UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Camila Santos Pereira

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Unimontes

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RESUMO

O presente estudo avaliou o índice de proliferação e a expressão imunohistoquímica de SIRT1 em lesões de nevos melanocíticos cutâneos (NMC) e melanoma maligno cutâneo (MMC). Através da técnica de imunohistoquímica foi analisado o índice de proliferação celular (Ki67) e SIRT1 na pele normal (17) e em lesões de nevos melanocíticos cutâneo benignos (40) e melanoma maligno cutâneo (22). A expressão de SIRT1 foi significativamente maior nas amostras de nevos melanocíticos quando comparado com as lesões de melanoma maligno cutâneo ($p = 0.035$). O índice de proliferação foi significativamente maior em amostras de melanoma maligno cutâneo quando comparado com a pele normal e o nevo melanocítico ($p<0.001$). No entanto, a expressão da proteína Ki67 não foi significativamente associada com a expressão de SIRT1 ($p > 0.05$). Em conclusão, uma baixa expressão de SIRT1 e alto índice proliferativo podem apresentar um importante papel a cerca do comportamento biológico dessas lesões. O segundo objetivo do presente estudo foi avaliar o efeito do resveratrol e sirtinol na viabilidade celular bem como a expressão do RNA mensageiro da SIRT1 na linhagem celular de melanoma maligno cutâneo (B16F10). As linhagens celulares, B16F10 (melanoma maligno cutâneo metastático) e Melan-A (melanócito murino normal) foram tratados com resveratrol e sirtinol para avaliação da viabilidade celular, fragmentação de DNA e expressão de SIRT1. O tratamento das células com resveratrol e sirtinol significativamente afetou a viabilidade celular ($p < 0.05$) e promoveu a fragmentação do DNA nas células B16F10 e Melan-A ($p < 0.05$). No entanto, o tratamento com resveratrol e sirtinol nas concentrações estudadas não alterou a expressão do transcrito primário da SIRT1. Os resultados do presente estudo revelam que o uso dessas drogas pode representar uma promissora estratégia quimioterápica no combate ao melanoma, mesmo não ocorrendo alterações no transcrito primário da SIRT1. Além disso, foi avaliada a expressão de endoglina e PECAM-1 no melanoma maligno cutâneo correlacionando com fatores clínico-patológicos. Para isso, foi realizada a análise da expressão de endoglina e PECAM-1 em amostras de pele normal (12), nevos melanóciticos (48) e melanoma maligno cutâneo (44) para avaliação da densidade microvascular. Nossos resultados mostraram um aumento da densidade microvascular para endoglina ($p < 0.001$) e PECAM-1 ($p < 0.001$) nas amostras de melanoma maligno cutâneo quando comparado com as amostras de nevos melanocíticos cutâneos. Esse aumento da densidade microvascular também foi constatado no nevo melanocítico quando comparado com o controle ($p < 0.001$). Indivíduos com melanoma maligno metastático apresentaram alta densidade microvascular para Endoglina ($p = 0.015$) e PECAM-1 ($p = 0.036$) quando comparados com indivíduos sem doença metastática. O desenvolvimento de uma rica rede vascular no melanoma maligno cutâneo permite o suprimento das demandas metabólicas e energéticas das células tumorais a fim de alcançarem um perfil metastático.

Palavras-chave: Melanoma Maligno Cutâneo; Melan-A; Nevo melanocítico; SIRT1; Ki67.

ABSTRACT

The current study evaluated the proliferation index and immunohistochemical expression of SIRT1 on cutaneous melanocytic nevi (CMN) and cutaneous malignant melanoma (CMM). Formalin-fixed paraffin-embedded tissue samples from 43 CMN, 22 CMM and 17 normal skin were obtained and clinical data were abstracted from the electronic medical record. SIRT1 and Ki67 proteins expressions were evaluated regarding to clinic pathological behavior in CMM. The level of significance was set at $\alpha = 5\%$ ($p < 0.05$). Our findings showed that SIRT1 positivity was significantly higher in benign melanocytic nevi than that in cutaneous malignant melanoma ($p = 0.035$). As expected, the proliferation index was significantly higher in samples of cutaneous malignant melanoma as compared to the normal skin and melanocytic nevi ($p < 0.001$). However, the expression of Ki67 protein was not also significantly related to the expression of SIRT1 ($p > 0.05$). In conclusion, low expression of SIRT1 and high proliferation index may play an important role in progression of cutaneous melanoma. The second objective aimed to evaluate the effect of resveratrol and sirtinol, activator and inhibitor of sirtuins, respectively, on cell viability and SIRT1 mRNA expression in murine metastatic skin melanoma (B16F10) cell line. B16F10 and non-tumoral murine melanocytes (Melan-A) cell lines were treated with resveratrol and sirtinol and evaluated for cell viability, DNA fragmentation and SIRT1 gene expression. All experiments were performed in triplicate and submitted to specific statistical tests with significance level at $\alpha = 5\%$ ($p < 0.05$). Treatments with resveratrol and sirtinol significantly affected the B16F10 cell viability ($p < 0.05$) and promoted DNA fragmentation ($p < 0.05$). Significant reductions in the viability and DNA fragmentation after exposure to resveratrol and sirtinol were also observed in Melan-A cells. However, both treatments did not change the SIRT1 expression on the same studied conditions. Results of the present study revealed that use of these drugs may be a promising chemotherapeutic strategy against melanoma cancer, even in the absence of changes in the transcription of the SIRT1 gene. Moreover, it was evaluated expression of PECAM-1 and endoglin for a correlation with clinicopathological behavior in CMM. Control ($n = 12$), CMN ($n = 48$), and CMM ($n = 44$) samples were submitted for immunohistochemistry. PECAM-1 and endoglin expression were counted in the stroma (hot spots) of all samples in order to calculate the MVD. Data analyses were performed using univariate statistical tests, with significance set at $p<0.05$. Our findings showed that CMM exhibited higher MVD estimates for both PECAM-1 and endoglin compared to control and CMN samples ($p < 0.001$, for all associations). Moreover, CMN samples exhibited higher MVD compared to control samples ($p<0.001$ for all associations). CMM from subjects with metastatic disease showed higher MVD by PECAM-1 ($p=0.036$) and endoglin ($p=0.015$) compared to non-metastatic CMM. In conclusion, increasing MVD from normal skin to benign and malignant melanocytic tumors suggest the importance of a rich vascular network in the peritumoral stroma to support greater metabolic and energetic demands, which favors the dissemination of melanocytic tumor cells.

Keywords: Cutaneous Malignant Melanoma; Melan-A; melanocytic nevi; SIRT1; Ki67.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

MMC	Melanoma Maligno Cutâneo
NMC	Nevo Melanocítico Cutâneo
TRP1	Proteína 1 Relacionada a Tirosinase
MATP	Proteína de transporte associada à membrana
CDK	Quinase Dependente de Ciclinas
MITF	Fator de transcrição associado à microftalmia
PTEN	Fosfatase Homóloga da Tensina
BCL2	Célula B de linfoma 2
NF-κB	Fator nuclear Kappa B
AKT	Proteína Quinase do tipo B
OMS	Organização Mundial de Saúde
UV	Ultravioleta
AJCC	Comitê de União Americana do Câncer
DTIC	Dacarbazina
NAM	Nicotinamida
Sir	Informação silenciosa
HDACs	Histonas Desacetilases
NAM	Nicotinamida
SIRT1	Sirtuina 1
AROS	Regulador Ativo da SIRT1
E2F	Fator Transcricional do tipo 2
BAX	Proteína X associada a BCL2
HUR	Antígeno Humano do tipo R
ATM	Ataxia Telangiectasia Mutada
ChK2	Checkpoint Quinase do tipo 2
DNA	Ácido Desoxirribonucléico
RB	Proteína Retinoblastoma
LPS	Lipopolissacarídeos
ATP	Trifosfato de Adenosina
NADPH	Fosfato de Dinucleótido de Nicotinamida e Adenina

kDa	Kilodalton
CDKN1A	*Do inglês: cyclin dependent kinase inhibitor 1A -inibidor de quinase dependente de ciclina 1A*
CDKN2A	*Do inglês: Cyclin – dependent kinase inhibitor 2A*
CDK4	*Do inglês: Cyclin – dependent kinase 4*
pRB	Proteína Retinoblastoma
MDM2	*Do inglês: Murine Doble Minute 2*
MITF	Fator de Transcrição Associado à Microfitalmia
MAPK	*Do inglês: Mitogen – Activated Protein Kinase*
KIT	Receptor Tirosino Quinase
DMV	Densidade microvascular
PECAM	Molécula de adesão celular endotelial plaquetária
TGF	Fator transformador de crescimento

SUMÁRIO

1 Introdução

1.1 Melanoma

Em todo o mundo, o câncer de pele representa o terceiro tipo de neoplasia mais comum, sendo que sua incidência tem aumentado em proporções alarmantes nos últimos anos. As formas mais comuns desses tumores são carcinoma basocelular, carcinoma espinocelular e melanoma. A cada ano, estima-se a incidência de 2-3 milhões de casos de câncer de pele e apesar do melanoma representar apenas 132.000 desses casos, a maioria das mortes é provocada por esse tipo de neoplasia (1). No Brasil, o câncer mais frequente é o de pele, correspondendo a 25% de todos os tumores diagnosticados. Dentre todos os tumores de pele, a incidência de melanoma maligno cutâneo é de apenas 4%, no entanto, é um tipo de neoplasia altamente agressiva e com altas taxas de mortalidade (2-7).

Melanoma cutâneo forma-se a partir dos melanócitos que, por sua vez, migraram da crista neural para toda a epiderme durante a embriogênese (8). A lesão origina-se na pele, mas podem surgir também a partir de mucosas ou em outros locais para os quais migraram as células da crista neural (9). A maioria dos melanócitos é encontrada na junção epiderme - derme da pele, podendo ser encontrados em torno das glândulas sebáceas ou próximos aos ductos lactíferos dos mamilos. Entretanto, os melanócitos também podem ser encontrados em sítios não cutâneos como a mucosa oral e gastrointestinal, trato respiratório, urinário, olhos e sistema nervoso central (10, 11). O melanócito sintetiza melanina, um pigmento castanho denso, insolúvel e de alto peso molecular, o qual assume aspecto enegrecido, quanto mais concentrado. Esse pigmento é responsável pela cor da pele que é a característica polimórfica mais marcante nos seres humanos. Além de seu papel na definição da etnia, a melanina desempenha papel importante na defesa do organismo contra raios UV (12).

A incidência da doença aumenta proporcionalmente com a idade, apresentando maior prevalência entre a quinta e a sétima década de vida, afetando ambos os sexos indistintamente (13). A detecção precoce e tratamento adequado evitam a progressão da lesão, sendo que a taxa de sobrevida nesses casos é de 90-97%. No entanto, pacientes com tumores primários com elevado grau de invasão da derme ou que apresentam metástases em linfonodos regionais frequentemente desenvolvem metástases à distância. Nesses casos, a sobrevida cai para 10 a 15% em cinco anos quando diagnosticada em estágios mais avançados (14, 15).

Estudos epidemiológicos apontam a presença de histórico familiar, exposição solar à radiação, presença de nevos congênitos e displásicos na pele como principais fatores de risco para a ocorrência de melanoma (16, 17). Em indivíduos que apresentam uma quantidade superior a 50 nevos ou a ocorrência de grandes nevos congênitos têm um risco cinco vezes maior de desenvolverem a doença (18).

Aproximadamente 10% dos melanomas apresentam origem hereditária (19). Nesses casos, a doença manifesta-se em dois ou mais parentes de primeiro grau ou em três ou mais membros de um mesmo lado da família independente do grau de parentesco (20). Pacientes com melanoma de origem familiar tendem a apresentar tumores múltiplos e em idades mais precoces quando comparados a pacientes sem histórico da lesão na família. Nesse contexto, a suscetibilidade genética é importante para a patogênese da doença. Alterações em grupos específicos de genes representa um fator de risco para o surgimento da doença, uma vez que, o crescimento tumoral é o resultado de alterações genéticas e epigenéticas em genes chaves controladores da proliferação celular, apoptose, senescência e resposta aos danos no DNA (21).

De acordo com Organização Mundial de Saúde (OMS), nas últimas décadas, a exposição excessiva a radiação ultravioleta foi a principal causa do aumento do número de casos de câncer de pele em todo o mundo (22). A frequente exposição solar desde o período da infância associado a queimaduras solares aumenta o risco de transformação de melanócitos benignos para um fenótipo maligno (23-25). Pessoas que apresentam pele clara, com sardas, que não bronzeia ou bronzeia muito lentamente, acompanhado de olhos e cabelos claros apresentam um risco aumentado para o desenvolvimento de câncer de pele (26). Nessas pessoas, a fotoproteção é menos eficiente, e nesse caso, o DNA dos melanócitos pode sofrer efeitos potencialmente mutagênicos que podem atuar como iniciadores das lesões tumorais (27).

