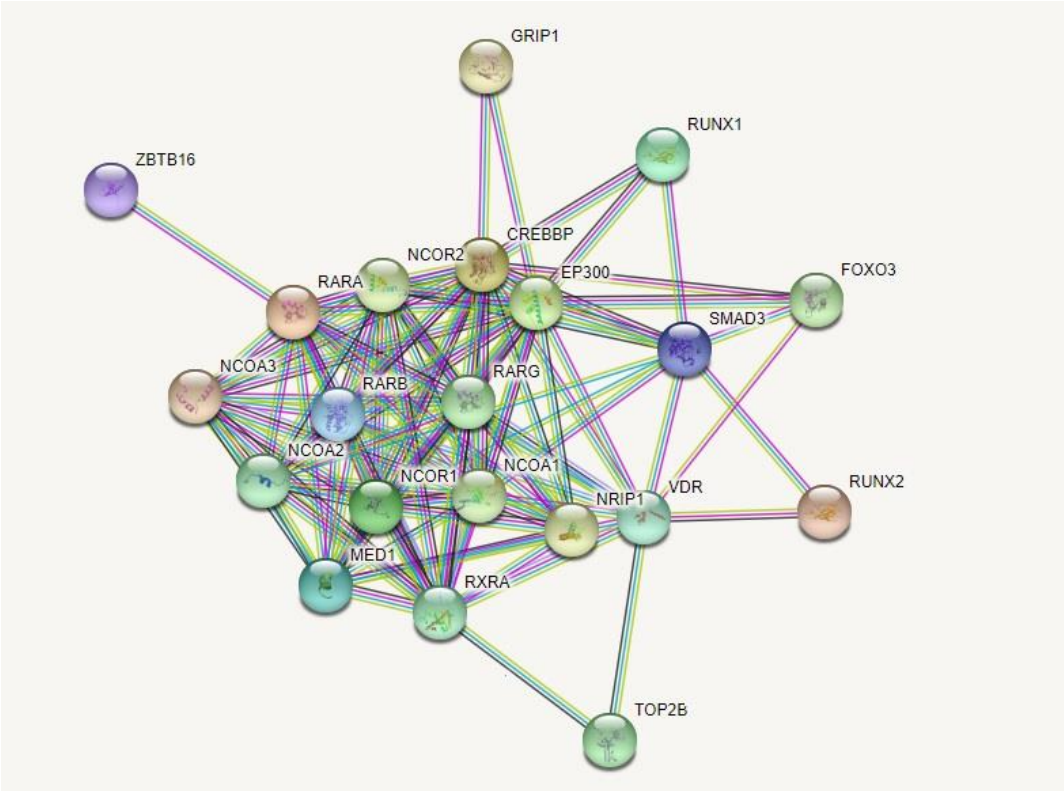


Figura 6: Malha de interações entre genes usando como descritor o VDR. Quanto maior o número de interações, maior a quantidade de linhas entre os termos. (Busca realizada no site genecards.org em julho de 2019)

Foram excluídos genes que, apesar de ter interação com VDR e RXRα estivessem pouco expressos na pele, ou com pouca associação descrita na literatura em relação às lesões de pele de interesse, segundo os dados disponíveis no mesmo site, Genecards.

Abaixo, segue tabela dos RNAm analisados:

Tabela 1 - Lista dos 24 RNAm analisados no estudo, seu número referência (disponível em https://www.ncbi.nlm.nih.gov/gene/) e sua principal função na pele

RNAm	Nome do gene referência	Número referência	Função
VDR	Receptor de Vitamina D	NM_ 000376.2	Estimula ou inibe transcrição gênica pela presença da vitamina D
NCOA1	Gene Coativador 1	NM_003743.5	Garante estabilidade ao VDR na sua fase ativa
NCOA2	Gene Coativador 2	NM_001321703.2	Garante estabilidade ao VDR na sua fase ativa
NCOA3	Gene Coativador 3	NM_001174087.1	Garante estabilidade ao VDR na sua fase ativa
NCOR1	Gene Correpressor 1	NM_006311.4	Inibem a transcrição gênica mediada por VDR
NCOR2	Gene Correpressor 2	NM_006312.6	Inibem a transcrição gênica mediada por VDR
MED1	Subunidade do complexo mediador 1	NM_004774.4	Regula as funções de VDR de controle e proliferação queratinócitos
SMAD3	Proteina da família SMAD 3	NM_005902.4	Medeia a relação entre VDR e TGF-beta
FOXO3	Fator de transcrição da familia ForkHead	NM_001455.4	Fator relacionado com a apoptose secundária a stress oxidativo
CREBBP	Proteina coativadora da acetiltransferase CREBBP	NM_004380.2	Liga-se ao RAR ou RXR, regulando eventos do ciclo celular
EP300	Proteina Histona acetiltransferase EP300	NM_001429.4	Liga-se ao RAR ou RXR, regulando eventos do ciclo celular
RARA	Receptor de Ácido Retinoico Alfa	NM_000964.4	Podem se ligar ao VDR, porém a ligação VDR/RXR é preferencial
RARB	Receptor de Ácido Retinoico Beta	NM_000965.4	Podem se ligar ao VDR, porém a ligação VDR/RXR é preferencial
RARG	Receptor de Ácido Retinoico Gama	NM_000966.6	Podem se ligar ao VDR, porém a ligação VDR/RXR é preferencial
RXRA	Receptor Retinoide X Alfa	NM_002957.6	Forma heterodímero com VDR, e atua no controle da transcrição gênica
RXRG	Receptor Retinoide X Gama	NM_006917.5	Outro membro da familia dos retinoides X, observado em células neurais
TP53	Gene supressor tumoral p53	NM_000546.5	Interfere diretamente no ciclo celular, reparo de DNA e apoptose
XPA	Haplótipo A do Xeroderma Pigmentoso	NM_000380.3	Haplótipo de síndrome com defeito de reparo dos nucleotídeos
XPC	Haplótipo C do Xeroderma Pigmentoso	NM_004628.4	Haplótipo de síndrome com defeito de reparo dos nucleotídeos
CYP27B1	Enzima 25-Hidroxi-1-alfa-hidroxilase	NM_000785.4	Enzima responsável pela produção de vitamina D pela forma clássica
CYP24A1	Enzima Vitamina D3-24-Hidroxilase	NM_000782.5	Enzima associada à degradação de vitamina D
AhR	Receptor Aril-Hidrocarbono	NM_001621.5	Receptor de vitamina D da via não-clássica
GAPDH	Gliceraldeido-fosfato-desidrogenase	NM_002046.7	Enzimas de metabolismo dos carboidratos. Utilizada como gene housekeeping de amostras de pele.
B-ACTIN	Beta-actina	NM_001101.5	Proteinas de citoesqueleto, bem conservadas. Utilizada como gene housekeeping de amostras de pele.

4.7 Exame molecular em plataforma Nanostring NCounter®

4.7.1 – Extração do RNAm

Os materiais parafinados foram cortados e dispostos em lâmina de vidro, para então serem raspados com uso de lâmina de Bisturi e armazenados em temperatura ambiente em um eppendorf para cada amostra.

Foram submetidos então à extração de RNA através do reagente RecoverALL da Thermofisher® seguindo protocolo da empresa, detalhado a seguir:

O eppendorf é centrifugado a 14 mil rotações por minuto (rpm)

Em seguida acrescenta-se xilol (1 ml por frasco)

Banho seco no termobloco por 3 min a 50 ºC, 700 rpm

Nova centrifugação a 14000 rpm, retirando o sobrenadante após cada centrifugação

Lavagem com 1 ml álcool absoluto, centrifugando e retirando o sobrenadante após, 2 vezes

Repetir mais uma vez a centrifugação, sem o álcool. Retirar o sobrenadante, deixando apenas o “pallet”

Colocar no Thermomixer ® a 37ºC por 5 min

Digestão da protease

Colocar 200 microlitros de buffer em cada amostra, fazendo movimentação up and down

Homogeneizar a amostra

Adicionar 4 microlitros de protease, mantida em estante gelada

Deixar no Thermomixer ® a 50º C overnight – 14 horas

Após preparo do kit, com complementação com álcool conforme orientação do fabricante, colocar 790 microlitros do kit recém preparado em cada amostra, e separar 700 microlitros a serem colocados em novo eppendorf com filtro de coluna

Centrifugar o novo frasco com filtro a 10000 G por 30 segundos.

Desprezar o líquido do fundo, pegar o restante que sobrou do eppendorf antigo e colocar no novo eppendorf com filtro

Nova centrifugação, desprezar o líquido do fundo

Colocar 700 microlitros do tampão 1 em cada eppendorf

Centrifugar novamente a 10000 G por 30 segundos, desprezar o liquido de fundo

Colocar 500 microlitros do tampão 2

Centrifugar novamente a 10000 G por 30 segundos, desprezar o liquido de fundo

Agitar brevemente na centrifuga

Digestão DNA

Acrescentar 6 microlitros de DNA buffer, 4 microlitros de DNAse, 50 microlitros de água livre de nucleotídeos em cada frasco, com 5% a mais para margem de erro

Deixar 30 min em ar ambiente

Acrescentar 700 microlitros do buffer 1

Centrifugar novamente a 10000 G por 30 segundos, desprezar o liquido de fundo

Acrescentar buffer 2

Centrifugar novamente a 10000 G por 30 segundos, desprezar o liquido de fundo

Acrescentar buffer 3

Centrifugar novamente a 10000 G por 30 segundos, desprezar o liquido de fundo

Agitar rapidamente no vórtex

Transferir a coluna para um novo eppendorf

Adicionar água livre de nucleases

Incubar por 1 minuto

Centrifugar por 1 min a 14000 rpm

Manter na rack refrigerada

4.7.2 – Avaliação da quantidade e qualidade do material extraído

Posteriormente à extração do material genético, o fluido com o material foi submetido a análise no aparelho Nanodrop® para avaliar se os níveis de RNAm eram suficientes para análise e se comportavam dentro da curva padrão do aparelho. Essa curva atesta a boa qualidade do material genético da amostra (Figura 7 abaixo, disponível em Nanostring® User Manual).

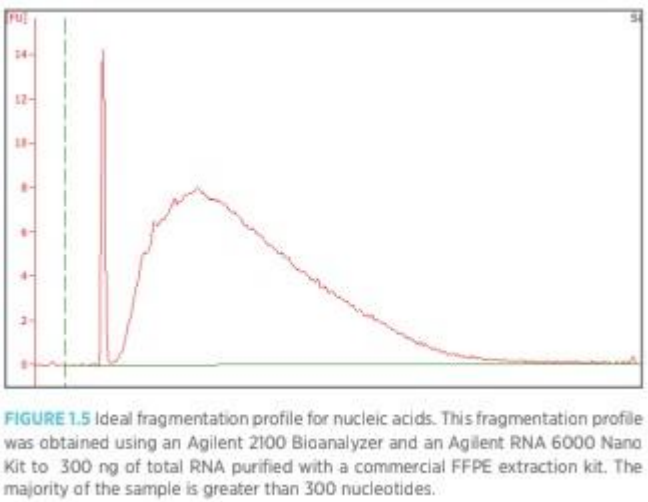


FIGURE 1.5 Ideal fragmentation profile for nucleic acids. This fragmentation profile was obtained using an Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Nano Kit to 300 ng of total RNA purified with a commercial FFPE extraction kit. The majority of the sample is greater than 300 nucleotides.

Figura 7: Curva padrão de RNAm detectado na amostra através do aparelho Nanodrop®

Na figura 8, observamos a concentração mínima de massa para análise do aparelho circulada em vermelho, também disponível em Nanostring ® User Manual.

TABLE 1.1 Recommended sample input mass for nCounter Elements XT reagents.

Sample Type	nCounter Analysis System (MAX/FLEX)
mRNA	100–300 ng
Fragmented DNA	300 ng
ChIP DNA (unamplified)	10 ng
ChIP DNA (whole genome amplification)	100 ng

Figura 8: Amostras parafinadas são consideradas DNA fragmentado. Para estas, a concentração mínima de RNAm deve ser de 300ng.

4.7.3 Resultados de estudo piloto

Foi realizado estudo piloto seguindo o método de extração e avaliação de quantidade de RNAm extraído conforme descrito nos itens 4.7.1 e 4.7.2.

Neste estudo piloto já descartamos a possibilidade de analisar as queratoses actínicas e nevos displásicos, pela baixa contagem de material genético nos blocos (vide tabela 1). O aumento nos cortes levaria ao esgotamento do bloco sem ainda atingir o mínimo detectável para a realização do exame molecular. Além disso, estas lesões são impossíveis de serem destacadas da epiderme adjacente a olho nu, o que comprometeria também o resultado da análise.

No estudo, também observamos, que a única forma de analisar os controles seria através de pool com 4 amostras distintas, pela baixa contagem de material genético.