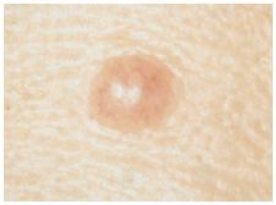
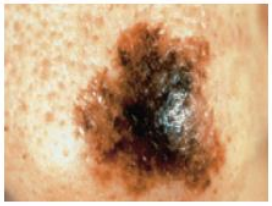
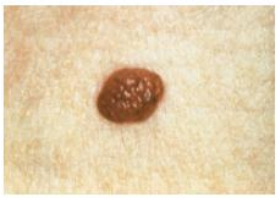
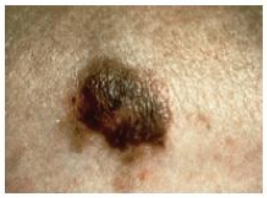
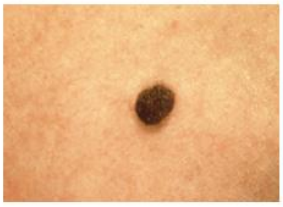
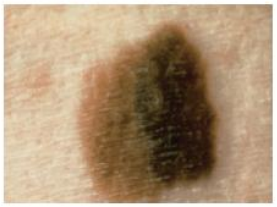
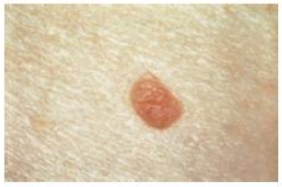
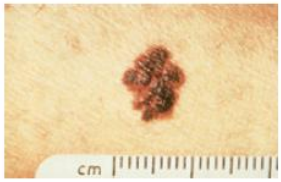
Os raios ultravioletas (UV) são classificados, de acordo com seu comprimento de onda, em UV-A (320-400 nm), UV-B (280-320 nm) e UV-C (100 a 280 nm). Os raios UV-C tem sua penetração bloqueada pela atmosfera terrestre. Dos raios ultravioleta que alcançam a terra, UV-A (95%) e UV-B (5%) são responsáveis por promover efeitos deletérios em proteínas e ácidos nucléicos. Os raios UV-B induzem a formação de dímeros de pirimidina (28) enquanto os raios UV-A produzem danos por estresse oxidativo (29). Além disso, as lesões provocadas

pelos raios UV-B também chamadas de mutações por “assinatura” podem provocar mutações do tipo C para T e transições do tipo CC para TT (30, 31).

1.2 Diagnóstico

O diagnóstico de melanoma deve ser realizado em toda lesão melanocítica que apresentar alteração de cor, tamanho ou forma (32). Nas décadas de 1960-1970, o melanoma maligno apresentava um pior prognóstico comparado aos dias atuais, pois era diagnosticado clinicamente em um estágio muito avançado, baseado em sintomas como sangramento, prurido ou ulceração da lesão (33). Na década de 1980, surgiu a regra do ABCD (assimetria, borda irregular, variações na cor e diâmetro maior que 6 mm), que é mundialmente utilizada no exame clínico do melanoma e que possibilitou o diagnóstico mais precoce da doença (33-35) Quadro 1. Quando, adotando-se os critérios acima citados, esta lesão deve ser avaliada por dermatologista, visando à realização de exame chamado dermatoscopia (36). Esta última consiste na visualização ampliada da lesão com aparelhagem especial. A partir daí, o dermatologista, através de índices específicos, determina se a lesão melanocítica é passível de biópsia excisional. O diagnóstico precoce e tratamento cirúrgico continuam a ser a melhor estratégia no tratamento da doença (35).

Quadro 1: Diagnóstico do melanoma através da regra do ABCD.

Regra ABCD	Benigno	Maligno
A = Assimetria suspeita a melanoma: O tumor é dividido ao meio, e as metades não são semelhantes		
B = Borda irregular suspeita de melanoma: Bordas desiguais ou irregular.		
C = Variação de cor suspeita de melanoma:existe mais de uma cor de pigmento		
D = Diâmetro suspeito a melanoma: Caso o diâmetro seja maior que 6 mm		

Fonte: Jerant et al, 2000 (37).

1.3 Histopatologia

O melanoma cutâneo teve seu estadiamento revisado em 2002 pelo *American Joint Commitee of Cancer* (AJCC) (38), sendo levada em consideração, atualmente, a presença de ulceração ou não, espessura em milímetros da lesão (Breslow) e informações sobre taxa de mitose (Breslow) (39, 40). Várias características histológicas ajudam na determinação do prognóstico da doença. Os níveis de Clark, propostos em 1969 por Wallace Clark Jr. definiram uma classificação histopatológica que analisa níveis de microinvasão das células tumorais. Amplamente utilizado nos dias atuais, esse sistema de gradação leva em consideração o nível de invasão da lesão melanocítica, iniciando-se na epiderme e estendendo-se até a hipoderme cutânea (41, 42). Os níveis de invasão de acordo com o método de Clark permite categorizar o melanoma primário de acordo com o grau de invasão das células neoplásicas. O nível I assegura a presença de células tumorais somente na região da epiderme. O nível II corresponde à invasão parcial da derme papilar (Camada mais superficial da derme). O nível III indica à invasão definitiva da derme papilar, estendendo-se até a interface derme papilar-

derme reticular. O nível IV corresponde à invasão definitiva da derme reticular (Camada mais profunda e densa da derme), e o nível V corresponde à invasão do tecido subcutâneo.

A espessura de Breslow proposta em 1970 por Alexander Breslow e complementar aos níveis de Clarck determina a espessura do tumor como um critério de prognóstico. A espessura tumoral medida em mm (Breslow) é o fator mais importante na determinação do risco de recorrência e metástases, bem como é o principal determinante no manejo dos pacientes. A análise do grau de aprofundamento das células tumorais medidas em milimetros com um micrometro ocular é utilizado para correlacionar grau de invasidade e sobrevida (43, 44). A espessura da lesão maligna varia entre abaixo de 0,75 mm, entre 0,76 mm a 1,5mm, entre 1,5mm a 3mm, e acima de 3 mm. Pacientes com lesões menores que 0,75 mm de espessura histológica têm um bom prognóstico e poucos apresentam recorrência do tumor (43).

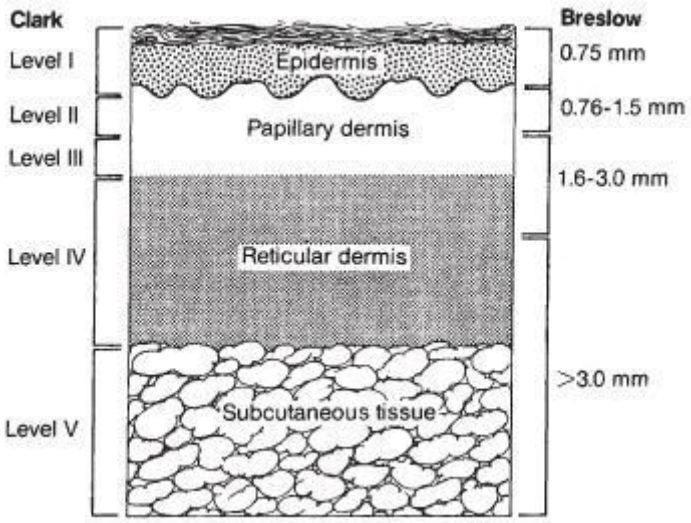


Figura 1: Critérios de Invasão por Clarck´s e Breslow. Os níveis de invasão de acordo com o método de Clark´s permite categorizar o melanoma primário de acordo com o grau de invasão das células neoplásicas. De acordo com Breslow, a medida tumoral é feita em mm e é o fator mais importante na determinação do risco de recorrência e metástases, bem como é o principal determinante no manejo dos pacientes.

1.4 Critérios anatomopatológicos para diagnóstico de melanoma maligno cutâneo.

Os patologistas utilizam alguns critérios anatomopatológicos para diagnóstico do MMC como tamanho da lesão, ocorrência de infiltração pagetóide, atividade mitótica com mitoses atípicas e reação inflamatória na derme para diferenciação das lesões melanocíticas malignas das benignas. Existe uma concordância na literatura mundial de que as lesões melanocíticas

benignas costumam ter tamanho inferior a 10 mm, enquanto os melanomas costumam apresentar tamanhos maiores (45, 46).

A infiltração pagetóide é outro critério utilizado e é definido como a presença de melanócitos isolados ou em grupos nas camadas superiores à basal da epiderme (47). Embora não esteja presente em todos os casos de melanoma, e também possa ser vista em algumas lesões melanocíticas benignas, é considerado um dos critérios mais importantes no diagnóstico do melanoma (45).

Outro critério de diagnóstico são as mitoses atípicas. Qualquer lesão melanocítica em fase de proliferação pode apresentar mitoses. De uma maneira geral, quando os nevos compostos ou intradérmicos (principalmente em crianças e mulheres grávidas) e o nevo de Spitz (48) exibem mitoses, essas estão situadas no componente intradérmico ou na derme superficial, e são em pequeno número. Como regra geral, mitose situada profundamente em lesão de aspecto névico exige avaliação cuidadosa, alertando para a possibilidade de melanoma. Alguns melanomas convencionais podem apresentar índice mitótico surpreendentemente baixo, porém de uma maneira geral, as mitoses são mais frequentes nos melanomas do que nos nevos (49, 50). Apesar dessas exceções, a presença de mitoses profundas e atípicas continua sendo um critério muito útil na avaliação desses tumores (51).

Quanto ao infiltrado inflamatório, os nevos adquiridos (com exceção do nevo displásico) raramente apresentam-se acompanhados de infiltrado inflamatório na derme. Já os melanomas mostram graus variados de infiltrado linfocitário, principalmente na fase radial de crescimento, situado ao redor de vasos do plexo superficial ou formando faixa na derme papilar (infiltrado liquenóide). Quando a lesão atinge a fase vertical de crescimento, a densidade do infiltrado tende a diminuir, podendo estar limitada à periferia do tumor (peritumoral) (45).

As atipias celulares representam um dos critérios mais valorizados no diagnóstico dos melanomas para diferenciação de algumas lesões benignas e malignas. A definição de atipia celular não é muito clara, e está relacionada com o tamanho e forma da célula, do núcleo e do nucléolo; a relação núcleo citoplasma também é valorizada. Uma lesão melanocítica com atipias leves ou moderadas costuma ser benigna, enquanto a presença de atipias severas favorece o diagnóstico de melanoma. A atipia severa foi definida pelo Cancer research

Campaign Melanoma Pathology Panel (47), como os melanócitos malignos apresentando aumento do tamanho do núcleo, com variação de sua forma, com contornos irregulares e com espessamento da carioteca; a cromatina costuma ser grosseira e o nucléolo grande, eosinofílico e proeminente (45). Deve ser lembrado que os melanomas apresentam alteração no grau de atipia de uma lesão para outra, e também uma inconstância na proporção de células atípicas dentro de uma mesma lesão.

1.5 Tratamento

A biópsia do tipo incisional representa um método padrão no diagnóstico do melanoma maligno. Isso é feito através de uma excisão completa com 1-2 mm de margem, incisando-se no sentido da corrente linfática (52). Após confirmação histológica, é realizada uma excisão mais extensa para permitir a retirada de possível tumor residual e diminuir as taxas de recorrência tumoral (52, 53). O acompanhamento clínico dos linfonodos loco-regionais é a melhor conduta para pacientes com lesões menores que 0,76 mm. Para as lesões maiores está indicada a pesquisa do linfonodo sentinela. Linfonodo sentinela (LS) é o primeiro linfonodo de drenagem na área entre tumor primário e cadeia linfática (54). A biópsia do linfonodo sentinela é uma opção para pacientes com melanoma sem linfonodos clinicamente evidentes que têm risco significativo para micrometástases, tais como: lesões primárias de 0,76 mm ou mais de espessura ou menores do que 0,76mm, porém, associadas a nível IV de Clark, e/ou regressão e/ou ulceração. A biópsia do LS está indicada por ser de grande ajuda na definição da terapia adjuvante e na definição anatômica da drenagem linfática do tumor primário (53, 55).