Tabela 2 – Resultados do Estudo Piloto

Material referência	Contagem de material genético, à partir do dispositivo Nanodrop ®
CONTROLE 3	-3,68
QUERATOSE ACTÍNICA 1	22,54 – Excluído pois provável contagem do folículo piloso e não da queratose (couro cabeludo)
QUERATOSE ACTINICA 7	-3,563
CARCINOMA BASOCELULAR 4	1,47
CARCINOMA BASOCELULAR 9	3,03
CARCINOMA ESPINOCELULAR 3	-1,455
CARCINOMA ESPINOCELULAR 10	4,154
NEVO INTRADERMICO 2	4,67
NEVO DISPLASICO 2	-1,75
NEVO DISPLASICO 68	-2,359
MELANOMA 3	1,179
MELANOMA 8	2,68

A partir desta avaliação, aumentamos a espessura do corte das lâminas para 10 micras e calculamos proporcionalmente ao tamanho da lesão uma quantidade média de cortes a serem realizados, variável de acordo com o tamanho da lesão, mas sempre proporcionalmente às lesões do projeto piloto. A média da quantidade de cortes na espessura de 10 micra foi de 27,5 lâminas por caso.

4.7.4 Resumo do método de avaliação do RNA na plataforma Nanostring ®

Figura 9: Fotografia do aparelho no momento de colocação de cartuchos para leitura. Fonte: próprio autor

O aparelho, automatizado (figura 9), realiza as análises em grupos de 12 amostras. Cada amostra de RNA é ligada a 2 probes, ou sondas, personalizadas, que se ligam a um gene especifico. Uma destas sondas

apresenta código de barras fluorescente que é lido pela máquina posteriormente, em mecanismo de espectrofotometria, que determina a presença do gene alvo (figura 10).

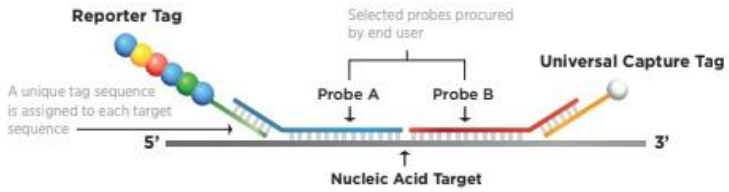


Figura 10: Ilustração da hibridização das duas sondas ao RNAm, disponível em Nanostring® User Manual.

O sistema também apresenta mecanismo “wash-out” para remoção de outros fragmentos de RNA que não sejam de interesse, através de “beams” metálicos em mecanismo de purificação eletromagnético (Figura 11).

Figura 11: Fotografia em que visualizamos o resíduo (RNA não desejado) ligado a esfera metálica no fundo da placa.

4.8 Hibridização com as sondas

4.8.1 Masterstock

Primeiramente, realizamos uma primeira diluição do probe A, para obtenção do Masterstock Probe A

Acrescentar 5 µl de cada probe A (concentração 1 µM) a volume de TE (10 µM Tris, Ph 8, 1 µM EDTA) até completar 1 ml

Reservar em temperatura de -20 a -80 ºC

Depois, realizamos uma primeira diluição do probe B, para obtenção do Masterstock Probe B

Acrescentar 5 µl de cada probe B (concentração 5 µM) a volume de TE até completar 1 ml

Reservar em temperatura de -20 a -80 ºC

4.8.2 Working pool – probes para uso imediato

Probe A – Acrescentar 4 µl do MasterStock A a 29 µl do TE

Concentração final = 0,6 nM

Probe B – Acrescentar 4 µl do MasterStock B a 29 µl do TE

Concentração final = 3 nM

4.8.3 –Hibridização de fato

Remover 1 Tagset do freezer e deixar em temperatura ambiente (“estante” onde as reações serão realizadas dentro da máquina)

Adicionar 70 µl do buffer de Hibridização e 7 µl do probe A Working pool a 7 µl do probe B Working pool. Adicionar água RNAse free até volume suficiente para 14 reações (considerar 2 perdas). Este é o Mastermix.

Nomear os tubos de hibridização

Adicionar 8 µl do Mastermix em cada um dos 12 tubos

Adicionar 7 µl de cada amostra de RNA, até volume mínimo de 15 µl (adicionar água RNAse free se necessário)

Feche os tubos e mexa gentilmente.

Previamente, aquecer o termociclador a 67ºC e colocar ali as amostras por no mínimo 16h (16 a 21h).

No dia seguinte, abaixar para 4ºC e realizar a analise em no máximo 24h.

4.9 Avaliação do RNA feita pelo Nanostring®

Após leitura por mecanismo de espectrofotometria, o aparelho gera os dados da leitura em software disponível no mesmo. O resultado é dado na forma de número absoluto de moléculas detectadas. Neste mesmo software, são listadas as amostras de baixa qualidade a serem excluídas, de forma automática (sinalizados com bandeirinhas – “flags”).

Além disso, podem ser analisados os dados no próprio software, através de análise estatística comparando as informações das amostras. Os dados são normalizados levando-se em consideração o gene housekeeping, como um padrão positivo para as amostras analisadas.

5.0 Análise Estatística

Os dados foram submetidos a análise estatística, e de acordo com sua distribuição, submetidos a testes paramétricos e não-paramétricos. Análise de variância e teste de Tukey foram utilizados para avaliar idade. Testes de comparação múltipla de Poisson e Wald foram utilizados para avaliar as variáveis discretas nos primeiros dois artigos. Também foram aplicados testes de correlação por coeficiente de Spearman.

No terceiro artigo, o teste de Kruskall-Wallis foi utilizado para avaliar idade e o de Qui-Quadrado para avaliar gênero e exposição solar. A comparação de medianas dos grupos para as variáveis discretas (contagens de RNAm) com o grupo controle e em duplas (CBC X CEC e NI X MM) foi realizada pelo teste de Mann-Whitney com correção pelo teste de Holm-Bonferroni.

Valor de p abaixo de 5% foi considerado estatisticamente significante.

6.0 Aspectos éticos

O comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu (UNESP) aprovou o estudo, com o número de protocolo

03058918.4.0000.5411 e dispensou o uso de Termo de Consentimento Livre e Esclarecido. (Vide anexo 1)

CAPÍTULO II – Artigos científicos

Artigo 1. Ocanha-Xavier JP, Xavier JCC Jr, Guimarães da Silva M, Marques MEA. Impact of VDR and RXR expression in non-melanoma skin cancer pathogenesis. Exp Dermatol. 2022; 31(8):1202-1207.

Artigo 2. Ocanha-Xavier JP, Xavier JCC Jr, Guimarães da Silva M, Marques MEA. VDR and RXRα in melanocytic benign lesions and melanoma. Am J Dermatopathol. 2023; 45(9):619-625.

Artigo 3. Ocanha-Xavier JP, Xavier-Jr JCC, Guimarães da Silva M, Marques MEA. Transcriptomic analysis of genes associated with vitamin D receptor signalling reveals differences between skin cancers. Exp Dermatol. 2024; 33(10):e15160.

1. Artigo 1

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RESEARCH ARTICLE

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Impact of VDR and RXR expression in non-melanoma skin cancer pathogenesis

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Abstract

1,25$(OH)_2D_3$, the active form of vitamin D, has been extensively studied for its putative protective activities against tumors. It does biological work by connecting to a nuclear receptor called VDR, which heterodimerizes itself to another nuclear receptor, RXR. The study observed differences in VDR and RXR expression in non-melanoma skin cancer a actinic keratosis and compared it with normal skin. We performed VDR and RXR immunohistochemistry of 76 controls (normal skin), 49 actinic keratosis, 99 basal cell carcinomas and 96 squamous cell carcinomas from formalin-fixed paraffin-embedded, resulting from surgical procedures. There was a clear pattern in the control group ($p < 0.001$), with the positivity of both receptors, VDR and RXR. Actinic keratosis differed from the basal cell carcinoma and control groups concerning RXR expression ($p < 0.001$). SCC was negative for both receptors, differing in all groups ($p < 0.001$). The site of positivity (nuclear, cytoplasmatic or both) of VDR differed between all groups ($p < 0.001$). To date, our series is the largest of VDR and RXR immunohistochemistry concerning non-melanoma skin cancer. Our findings reinforce the need to understand the pathways involving VDR and RXR to direct therapies and prevention manoeuvres.

KEYWORDS
actinic, basal cell, calcitriol, carcinoma, keratosis, receptors, retinoid X receptor alpha, squamous cell

1 | INTRODUCTION

Ultraviolet radiation is the primary cause of preneoplastic and cancer lesions in the skin. Ultraviolet B (UVB) at 290–320 nM transforms 7-dehydrocolesterol to lumisterol and taquisterol and secosterol. They suffer hydroxylation systemically (especially in the kidney and liver) and locally (in the skin), resulting in the active form of vitamin D: 1,25$(OH)_2D_3$.[1]

1,25$(OH)_2D_3$ initiates biological responses when it binds to its receptor (VDR), a nuclear receptor that heterodimerizes preferentially to a retinoid receptor (RXR). This heterodimer recognizes vitamin D-responsive elements (VDRE) located in vitamin D-regulated genes and co-regulates the transcription or suppression of many genes.[2] The VDR receptor is generally positive in normal skin and is more robustly expressed in basal cells and the outer sheath of hair follicles than in upper cells.[3] It has been studied in benign and malignant lesions of the skin, breast, bones, prostate and the gastrointestinal tract.[4–6] Basal cell carcinomas (BCCs) express VDR positivity in the nucleus that was generally stronger than adjacent normal skin according to immunohistochemistry and polymerase chain reaction (PCR) assays.[7,8] Slominski et al. reported less intense (but positive) VDR receptors in squamous cell carcinomas (SCCs) than in normal

cells and BCCs.[1] Melanomas and their metastases showed a reduction in cytoplasmic VDR receptors and enhancement of nuclear VDR receptors, compared to benign and normal skin.[2] This association varied according to subtype, the thickness of the tumor, and sun exposure, being more positive in non-exposed areas than in sun-exposed areas.[2]

Retinoid receptors are a superfamily of nuclear receptors. The preferred receptor that attaches to VDR is RXR, with its subunits α, β and γ.[10,11] RXR α is more frequent in the skin than other tissues, while β occurs in internal organs and γ is frequently related to neural disorders.

A study with RXR-suppressed mice showed differences in size, proliferation rate and enhanced vascularization in melanoma, demonstrating that RXR is a protective factor.[12] T- cell cutaneous lymphomas treated with RXR agonists showed a better response to treatment, with the induction of apoptosis and decreased cell proliferation.[13] RXR agonists inhibit cell proliferation in SCCs,[14] and RXR is progressively decreased in actinic keratosis (AK) and SCC.[15] Previous data suggested that RXRs inhibit cell growth by affecting the MAPKinase pathway.[16]

Some studies have suggested the use of retinoid agonists even though governmental agencies do not yet regulate them. These include isotretinoin and acitretin for the chemoprevention of non-melanoma skin cancers (NMSC). A study showed a reduction of 63% NMSC in patients with genetic syndromes,[17] and other studies reported this protection to patients after kidney transplantation.[18,19]

The pathway to NMSC pathogenesis appears to involve VDR and RXR; nevertheless, the mechanism is largely unclear. Therefore, this study aimed to contribute the most extensive series of VDR and RXR immunohistochemistry analyses of epithelial skin tumors to fill knowledge gaps regarding the role of these receptors in NMSC.

2 | MATERIALS AND METHODS

2.1 | Sample selection

Formalin-fixed paraffin-embedded skin specimens, collected from skin surgeries conducted in Botucatu Clinical Hospital, Faculty of Medicine from 2012 to 2018, were included in this retrospective cross-section study. They were randomly allocated to four groups: AK, BCC, SCC and control. The smallest group was AK because of the lower demand for surgical treatment. The control group was obtained from a free margin of melanoma or dysplastic nevi that did not show any sign of remaining lesions.

Haematoxylin and eosin-stained slides of all samples were reviewed to exclude BCCs that were not of the nodular subtype and samples without appropriate technical conditions (eg lack of material after the first section or unsatisfactory tissue quality). The SCC was all well- and moderately-differentiated types, and in situ lesions were excluded.

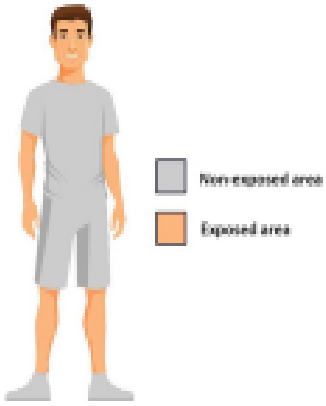


FIGURE 1 Sunlight-exposed and non-exposed areas

Pathological reports were analysed to record gender, age and site of occurrence (exposed or non-exposed sunlight areas). The classification according to sunlight-exposure followed the orientation in Figure 1.

2.2 | Immunohistochemistry analysis

Paraffin samples underwent immunohistochemistry for VDR (Rabbit, Abcam - UK, 1:1500) and RXRα (NR2B1 Antibody, Mouse, Novus Biologicals[3]), according to manufacturer's protocols of Dako[4] Immunohistochemistry Autostainer Plus. Subsequently, they were analysed by two dermatopathologists and one dermatologist that defined the positivity level of markers.

We employed a score that classifies the staining strength from 0 to 3 (0 = negative, 1 = weak, 2 = moderate and 3 = strong) and another score that classifies the number of positive cells, from 0 to 4 (0 = below 5% cells, 1 = from 6 to 25% cells, 2 = from 26 to 50% cells, 3 = from 51 to 75% cells and 4 = from 76 to 100% cells). A final score from 0 to 12 was obtained by multiplying these factors. Staining was considered positive when the final score was greater than 4. Many scores have been utilized; however, this score, which splits groups into equal quartiles, was preferred.[20]

The VDR marker had two different scores according to location site: nuclear and cytoplasmic. They were classified into the following four categories: negative, nuclear, cytoplasmic and both (when both places had a positive score and it was not possible to determine the predominant site of location). Normal skin surrounding the lesion site was an internal control. Lesions with adjacent normal skin negative to immune markers were excluded.

Data were subjected to statistical analysis, and according to their distribution, they were subjected to parametric and non-parametric tests. Analysis of variance and Tukey tests were used to analyse age. Poisson and Wald multiple comparison tests were used

to analyse discrete variables. A p-value below 5% was considered statistically significant.

The Ethics Research Committee of our Institution approved the study with acceptance number 03058918.8.4.0000.5411.

3 | RESULTS

After applying the exclusion criteria, the tissue samples included 76 controls, 49 AK, 99 BCCs, and 96 SCCs; 47.6% were females and 52.34% were males. The control group was younger than the other groups, as described in Table 1 (analysis of variance, $p < 0.001$). Groups differed regarding sunlight exposure ($p < 0.001$) because the control group primarily consisted of samples from non-sun-exposed areas, as opposed to AK, BCCs and SCCs in sun-exposed areas, as expected.

3.1 | VDR- and RXR-positivity

There was a clear pattern of positivity in the control group: 85.5% were VDR-positive, located primarily in the nucleus and in the cytoplasm, and 93.8% were RXR-positive. The BCC group was 50.5% VDR-positive, primarily in the cytoplasm, and 73.5% were RXR-positive. The AK group was 85.7% VDR-positive, primarily in the nucleus and 81.3% were RXR-negative. The SCC group was 90.6% VDR-negative, and 58.5% were RXR-negative. This group significantly differed from the control, AK, and BCC groups for both markers (Poisson, $p < 0.001$). When considering VDR expression separately (nucleus and cytoplasmic sites), nuclear expression showed no significant difference between BCC and SCC groups and they differed from other groups. The BCC group was primarily positive in the cytoplasm. VDR expression sites are represented in Graphic 1.

The most frequent presentation of immunohistochemistry of each lesion is shown in Figure 2. After analysing the final results, we generated a figure that schematically summarizes changes in immunohistochemistry between groups (Figure 3).

4 | DISCUSSION

We found significantly reduced VDR and RXR expression in SCC. AK showed a significant reduction of RXR, reinforcing the notion of progression of AK to SCC, while BCC changed the site of VDR location compared with the control group. The downregulation of these receptors may represent a reduction in protective strategies during NMSC development, as discussed below.

Vitamin D3 and its active forms have been related to cancer protection by several mechanisms.[15–18] In keratinocytes, it protects against UVR-induced DNA damage, reduces pro-inflammatory response, increases p53 expression and minimizes UV immunosuppression.[19–21]

In the experimental mouse model, VDR knockout mice ($VDR^{-/-}$) demonstrated a compromised DNA repair pathway after radiation exposure. $VDR^{-/-}$ showed the same growth rate under UV exposure, suggesting a possible failure in eliminating damaged cells via apoptosis. $VDR^{-/-}$ mice also showed an inability to thicken the epidermis, an adaptive mechanism of keratinocyte DNA protection. The results of this study did not suggest the influence of circulating $1,25(OH)_2D_3$ during tumorigenesis, while changes in VDR did exert an influence.[22]

RXR-α knockout mice demonstrated destruction in the hair follicle architecture, resulting in alopecia and epidermal interfollicular hyperplasia with keratinocyte hyperproliferation and aberrant terminal differentiation, reinforcing its importance to cell growth control.[23]

RXR agonists—but not all-trans retinoid acids—induced hepatoma cell apoptosis in vitro.[24,25] Suppression of RXR-α levels in hepatocytes increased Wnt signalling and enhanced tumorigenicity, whereas ligand activation of RXR-α achieved the opposite.[26] Several studies indicated the importance of RXRs in colon[27] and breast

TABLE 1 Immunohistochemistry data

Group	Control	BCC	AK	SCC	Statistical analysis
Categories					
Male	53.8%	52.5%	50%	52.1%	$p = 0.9820$
Female	46.2%	47.5%	50%	47.9%	
Age	50.22 ± 16.8	69.59 ± 12.52	71.56 ± 11.73	72.65 ± 13.79	$p < 0.001$[1]
Sun exposure	90% non-exposed	88.8% exposed	95.7% exposed	85.9% exposed	$p < 0.001$[2]
VDR nuclear score	7.72 ± 3.19[a]	1.34 ± 2.43[b,c]	8.76 ± 2.92[a]	1.22 ± 2.02[b,c]	$p < 0.001$[3]
VDR cytoplasmic score	4.12 ± 1.1[a]	5.01 ± 2.65[b]	5.06 ± 1.78[b]	3.29 ± 2.08[a,c]	$p < 0.001$[3]
RXR score	8.86 ± 2.45[a]	8.01 ± 3.85[b]	3.29 ± 2.99[c,d]	4.87 ± 3.94[c,d]	$p < 0.001$[3]

[a,b,c,d]Demonstrates differences between groups.

[1]Anova test.

[2]Wald test.

[3]Poisson test.

‡Scores considered negative according to cut-off.

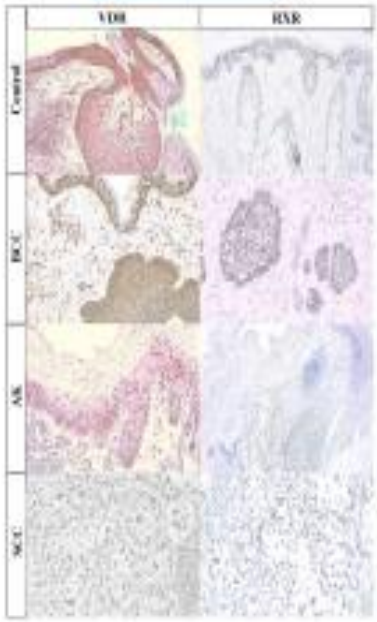
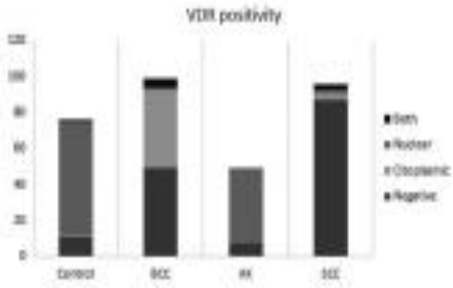


FIGURE 2 Most frequent presentation of immunohistochemistry in each group. VDR immunostaining is presented; in the right column, RXR immunostaining is observed. In the first line, we see the control group, with VDR-positivity in the cytoplasm and nuclei (200×) and RXR-positivity (200×). In the second line, we see the BCC group, with VDR-positivity mainly in the cytoplasm and RXR-positivity (both in 200×). In the third line, we see the AK group, with VDR-positivity in both sites and RXR-negativity (both in 200×). Finally, in the fourth line, we see the SCC group, both VDR- and RXR-negative (in 200×)



GRAPHIC 1 VDR positivity sites in different lesions

cancer[18,19] development; however, little information is found regarding skin cancer, although RXRα has been identified in skin tissue.

Although our series was the largest of VDR and RXR immunohistochemistry in NMSC, our control group was primarily composed of skin from non-exposed areas, and some studies showed a difference in positivity according to sunlight exposure.[4]

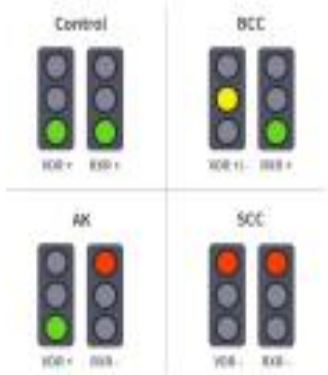


FIGURE 3 Immunohistochemistry results. Green lights represent when the receptor is significantly positive, and red lights represent significantly negative. Yellow lights represent a tendency to change in receptors, with the positive/negative ratio near 50/50 (however, not statistically significant)

Our results in the BCC group agree with those previously demonstrated in the literature[5,13]; nevertheless, in our series, positive staining was located in the cytoplasm (50.5%). The strength of VDR marker staining was higher in the BCC group than the control group, only in the cytoplasm but not in the nucleus.

Some studies discussed the exact localization of VDRs and RXRs. Barsony et al[40] utilized the multicolour fluorescent protein chimeras of nuclear receptors. Using microscopy in living cells, they demonstrated that VDR may be shuttled from the nucleus to the cytoplasm and are found in both sites, while RXRs were predominant in the nucleus even in living cells.[40] Little is known about VDR cytoplasmic interactions. Pensel et al observed 44 new interactions, including p53 that may have tumor-suppressing activity[41]; nevertheless, further studies are required to understand the biological importance of the shuttle outside the nucleus.

There are few studies on the correlation of AK to VDR and RXR pathways; however, some studies demonstrated that vitamin D administration followed by photodynamic therapy reduced the risk of recurrence.[42] The AK group showed an apparent loss of RXR, consistent with being an intermediate state between the control and SCC groups that presented a loss of both receptors.

The loss of RXRs in the SCC group is consistent with the protective effect of RXRs reported previously[12–15] and reinforces previous attempts to administer RXR agonists to risk populations developing SCCs. Knowledge of the VDR/RXR pathway to developing NMSC may direct therapies that could decrease the persistent rise of the most common cancer in humans and minimize the social and economic burden.

Our group initiated molecular analysis (PCR) to confirm immunohistochemistry results; however, there were some limitations in using skin lesions such as AK. Because of the small number of cells, the RNA yield after RNA extraction was insufficient for molecular analysis, resulting in additional costs to amplify RNA material. These

small lesions will be more easily analysed using molecular methods as technologies develop. However, immunohistochemistry analysis remains a feasible way to demonstrate these receptor interactions in small lesions, which are currently the most frequent type of lesions.

In conclusion, SCC decreased VDR and RXR levels, while AK only decreased RXRs. This observation demonstrates that RXR alteration occurs before VDR alteration in SCC pathogenesis, reinforcing these receptors' role in the process of carcinogenesis. BCCs have only shown a significant difference in VDR intracellular location; however, further studies are needed to ensure that this phenomenon has biological outcomes for the tumor. Therefore, VDR and RXR have possible protective effects against NMSC that require more investigation to enable targeted treatment therapies focusing on vitamin D and retinoid receptors.

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CONFLICT OF INTEREST

Nothing to declare.

AUTHOR'S CONTRIBUTIONS

JPOX involved in conception of the work, review of patient records, review of haematoxylin and eosin staining, immunohistochemistry assay, and analysis, review of the literature, writing and manuscript revision. JCCKJr involved in review of haematoxylin and eosin staining, immunohistochemistry, analysis, review of the literature, writing and manuscript revision. MGS and MEAM involved in conception of the work and manuscript revision.

DATA AVAILABILITY STATEMENT

The datasets used or analysed during the study are available from the corresponding author on reasonable request.

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2. Artigo 2

Vitamin D Receptor and Retinoid X Receptor Alpha in Melanocytic Benign Lesions and Melanoma

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Abstract: Vitamin D receptor (VDR) exerts its biological effects when it heterodimerizes to a nuclear receptor of the retinoid family called retinoid X receptor α (RXRα), stimulating or inhibiting DNA transcription. VDR stimulation by vitamin D analogs led to in vitro antiproliferative effects, and experimental RXRα knockout led to loss of proliferation control in melanoma cells. The aim of this study was to determine VDR and RXRα positivity in melanocytic lesions, compared with normal skin species. By immunohistochemistry assays, nuclear VDR, cytoplasmic VDR, and RXRα and RXRα in keratinocytes surrounding melanocytes were evaluated in 77 controls, 92 intradermal nevi, 54 dysplastic nevi, and 83 melanomas in this retrospective cross-sectional study. Nuclear VDR, cytoplasmic VDR, and RXRα were less expressed in exposed areas ($P < 0.001$, $P = 0.0006$, and $P < 0.001$, respectively) than covered areas. All melanocytic lesions had loss of VDR and RXRα comparing with the control group. In the melanoma group, nuclear VDR tended to inversely correlate with the Breslow index ($r = -0.11$, $P = 0.29$) but directly correlated with histological regression ($P = 0.0293$). RXRα inversely correlated with mitosis ($r = -0.245$; $P = 0.0263$). We can suggest that sun exposure affected VDR and RXRα immunopositivity. Nuclear VDR tendency of inverse correlation with the Breslow index showed that worse melanomas have a greater loss of VDR. RXRα inversely correlated with mitosis, indicating that RXRα can have a role in proliferation control. VDR and RXRα may participate in the development of melanocytic lesions and be a future target of new studies and directed therapies.