A quimioterapia consiste na aplicação de drogas citotóxicas e representa a forma primária de abordagem do melanoma metastático. As drogas mais utilizadas atualmente são representadas pela Dacarbazina (DTIC), Cisplatina (CDDP), Nitrosouréias (Carmustina e Lomustina) e agentes que atuam sobre os microtúbulos (Alcalóides da Vinca e Taxanes). O principal e mais ativo quimioterápico no tratamento do melanoma é representado pela Dacarbazina, agente alquilante que isoladamente proporciona taxas de resposta de 14 a 20% com duração mediana de resposta de quatro a seis meses (56). Estudos clínicos mostram que somente 2% dos pacientes que recebem quimioterapia com DTIC isolado estarão vivos em seis anos e a associação de drogas (Poliquimioterapia) não mostrou benefício adicional na taxa de sobrevida (57).

O interferon alfa 2b é uma citocina que apresenta maior controvérsia para o uso adjuvante em pacientes com melanoma. Apesar de não existir consenso, a terapia com Interferon alfa 2b administrado em altas doses pode aumentar o tempo livre de doença bem como proporcionar um discreto aumento de sobrevida (58, 59). A administração de interferon alfa 2b mostra benefícios reprodutíveis quanto a tempo livre de doença e sobrevida em pacientes com alto risco, ou seja, com 40 a 50% de risco de recidiva e morte (60). Não existem evidências que suportem qualquer benefício do Interferon alfa 2b administrado em baixas doses (52).

O melanoma é um tumor resistente à radioterapia. A radioterapia é indicada para casos de melanoma malignos inoperáveis e pode ser utilizado de forma paliativa em casos de metástases principalmente ósseas (61).

1.6 Nevos melanocíticos cutâneos

Os nevos melanociticos são tumores pigmentados e benignos da pele e são formados pela proliferação de melanócitos na junção dermoepidérmica. A sua formação se dá por melanócitos pré-existente ou de células chamadas de nevo melanócitos que se originam de nevomelanoblastos (62). São lesões comuns que apresentam tonalidade marrom podendo variar na forma e tamanho. Alterações em gene regulatórios de crescimento, produção de fatores de crescimento autócrinos e dos receptores de adesão contribuem para o rompimento da sinalização intracelular dos melanócitos e, consequentemente deixam de ser controlados pelos queratinócitos, assim, o melanócitos podem proliferar formando nevos ou verrugas (63).

Para diagnóstico, deve-se inicialmente identificar se a lesão pigmentada da pele é melanocítica ou não melanocítica. A presença de rede pigmentar, glóbulos ou pontos caracterizam as lesões melanocíticas e em relação ao nevo azul, a presença de áreas homogenias azuis acinzentadas determina seu diagnóstico. Os nevos são classificados e descritos na patologia, dependendo da localização da derme, em nevo juncional, apresentando ninhos de células névicas confinadas a junção dermo-epidérmica; Nevo intradérmico possuindo ninhos de células somente na derme e Nevo composto, evidenciando células em ambas as localizações (64).

No nevo composto encontramos três tipos celulares na derme: 1) células do tipo A, situadas em contato com epiderme, dispostas em ninhos de células coesas e pigmentadas, com citoplasma amplo, eosinofílico e núcleos uniformes, com carioteca delicada e nucléolo

pequeno e distinto; 2) células do tipo B, linfocitóides, situadas em posição intermediária na espessura da lesão, caracterizadas por citoplasma escasso, núcleos pequenos e hipercromáticos, quase não havendo síntese de melanina; 3) células do tipo C, situadas na porção profunda do nevo, arredondadas ou ligeiramente fusiformes, de aspecto fibroblástico ou schwannóide, isoladas ou em pequenos grupos, e sem melanina (45).

O nevo de Spitz é uma forma peculiar de nevo composto que apresenta algumas características microscópicas de melanoma maligno, mas, ao contrário, não evolui com metástases (65). Apresenta-se como pápulas ou nódulos avermelhados na face, tronco ou extremidades. Microscopicamente, notam-se conglomerados de células névicas epitelióides ou fusiformes, localizadas na junção dermo - epidérmica e na derme superior que podem fundir-se e formar células gigantes (66).

O nevo congênito ocorre em aproximadamente 1% dos recém-natos. Geralmente solitário, tem predileção pela região do tronco. A maioria aparece logo após o nascimento e mede menos que 10 mm de diâmetro. O nevo congênito gigante corresponde a um tipo de nevo congênito que mede em torno de 20 cm de diâmetro que evolui para melanoma maligno em 5% dos casos (67).

Nevos melanocíticos displásicos apresentam atipia arquitetural e celular das células névicas. Este grupo especial de nevos melanocíticos pigmentares, podem ser ou não hereditários e pacientes portadores desses nevos possuem maior risco de desenvolver melanoma em idades precoces, sob estímulos como à luz solar, hormônios e alterações imunológicas. Este risco é maior do que o risco associado a nevos clinicamente normais (64). Pacientes com nevos displásicos e história familiar de melanoma em dois ou mais familiares tem um risco muito alto de desenvolvimento tumoral (68).

As principais alterações arquiteturais nos nevos displásicos são hiperplasia melanocítica lentiginosa, fusão de ninhos de melanócitos e alongamento de cones interpapilares. Na hiperplasia melanocítica lentiginosa observa-se a proliferação de células névicas de forma isolada ou em ninhos ao longo da camada basal e da epiderme. Esses ninhos de células podem apresentar irregularidades quanto à forma e distribuição dessas células e geralmente tendem a não ocupar as regiões próximas as cristas epiteliais como acontece nas outras lesões (62). As alterações citológicas nos melanócitos são o aumento da relação núcleo e citoplasma, aumento

da pigmentação nuclear e presença ocasional de figuras de mitose. A presença dessas atipias, provavelmente ligadas a anormalidades cromossômicas, é consistente com a hipótese de que os nevos displásicos se constituem em um reservatório de melanócitos displásicos e com riscos de sofrer mutações adicionais (69).

O surgimento do melanoma cutâneo pode ser precedido pelo nevo melanocítico cuja progressão pode ocorrer de forma lenta até alcançar a forma de melanoma in situ (FIG. . Aproximadamente 25% dos casos de melanoma surgem a partir de um nevo melanocítico que adquiriu sucessivas alterações genéticas antes de formar tumores e metástases (70). Os nevos são tumores benignos, no entanto, sob estímulos proliferativos podem progredir para melanoma em fase de crescimento radial, uma lesão intra - epitelial com microinvasões na derme, sendo considerado um estágio primário do melanoma (71). Esse tipo de crescimento a partir dos nevos melanocíticos é chamado de fase radial e é caracterizado por ocorrer de forma horizontal e por toda a epiderme (72). A fase radial pode progredir para uma fase vertical, com nódulos ou ninhos de células invadindo a derme (73). Com o decorrer do tempo, esta invasão pode atingir o sistema vascular e linfático, invadindo camadas dérmicas mais profundas na forma de massa em expansão, porém sem maturação celular. A probabilidade de metástase a partir dessa fase pode ser inferida através da medida do índice de Breslow, que consiste em medir, em milímetros, a profundidade de invasão da lesão em fase de crescimento radial ou vertical a partir do topo ou porção mais superficial da camada de células granulares da epiderme sobrejacente (74). Nem todo melanoma é precedido pelas fases de crescimento radial ou vertical. Muitas vezes, a lesão pode iniciar-se diretamente a partir de melanócitos isolados ou nevos podendo progredir diretamente para o melanoma maligno metastático (73).

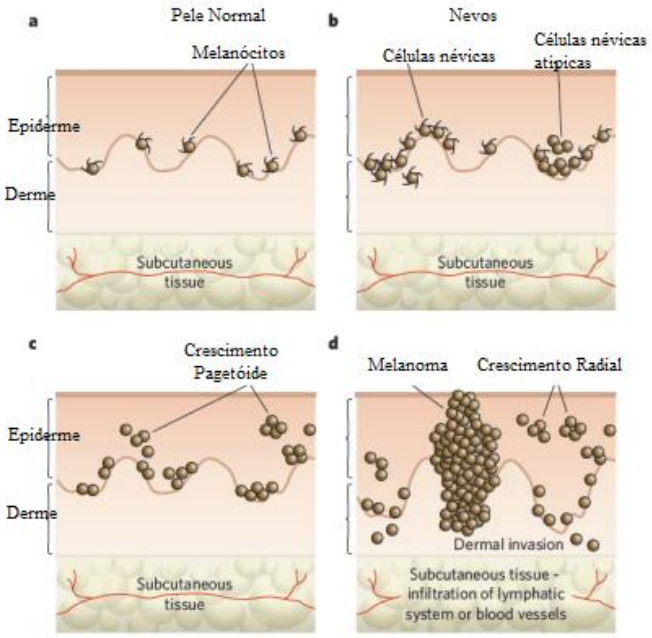


Figura 2: Estágios da transformação maligna a partir do nevo melanocítico. Cada estágio de transformação da lesão melanocítica está marcado por um novo clone de células que apresentam vantagens de crescimento ao longo dos tecidos circundantes. A) Pele normal: Há uma distribuição uniforme dos melanócitos dendríticos no interior da camada basal da epiderme. B) Nevos: nos estágios iniciais, observa-se um aumento do número de melanócitos dendríticos. De acordo com sua localização, os nevos podem ser classificados como juncionais, intradérmicos ou compostos. Alguns nevos são displásicos, pois os melanócitos apresentam alterações morfológicas. C) Fase de crescimento radial: lesão intra-epidermal que pode envolver algumas micro - invasões na região da derme. D) Fase de crescimento vertical: Representa uma fase de grande potencial maligno por apresentar infiltração de melanócitos nos sistemas vasculares e linfático. Observa-se a propagação pagetóide com migração ascendente dos melanócitos na epiderme e o empilhamento vertical dos melanócitos, característico das lesões de melanoma. Fonte: Gray-shopfer et al, 2007 (1).

1.7 Etiopatogênese no melanoma maligno cutâneo.

O crescimento tumoral é resultante de alterações genéticas e epigenéticas em genes chaves controladores da proliferação, apoptose, senescência e resposta aos danos no DNA (75, 76). Mutações deletérias em proteínas reguladoras do ciclo celular como, por exemplo, a Cyclin – dependent kinase inhibitor 2A (CDKN2A) e Cyclin – dependent kinase 4 (CDK4) conferem um risco aumentado de desenvolvimento de melanoma maligno (77, 78) (70). Esses genes estão envolvidos na parada do ciclo celular e senescência celular. CDKN2A está localizado no cromossomo 9, na região p21 (braço curto) sendo responsável por codificar duas proteínas supressoras, p16 e p14. Mutações no gene CDKN2A são encontradas em 20 a 40% dos casos de melanoma com herança familiar (79). Essas mutações podem afetar as proteínas p16 e p14 (80). A perda de função da p16 promove ativação de CDK6 e CDK4, resultando na hiperfosforilação de pRB e ativação do fator transcricional E2F. Esse fator transcricional media a ativação de genes da fase S do ciclo celular, promovendo, dessa forma, a proliferação celular (70). A importância do p16 na etiopatogênese do melanoma humano verifica-se a partir de estudos que exibem perda do gene em 50% dos casos, inativação por mutações pontuais em aproximadamente 9% dos tumores e metilação na região promotora de seu gene em 10% dos casos de melanoma (81). O gene p14 é um supressor tumoral que previne a degradação da p53 pelo complexo MDM2. Esse gene encontra-se frequentemente inativo no melanoma maligno (82).

MITF (Fator de transcrição associado à microfitalmia) é outro gene relacionado ao melanoma (83, 84). Esse gene é responsável pela regulação biológica dos melanócitos e suas atividades incluem: sobrevivência dos melanoblastos, regulação da expressão de proteínas melanogênicas e de proteínas envolvidas no controle do ciclo celular como a p16 e p21 (85). Esse gene atua na diferenciação celular, controle da proliferação e sobrevivência dos melanócitos (86). Amplificação do gene MITF é encontrada em 10% dos melanomas primários e em 20% dos melanomas metastáticos (87, 88). A expressão de MITF aumenta as propriedades invasivas e migratórias das células de melanoma bem como habilita essas células para formar colônias de melanócitos imortalizados (89)

A família de serina/ treonina quinase (RAF) apresenta três isoformas: ARAF, BRAF e CRAF que atuam na formação da via da Mitogen – activated protein kinase (MAPK), via primária de sinalização de controle do crescimento celular (90). O gene BRAF produz uma proteína

quinase/ serina/treonina (B-Raf) que está envolvida no envio de sinais para o interior das células, regulando o crescimento, diferenciação, proliferação, senescência e apoptose das células (91). Mutações no gene BRAF são descritas na progressão inicial do melanoma humano, sendo que 82% dos nevos adquiridos apresentam mutações nesse gene (92). O gene BRAF é mutado em 50 a 70% dos casos de melanomas, sendo que a mutação mais comum é resultante da substituição do ácido glutâmico pela valina (93). Essa porcentagem de melanomas com mutações no gene BRAF é relativamente alta quando comparada com tumores de tireóide (30%), pulmão (3%), e câncer colorretal (12%) (93-95). Essas mutações fazem com que o gene permaneça ativo independente da ativação prévia pelo oncogene RAS ou por estímulos externos (70). A droga vemurafenib é considerada um alvo terapêutico promissor, pois tem exibido benefícios clínicos significantes no tratamento de pacientes com melanoma que apresentam mutações no gene BRAF (96-98).