Key Words: calcitriol, VDR, retinoid X receptor alpha, melanoma, nevus

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INTRODUCTION

Several studies associated ultraviolet (UV) exposure with melanoma; however, this association is not dose-dependent because continuous exposure (eg, among outdoor workers) was associated with lower risk than sunbathing and burning episodes.[1,2] Hypothetically, sunburn leads to immune suppression and loss of DNA repair mechanisms.[2–4] Intermittent sun exposure can thicken the epidermis, whereas covered areas (which suffer occasional exposure) have more chance to develop melanoma.[2]

UVA seems less carcinogenic because it generates guanine oxidation in DNA. Ultraviolet radiation B (UVB) generates pyrimidine or cyclobutene dimers, which are more oncogenic; nevertheless, it is not clear which wavelength is predominant in melanoma pathogenesis.[5]

The primary source of vitamin D is UVB exposure.[1,2] There is no consensus regarding the ideal serum vitamin D levels because this determination depends on phototype, body mass index, dietary intake, supplementation, latitude, and genetics.[2] Low vitamin D levels have been associated with worse outcomes in melanoma.[1,2] A prospective study involving 47,800 men associated low vitamin D serum levels with only a tendency of developing melanoma, whereas higher levels were not associated with protection.[6]

Many experimental studies demonstrated the effects of vitamin D on melanoma cells in vitro. In their classical study, Reichrath et al[7] reported that MeWo, SkMel-28, and SM melanoma cell lines demonstrated antiproliferative effects mediated by vitamin D and its analogs. Vitamin D effects in vitro depend on the cell line, because SkMel-5, SkMel-25, Institut Gustave Roussy, and MelJuso did not respond to vitamin D. Other studies demonstrated the potential effects of vitamin D in melanoma.[5,8,9] These include inducing differentiation and senescence,[10,11] apoptosis,[12,13] inhibiting angiogenesis,[14] and affecting inflammation[12,15] depending on the vitamin D receptor (VDR). VDR attaches to retinoid X receptor α (RXRα), an intranuclear receptor from the retinoid receptor family, which binds to vitamin D–responsive elements in target genes activating or repressing transcription.[1]

After many conflicting studies, a recent meta-analysis involving 14 studies considered VDR polymorphisms responsible for a lower or higher risk of developing melanoma.[16] The Fok1-dominant allele increased the risk of melanoma by 22%. The Apa-I–dominant allele raised the risk by 20%, while BsM-1 lowered the risk by 15%. Other variants (ie, A1012G, Cdx2, Bg-III, and Taq 1) showed no significant associations with the risk of melanoma. Varying distributions of polymorphisms may explain the previous conflicting results according to the population or bias during these studies.

Studies also showed that loss of RXRα was more significant in melanoma than in benign melanocytic cells.[17]

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Retinoid analogs inhibited cell proliferation in melanoma cells.[18–21] RXRα is the most frequent retinoid receptor expressed in melanoma; however, some studies discuss which retinoid receptor of the retinoid family is most responsible for the antiproliferative effect. A recent experimental mouse model study using adapalene (a topical retinoid) in A375 melanoma cells showed a dose-dependent cytotoxic effect, arrest in the S phase of the cycle cell, and induction of apoptosis.[21] Trigenic knockout mice (RXRα^{-}/NRAS^{+}/CDK4^{+}) exposed to a single neonatal UVB dose showed higher cell proliferation, higher expression of tumoral markers, higher vascularization, and lymph node invasion than mice without the knockout.[22] Because RXRα is expressed at lower levels in melanoma cells than in keratinocytes, the authors speculated that RXR in the surrounding keratinocytes might exert paracrine effects on melanogenesis because its knockout enhanced melanocytic proliferation.[22]

The purpose of this study was to compare VDR and RXRα expression in melanocytic lesions, such as melanocytic nevi (intradermal type), dysplastic nevi, and melanomas, with normal skin, and we investigated variations that could affect this expression, as described previously in the literature.

MATERIALS AND METHODS

Sample Selection

This retrospective cross-sectional study considered formalin-fixed paraffin-embedded skin specimens collected from skin surgeries conducted at Botucatu Clinical Hospital, Faculty of Medicine, from 2012 to 2018. Samples were allocated to the following groups: intradermal nevi (IN), dysplastic nevi (DN), melanoma (MM), and control group. The smallest group was DN, and it defined the minimum sample size. The maximum sample size was arbitrary and consisted of 100 cases. The control group was obtained from re-excisions to obtain wider free margins of melanoma or DN that had no signs of residual lesions.

Hematoxylin/eosin-stained slides of all samples were reviewed to exclude nevi that were not the intradermal subtype, confirm DN and MM diagnosis, and exclude samples without technical conditions to join the study (ie, insufficient material or unsatisfactory tissue quality). This review considered World Health Organization criteria for diagnosing DN and excluded cases with no agreement between the 3 observers (2 dermatopathologists and 1 dermatologist).

Considering normal skin surrounding the melanocytic lesion as an internal control of immunohistochemistry positivity, we excluded cases with negative normal skin surrounding the lesion. Melanoma cases were classified as superficial spreading (SS), lentigo maligna (LM), acral (AC), and nodular (ND) subtypes from all staging classifications.

Anatomopathological reports were analyzed to gather information about sex, age, and site of occurrence (exposed or nonexposed sunlight areas). The Breslow index, mitotic index, presence of ulceration, and histological regression were also recorded for melanomas. The classification according to sunlight exposure is presented in Figure 1.

Immunohistochemistry Analysis

Paraffin samples were subjected to VDR immunohistochemistry (Rabbit, Abcam, United Kingdom, 1:1500) according to the guidelines of Dako Immunohistochemistry Autostainer Plus. For RXRα (NR2B1 Antibody, Mouse, Novus Biologicals, Centennial, CO, 1:800), we performed a double staining with monoclonal mouse antibodies against a nuclear (RXRα) and a cytoplasmic target (Melan-A; Dako), staining with 3,3'-diaminobenzidine (DAB) and horseradish peroxidase (HRP) Magenta (Dako), respectively. Cases with high amounts of melanin that disturbed differentiation with positive cytoplasmic VDR were subjected to Giemsa counterstaining. The normal skin surrounding lesion sites was the internal control for immunostaining, and lesions with adjacent normal skin negative to any immune markers were excluded.

Sections were analyzed by the 3 observers who defined the positivity level of markers. We used a score classifying the strength of the marker from 0 to 3 (0 = negative, 1 = weak, 2 = moderate, and 3 = strong) and another score classifying the number of positive cells from 0 to 4 (0 = below 5% cells, 1 = from 6% to 25% cells, 2 = from 26% to 50% cells, 3 = from 51% to 75% cells, and 4 = from 76% to 100% cells). A final score from 0 to 12 was obtained by multiplying these 2 factors. Final scores >4 were considered positive. Many scores have been used; however, this score that splits groups into equal quartiles was preferred.[23]

The VDR marker had 2 scores according to the location site (nuclear or cytoplasmic). RXRα had 1 score expressed in the nucleus of melanocytes (RXRα); however, we also analyzed

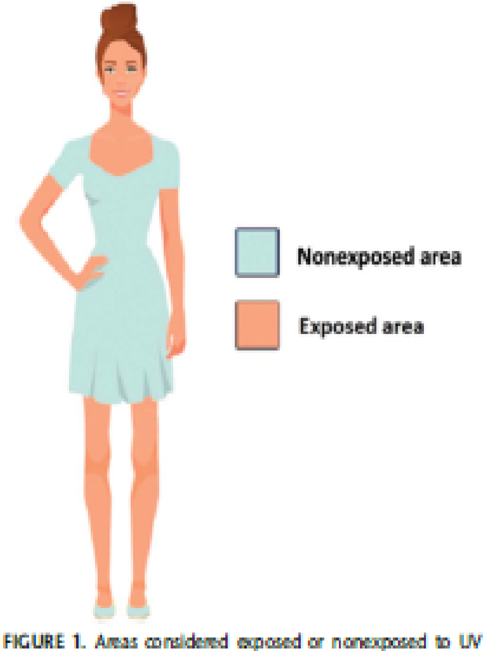

FIGURE 1. Areas considered exposed or nonexposed to UV radiation.

RXRα in the nucleus of the keratinocytes surrounding the melanocytic lesions (RXRα in surrounding keratinocytes [SK]).

Data were subjected to statistical analysis, and according to their distribution, they were subjected to parametric and nonparametric tests. Analysis of variance and Tukey tests were used to analyze age. Poisson and Wald multiple comparison tests analyzed discrete and continuous variables. Spearman correlation tests were performed for multiple comparisons. A P value below 5% was considered statistically significant.

The Ethics Research Committee of our institution approved this study (Acceptance Number 03058918.4.0000.5411).

RESULTS

After applying the exclusion criteria, there were 77 controls, 92 IN, 54 DN, and 83 MM for 306 specimens.

General overview of the Sample

There were 54.8% from female and 45.2% from male patients. IN differed from other groups because there were more women than men (72.5%, $P = 0.0003$). Patients in the MM group were older than those in the other groups (Table 1; analysis of variance followed by the Tukey test, $P < 0.0001$). All groups differed regarding sunlight exposure ($P < 0.0001$), and the IN group was the only one that occurred primarily in exposed areas.

The MM group is summarized separately in Table 2, considering subtype, Breslow index, ulceration mitotic index, and histological regression. The group comprised 57 SS, 9 LM, 8 AC, and 9 ND. All groups had Breslow indexes media higher than 1 mm; however, the ND and AC groups had thicker lesions than the SS and LM groups. Ulceration was seen in 10.7% of SS, 2.5% of LM, 62.5% of AC, and 66.7% of ND groups, with significant differences between the SS and the AC and ND groups ($P = 0.0043$). The SS group showed less mitosis than the AC and LM groups, which did not differ from one another and showed less mitosis than the ND group ($P < 0.001$). SS had higher levels of histological regression ($P = 0.002$).

VDR and RXRα Positivity

The control group showed nuclear and cytoplasmic VDR positivity and RXRα positivity (Table 1), whereas other groups were negative, considering the cutoff value of 4. Age did not significantly correlate with nuclear and cytoplasmic VDR, nor RXRα and RXRα in SK. Although all groups were different in sunlight exposure (Table 1), the body site inversely correlated with VDR and RXRα positivity. Nuclear VDR, cytoplasmic VDR, and RXRα were lower in exposed areas ($P < 0.001$, $P = 0.0006$, and $P < 0.001$, respectively) (Table 3) while RXRα in SK did not show a significant correlation with exposure ($P = 0.5738$). When groups were analyzed separately, the control group validated this information, considering nuclear VDR and exposition ($P = 0.0207$, χ^2 test) while other groups did not show statistical significance (P-value not displayed).

Nuclear VDR showed no difference between melanocytic groups and was higher in the control group ($P < 0.001$). Cytoplasmic VDR tended toward higher levels in the MM and DN groups than in the IN group, although it was not statistically significant. Surprisingly, the DN group had higher levels of RXRα than the MM and IN groups; however, the difference was not statistically significant. RXRα in SK was not statistically different between groups. Nuclear VDR had a positive correlation with cytoplasmic VDR ($r = 0.49$, $P < 0.001$) and a highly positive correlation with RXRα ($r = 0.81$, $P < 0.001$) (Table 3).

Inside Melanoma Group

Considering only the MM group, all subtypes were negative for nuclear VDR (Table 2). Some positivity of nuclear VDR was seen only in the SS group. Cytoplasmic VDR was higher in LM and ND groups without significant differences. RXRα scores were negative in all subtypes. RXRα in SK was higher in the SS group than in the LM and AC groups ($P = 0.0413$). There was no significant difference between all groups and ND.

Nuclear VDR showed a tendency to correlate inversely with the Breslow index, although it was not statistically significant ($r = -0.11$, $P = 0.29$) but correlated with

TABLE 1. Distribution of Data Considering Various Melanocytic Lesions

Group	Control (n = 77)	IN (n = 92)	DN (n = 54)	MM (n = 83)	Statistical Analysis
Categories, %					
Male	51.3	27.5	46.3	58.5	$P = 0.0003$
Female	48.7[a]	72.5[b]	53.7[a]	41.5[a]	
Age	49.76 ± 17.22[a]	48.88 ± 17.01[a]	42.61 ± 14.66[a]	61.31 ± 12.63[b]	$P < 0.001$[a]
Sun exposure	93.3% nonexposed[a]	76.9% exposed[b]	98.1% nonexposed[a]	65.5% nonexposed[c]	$P < 0.001$†
VDR nuclear score	7.68 ± 3.19[a]	0.11 ± 0.35[b]	0.02 ± 0.14[b]	0.04 ± 0.24[b]	$P < 0.001$†‡
VDR cytoplasmic score	4.12 ± 1.09[a]	2.37 ± 1.3[b]	2.81 ± 1.83[b]	2.67 ± 1.67[b]	$P < 0.001$†‡
RXRα	8.9 ± 2.51[a]	0[b]	1.41 ± 2.98[c]	0.19 ± 0.74[d]	$P < 0.001$†‡
RXRα in SK	Not applicable	6.24 ± 2.57[a]	6.17 ± 2.57[a]	5.59 ± 3.05[a]	$P = 0.1772$

*Are Demonstrate differences between groups.
*ANOVA test.
†Wald test.
‡Poisson test.

TABLE 2. Distribution of Data Considering Only the Melanoma Subgroup

Subtype	SS	LM	AC	ND	Statistical Analysis
Samples	57	9	8	9	
Breslow	1.43 ± 1.88[a]	2.27 ± 1.39[a]	3.84 ± 3.46[b]	5.02 ± 2.91[b]	$P < 0.0001$*
Presence of ulceration, %	10.7[a]	25[ab]	62.5[b]	66.7[b]	$P = 0.0043$†
Mitosis presence	1.65 ± 3.82[a]	3.11 ± 3.52[b]	4 ± 3.02[b]	17.78 ± 5.33[c]	$P < 0.0001$†
Histological regression, %	41.1[a]	11.1[b]	0[b]	11.1[b]	$P = 0.002$†
VDR nuclear score	0.05 ± 0.29	0	0	0	$P = 0.5121$*†
VDR cytoplasmic score	2.49 ± 1.65	3.11 ± 1.62	2.38 ± 1.41	3.44 ± 2.01	$P = 0.3299$*†
RXRα	0.21 ± 0.73	0	0	0	$P = 0.9999$*†
RXRα in SK	6.05 ± 3.34[a]	4.33 ± 2.18[b]	4.13 ± 1.64[b]	5.44 ± 2.46[ab]	$P = 0.0413$†

a–c Demonstrates difference between groups.
*Wald test.
†Poisson test.

histological regression ($P = 0.0293$) (Table 3). Cytoplasmic VDR did not significantly correlate with the Breslow index ($P = 0.0589$), ulceration, mitosis, or regression. Cytoplasmic VDR and nuclear VDR were directly correlated ($r = 0.49$, $P < 0.001$) (Table 3). RXRα and RXRα in SK did not correlate with the Breslow index, ulceration, or regression. Notably, RXRα inversely correlated with mitosis ($r = -0.245$; $P = 0.0263$) (Table 3).

Figures 2 and 3 show the primary immunohistochemistry findings. Figure 4 schematically shows VDR and RXRα presentations in melanocytic lesions.

DISCUSSION

Our study showed that most melanocytic lesions were negative for VDR and RXRα. This can suggest that the loss of these receptors is a primary change for developing melanocytic lesions. Nuclear and cytoplasmic VDR and RXRα are less expressed in exposed sites, while RXRα in SK seems to be not affected by sun exposure. These data were endorsed by statistically analyzing the receptors separately in the control skin.

Our population (compared with North Americans and Europeans) tends to be more ethnically mixed, with higher levels of skin pigmentation and higher UV exposure. Some studies suggested that pigmentation in melanocytic cells reduced VDR and RXRα expression and responsiveness to vitamin D analogs.[1,24] However, melanogenesis affects this responsiveness in a complex mechanism based on results that change dramatically according to the intensity of pigmentation.[24]

Lower levels of VDR and RXRα in exposed areas than in nonexposed areas can explain why our levels of positivity were lower than those in other studies with other levels of sun exposure.[25,26] We had an IN population in primarily exposed areas, which could influence VDR positivity in this group.

There was a loss of nuclear VDR in all melanocytic lesions and a tendency toward higher cytoplasmic VDR in the DN and MM groups than IN in our study (although it was not statistically significant) which disagrees with the previous data.[25,26] Cytoplasmic VDR did not correlate with the Breslow index, mitosis, ulceration, or regression, whereas nuclear VDR directly correlated with histological regression in melanomas.

A previous study showed that cytoplasmic VDR was higher in melanocytic nevi (not specified) than in melanomas.[25] However, this study had significant differences from ours because it considered positivity to be above 31% of cells; in our group, this positivity level could be negative, depending on the strength. Another study of 25 nevi, 70 melanomas, 35 metastases, 5 re-excisions, and 5 controls demonstrated both nuclear and cytoplasmic VDR with higher levels in nevi than melanoma and metastasis.[26] However, most cases were VDR-absent or VDR-low, and their nevi group consisted of

Table 3. Spearman Correlation Coefficients—Only Main Results

	Nuclear VDR	Cytoplasm VDR	RXRα
Age	−0.0268	0.01397	−0.0578
P value	0.6483	0.8121	0.325
Sun exposure	−0.2829	−0.19479	−0.40916
P value	<0.0001	0.0006	<0.0001

	Melanoma and VDR		
	Histological Regression	Nuclear VDR	Cytoplasm VDR
Ulceration	−0.11255	−0.08882	−0.04374
P value	0.314	0.438	0.6964
Mitosis	−0.15039	−0.13816	0.11844
P value	0.1775	0.2158	0.2892
Histological regression	—	—	0.10317
P value	—	—	0.3533
Nuclear VDR	0.23932	—	—
P value	0.0293	—	—
Cytoplasm VDR	0.10317	0.49195	—
P value	0.3533	<0.0001	—

	Melanoma and RXRα		
	Ulceration	Mitosis	Histological Regression
RXRα	−0.1542	−0.24537	0.12088
P value	0.1666	0.0263	0.2763

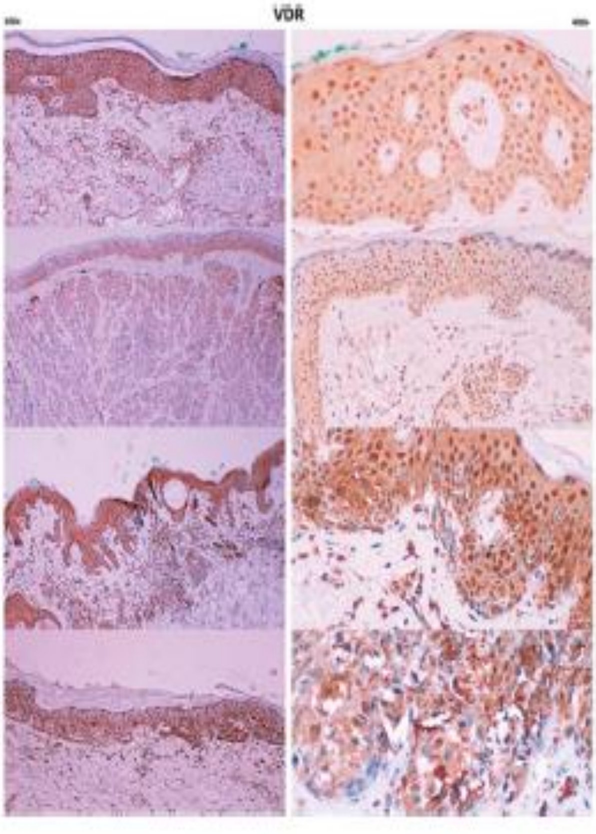

FIGURE 2. VDR's most frequent presentations among melanocytic lesions. Left row: images at 100×; right row: images at 400×, revealing details. Most of the positive marking is observed in keratocytes nuclei but not in melanocytes. Coarse melanin granules are observed in the cytoplasm of DN and MM lesions.

junctional, intradermal, compound, and DN, mixing a heterogeneous group of nevi lesions. Data were scored in arbitrary units, from 0 to 3, considering only the strength of positivity. They did not consider the proportion of positive cells (which, in our opinion, can lead to inaccurate conclusions) and neither established a cutoff number to define VDR positivity. Thus, considering the different sample selection, different antibody clones in immunohistochemistry, and distinct scores may explain the differences between their results and ours.

Previous studies showed inverse associations between VDR and Breslow index.[1,26,27] A previous study of 30 SS, 37 ND, and 2 AC melanomas showed that cytoplasmic VDR positivity had an inverse correlation with VDR and TNM staging system - classification of malignant tumors, being more positive in stages pT1 and pT2 than in the more advanced lesions.[27] Our study showed a tendency toward an inverse correlation between nuclear VDR and Breslow index that was not statistically significant ($r = -0.11$, $P = 0.29$). All melanoma subtypes had mean Breslow indexes above 1 mm in our sample, characterizing a population with poor outcomes, and perhaps because of that, VDR (nuclear and cytoplasmic) was predominantly negative.

Some studies correlated low VDR levels with nodular type[26]; however, our study did not confirm these data when matching Breslow indexes. Although Breslow indexes were statistically comparable between AC and ND, these groups had no significant difference according to VDR positivity. VDR had a direct, significant correlation with histological regression. We have not found a similar conclusion in the literature, and future studies are needed to explain this finding. Histological regression remains a phenomenon that divides specialists if it is a progression marker or an enhanced immune response marker.

Our study showed a zero score of RXRα in IN. All groups were significantly different considering RXRα positivity in a progressive order of IN < MM < DN < control. Surprisingly, the DN group had higher RXRα levels than the MM and nevi groups. Two possibilities can be suggested: First, RXRα levels can be lower in the IN group because of higher sun exposure, but we also can hypothesize that this might be a sign of the maintenance of cell proliferation

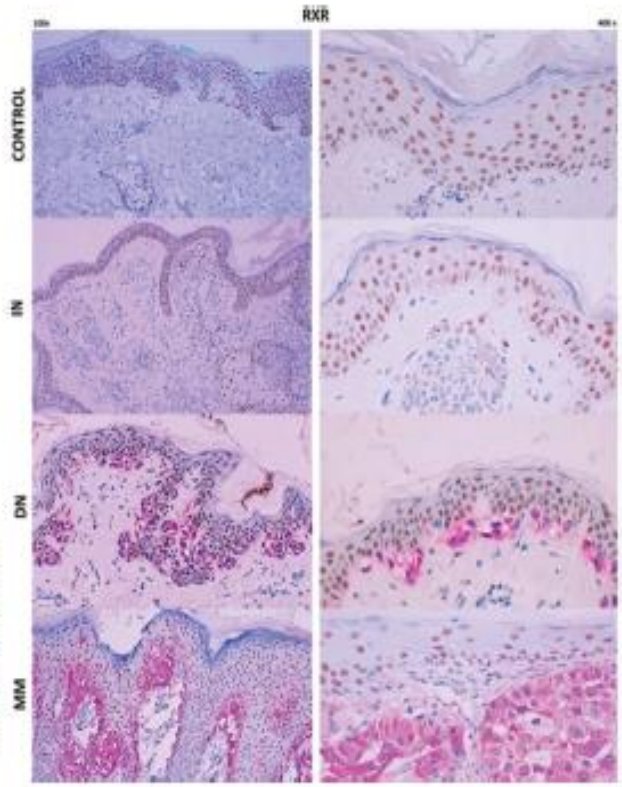

FIGURE 3. RXRα immunohistochemistry displaying the most frequent presentations among melanocytic lesions. In the left row, images at 100x and the right row pictures at 400x, revealing details. A double staining with monoclonal mouse antibodies against a nuclear (RXRα) and a cytoplasmic target (Melan-A), staining with DAB and HRP magenta, respectively, was performed, which resulted in beautiful pinkish pigmentation of melanocytes, allowing their clear differentiation from keratinocytes.

control effect. According to previous dermoscopy studies, IN lesions could be considered older than compound nevi or junctional nevi that are silent regarding cell proliferation.[28,29] At the same time, DN maintain proliferation control, evolving as a benign lesion, and MM lost this control.

A previous study of 130 melanomas, 47 metastases, and 49 benign nevi showed more cytoplasmic RXRα positivity in MM than nevi lesions and more nuclear RXRα positivity in nevi.[17] However, in that study, the nevi groups were not detailed; the antibody used was polyclonal, RXRα was higher in thicker lesions (cytoplasmic), with regression areas (nuclear), and loss of RXRα was associated with worse survival. In this study, neither RXRα nor RXRα in SK correlated with one another ($P = 0.11$) or the Breslow index ($P = 0.09$), ulceration, or regression. Nevertheless, RXRα inversely correlated with mitosis, reinforcing the RXRα role in proliferation control hypothesis. Little literature is available on RXRα and melanomas, with mostly experimental studies. We believe further studies are needed to fully understand the influence of RXRα in melanoma pathogenesis and prognosis.

We encountered difficulties in determining VDR positivity, such as differentiating cytoplasmic VDR from melanin in highly pigmented lesions. This phenomenon compelled us to perform different counterstaining to resolve this confusion. We have already initiated molecular tests to ensure these immunohistochemistry findings; however, we highlight that immunohistochemistry can be performed in smaller lesions, and it is easily reproduced, once it uses routine techniques and is available in more health care unities (not only research centers).

These findings suggest that VDR is less expressed in melanocytic lesions than in surrounding keratinocytes. This phenomenon suggested some VDR role to develop melanocytic lesions (benign or not) and that can be affected by environmental factors such as UV exposure. Moreover, loss of RXRα expression may affect lesion evolution because it is statistically different among IN, DN, and MM. Although there is no clear explanation for these findings, the maintenance of RXRα expression in DN can explain why these lesions are associated with better outcomes than melanoma. We hope these findings can provide support to future studies

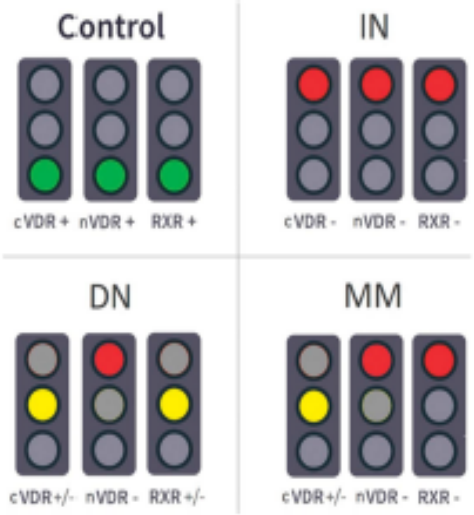

FIGURE 4. Illustration schematically shows differences between VDR and RXRα presentations. Green light shows positive results, yellow light represents results that reached border scores between negativity/positivity, and red light represents negative results (Table 1 for these results). cVDR, cytoplasmic VDR; nVDR, nuclear VDR.

regarding VDR and RXRα as potential physiopathogenesis pathways and therapeutic targets and might substantiate an approach to melanoma studies.

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3. Artigo 3.