O gene KIT (receptor tirosina quinase transmembrana) é responsável por várias funções celulares, dentre as quais proliferação, adesão, apoptose e diferenciação celular (99). Mutações no gene KIT resultam em um receptor constantemente ativo que promove o crescimento tumoral (99). Há um espectro de mutações encontrada no gene KIT em vários tipos de melanomas, isso possibilita o estudo de alvos terapêuticos visando esse gene (100, 101). Em tumores de estroma gastrointestinal, mutações no gene KIT são observadas em aproximadamente 85% dos tumores (102). O Imatinibe foi o primeiro inibidor de tirosina-quinase utilizado em câncer sólido, evidenciado em um caso clínico, no qual um paciente com tumores de estroma gastrointestinal metastático obteve resposta clínica favorável com o uso dessa terapia (103). A droga imatinibe para o receptor de KIT tem apresentado resultados promissores no tratamento de pacientes com melanomas que apresentam mutações nesse gene (104, 105). A droga sunitinibe também tem apresentado resultados efetivos no tratamento de pacientes com melanoma, pois além de atuar nos receptores para KIT, apresenta ação sobre os receptores para VEGF (106, 107).

P53 é um gene supressor tumoral que age reprimindo ou ativando genes alvos em resposta ao estresse celular e danos no DNA. Em condições normais, a proteína p53 apresenta-se em baixos níveis na célula, isso é mantido através da degradação de p53 mediada pela ubiquitinase MDM2 (108). Em resposta ao estresse celular, p53 induz parada do ciclo celular para reparar danos na molécula de DNA via p21. Caso não ocorra o reparo do DNA, p53 ativa a apoptose através da ativação dos genes da via BAX (109, 110). Aproximadamente 80% das

lesões de melanoma expressam p53 na sua forma selvagem (111), no entanto, a maioria dessas lesões falha em induzir parada do ciclo celular e apoptose (112). Uma possível explicação para a perda da função de p53 ocorre devido a mutações no gene p14 (113). Essas mutações diminuem a habilidade da proteína p14 de inibir a atividade de MDM2, uma ubiquitina ligase, que apresenta como alvo a degradação da p53 (114). Outra explicação é que a acetilação de p53 representa uma modificação pós-transcricional importante para a sua atividade. Dessa forma, a desacetilação de p53 resulta na perda da habilidade dessa proteína em parar o ciclo celular e desencadear a apoptose (115).

1.8 SIRTUINAS

Os genes reguladores da informação silenciosa (Sir) constituem uma família de proteínas altamente conservadas, com um ou mais genes presentes em todas as espécies desde bactérias até mamíferos. Em mamíferos, estes genes são conhecidos por sirtuínas que codificam sete enzimas distintas que atuam como desacetilases ou mono-ADP-ribosiltransferases. As sirtuínas pertencem à classe III de histonas desacetilases (HDACs) que se diferencia das outras classes por seu mecanismo de ação ser dependente de NAD^+, tendo como principal papel a reverção da acetilação regulatória das histonas e outras proteínas influenciando assim na transcrição gênica e regulando a atividade de seus substratos (116). As sirtuinas de classe III removem o grupo acetil de proteínas e a transfere para o agrupamento NAD^+, gerando dois metabólitos: O-acetil-ADP ribose e a nicotinamida (NAM) (117).

Foram identificadas sete isoformas (SIRT 1-7) em mamíferos, distribuídas em três compartimentos dentro da célula (118). SIRT 1, 2, 6, 7 encontram-se no núcleo; as isoformas SIRT1 e SIRT2 são também reconhecidas no citoplasma e as SIRT 3, 4 e 5 estão localizadas na mitocôndria (119, 120). Apesar de todas terem um domínio catalítico comum, as diferentes sirtuínas possuem atividade enzimática distinta, nomeadamente desacetilases de histonas (SIRT1, SIRT2, SIRT3, SIRT5 e SIRT7) e monoribosiltransferase (SIRT4 e SIRT6) (121, 122).

As sirtuínas são consideradas genes reguladores, ou seja, capazes de influenciar outros genes, além de responder a uma variedade de fatores epigenéticos. Estas enzimas executam um importante papel na resposta do organismo a vários tipos de estresse e toxicidade. As sirtuínas regulam a expectativa de vida cronológica e reprodutiva de organismos inferiores, como

leveduras e bactérias, e parecem afetar aspectos biológicos envolvidos em doenças metabólicas e do envelhecimento em mamíferos (123). Diferentes autores sugerem que a modulação das sirtuínas se tornará um possível caminho para amenizar os efeitos e complicações do envelhecimento e de doenças como a diabetes, o câncer, doenças cardiovasculares e neurodegenerativas (124). Uma vez que as sirtuínas participam na regulação de diversos processos celulares e na patogenia de diversas doenças, existe um interesse crescente em descobrir moléculas que modifiquem a sua atividade.

1.9 Sirtuina 1 (SIRT1)

O primeiro membro da família das sirtuínas foi identificado em *Saccharomyces cerevisiae*, sendo chamado regulador da informação silenciosa-2 (Sir2). A Sir2 foi posteriormente encontrada na mosca da fruta (*Drosophila melanogaster*) e lombriga (*Caenorhabditis elegans*). A Sir2 está envolvida em extender por mais de 70% a vida útil desses organismos através da desacetilação das histonas mantendo o silenciamento da cromatina. Em mamíferos, o primeiro gene homólogo da Sir2 foi identificado como Sirtuína 1 (SIRT1). A SIRT1 é uma histona desacetilase de classe III pertencente à família das sirtuinas que dependem de NAD^+ para exercer sua atividade catalítica (116). Ela remove o grupo acetil do substrato transferindo-o para clivar NAD^+, gerando O-acetil-ADP ribose e a nicotinamida (NAM) (125). A SIRT1 encontra-se predominantemente no núcleo e está relacionada com estabilidade do genoma, longevidade, proliferação celular (126), respostas ao estresse, envelhecimento e metabolismo do câncer (127, 128).

O fator transcricional E2F pode impedir a transcrição de SIRT1 através de sua ligação à região promotora do gene. Esse fator pode induzir a apoptose após danos no DNA através de mecanismos dependentes e independentes da p53 (129). A SIRT1 pode desacetilar E2F inibindo sua atividade como fator transcricional. A p53 também pode reprimir a transcrição de SIRT1 através da ligação de elementos em sua região promotora. Por outro lado, a SIRT1 desacetila p53 inibindo sua ação sobre CDKN1A e BAX após dano no DNA (130). A acetilação de p53 é indispensável para sua habilidade em inibir o crescimento celular e induzir apoptose após dano celular (115).

A expressão de SIRT1 pode ser modulada através da participação de fatores que podem agir na região promotora do gene ou impedir a síntese proteica. HUR (Antígeno Humano do tipo

R) é uma proteína codificada pelo gene ELAVL1. Essa proteína apresenta domínios de ligação na região 3’ do RNA mensageiro da SIRT1, principalmente em regiões ricas em adenina e uracila. HUR pode impedir a tradução do mRNA regulando a expressão de SIRT1 (131). Isso pode resultar em uma diminuição dos níveis de SIRT1 levando a apoptose da célula mediada por p53 (132). O microRNA, miR-34a, também é um regulador downstream de SIRT1 que se liga na região 3’ UTR do mRNA de sua molécula impedindo a tradução de SIRT1 e, consequentemente sua atividade desacetilase (133). A proteína DBC1 (proteína deletada no câncer de mama) também interagi com o domínio catalítico da SIRT1 regulando negativamente sua atividade desacetilase (134). Por outro lado, a proteína nuclear AROS pode ligar-se diretamente à SIRT1 aumentando sua atividade desacetilase sobre outras proteínas (135). Além disso, outros eventos pós-transcricionais como a sumoilação de SIRT1 no resíduo 734 da lisina facilita sua atividade catalítica (136) e a fosforilação de SIRT1 por CDK1 também contribui positivamente para sua atividade (137, 138).

A acetilação e desacetilação têm sido reconhecidas como eventos cruciais para a regulação da atividade, estabilidade e localização de proteínas. A SIRT1 pode induzir o silenciamento da cromatina através da desacetilação das histonas H1, H3 e H4 (139). A SIRT1 também altera a atividade biológica de muitas proteínas não histonas, que atuam em resposta a danos no DNA após estresse celular (140). A enzima regula a sobrevivência celular através da modulação de p53, Ku70 (141), NF-kB (142), proteínas FOXO (143, 144) e p300 (145).

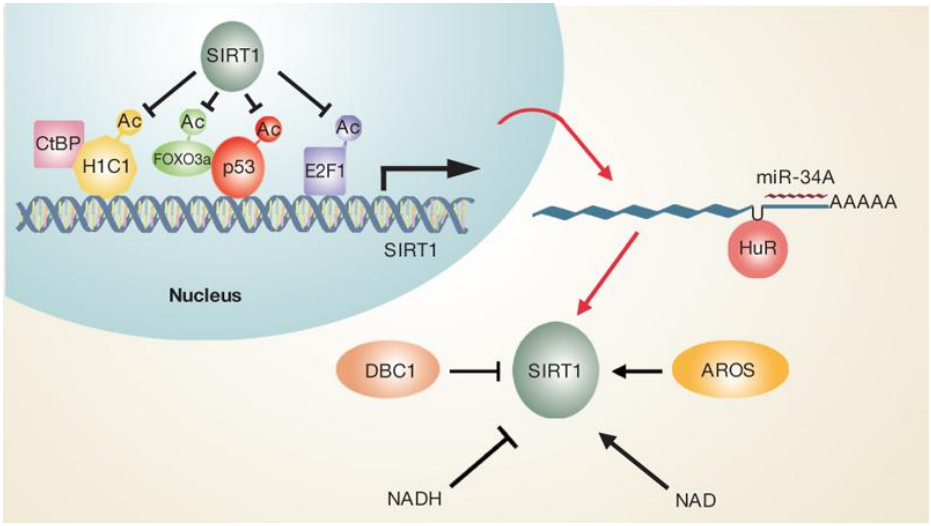


Figura 3: Regulação da SIRT1. Vários fatores transcricionais, como por exemplo, H1C1, E2F, CtBP, FOXO3a podem inibir a transcrição de SIRT1 através de sua ligação à região promotora do gene. A acetilação desses fatores é controlada pela SIRT1. HUR e miR-34a ligam-se a molécula de RNA mensageiro da SIRT1 estabilizando o transcrito primário. As

proteínas ARO e DBC1, respectivamente, podem ativar ou inativar o funcionamento da SIRT1. Finkel et al, 2009 (146).

Vários estudos tem tentado esclarecer o papel de SIRT1 como supressor ou promotor tumoral (147). Algumas evidências sugerem a participação de SIRT1 como um promotor de tumor, devido a sua elevada expressão em alguns tipos de câncer (148-151). Sua atividade oncogênica estaria relacionada com a sobrevivência das células em resposta ao estresse celular. A SIRT1 desacetila e, consequentemente inativa p53. Além disso, ela previne a apoptose em resposta aos danos ou estresse celular por interferir com a atividade de fatores transcricionais, como FOXO, BAX, Rb e E2F (152). Por outro lado, com a perda da SIRT1 as células parecem perder o controle da integridade genômica, apresentando um maior risco para o desenvolvimento de tumores. Nese contexto a SIRT1 parece agir como um supressor de tumor (128). A SIRT1 também exerce um papel positivo sobre outras proteínas e processos que resultam na supressão do crescimento tumoral e aumento do reparo de DNA (153-156).

SIRT1 ativa proteínas que protegem as células da inflamação e estresse oxidativo, duas das principais causas de envelhecimento prematuro das células e doenças degenerativas. A atuação desse gene representa um mecanismo de defesa que pode ser ativado no período de restrição calórica e pelo resveratrol (157). SIRT1 protege as células dos danos provocados pelos radicais livres e inibe substâncias inflamatórias como NFk-β e TNF-α (142). Com esses efeitos anti-inflamtórios e antioxidantes, SIRT1 pode retardar o processo de envelhecimento celular, estender a vida útil das células e reduzir o risco de muitas doenças degenerativas.

Sabe-se também que a inflamação crônica está associada aos estágios de promoção e progressão da carcinogênese (158). A SIRT1 pode inibir a inflamação provocada por várias citocinas pró-inflamatórias, como o fator de necrose tumoral (TNF-α), fator NFkB, interleucinas e moléculas de lipopolissacarídeos (LPS) (159). A SIRT1 é capaz de regular o processo inflamatório através da desacetilação em resíduos específicos da lisina do fator NFkB. A ativação de SIRT1 pelo resveratrol promove uma inibição dos mediadores inflamatórios que são ativados por NFkB o que pode ser útil na intervenção de doenças inflamatórias como diabetes, enfisema pulmonar e colites (160-163). Expressão reduzida de SIRT1 foi encontrada em pacientes com obstrução pulmonar crônica, doença relacionada à inflamação crônica (164). Outro meio regulatório do processo inflamatório pode ocorrer quando a SIRT1 suprime a atividade transcricional da proteína de ativação (AP-1) levando a uma diminuição da expressão da ciclooxigenase do tipo 2 (165).

1. 10 Resveratrol e Sirtinol

Polifenóis são metabólitos secundários provenientes de plantas e são caracterizados pela presença de um ou mais grupos hidroxila em sua estrutura. O resveratrol (trans-3,5,4'-trihidroxiistilbeno) é um polifenol muito estudado por apresentar propriedades antioxidantes e anti-inflamatórias. O resveratrol pertencente ao grupo dos flavonoides e pode ser encontrado nas uvas (*Vitis vinifera* e *Vitis labrusca)* principalmente nas cascas, amendoins (*Arachis hypogea)* e amoras em resposta ao estresse, radiação ultravioletra (UV) e infecções virais (166-168). O resveratrol foi identificado pela primeira vez em 1940 como componente das raízes do hellebore branco (*Veratrum grandiflorum O. Loes*) e mais tarde nas raízes secas de Polygonum cuspidatum, conhecido como Ko-jo-kon em japônes para tratamento de dermatites e hiperlipidemia (169-172).

Achados epidemiológicos encontraram uma relação inversa entre o consumo de vinho tinto e a incidência de doenças cardiovasculares. Os principais estudos envolvendo estas ações iniciaram na década de 1990, em virtude de uma possível associação entre o resveratrol e o "Complexo Francês" (167, 173). Apesar de terem as mesmas taxas de colesterol que os americanos, a taxa de mortalidade dos franceses em doenças cardiovasculares, correspondem a um terço da mesma taxa observada nos Estados Unidos. Segundo especialistas, a causa dessa aparente contradição é muito simples: o hábito frânces de desfrutar algumas taças de vinho às refeições (174). Entre os efeitos provocados pela ação do resveratrol incluem-se diminuição dos níveis de lipídeos no soro sanguíneo, supressão da peroxidação lipídica e síntese de eicosanóides, inibição da agregação de plaquetas, ações antioxidantes, anti-inflamatórios e relaxamento dos vasos sanguíneos (175).

Pouco se sabe ainda sobre os mecanismos de ação do resveratrol sobre a molécula SIRT1. Alguns estudos consideram que resveratrol aumenta a atividade desacetilase da SIRT1 para substratos acetilados através da diminuição da constante Michaelis (K_m) (176-178). Quanto menor for o valor de *Km*, maior será a afinidade da enzima pelo substrato. Outros estudos já propõem que o resveratrol atua por mecanismos indiretos para ativar SIRT1, através da ativação de outras moléculas como AMPK. AMPK ativa SIRT1 pela elevação dos níveis intracelulares de NAD^+ que é um substrato importante para a atividade desacetilase da SIRT1 (179-181). A capacidade do resveratrol em ativar AMPK tem sido observada em experimentos in vivo e com cultura de células (182, 183). SIRT1 e AMPK atuariam de forma dependente e com muitas funções similares incluindo habilidade em responder ao status

nutricional e estresse celular, biogênese mitocondrial, regulação da homeostase da glicose e controle da atividade de importantes reguladores transcricionais como FOXO, PGC-1α e p300 (180).

Espécies reativas de oxigênio são capazes de atacar a molécula de DNA induzindo mutações e modificações nas bases do DNA. Esses danos oxidativos representam o passo inicial da carcinogênese quando o reparo de DNA não é eficaz na correção desses danos. Isso pode resultar em efeitos transitórios quando ocorrem mudanças epigenéticas que modificam a expressão de genes ou permanentes quando resultam em mutações somáticas e rearranjos cromossomais (184, 185). O resveratrol é um potente ativador de enzimas de detoxificação de fase II como NADPH, quinona oxidoredutase (186). Essa atividade antioxidante protege as células dos efeitos danosos dos radicais livres, prevenindo a formação inicial de tumores (187, 188). Além disso, essa substância inibe a atividade proliferativa em várias linhagens de células tumorais (189). O resveratrol induz uma parada do ciclo celular na fase S através da inibição da síntese de DNA. Esse efeito sobre a replicação do DNA parece envolver a inibição da ribonucleotídeo redutase e a atividade da DNA polimerase (190, 191).

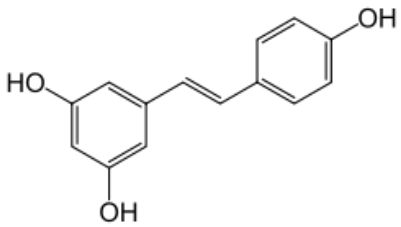


Figura 4. Estrutura química do resveratrol

O sirtinol (2-[(2-hidroxi-1-naftalenilmetileno) amino]-N-(1-feniletil)benzamida) é um inibidor da atividade das histonas desacetilases dependentes de NAD^+ . O seu efeito inibitório no crescimento celular foi descrito no cancro da mama e pulmão de seres humanos e foi capaz de aumentar a quimiosensibilidade de várias linhagens celulares para a camptotecina e cisplatina (192). O sirtinol inibe o crescimento de células cancerígenas e suprime a sinalização de partículas inflamatórias em células endoteliais (192, 193).

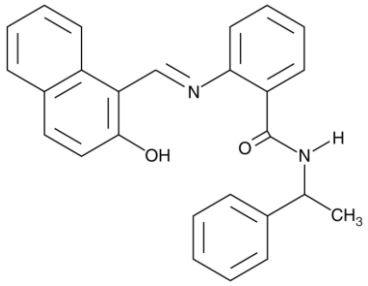


Figura 6: Estrutura química do sirtinol

1.11 Ki67

A proliferação celular engloba uma cascata de eventos, processados de maneira ordenada, assegurando a duplicação fiel dos componentes celulares em uma seqüência lógica e a divisão destes componentes em duas células filhas (194). Existem pelo menos quatro fases distintas no ciclo celular: o período antes da síntese de DNA (G1), a fase de síntese de DNA (S), o período após a replicação do DNA (G2) e a fase mitótica (M). As células que não estão no ciclo celular estão na chamada fase G0 e podem permanecer nesta fase por tempo indeterminado (195).

A atividade proliferativa de qualquer tecido, ou neoplasia, pode ser determinada pelo taxa de crescimento isto é, o número de células em proliferação e o tempo que estas levam para completar este ciclo (196). A descoberta de inúmeras proteínas que desempenham papel fundamental na fase replicativa do DNA permite, através de sua detecção e contagem, tanto a identificação objetiva das células em proliferação como uma estimativa da taxa de crescimento do tecido ou neoplasia (194).

Ki67 é uma proteína nuclear de aproximadamente 360 kDa que é comumente utilizada para avaliar e quantificar células em estágio proliferativo (197, 198). Sua localização é complexa e específica dentro do núcleo, e se altera durante o ciclo celular, isto é, movendo-se do interior do nucléolo para a camada pericromossomal durante a mitose (199). Sua expressão é induzida quando células quiescentes são estimuladas para sair da fase G0 e alcançar a fase G1 do ciclo celular. A proteína Ki67 está presente nas fases G1, S e G2 do ciclo celular alcançando um pico durante a fase da mitose. Células em fase G0 não expressam Ki67 (200).

A função de Ki67 no ciclo celular ainda não foi totalmente elucidada. Ela é descrita como uma proteína possivelmente associada ao nucléolo e aos componentes fibrilares, bem como um fator essencial a síntese de ribossomos durante a divisão celular (199). Ki67 atuaria na camada pericromossomal estabilizando o fuso mitótico e recrutando proteínas para os eventos ocorridos no ciclo celular (201).

A expressão de Ki67 é alta em uma variedade de tumores sendo que há uma correlação inversa entre a taxa de expressão da proteína e sobrevida dos pacientes com câncer (197, 202, 203). Além disso, a expressão de Ki67 tem sido correlacionada com comportamento tumoral, grau histopatológico e recidiva inicial em vários carcinomas (204-208).

1.12 PECAM-1 e CD105

O crescimento do tumor é dependente da angiogênese, processo de formação de novos vasos sanguíneos a partir da proliferação e migração de células endoteliais preexistentes. A angiogênese representa um processo crítico para o crescimento tumoral e formação de metástases (209). A formação de novos vasos sanguíneos no tecido tumoral visa o fornecimento de nutrientes e oxigênio permitindo a proliferação das células e consequente crescimento e progressão do tumor (210, 211). Além disso, permite a retirada do gás carbônico (CO2) e dos resíduos metabólicos, e representa uma importante via de disseminação metastática (212). A angiogênese tumoral é um processo complexo que envolve a produção de fatores de crescimento e proteínas da matriz extracelular. VEGF (Fator de crescimento endotelial vascular), Molécula de Adesão Celular Endotelial Plaquetária (PECAM-1), endoglina e fatores de crescimento do fibroblasto são liberados pelo tumor e células do estroma como potentes indutores da angiogênese (213).

A identificação da angiogênese em tumores pode ser realizada através de diversos métodos, sendo a identificação por meio da densidade microvascular (DMV) um dos mais utilizados. Avaliação imunohistoquímica da densidade microvascular por unidade de área está associada com grau de neovascularização intratumoral, capacidade metastática do tumor e prognóstico para pacientes que apresentam tumores sólidos (214, 215). Os vasos corados pelo método são contados em microscopia de grande aumento nas áreas de maior concentração vascular, os chamados “hot-spots.” Diversos anticorpos podem ser usados para identificação da microvascularização, sendo os mais estudados PECAM-1 e endoglina (216).

A molécula de adesão celular endotelial plaquetária (PECAM-1) também chamada de CD31 é uma glicoproteína transmembrana que pertence à superfamília das imunoglobulinas. Seu gene está localizado no braço longo do cromossomo 17 e codifica uma glicoproteína de 130 kDa (217). Está presente em células endoteliais, plaquetas, monócitos e células polimorfonucleares (218). PECAM-1 funciona como um receptor de adesão celular apresentando um papel importante na ativação de integrinas e na migração de leucócitos, monócitos e linfócitos durante a inflamação (219). PECAM-1 atua entre as junções celulares do endotélio contribuindo para a integridade da permeabilidade vascular (220). Além disso, PECAM-1 pode apresentar papel fundamental durante a angiogênese tumoral, pois essa proteína tem sido altamente expressa no endotélio de vários tecidos tumorais (221-223). Esse marcador tem sido relacionado com pior prognóstico em muitas malignidades, pois atua na adesão e crescimento da célula tumoral bem como nos fenômenos metastáticos (224, 225).

Endoglina também conhecida por CD105 é uma glicoproteína da membrana celular que pode ser encontrada em células endoteliais, células musculares lisas das paredes vasculares (226), fibroblastos (227) e macrófagos (228, 229). O gene da endoglina está localizado no braço longo do cromossomo 9 sendo que sua região codificante possui 14 éxons**.** Sua proteína apresenta um peso molecular de 180 kDa e um domínio externo que se liga aos receptores tipo I e II do fator transformador de crescimento β (TGF-β) (230). TGF-β é uma citocina pleiotrópica envolvida na proliferação, diferenciação e migração celular. A expressão da endoglina é importante para fosforilação dos receptores TGF-βR1 e TGF-βR2, o que permite a modulação da sinalização de TGF-β e de suas proteínas alvos. A inibição da expressão de endoglina faz com que TGF-β1 diminua o crescimento, migração e capacidade para formação de tubos capilares em culturas de células (231, 232). Isso mostra que endoglina é um importante componente do complexo TGF-β/receptores e que sua expressão modula a função desse fator e de seus receptores.