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RESEARCH ARTICLE

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Transcriptomic analysis of genes associated with vitamin D receptor signalling reveals differences between skin cancers

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Abstract

Vitamin D activates the vitamin D receptor (VDR), which dimerizes preferentially with the retinoid X receptor-α (RXRα). This heterodimer connects with genetic elements responsive to vitamin D, inhibiting or stimulating gene activity. We performed Nanostring® analysis of VDR/RXRα to compare the mRNA expression of this heterodimer and their correlated transcriptomes in non-melanoma skin cancer (basal cell carcinomas (BCC) and squamous cell carcinomas (SCC)) and melanocytic lesions (intradermal nevi (IN), and melanomas (MM)) with control skin. To evaluate VDR, RXRα and other 22 correlated genes in BCC, SCC, IN and MM, paraffin samples had their transcriptomes analysed using Nanostring®, a platform that allows multiple mRNA analyses. There were 46 samples, including 11 BCC, 10 SCC, 10 IN, 12 MM and 3 pools of control skins. Most mRNAs differed between the lesion groups and the control group. BCC and SCC NCOR2 were upregulated; in MM and IN, RXRγ was higher than in the control group. TP53, FOXO3 and MED1 showed a significant difference when we compared the BCC group to the SCC group. Melanoma and intradermal nevi differed only in AhR. VDR and RXRα were lower than the control in all groups. The panel shows a clear difference between the non-melanocytic cancers and, on the other hand, a slight difference between the melanocytic lesions. The study of vitamin D's influence through its receptor and RXRα is an exciting issue for understanding the importance of this pathway, and the present study can impact the prevention and treatment strategies, mainly in non-melanocytic tumours.

KEYWORDS
calcitriol, carcinoma, squamous cell, melanoma, retinoid X receptor alpha, VDR

1 | INTRODUCTION

Vitamin D and its analogs have antiproliferative activities in melanoma cells in vitro.[1,2] They work by attaching to the vitamin D receptor (VDR), which heterodimerizes with the nuclear retinoid X receptor (RXRα), and they associate with vitamin D-responsive genes, activating or inhibiting transcription. Retinoid analogs inhibit proliferation in melanoma cells.[3,4] It remains unclear which retinoid receptor would be the most essential in neoplastic cells because they are a large family, including retinoic acid receptors (RARα, β and γ), retinoid-orphan-receptors and (RXRα, β and γ); however, RXRα is cited as the primary retinoid receptor in melanoma cells.[5]

Our previous immunohistochemistry assays showed that basal cell carcinoma (BCC) cells change the location of VDR from the

nucleus to the cytoplasm. Actinic keratoses showed RXRα loss without loss of VDR. Squamous cell carcinoma (SCC) cells showed loss of VDR and RXRα compared to the control group.[6] Melanocytic lesions, such as intradermal nevi (IN), dysplastic nevi (DN) and melanoma (MM), were VDR- and RXRα-negative.[7] VDR and RXRα were less evident in exposed than covered areas. In the melanoma group, VDR directly correlated with histological regression, and RXRα inversely correlated with mitosis[7] in agreement with previous data, suggesting that RXRα has a role in proliferation control.[8]

Few studies used Nanostring NCounter® to analyse cutaneous neoplasms. Nanostring sequencing differs from bulk and single-cell RNA sequencing because it can detect tumour heterogeneity. Second, it is not single-cell-based, so it can provide insight into regulatory networks between cancer cells that are important for tumour development and maintenance.

Previous studies analysed BCC responses to vismodegib.[9] Some studies regarding the SCC response to pembrolizumab[10] and pathophysiological studies in oral lesions considering SCC[11] exist. A previous study of nine SCCs and seven healthy skin samples generated a database of 105 transcription factors that displayed altered levels in SCCs, including fundamental regulators of skin development.[12] Another study showed 81 potential genes involved in ultraviolet-induced mitogen-activated protein kinase signalling through microRNA analysis of SCC.[13]

In melanoma studies utilizing Nanostring NCounter®, some investigators analysed cancer markers concerning response to immunotherapy, such as pembrolizumab,[14] and others tried to identify genes that could be therapeutic targets, such as CSAG2, MAGEA3, MAGEC2, IL13RA2, PRAME, CSPG4 and SOX10.[15] A study compared 12 benign and malignant melanocytic lesions regarding 1000 genes and proposed S100A8 as a melanoma marker expressed by keratinocytes among melanoma cells.[16]

We performed Nanostring® analysis of VDR/RXRα to understand and compare to control skin, the mRNA expression of this heterodimer and their correlated transcriptomes in non-melanoma skin cancer (BCC and SCC) and melanocytic lesions (IN and MM). This approach has not been seen in these other studies so that this study may yield new data on VDR/RXRα expression in BCC, SCC or MM lesions.

2 | MATERIALS AND METHODS

2.1 | mRNA selection

Using the Genecards database (www.genecards.org), the most frequent genetic relations to VDR were recorded, and 20 other genes were considered. GAPDH and β-actin were selected as housekeeping genes. The relevant interactions had the highest confidence level (0.900). The less statistically significant interactions and genes not expressed in the skin were excluded, in agreement with the Genecards® database. The genes selected were submitted to bioinformatic analysis by IDT® staff in March 2022, and the genes followed by their correspondent mRNA reference number (available at https://www.ncbi.nlm.nih.gov/gene/) enrolled are displayed in Table 1.

2.2 | Sample selection

Formalin-fixed, paraffin-embedded skin specimens collected from skin surgeries at Botucatu Clinical Hospital, Faculty of Medicine, from 2012 to 2018 were included in this retrospective cross-sectional study. There were five groups: control, BCC, SCC, IN and MM; the control group was obtained by margin amplation in melanomas, whose specimens were lesion-free and contained unremarkable epidermis. The selection of margin ampliation in melanomas as control was based on the following characteristics: wider size of clear skin than other tumour's margin, higher probability of a non-exposed area and availability of good material in our service. Two dermatopathologists and one dermatologist previously reviewed cases in H&E stain to ensure the diagnosis. BCCs were only nodular subtypes; SCCs were well or moderately differentiated; in situ, lesions and poorly differentiated were excluded. MM included superficial spreading, acral, nodular and lentigo maligna subtypes.

2.3 | Pilot study

A pilot study was performed to determine the necessary sections, utilizing two samples randomly selected from each group. The results are shown in Table S1.

This analysis showed that some lesions could not be included because several slice cuts would necessitate reaching the end of the material without achieving the minimum necessary for mRNA counting using Nanostring NCounter® analysis. For these reasons, there was a limitation based on the lesion size.

The number of slide cuts was based on this pilot study. The cases varied between 20 and 50 slide cuts, depending on the lesion size. We also observed that it would be difficult to obtain the control group. We then decided to pool four samples in the same analysis, resulting in four controls with four different samples each.

2.4 | Sample size and mRNA extraction

The control group consisted of four pools of four different skin samples each. Eleven cases were enrolled in each group (i.e. BCC, SCC and IN). MM had an extra case, totalling twelve cases.

The number of sections varied according to size. Macroscopically, the material was spared from surrounding keratinocytes and stored at room temperature. All samples underwent mRNA extraction utilizing the ThermoFisher RecoverALL® kit. The extracted mRNA was subjected to Nanodrop® analysis to determine if the amount of mRNA was sufficient for analysis (which, in our case, was a minimum of 300 ng) and to certify the quality of the material if it followed the pattern curve presentation of mRNA.

TABLE 1 List of the 24 mRNA analysed, their reference number (available at https://www.ncbi.nlm.nih.gov/gene/), and their primary function in the skin.

mRNA	Reference gene name	Reference number	Function
VDR	Vitamin D receptor	NM_000376.1	Stimulates or inhibits gene transcription by the presence of vitamin D
NCOA1	Coactivator gene 1	NM_003743.5	Guarantees VDR stability in its active form
NCOA2	Coactivator gene 2	NM_001321705.2	Guarantees VDR stability in its active form
NCOA5	Coactivator gene 5	NM_001174087.1	Guarantees VDR stability in its active form
NCOR1	Corepressor gene 1	NM_006311.4	Inhibits gene transcription mediated by VDR
NCOR2	Corepressor gene 2	NM_006312.6	Inhibits gene transcription mediated by VDR
MED1	Mediator complex subunit 1	NM_004774.4	Regulates VDR function of control and proliferation of keratinocytes
SMAD3	SMAD 3 protein	NM_005902.4	Mediates relation between VDR and bTGF
FOXO3	Forkhead transcription factor O3	NM_001455.4	Related to apoptosis secondary to oxidative stress
CREBBP	CREBBP acetyltransferase coactivator protein	NM_004380.2	Binds to RAR or RXR, regulating cycle cell events
EP300	EP300 acetyltransferase histone protein	NM_001429.4	Binds to RAR or RXR, regulating cycle cell events
RARA	Alpha retinoid acid Receptor	NM_000964.4	Can heterodimerize with VDR, but VDR/RXR binding is preferential
RARB	Beta retinoid acid Receptor	NM_000965.4	Can heterodimerize with VDR, but VDR/RXR binding is preferential
RARG	Gama retinoid acid Receptor	NM_000966.6	Can heterodimerize with VDR, but VDR/RXR binding is preferential
RXRA	Retinoid X receptor alpha	NM_002957.6	Heterodimerizes with VDR and acts in the control of gene transcription
RXRG	Retinoid X receptor gamma	NM_006917.5	Another member of the retinoid X family, described in neural cells
TP53	P53 tumour suppressor gene	NM_000546.5	Directly affects cycle cell, DNA repair, and apoptosis
XPA	Xeroderma Pigmentosum A haplotype	NM_000380.3	Nucleotide repair defect-related syndrome haplotype
XPC	Xeroderma Pigmentosum C haplotype	NM_004628.4	Nucleotide repair defect-related syndrome haplotype
CYP27B1	25-hydroxi-alpha-hydroxilase	NM_000785.4	Enzyme responsible for Vitamin D production by classic or canonical pathway
CYP24A1	Vitamin D3-24-hydroxylase	NM_000782.5	Enzyme related to Vitamin D degradation
AhR	Aryl-Hydrocarbon receptor	NM_001621.5	Vitamin D receptor from non-canonical pathway
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046.7	Glycolysis enzymes are utilized to housekeeping genes in the skin samples
B-actin	Beta-actin	NM_001101.5	Cytoskeleton proteins utilized as housekeeping genes in the skin sample

2.5 | Nanostring NCounter® analysis

We followed the Nanostring NCounter® user manual to perform hybridization and spectrophotometry. Each mRNA sample was hybridized into two personalized probes that targeted a specific gene. Probes were designed by IDT® (Integrated DNA Technologies, Coralville—Iowa). One probe possessed a universal capture tag, while the other had a fluorescent barcode read by the machine using a spectrophotometry method that determined the presence of the mRNA target. The system also used a wash-out mechanism to remove unwanted RNA fragments using metallic beams in an electromagnetic purifying mechanism.

The automatized device analysed 12 samples per run. Data were generated and analysed using the same device's software. Low-quality samples were flagged and excluded automatically.

2.6 | Statistical analysis

Statistical analysis was performed using the software and confirmed by a statistician later. The Kruskall–Wallis test was performed to analyse age, and the Chi-Square test was used to investigate gender and sun exposure. Median comparisons between the groups (mRNA counting) and between groups and the control were made using the multiple-comparison Mann–Whitney test followed by a Holm–Bonferroni correction.

3 | RESULTS

Forty-nine samples were studied. One IN sample was automatically excluded by the Nanostring® platform (flagged), and two cases were

excluded as they were considered outliers (one sample from the group control and another from the SCC group), totalling 46 cases.

GAPDH performed better than β-actin as a housekeeping gene. The sample characteristics are displayed in Tables 2 and 3. By analysing age, gender and sun exposure, we concluded that the groups were heterogeneous. The melanoma subgroup is described in Table 3, characterizing a sample of advanced melanomas.

Figure 1 contains a heatmap and dendrograms showing all lesion profiles. Figure 2 contains four Forest plots drawn to display the differences between groups and comparisons to the control group. The plots reveal an initial presentation of the fundamental differences between groups. Almost all mRNAs were higher in the control group, except for NCOR2 (higher in the BCC and SCC groups) and $RXR\gamma$ (higher in the IN and MM groups). VDR and $RXR\alpha$ were lower in all groups than in the control group.

Table 4 shows that most mRNA counts significantly differed between the groups and the control group. VDR and $RXR\alpha$ were substantially lower in all groups. AhR, CYP24A1, NCOA 1–3, $RAR\alpha$, $RAR\gamma$, SMAD3, EP300 and XPA were lower in all groups, while FOXO3, MED1 and TP53 were significantly lower only in the SCC, MM and IN groups.

Table 5 compares the epithelial lesions between each other and melanocytic lesions as well. BCC differed from SCC in TP53, FOXO3 and MED1 genes, while MM differed from IN only in AhR.

4 | DISCUSSION

VDR and $RXR\alpha$ were less positive in all lesions than in control skin. BCC had higher mRNA counts for NCOR2, and TP53 was not significantly different from the control with low VDR and $RXR\alpha$. SCC had higher counts for NCOR2 but the loss of TP53, FOXO3, VDR and $RXR\alpha$. Melanoma had higher counts for $RXR\gamma$ and lower FOXO3, TP53, VDR and $RXR\alpha$; IN only differed from MM by AhR.