A endoglina participa do desenvolvimento vascular e manutenção da integridade dos vasos sanguíneos (233, 234). A atuação da endoglina na formação vascular foi observada em experimentos envolvendo camundongos knockout para essa proteína. Nesses animais, foi observada a presença de múltiplos defeitos cardíacos e vasculares que resultou em morte nos estágios iniciais da formação embrionária (235). A endoglina tem sido altamente expressa no endotélio de vários tecidos tumorais em comparação aos tecidos normais (236). A expressão

de endoglina por meio da densidade microvascular tem sido correlacionada com pior sobrevida, ocorrência de recidiva e presença de metástases em diferentes tumores (237-240).

2 OBJETIVOS

2.1 Objetivo geral

- Analisar a expressão de marcadores moleculares em lesões melanocíticas humanas e o efeito do resveratrol e sirtinol na viabilidade celular em linhagem celular de melanoma maligno cutâneo.

2.2 Objetivos específicos

- Analisar a expressão imunohistoquímica da SIRT1 e o índice de proliferação celular (Ki67), Endoglina e PECAM-1 entre as amostras de melanoma cutâneo humano, nevo melanocítico e pele normal.
- Verificar uma possível associação entre a expressão dessas proteínas e fatores clínico – patológicos e estadiamento clínico e morfológico das lesões.
- Verificar o efeito do tratamento com resveratrol e sirtinol sobre a viabilidade de linhagens celulares murinas de melanoma cutâneo metastático e melanócitos normais, bem como sobre a expressão gênica da SIRT1.

3 PRODUTOS

3.1 Produto 1: *SIRT1 and Ki67 immunohistochemical expression in progression of cutaneous malignant melanoma,* formatado segundo as normas para publicação do periódico Melanoma research, enviado nesse periódico.

3.2 Produto 2: *Resveratrol and sirtinol decrease the viability of cutaneous malignant melanoma cells without changes of SIRT1 mRNA expression,* formatado segundo as normas para publicação do periódico Melanoma research enviado nesse periódico.

3.3 Produto 3: *Analysis of immunohistochemical expression of PECAM-1 and endoglin in normal skin, benign melanocytic nevi, and cutaneous malignant melanoma* formatado segundo as normas para publicação do periódico Melanoma research enviado nesse periódico.

3.1 PRODUTO

SIRT1 and Ki67 immunohistochemical expression in progression of cutaneous malignant melanoma

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Abstract

The current study evaluated the proliferation index and immunohistochemical expression of SIRT1 on normal skin and cutaneous melanocytic nevi (CMN) and cutaneous malignant melanoma (CMM). Formalin-fixed paraffin-embedded tissue samples from 43 CMN, 22 CMM and 10 normal skin were obtained and clinical data were abstracted from the electronic medicalrecord.SIRT1 and Ki67 proteins expressions were evaluated regarding to clinic pathological behavior in CMM. The level of significance was set at $\alpha = 5\%$ ($p < 0.05$).Our findings showed that SIRT1 positivity was significantly higher in benign melanocytic nevi than that in cutaneous malignant melanoma (p=0.002). As expected, the proliferation index was significantly higher in samples of cutaneous malignant melanoma as compared to the normal skin and melanocytic nevi (p=0.001). However, the expression of Ki67 protein was not also significantly related to the expression of SIRT1 ($p > 0.05$). In conclusion, low expression of SIRT1 and high proliferation index may play an important role in progression of cutaneous melanoma.

Keywords: melanocytic nevi; skin lesions; proliferation; sirtuins.

Introduction

Skin cancer is the third most common human malignancy and its global incidence is rising at an alarming rate, with basal cell carcinoma, squamous cell carcinoma and melanoma being the most common forms [1].There are an estimated 2–3 million cases of skin cancer across the world each year, and although cutaneous malignant melanoma (CMM) only accounts for about 200,000 of these (World Health Organization), it is the most dangerous form, accounting for most skin cancer deaths [2]. CMM diagnosed in early stage can be cured by surgical resection, and about 80% of cases are dealt with in this way [3]. However, metastatic malignant melanoma is largely refractory to existing therapies and has a very poor prognosis, with a median survival rate of 6 months [4].

Malignant melanoma is a tumor that originates from the melanocytes and manifests mainly on the skin. UVR exposure is a major risk factor, especially in light skin populations [5, 6]. Lightly pigmented skin and a large number of melanocytic nevi are associated with increased risk of developing malignant melanoma [6-8]. Cutaneous melanocytic nevi (CMN) are benign proliferations of melanocytes that are postulated to resultfrom sun-induced mutations [9], typically BRAF [10] and genes associated melanocytic system [11]. In the majority of such neoplasms, subsequent melanocyte senescence is induced by tumor suppressor proteins such as p16, and the nevus therefore ceases to grow and becomes stable or even involutes [12]. The accumulation of alterations in key genes controlling processes such as proliferation, apoptosis, senescence, and response to DNA damage can favor the formation morphologically atypical melanocytes predisposing the risk of developing melanoma [13, 14]. Cutaneous melanocytic nevi can progress to the intra-epidermal lesion that can involve some local microinvasion of the dermis. The next phase, the cells can progress invading the dermis, a more dangerous stage in which the cells have metastatic potential, with nodules or nests of cells. Not all melanomas pass through each of these individual phases but develop directly from isolated melanocytes or nevi, and both can progress directly to metastatic malignant melanoma [15].

Silent mating-type information regulation 2 homologue 1 (SIRT1) is a protomember of the sirtuin family (SIRT1-7) that is involved in a variety of biological processes, including genetic control of aging, regulating transcription, apoptosis, stress resistance and energy efficiency during low-calorie conditions [16, 17]. To date, the role of SIRT1 remains controversial as previous data suggest that SIRT1 can act as an oncogene or a tumor suppressor, likely depending on cell type, its distribution and biological targets [18-20].

Recent studies demonstrated that SIRT1 levels are reduced in some types of cancers, and that SIRT1 deficiency results in genetic instability and tumorigenesis [21-23]. SIRT1-deficiency resulted in an increased tumor formation in p53-null mice [24]. SIRT1 inhibits proliferation of pancreatic cancer cells expressing pancreatic adenocarcinoma up-regulated factor [24]. On the other hand, SIRT1 has been considered as a tumor promoter because of its increased expression in some types of cancers and its role in inactivating proteins that are involved in tumor suppression and DNA damage repair [25, 26].

The role and functional significance of SIRT1 in cancer development and progression is currently an intense area of research investigation. SIRT1 has been shown to be upregulated in several cancers such as prostate cancer, cutaneous T-cell lymphoma, colorectal cancer and pancreatic cancer [17, 27-30]. SIRT1 is also overexpressed in non-melanoma skin cancers, including squamous and basal cell carcinomas, actinic keratosis, and especially in Bowen's disease [31]. However, several studies have shown that both the overexpression and low expression of SIRT1 has been linked to poor disease prognosis and survival depending on the type of cancer [32-34] [24, 35, 36]. In spite of the controversial role of SIRT1 in tumorigenesis [37, 38], it is evident that SIRT1 is significantly involved in the process of tumorigenesis, however, its expression status in melanoma is poorly defined and are required further investigation. In this research, we observed the expressions of SIRT1 and index proliferation in skin lesions, and investigated the association between the expressions and clinicopathological characteristics.

Methods

Patients

This retrospective and cross-sectional study analyzed samples of human tissues in 17 normal skins (control), 40 benign cutaneous melanocytic nevi (CMN) and 22 cutaneous malignant melanoma (CMM) with confirmed histopathological diagnosis. Clinic data were obtained from medical records of patients attended at public health centers for Oncology treatment at Montes Claros city, Minas Gerais state, Brazil. The normal human skin samples were obtained from patients who experienced aesthetic or corrective surgical procedures in women's breasts.

Ethical approval for this study was obtained from a relevant local ethic committee (Committee on ethic in research – Faculdades Integradas Pitágoras: protocol no: 714.865/2014).

Clinic and pathological analyses

All CMM cases were classified according to the American Joint Committee on Cancer melanoma staging [39]. CMM patients were categorized as T1/T2 (≤ 2 mm thickness, n = 5 (22.72%) and T3/T4 (> 4 mm thickness, n = 17 (77.27%). Metastatic diseases were diagnosed in 7 (31.8%) CMM patients. Furthermore, cases of CMM presented the following clinical aspects: superficial spreading (n = 11, 50.0%), nodular (n= 3, 13.63%), lentigo malignant (n = 5, 22.72%), and acral melanomas (n = 3, 13.63%).

According to anatomical sites, CMM samples were classified as low-risk (lower trunk, thigh, lower leg, foot, lower arms, hands, and face) and high-risk (back and breast/thorax, upper arm, neck, and scalp) for death caused by CMM [40, 41].

Formalin fixed and paraffin embedded samples were submitted to histopathological analysis. Tissue sections were cut at a thickness of 3–5μm and stained with hematoxylin and eosin (H&E). CMM samples were subjected to analysis of tissue invasion of melanoma cells by Breslow's thickness [42] and Clark's level [43] criteria. According to Breslow's thickness grade, CMM samples were categorized as follows: TI (up to 0.75mm, n = 3, 13.6%), TII (from 0.75 to 1.5mm, n = 7, 31.8%), TIII (1.5 to 4mm, n = 11, 50.0%), and TIV (≥ 4mm, n = 1, 4.5%). According to Clark's level (degree of invasion), CMM samples were categorized as follows: level I (limited to the epithelium, n = 2, 8.3%), level II (invasion up to the papillary dermis, n = 6, 25.0%), level III (invasion fills the entire reticular dermis, though without invading it, n = 11, 45.8%), level IV (invasion of the reticular dermis, n = 5, 20.8%), and level V (invasion of the hypodermis, n = 0).

Immunohistochemical reactions

The 5-μm tissue sections were deparaffinized, hydrated and the antigen retrieved. The tissue sections were incubated with 3% (v:v) hydrogen peroxide for 30 min at room temperature to quench the endogenous peroxidase. After blocking in normal goat serum, the tissue sections were incubated with the primary antibodies anti-Ki67 (Mouse monoclonal, Dako, 1:200 dilution, clone MIB-1, Glostrup, Denmark) and anti-SIRT1 (Clone sc-15404, Santa Cruz Biotecnology, Dallas - TX) overnight at 4°C. The slides were then washed in PBS and incubated with LSABTM-Kit Plus Peroxidase® for 1h. Tissues were stained with a chromogen amino-etil-carbazol, counterstained with Mayer’s hematoxylin, cover slipped, and visualized under an optical microscope Olympus® BH2 microscope (model: CX31; RTSF, Miami, USA).Positive and negative controls were applied according to the manufacturer’s

instructions (Dako Cytomation, Glostrup, Denmark).All control, CMN, and CMM samples were examined by two independent investigators who were blind to the clinical data.

Counting of immunostained cells with Ki67and SIRT1

The immunohistochemical staining for Ki67 and SIRT1 were measured manually using custom software ImageJ®, version 1:44, for Windows® to assist in performing the cells counts. Ten images were acquired per case at a total magnification of ×400 using an optical inverted Olympus® FSX100 microscope (model: CX31; RTSF, Miami, USA). Selected fields were those with highest density of Ki67 or SIRT1 positive cells. Ki67 labeling index was performed as follows: % marking = (positive nuclei/[positive nuclei + negative nuclei]) [44, 45].

The immune reactivity of SIRT1 was evaluated in the normal epithelial /nevi/neoplastic cells considering the cytoplasmatic and/or nuclear staining, or even absent. It was estimated the proportion of cells labeled with both cytoplasmic and nuclear expression in each one of the photomicrographs. Next, the average of the ratios was calculated for each case, considering individual nuclear and cytoplasmic expression for statistical analysis. It aimed to further determine the best cutoff point to define the expression of the protein as positive or negative in samples to the lesions types and location of this protein, using the receiver operating characteristic curve (ROC curve). In cytoplasmic expression, it was found that any ratio of higher than 36.1% represented a good cut-off for positive cytoplasmatic staining ($p < 0.021$). In the evaluation of nuclear expressions identified that positivity would be better represented in values above 1.8150% markup ($p = 0.198$). In the final evaluation of the positive immunohistochemical expression of SIRT1, cases were further ranked as cytoplasmic or nuclear staining to study statistical inferences.

*Statistical analysis*Statistical analyses were performed using SPSS® 18.0 (SPSS Inc., Illinois, USA) and Graph Pad Prism®5.0 Softwares. Results were expressed as mean ± SE or as percentages. P values ≤ 0.05 were considered statistically significant.

Comparisons of immunohistochemical expressions of studied proteins between the lesions were evaluated using Mann-Whitney and Fisher's exact test. Kruskal-Wallis was used to evaluate the differences between SIRT1 and Ki67 expressions and clinicopathological characteristics of melanoma.The analysis immunohistochemical of the expression of SIRT1 and variables clinicopathological lesion and comparing of the expression of SIRT1 between the types of study samples was performed by Chi-square test and Fisher's exact test with

application of ROC ("Receiver-operator curves") curve. The curve ROC was used to assess the sensitivity and specificity as the cutoff point for analysis of SIRT1 expression.

Results

SIRT1immunohistochemical expression is reduced in human cutaneous malignant melanoma

SIRT1 immunohistochemical staining was localized in tumor and normal cells (lymphocytes and fibroblasts) with varying intensities.The immunohistochemical staining of SIRT1 in CMM, CMN and normal skin is shown in Fig. 1.

Based on the ROC curve, it was simulated a cutoff point to distinguish samples with low and high staining of SIRT1, according to the diagnosis of the sample. Applying the values on a ROC curve, the area under the curve [46] was 0.684 (95% CI) with best estimates occurring in the amount of 36.1, which was a sensitivity of 59.1% and specificity of 23.5% (P= 0.021). Tumors with scores above the 36.1 cut-off values were considered positive for the cytoplasmic expression of SIRT1 protein. According to ROC curve analysis, expression percentage for nuclear SIRT1 above the critical value 1.8150% was defined as positivity. Applying the values on a ROC curve, the area under the curve [46] was 0.602 (95% CI) with a sensitivity of 27.4% and specificity of 5.9% (p= 0.198).

Normal skin samples (control) showed weak or negative cytoplasmic staining to SIRT1. In normal skin, cytoplasmic SIRT1 staining was weakly and diffusely expressed in suprabasal epidermal keratinocytes, with only faint and focal staining in the granular layer and stratum corneum. The SIRT1 protein was detected in the normal epithelial tissues in 35.29% of normal skin (Table 1).

The SIRT1 protein was present in both cytoplasmic and nuclear compartments of the cutaneous melanocytic nevi. Therefore, SIRT1-positive cases were classified into two categories (nuclear or cytoplasmic SIRT1). SIRT1 was positive in 76.74% of benign melanocytic nevi cases. Among the SIRT1-positive cases, 63.63% were cytoplasmic positive and 36.36% were nuclear positive. In particular, the junctional component of benign melanocytic nevi was positive in the most cases. SIRT1 positivity is observed in the majority melanocytes, especially those arranged in bridging nests at the dermoepidermal junction and the intradermal component.

The SIRT1 protein was present in cytoplasmic compartment of the malignant melanocytic cells. SIRT1 was positive in twelve of 23 MMC (52, 17%), and all SIRT1-positive MMC cases showed cytoplasmic positivity. SIRT1 positivity was significantly higher in benign CMN than that in MMC (p= 0.002). The invasive component of melanoma shows a

weak and diffuse SIRT1 cytoplasmic staining. Most MMC displayed high rate of melanocytes expressing SIRT1 in intradermal component. SIRT1 expression was not significantly related to any of the clinicopathological parameters (Table 2).

Ki67 immunostaining was nuclear and nucleolar (Fig.1). The average number of Ki67-positive cells was significantly higher in samples of cutaneous malignant melanoma as compared to the normal skin and melanocytic nevi ($p < 0.001$) (Table1). The normal skin and benign CMN displayed positive Ki67 immunostaining in basal keratinocytes whereas benign CMN was absent in the majority of nevi cells. The expression of Ki67 it found in intradermal component of MMC. The associations between the Ki67 expression and clinicopathological factors did not have statistical significance (Table 2). Furthermore, the expression of Ki67 was not also significantly related to the expression of SIRT1 ($p > 0.05$).

Discussion

This study investigated the immunohistochemical expression of Ki67 and SIRT1 in normal skin, CMN and CMM samples. In this study, we noted a significant decrease of staining of SIRT1 from CMN to CMM samples. Also, normal epithelial cells showed weak or negative staining to SIRT1 while CMN samples exhibited a higher SIRT1 expression as compared to the CMM samples. Comparatively, the SIRT1 expression was gradually decreased during carcinogenesis and tumour progression of colorectal adenocarcinoma [36]. This suggests that loss of SIRT1 expression in tumoral lesions may be associated with a more aggressive phenotype. However, the role of SIRT1 in human malignant tumors is controversial. Some previous studies have reported that SIRT1 overexpression was associated with shorter overall survival or poor prognostic indicators in breast and gastric carcinoma [33, 47]. The expression of SIRT1 is relatively higher in hepatocellular carcinoma, breast cancer, and thyroid cancer [48-50] but lower in colon and lung cancer [23, 51, 52] compared with their corresponding normal tissues. In cancer, SIRT1 has been reported as either an oncogenic or a tumor suppressive role, depending on the type of cancer and the context of the analysis [22, 33, 53]. These results suggest that SIRT1 may acts differently depending on the specific organ or type of tumor involved.

We demonstrated that SIRT1 is predominantly localized in the cytoplasm of CMM. SIRT1 cytoplasmic localization is not commonly identified in cancer cells and it is unclear if SIRT1 localization has any changes during carcinogenesis. Similar results have also identified

aberrant cytoplasmic localization in human cancer cells [54-56]. This finding may suggest a new mechanism for SIRT1 function as a cancer-specific survival factor by targeting cytoplasmic proteins. In contrast to its well-described role in the nucleus, the deacetylation function of cytoplasmic proteins caused by SIRT1 provides important insights into the function of cytoplasmic SIRT1. Zhang, 2007 showed that SIRT1 enhanced IGF-1 signaling by deacetylating the ISH-2 cytoplasmic protein [57]. SIRT1 also deacetylates cytoplasmic cortactin and promotes cancer cell migration [58]. In addition, SIRT1 was found to promote the activation of cytoplasmic kinases, including AMPK, Ras-MAPK, Erk and S6K1 [26, 59, 60].

In the case of certain cancers, including prostate cancer, lung cancer, breast cancer, and melanoma, SIRT1 has been shown to localize to the cytoplasm, while being located predominantly in the nucleus in the corresponding normal tissues [54, 61].This change in localization could theoretically minimize the deacetylation of TP53 in the nucleus by SIRT1 while still allowing TP53 to regulate its transcription. Thus, in cancers under these conditions, the oncogenic role of SIRT1 overexpression through TP53 might be minimized, allowing for other targets of SIRT1 to play a more significant role, especially those that are localized to the cytoplasm [62]. Cellular localization of SIRT1 also has been shown to differ among different tissue types in mice [63], which could explain why SIRT1 sometimes exhibits tumor suppressor properties in certain types of cancer but not in others.

In current study, there was no association significant between SIRT1 and Ki67 immunostaining, however, further functional study will be needed to investigate the relationship between SIRT1 and cellular proliferation. Melanoma is known to exhibit aberrant expression of proliferation markers, and these abnormalities are considered important steps in the genesis and progression of melanoma [64]. An increasing literature describes the role of proliferation markers in the evaluation of melanocytic tumors [65]. Ki67 staining has been shown positive in multiple lesions, 5% of positivity on melanocytic cells in most benign nevi, although there have been reports of up to 15% positivity in Spitz and dysplastic nevi [66-71]. Conversely, Ki67 staining is reported as positive in 13–30% of the cells in a malignant melanoma, although individual cases can show almost 100% nuclear positivity [69, 71, 72]. In our study, we found a lower average than 5% staining in benign melanocytic nevi while in melanoma was greater than 15%. Therefore, Ki67 index was reported to be higher in malignant melanomas than in benign nevi. Correspondingly, no associations between Ki67 and measures of tumor size (thickness and diameter) and invasion (Clark’s level) were found. Other studies on cutaneous melanoma have suggested that increased Ki67 expression might

be associated with tumor thickness and tumor cell proliferation [73-75]. Our experiments have some limitations, such as small sample size. The tumor heterogeneity and staining scoring method also may to interfere the results. In summary, we need further study on the roles of SIRT1 and Ki67 on clinical and pathological behavior of melanoma.

Conclusion

Low expression of SIRT1 and high proliferation index may play an important role in progression of cutaneous melanoma.

Declaration of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

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LEGEND OF FIGURES

Figure 1. Morphological aspects of normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples (Figures A, B, and C, respectively. H&E; higher magnification of 100x). Immunohistochemical expression of Ki67 (Figures D, E, F) higher magnification of 200x in samples of control, CMN, and CMM. SIRT1 (Figures G, H, and I) higher magnification of 200x in samples of control, CMN, and CMM. Immunostaining: AEC; counterstaining: Mayer's hematoxylin).

Table

Table 1: Expression of Ki67 and SIRT1 in normal skin (control), benign cutaneous melanocytic nevi and cutaneous malignant melanoma.

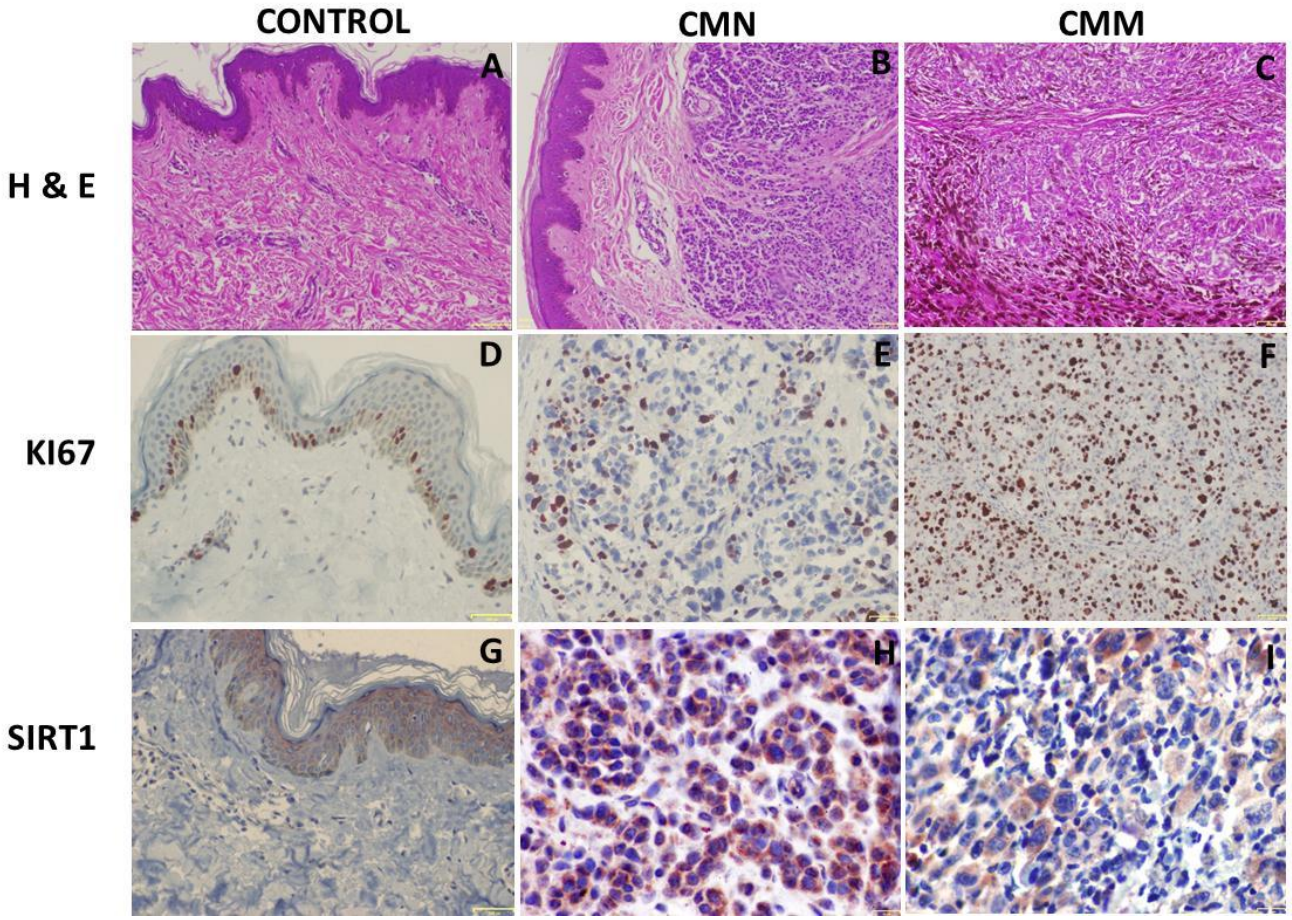
Variables	Ki67 Mean (SD)	p	SIRT1+ n (%)	p	SIRT1+ Cytoplasmic n (%)	SIRT1+ Nuclear n (%)	p
Control [a] (17)	0.93 (1.82)	$p^{ab} = 0.155$	6 (35.3%)	$p^{ab} = 0.001$ **	6 (100.0%)	0	$p^{ab} = 0.151$
Melanocytic Nevi [b] (43)	2.04 (4.28)	$p^{ac} = <0.001$*	33 (76.7%)	$p^{ac} = 0.334$	21 (63.6%)	12 (36.4%)	$p^{ac} = 0.529$
Melanoma [c] (22)	16.96 (14.54)	$p^{bc} = <0.001$*	12 (54.6%)	$P^{bc} = 0.035$	10 (83.3%)	2 (16.7%)	$p^{bc} = 0.287$

*Values bearing asterisks show significant association using Mann-Whitney. **Values bearing asterisks show significant association using Chi-square test and Fisher's exact. Level of significance was set at $\alpha = 5\%$ ($P < 0.05$).