NCOA1, NCOA2 and NCOA3 are a family of nuclear coactivators that close the VDR 12 and four DNA helices, ensuring VDR stability in their active form.[17] NCOR1 and NCOR2 are nuclear corepressor molecules; these elements inhibit gene activity and transcription. However, studies demonstrated that RXR liberates NCORs in a VDR-independent way, inhibiting VDR repression and activating VDR.[18] NCOA 1–3 were lower in all groups than the control group, in agreement with VDR downregulation. NCOR2 mRNA was lower in the melanoma group than in the control and higher in BCC and SCC.

Corepressor genes could counteract the protective effects of VDR and $RXR\alpha$; however, as previously hypothesized,[17,18] their overexpression can stimulate VDR release. That mechanism could explain why upregulation of NCOR2 causes antiproliferative effects mediated by VDR and why up- and downregulation are seen in neoplasms.

$RAR\alpha$, $RAR\beta$ and $RAR\gamma$ are the retinoic acid receptor family members. These receptors can heterodimerize with VDR; however, the VDR/$RXR\alpha$ interaction is preferred.[19] $RAR\alpha$ and $RAR\gamma$ were significantly lower in all groups than the control in agreement with $RXR\alpha$ downregulation, suggesting that retinoic acid receptors play a role in developing these lesions. Nevertheless, this mechanism remains unclear.

MED1 is a mediator complex subunit that mediates nuclear gene coactivation.[20] A study showed that MED1 ablation alters the hair follicle cycle and causes interfollicular keratinocyte hyperproliferation.[21] Another study found that MED1/MED21 blockade caused keratinocyte hyperproliferation, suggesting that this mediator regulates VDR proliferation and cell differentiation in keratinocytes.[22] MED1 was higher in BCCs than in SCC, IN or MM but still lower than the control. We hypothesize this happened because follicular basal cells proliferate in this neoplasm, and the cell origin can explain the higher count.

$RXR\gamma$ was lower in BCCs and SCCs and higher in IN and MM than in the controls. This phenomenon can be explained by $RXR\gamma$ detection in neural but not epidermic cells in mice.[23] Because melanocytes and neural cells derive from the exact embryogenic origin, we hypothesize that $RXR\gamma$ is less frequent in keratinocyte lesions.

SMAD3 is a coactivating protein mediating the interaction between VDR and TGFβ, acting in cell proliferation and differentiation.[24] All groups showed lower SMAD3 than the control group, suggesting that the interaction of VDR and TGFβ is critical for initiating these lesions.

FOXO3 is a transcription factor that controls proliferation and survival. It is a tumour suppressor linked to longevity. FOXO3 mRNA was less frequent in SCC, MM and IN than in controls and less frequent in MM than IN. FOXO3 suppression in melanoma is associated with impaired apoptosis and increased metastatic potential of melanoma cells.[25] When FOXO3 is inhibited, melanogenesis and melanin levels are increased.[26]

FOXO3 must be upregulated to induce apoptosis secondary to oxidative stress, and its downregulation has been associated with

TABLE 2 Demographic description of the sample.

Variac	Group					p-value
	Control (n = 8)	Basal cell carcinoma (n = 11)	Squamous cell carcinoma (n = 10)	Melanoma (n = 12)	Intradermal nevi (n = 10)	
Age (Mean ± SD)	65.18 ± 12.04^{ab}	71.27 ± 11.25^{ab}	79.50 ± 15.05^{b}	62.91 ± 9.95^{ab}	51.10 ± 16.91^{a}	p = 0.002
Gender (female/total)	$2/12^{a}$	$6/11^{ab}$	$8/10^{b}$	$2/12^{a}$	$8/10^{b}$	p < 0.01
Sun exposure (exposed/total)	$2/12^{a}$	$8/11^{b}$	$8/10^{b}$	$4/10^{ab}$	$6/10^{ab}$	a = p < 0.001 b = p < 0.02

Note: Letters a and b indicate statistical differences between groups.

TABLE 3 Demographic description of the melanoma group.

Subtype	
Superficial spreading	6/12
Nodular	5/12
Lentigo Maligna	2/12
Acral	8/12
Ulceration (Yes)	7/12
Breslow	4.79 mm (SD ± 5,17)
Mitosis rate	8.5 (SD ± 7,62)

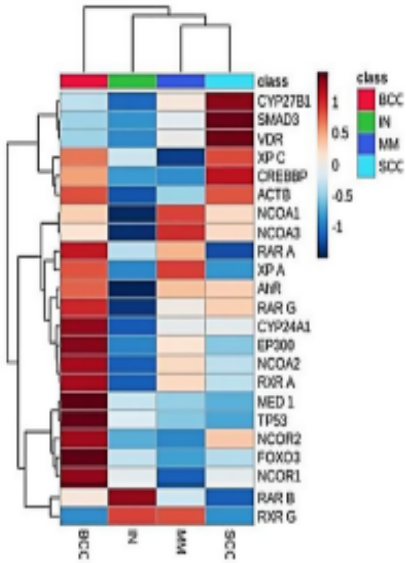

FIGURE 1 Heatmap and dendrograms showing the four lesions' (BCC, basal cell carcinoma; SCC, squamous cell carcinoma; IN, intradermal nevi; MM, melanoma) transcriptomic signatures. Genes are displayed upright.

fibroblast and keratinocyte senescence.[25] This latter phenomenon may explain why FOXO3 is also low in IN. FOXO3 downregulation in melanomas could be a mechanism for avoiding apoptosis and maintaining disease progression.

CREBBP and EP300 bind to RXR or RAR and regulate nuclear gene transcription.[27] They regulate cell cycle events such as senescence and apoptosis.[28] CREBBP/EP300 mutation leads to the rare Rubinstein-Taybi syndrome, predisposing to hematologic, and genitourinary neoplasms and benign skin lesions like pilomatrixomas.[29] CREBBP was lower in the IN group. Many skin alterations are related to Rubinstein-Taybi syndrome. However, our literature search did not find this association with IN.

CYP27B1 and CYP24A1 encode 1,25(OH)2D3 (vitamin D)-related enzymes through classic or canonical pathways. A study showed that while rats with VDR deletion had higher tumour indexes induced by ultraviolet radiation, rats with CYP27B1 deletion were resistant, indicating a preventive action mediated by VDR, independent from 1,25(OH)2D3.[30] A study demonstrated a correlation between CYP27B1 from keratinocytes and melanoma; it was less positive in the vertical growth phase and metastasis, and less positivity correlated with worse outcomes.[31] We did not find a significant relevance of CYP27B1 in the studied groups.

Higher counts of CYP24A1 in actinic keratosis, BCC and SCC were described.[32] However, the data are contradictory since higher levels of CYP24A1 were related to worse outcomes in colorectal cancer[33]; on the other hand, low CYP24A1 was linked to worse outcomes in breast cancer.[34] This enzyme is associated with vitamin D degradation from the canonic pathway. In our study, all groups showed fewer counts than the control group. Additional studies are necessary to understand these contradictions.

The AhR is a VDR from the non-classical pathway (20,23[OH[2D3) and depends on CYP11A1 activity.[35] AhR was linked to SCC susceptibility[36] in a genome-wide association study[37] and is related to photocarcinogenesis, immunosuppression and worsening nucleotide excision repair.[38] Some experimental data associated AhR deficiency with resistance to carcinogenesis, and this phenomenon might explain why it was lower in the IN group than in the other groups. However, AhR-deficient mice can toxify 7,12-dimethylbenz[a]anthracene and initiate skin carcinogenesis.[30]

TP53 is a tumour-suppressor gene involved in apoptosis, DNA repair and cycle cell control. Li Fraumeni syndrome results from mutations in the germline, enhancing susceptibility to cancers such as MM.[39] The importance of p53 includes its susceptibility to ultraviolet action, which accelerates BRAF mutations in some melanomas.[40] A population-based study found no correlation between TP53 polymorphisms and non-melanoma skin cancer.[41]

TP53 was less frequent in SCC and MM than IN; in BCC, there was no significant difference from the control. P53 is a critical tumour-suppressor gene mutated in over half of all human cancers.[40] Some studies showed that few TP53 mutations are common, even among healthy people.[42] Others showed that TP53 mutations accelerate MM formation in BRAF-expressing mice when subjected to ultraviolet light.[43] Deletion of p53 in melanomas was nearly 90% of cases, according to Rusu et al.[44] Nevertheless, its role in melanoma remains controversial.

XPC and XPA are genes related to xeroderma pigmentosum, a rare condition characterized by a deficiency in nucleotide excision repair, which enhances the risk of ultraviolet-induced cancers. One study associated less risk of developing MM in the presence of an XPC haplotype when compared to the reference gene.[45] In contrast, other studies associated a direct correlation between melanoma and XPA/XPD haplotypes. A study showed an enhanced risk of developing UVB-related tumours in XPA knock-out mice.[46] Our study showed loss of XPA in all types of lesions, which can represent a higher risk of developing neoplasms in our population. XPC was also lower in melanomas than in other groups; XPC is required for DNA damage detection and initiation of repair,[46] and XPC loss disfavors repair and favours MM progression.

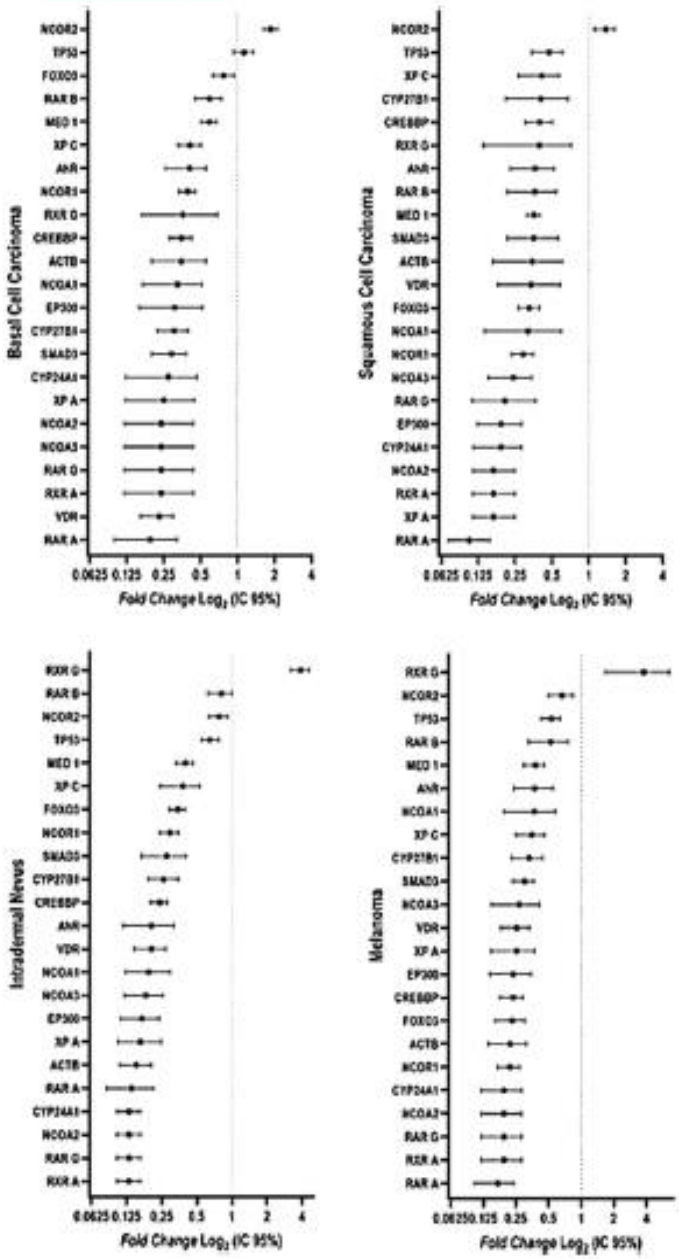

FIGURE 2 Forest plots showing mRNA fold-change in the groups compared to the control group. Values on the left side of the line show lower counting than the control group, while values on the right side show higher counting. Values that cross the line did not show statistical difference from the control group.

TABLE 4 Display of mRNA counting of the control group and *p*-value that was obtained by comparing all groups versus controls.

Gene	Control ($n = 5$) Mean ± standard deviation	BCC ($n = 11$) p-value (compared to control)	SCC ($n = 10$)	MM ($n = 12$)	IN ($n = 10$)
AhR	502.54 ± 928.16	**0.038**	**0.028**	**0.018**	**0.010**
CREBBP	5558.42 ± 1984.88	0.088	0.217	0.07	**0.028**
CYP24A1	168.81 ± 266.78	**0.022**	**0.003**	**0.002**	**0.003**
CYP27B1	558.92 ± 559.51	0.568	0.575	0.295	0.469
EP300	168.81 ± 266.78	**0.022**	**0.007**	**0.002**	**0.007**
FOXO3	2861.08 ± 1582.95	0.659	**0.014**	**0.009**	**0.007**
MED1	675.55 ± 548.16	0.088	**0.007**	**0.004**	**0.007**
NCOA1	168.81 ± 266.78	**0.022**	**0.028**	**0.046**	**0.007**
NCOA2	168.81 ± 266.78	**0.022**	**0.003**	**0.002**	**0.003**
NCOA3	168.81 ± 266.78	**0.022**	**0.007**	**0.002**	**0.003**
NCOR1	1660.02 ± 1812.1	0.291	0.161	0.101	0.187
NCOR2	2096.68 ± 1557.56	0.568	0.957	0.18	0.187
RAR_A	185.45 ± 261.15	**0.008**	**0.003**	**0.004**	**0.007**
RAR_B	704.15 ± 770.65	0.456	0.161	0.295	0.957
RAR_G	168.81 ± 266.78	**0.022**	**0.003**	**0.002**	**0.003**
RXR_A	168.81 ± 266.78	**0.022**	**0.003**	**0.002**	**0.003**
RXR_G	1052.01 ± 1178.65	**0.022**	0.077	0.84	0.469
SMAD3	1187.76 ± 1288.87	**0.005**	**0.028**	**0.004**	**0.014**
TP53	2021.2 ± 1649.55	0.555	**0.007**	**0.004**	**0.014**
VDR	5462.88 ± 4265.66	**0.038**	**0.049**	**0.031**	**0.014**
XP_A	168.81 ± 266.78	**0.022**	**0.003**	**0.002**	**0.003**
XP_C	550.5 ± 589.27	0.225	0.161	**0.031**	0.077

Note: Statistically significant differences are marked in bold, underlined numbers. GAPDH and β-actin were not described as they worked as housekeeping genes.

Abbreviations: BCC, basal cell carcinoma; IN, Intradermal Nevi; MM, melanoma; SCC, squamous cell carcinoma.

As expected, VDR was less positive in IN, MM, BCC and SCC than in normal skin. Some works suggested a higher positivity in BCCs[47]; however, our immunohistochemistry assays did not confirm this result; in the current study, mRNA analysis ruled out this possibility. Previous studies showed that VDR is less positive in melanocytes than keratinocytes and less positive in SCC than in normal skin.[6,48] VDR was less positive in melanocytic lesions as expected and less positive in SCC than in control skin, confirming previous results.[6,7]

RXRα was less positive in all groups. Our previous immunohistochemistry assays showed the same tendency of less positivity of RXRα in IN and a significant inverse correlation with mitosis, in agreement with previous studies that suggested that RXRα has a role in proliferation control.[7] Chakravarti et al. returned the opposite results; they considered 226 melanocytic lesions, 130 melanomas, 47 metastasis and 49 benign nevi (not specified).[49] These lesions were subjected to tissue microarray and immunohistochemistry. The authors reported significantly higher nuclear RXRα in IN than in MM and found a correlation between RXRα and histological regression. We believe a different methodology (not specifying the type of nevi and utilizing polyclonal antibodies) can explain the results.

Our previous immunohistochemistry studies did not see loss of RXRα in a sample of 99 BCCs. When analysing our data, we found that most of the BCCs selected for Nanostring® analysis were indeed RXRα-negative (6/11). We believe selection bias explains this unexpected result.

Finally, the MM and IN groups differed only in the AhR gene, suggesting that VDR and RXRα do not directly participate in melanoma carcinogenesis. This finding agrees with our previous immunohistochemistry assays, which found no significant VDR and RXRα expression differences between IN, DN and MM.

We pooled four skin samples in each control to utilize normal skin to perform the control group. Otherwise, we would have needed more to achieve the minimum mRNA counts to use the technique. We agree that this method is controversial, as it unites different skin samples and may be challenging to evaluate.

The advanced melanoma sample was influenced by the method of extracting mRNA. When paraffin samples were extracted macroscopically, the larger samples were preferred over the smaller ones. Larger samples with larger tumours tend to have a worse prognosis.

TABLE 5 Comparison between the BCC and SCC groups and the MM and IN groups.

	BCC versus SCC	MM versus IN
Gene	p-value	
AhR	0.809	**0.050**
CREBBP	0.605	0.918
CYP24A1	0.751	0.661
CYP27B1	1	0.628
EP300	0.517	0.960
FOXO3	**0.000**	0.069
MED1	**0.002**	1.000
NCOA1	0.768	0.527
NCOA2	0.751	0.661
NCOA3	0.615	0.516
NCOR1	0.099	0.080
NCOR2	0.061	0.456
RAR_A	0.272	0.501
RAR_B	0.061	0.069
RAR_G	0.751	0.661
RXR_A	0.751	0.661
RXR_G	0.605	0.254
SMAD3	0.705	0.628
TP53	**0.000**	0.258
VDR	0.705	0.559
XP_A	0.751	0.595
XP_C	0.975	0.628

Note: Statistically significant differences are marked in bold, underlined numbers.

Abbreviations: BCC, basal cell carcinoma; IN, intradermal nevi; MM, melanoma; SCC, Squamous cell carcinoma.

The outliers excluded were, first, a pool of controls consisting exclusively of non-exposed cases that can enhance receptor positivity and explain an aberrant presentation. Second, an SCC whose only differential characteristic was that it presented an acantholytic subtype. It was from a well-differentiated female patient that occurred in an exposed area.

In conclusion, many transcriptomes related to VDR and RXR_{α} were significantly different in the lesions compared to control skin. This finding appears to be critical to the development of non-melanoma skin cancers. However, we observed that IN and MM groups were quite similar (except for the AhR gene), suggesting that VDR and RXR_{α} loss may not directly participate in melanoma carcinogenesis, which differs from previous experimental data. These findings introduce novel perspectives for future studies and potential targeted therapies.

AUTHOR CONTRIBUTIONS

J.P.O.X. performed the concept study and design, and J.P.O.X. and J.C.C.X.Jr. developed methodology and writing, analysis and interpretation of data, and review and revision of the paper. H.A.M. performed data curation and statistical analysis. M.G.S. performed a concept study and funding acquisition. M.E.A.M. performed funding acquisition and supervision. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to declare.

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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

TABLE S1. Results of the pilot study.

How to cite this article: Ocanha-Xavier JP, Xavier-Junior JCC Jr, Miot HA, da Silva MG, Marques MEA. Transcriptomic analysis of genes associated with vitamin D receptor signalling reveals differences between skin cancers. Exp Dermatol. 2024;33:e15160. doi:10.1111/exd.15160

CAPÍTULO III – Conclusão

- VDR e RXRα são influenciados pela exposição solar. Esta diminui a expressão dos receptores.

- Nas lesões de câncer de pele não melanoma, houve alteração significativa no carcinoma espinocelular, com perda dos dois receptores, e perda apenas do RXRα na lesão pré-maligna correspondente, a queratose actínica

- Ambos os receptores foram negativos nas lesões melanocíticas em relação ao controle, tanto na análise imuno-histoquímica quanto molecular

- Nos melanomas houve correlação significativa entre VDR e regressão histológica. Houve correlação inversa entre RXRα e mitoses no melanoma.

- Na análise molecular, houve perda de VDR e RXRα em todas as lesões avaliadas, não-melanocíticas e melanocíticas. Quase todos os RNAm apresentaram relação de perda nas lesões com relação ao controle, exceto NCOR2 nas não-melanocíticas e RXRγ nas melanocíticas. Houve diferença estatística entre a maioria dos RNAm analisados comparando controle com as lesões.

- Houve diferença estatisticamente significante entre os grupos CBC e CEC com relação aos genes FOXO3, TP53 e MED1, com relação aos grupos NI e MM, houve diferença significante apenas para o gene AhR.

- **A maior compreensão desta via fisiopatogênica abre oportunidade para novos estudos e pesquisas.**

Anexos

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Presença dos receptores de Vitamina D e de Retinoide X nas lesões pré-malignas e malignas de pele: estudo comparativo

Pesquisador: JULIANA POLIZEL OCANHA XAVIER

Área Temática:

Versão: 1

CAAE: 03058918.4.0000.5411

Instituição Proponente: Faculdade de Medicina de Botucatu/UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.055.543

Apresentação do Projeto:

A 25-OH-vitamina D, forma ativada de vitamina D é substância alvo de discussão contínua acerca de suas propriedades antioxidativas, além de alterar a proliferação, diferenciação celular e apoptose.1 Alguns estudos demonstraram associação entre os níveis de vitamina D sérica e diminuição do risco de cânceres 2,3, enquanto outros já demonstram controvérsia sobre o tema.

Objetivo da Pesquisa:

Estudo da distribuição dos receptores VDR e RXR em material parafinado das seguintes lesões de pele: Carcinoma Basocelular, Carcinoma Espinocelular, Melanoma, Nevo, Nevo displásico, Ceratose actínica, em relação aos controles (pele proveniente de ampliação de margens, quando ausente de neoplasia residual).

Avaliação dos Riscos e Benefícios:

Metodologia Proposta: Estudo tipo caso-controle retrospectivo, com realização de análise imunohistoquímica de material previamente parafinado.

Critério de Inclusão: Incluímos todos os casos de melanoma provenientes do hospital das Clínicas da Faculdade de Medicina de Botucatu (UNESP) à partir do ano de 2012 até 2017. À partir deste número chegamos ao valor amostral por grupo (n=180). As demais amostras foram selecionadas de forma aleatória, até completar o valor de 180 lesões, dentro do período selecionado. Para o

grupo Carcinoma Basocelular foi selecionado o subtipo histológico nodular, para fins de padronização da amostra. Para o grupo Nevo foi selecionado o subtipo intradérmico para padronização amostral. Foram considerados controles materiais provenientes de ampliação de margem cirúrgica, quando não continham neoplasia residual.

Critério de Exclusão: Material em condições ruins de utilização para realização de análise imunohistoquímica Lesões de coalisão (presença de 2 lesões na mesma peça) No grupo do carcinoma basocelular, lesões de outros subtipos que não o nodular. No grupo Nevo as lesões congênitas e outros subtipos que não o intradérmico. Também foram excluídos os casos em que não havia informação sobre o local de acometimento, se exposto ao sol ou em área coberta.
Riscos: Risco de avaria do material analisado (lâminas e blocos de parafina), sendo que o risco se torna mínimo a partir de procedimentos operacionais padrão da instituição participante.

Benefícios: Ampliação do conhecimento sobre tema recorrente na literatura científica internacional e implicações no seguimento e tratamento dos pacientes, com possibilidade de novas terapias-alvo no futuro.

Metodologia de Análise de Dados: As lâminas serão avaliadas em microscópio multicabeças por 2 dermatopatologistas e 1 dermatologista. Serão aplicados escores de positividade dos marcadores já padronizados na literatura científica internacional. Os dados serão analisados em testes estatísticos paramétricos ou não paramétricos, com intervalo de confiança mínimo de 95%.

Comentários e Considerações sobre a Pesquisa:
Trata-se de estudo tipo caso-controle retrospectivo, com realização de análise imunohistoquímica de material previamente parafinado, com a participação de 1.260 pessoas distribuídas em grupos de 180 segundo os critérios em 7 grupos: Carcinoma Espinocelular, Nevo, Pele normal, Melanoma, Nevo displásico, Ceratose actínica, Carcinoma basocelular. Trata-se de pesquisa em nível de doutorado do Programa de Pós-graduação em Patologia da Faculdade de Medicina de Botucatu, e não há indicações de custos (custo zero) e terá financiamento próprio.

Considerações sobre os Termos de apresentação obrigatória:
Todas as documentações obrigatórias foram apresentadas corretamente.
Foi solicitado dispensa de TCLE, que justificou:
"Para a pesquisa, serão utilizados materiais provenientes de espécimes parafinadas já excisadas de pacientes anteriormente. Estes pacientes não sofreram qualquer intervenção no sentido de

mudança de conduta terapêutica devido à pesquisa e nem sofrerão, pois não serão recrutados posteriormente. Também não será utilizado prontuário dos pacientes para informações adicionais, apenas serão utilizadas as informações contidas nos laudos anatomopatológicos e, através de material oriundo de cirurgia prévia, serão efetuados exames imunohistoquímicos não realizados previamente para investigação científica."

Recomendações:

Apresentação dos resultados finais da pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Após análise em reunião ordinária, o Colegiado deliberou APROVADO o projeto de pesquisa apresentado.

Considerações Finais a critério do CEP:

Conforme deliberação do Colegiado em reunião ordinária do Comitê de Ética em Pesquisa da FMB/UNESP, realizada em 03 de dezembro de 2018, o projeto analisado encontra-se APROVADO, sem necessidade de envio à CONEP.

No entanto, informamos que ao final da execução da pesquisa, seja enviado o "Relatório Final de Atividades", na forma de "NOTIFICAÇÃO".

Atenciosamente,

Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu – UNESP

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1225671.pdf	13/11/2018 12:59:09		Aceito
Projeto Detalhado / Brochura Investigador	projetopb.pdf	13/11/2018 12:58:17	JULIANA POLIZEL OCANHA XAVIER	Aceito
Declaração de Instituição e Infraestrutura	TermoDeAnuenciaInstitucional.pdf	13/11/2018 11:16:16	JULIANA POLIZEL OCANHA XAVIER	Aceito
Folha de Rosto	FolhaDeRostoAssinada.pdf	13/11/2018 11:15:31	JULIANA POLIZEL OCANHA XAVIER	Aceito
Declaração de Manuseio Material	termo_anuencia.pdf	13/11/2018 11:15:01	JULIANA POLIZEL OCANHA XAVIER	Aceito

Biológico / Biorepositório / Biobanco	termo_anuencia.pdf	13/11/2018 11:15:01	JULIANA POLIZEL OCANHA XAVIER	Aceito
Declaração de Instituição e Infraestrutura	Anuenciahcfmb.Pdf	13/11/2018 11:12:23	JULIANA POLIZEL OCANHA XAVIER	Aceito
Cronograma	cronograma.doc	04/11/2018 15:22:50	JULIANA POLIZEL OCANHA XAVIER	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BOTUCATU, 04 de Dezembro de 2018

Assinado por:
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