2: Evaluations Ki67 and SIRT1immunohistochemical expressions in function of clinic and pathological parameters on melanoma malignant cutaneous.

Variables	KI67 Mean (SD)	p	SIRT1+ n (%)	p	SIRT1 Cytoplasmic n (%)	SIRT1 Nuclear n (%)	p
Anatomical Site							
Low risk (18)	13.82 (15.01)	0.631	9 (50.0%)	0.478	8 (88.9%)	1 (11.1%)	0.522
High risk (4)	17.22 (14.09)		4 (100.0%)		3 (75.0%)	1 (25.0%)	
TNM							
I/II (5)	16.89 (11.60)	0.990	2 (40.0%)	0.457	2 (100.0%)	0	0.488
III/IV (17)	16.98 (15.62)		10 (58.8%)		8 (80.0%)	2 (20.0%)	
Clinical Size							
Small (5)	16.89 (11.60)	0.990	2 (40.0%)	0.457	2 (100.0%)	0	0.488
Large (17)	16.98 (15.62)		10 (58.8%)		8 (80.0%)	2 (20.0%)	
Regional Metastasis							
Absent (15)	16.69 (15.74)	0.902	8 (50.0%)	0.867	6 (75.0%)	4 (50.0%)	0.273
Present (7)	17.54 (12.70)		4 (57.1%)		4 (100.0%)	0	
Recurrence							
Absent (18)	17.04 (14.61)	0.646	9 (56.2%)	0.422	7 (77.8%)	2 (22.2%)	0.371
Present (4)	20.86 (15.03)		3 (60.0%)		3 (100.0%)	0	
Level of Invasion							
I-III (17)	14.80 (13.57)	0.206	9 (50.0%)	0.781	7 (77.8%)	2 (22.2%)	0.371
IV-V (5)	24.31 (16.91)		3 (60.0%)		3 (100.0%)	0	
Tumor Thickness							
$\leq$ 2 mm (12)	15.60 (12.38)	0.642	8 (72.7%)	0.211	6 (75.0%)	2 (25.0%)	0.273
$\geq$2 mm (10)	18.59 (17.34)		4 (33.3%)		4 (100%)	0	

Figure 1



3.2 PRODUTO

Resveratrol and sirtinol decrease the viability of cutaneous malignant melanoma cells without changes of SIRT1 mRNA expression

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ABSTRACT

The current study aimed to evaluate the effect of resveratrol and sirtinol, activator and inhibitor of sirtuins, respectively, on cell viability and SIRT1 mRNA expression in murine metastatic skin melanoma (B16F10) cell line. B16F10 and non-tumoral murine melanocytes (Melan-A) cell lines were treated with resveratrol and sirtinol and evaluated for cell viability, DNA fragmentation and SIRT1 gene expression. All experiments were performed in triplicate and submitted to specific statistical tests with significance level at $\alpha = 5\%$ ($p < 0.05$).Treatments with resveratrol and sirtinol significantly affected the B16F10 cell viability ($p < 0.05$) and promoted DNA fragmentation ($p < 0.05$). Significant reductions in the viability and DNA fragmentation after exposure to resveratrol andsirtinol were also observed in Melan-A cells. However, both treatments did not change the SIRT1 expression on the same studied conditions. Results of the present study revealed that use of these drugs may be a promising chemotherapeutic strategy against melanoma cancer,even in the absence of changes in the transcription of the SIRT1 gene.

Keywords: B16F10; Melan-A; sirtuins; DNA fragmentation.

Introduction

Cutaneous malignant melanoma (CMM) is a tumor arising from melanocytes whose injuryis manifested mainly in the skin. The incidence of cutaneous malignant melanoma (CMM) has risen rapidly in the last decades and is responsible for the greatest number of skin cancer related deaths [1]. Although CMM accounts for only 4% of malignancies of the body, it presents high risk of metastasis [2]. Once metastasized to remote sites, it is characteristically unresponsive to treatment [3]. Prevention and early detection are the most effective measures against melanoma-related mortality [4].

Cutaneous malignant melanoma has multifactorial etiologies and the risk factors that contribute to the development of CMM include family history, presence of multiple dysplastic or benign nevi, skin type, eye color and hair, chronic exposure to chemical and physical mutagens, and genetic factors [3, 5]. Some of these factors such as the presence of numerous normal or atypical melanocytic nevi may confer a strong risk factor for the development of CMM [6]. In addition, overexposure to ultraviolet (UV) radiation, particularly to ultraviolet type B (UVB), has been linked to increased risk for the occurrence of CMM and other skin cancers [7]. The deregulation of certain genes responsible for cell proliferation, cell death and metastasis and its complex interaction with the acquired factors are associated with the development of the disease [8-10].

The knowledge of genes involved in senescence and cell proliferation processes can be exploited for the discovery of new therapeutic targets in cancer treatment. Sirtuins, also known as SIRTs, are NAD^+-dependent class III histone deacetylases, (HDACs) [11]. In mammals, seven SIRT homologues have been identified that primarily possess HDACs activities (SIRT1, SIRT2, SIRT3 and SIRT5) or monoribosyltransferase activity (SIRT4 and SIRT6), which target histone and various non-histone proteins in distinct subcellular locations [12-14]. The best identified and characterized among the human sirtuins is sirtuin1 (silent information regulator-1, SIRT1), a nuclear enzyme, that has been shown to regulate important metabolic and physiological processes that play a critical role in processes related to cancer[15-17]. SIRT1 determines gene expression by deacetylating either histones or transcription factors [18, 19]. SIRT1 participates in a variety of biological processes, such as metabolic reprogramming cell proliferation, differentiation, and senescence [20, 21].

The role of SIRT1 in cancers has been extensively studied in the past decade. However, there is controversy regarding cancer and sirtuins since it could act as either a

tumor suppressor or tumor promoter depending on the cellular context or its targets in specific signaling pathways or specific cancers [22-24]. Inhibition of SIRT1 activity has been demonstrated to elevate p53 acetylation and transactivation and results in enhanced apoptosis and cytostasis [25, 26], it has been suggested that SIRT1 has a promoting function in tumor development and progression [27-31].

In contrast, SIRT1 inhibits cell growth by targeting the Wnt/β-catenin, NF-κ B, or HIF-1 signaling pathway [32-34]. As β-catenin is constitutively activated in many human tumors including ovarian cancer and melanoma [35, 36], SIRT1 may inhibit the growth of these tumors by deacetylating and inactivating β-catenin [37]. In addition, SIRT1 deacetylates and inactivates NF-κB [38], which plays a beneficial role in cancer survival under stressful conditions like inflammation, and HIF-1α [39], which promotes cancer survival under hypoxic microenvironment. Accordingly, the inhibition of some of these signaling pathways may be associated with the inhibitory action of SIRT1 on cancer growth. These studies support the potential of SIRT1 as therapeutic target, and provide the rationale for medical research of activators and inhibitors of SIRT1 in the treatment of cancer. To test this hypothesis, we investigated the anticancer effect of resveratrol and sirtinol, activator and inhibitor of SIRT1, respectively, on some cell types, and if these drugs can modulate its expression in melanoma metastatic and normal murine melanocyte cell lines.

Methods

Ethical Aspects

Ethical approval for this study was obtained from a relevant local ethic committee (Committee on ethic in research - Unimontes: protocol number: 691.408/2014) and (Committee on ethic in research – Faculdades Integradas Pitágoras: protocol number: 714.865/2014).

Reagents

Resveratrol and sirtinol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute medium (RPMI), 3,4,5-dimethylthiazol-2,5 biphenyl tetrazolium bromide and propidium iodide were provided by Invitrogen (USA). Fetal bovine serum, hepes, $NaHCO_3$ amphotericin B, ampicillin and streptomycin were purchased from Gibco (USA). Phorbol 12-myristate 13-acetate (PMA) was obtained from Abcam (England). The reagents trizol, DNase, moloney murine leukemia virus reverse transcriptase M

(MLVRT) were purchased from Invitrogen and SYBR Green PCR Master Mix from Appllied Biosystems®, USA. The primary antibodies anti-Ki67 (clone MIB-1) and anti-SIRT1 (clone H300) were purchased of Dako and Santa Cruz, respectively. The peroxidase-conjugated secondary antibody kit used was LSABTM-Kit Plus Peroxidase® (Dako Cytomation, Glostrup, Denmark). Amino-etil-carbazol (AEC) chromogen was provided by Dako.

Cell lines

Both cell lines B16F10 (murine metastatic skin cell melanoma) and Melan A (non-tumoral murine melanocytes) were kindly provided by Dr. Lopes, M.T.P. (Department of Pharmacology, UFMG). The tumor cell line was propagated in cell culture medium Roswell Park Memorial Institute (RPMI) 1640, pH 7.4, supplemented with 10% (v:v) fetal bovine serum (FBS), ampicillin(0.27 mM), amphotericin B (5,41 µM) and streptomycin (0.06 mM). Melan-A cell line was grown under the same conditions mentioned above plus200 nMphorbol ester-myristate (PMA).

MTT cell viability assay

After resveratrol and sirtinol treatments, cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) yellow tetrazolium assay is which is enzymatically reduced to purple formazan in living cells. Briefly, 2 x 10^3 cells/well of B16F10 and 1 x 10^4 cells/well of Melan-A were seeded in 96-well plates for 24 hours. Next, cells were exposed at increasing concentrations of resveratrol (1.56 to 200 uM, dissolved in DMSO) and sirtinol (60-200 uM, dissolved in DMSO) for 48 hours. A control group was created with cells treated with phosphate-buffered saline (PBS, pH 7.4). Then, the cells were incubated with 0.5mg/ml of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dissolved in PBS and filtered through a 0,22 µm membrane) at 37°C for 4 hours. After clearance of MTT solution, formazan was extracted from the cells with dimethyl sulfoxide (DMSO). The absorbance (570 nm) was measured using a spectrophotometer [40]. Each concentration was tested in triplicate from three independent experiments. Results were expressed as percentage relative to control group. Concentrations of resveratrol and sirtinol below of IC50 were adopted for the next assays.

DNA fragmentation assay

We used the propidium iodide (PI) flow cytometric assay in order to evaluate apoptosis in B16F10 and Melan-A cells ($2x10^5$cells/well seeded in 24-well plates) after explosion of increasing concentrations of 5, 25 e 50 μM of resveratrol and sirtinol for 48 hours. The cells were harvested and re-suspended in a hypotonic fluorescent solution (50 mg/ml PI and 0.1% Triton X-100 in 0.1% sodium citrate buffer) for 4 h, at 4°C in the dark. Labeled cells were analyzed by flow cytometry (FACScan, BD, Biosciences, San Jose, CA) and the analyses were performed using FlowJo 7.6.5 software (Tree Star® Inc., San Carlos, CA). Fragmented DNA was identified in the sub-G1 (DNA content < 2n) population and calculated considering the totality of events [41].

Quantification of Sirt-1 expression using reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qPCR)

$6x10^5$B16F10 and Melan-A cells were harvested after treatments with resveratrol and sirtinol in concentrations of 5, 25 and 50 μM for48 h. The total RNA of cells was extracted using Trizol reagent (Invitrogen Corp., San Diego, CA, USA). The RNA extracted was treated with DNAse and the cDNA was synthesized by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT) using random hexamer primers. The endogenous β-actin (internal control) and SIRT1 mRNA were determined by quantitative real-time reverse transcriptase PCR (qRT-PCR) using SYBR Green reagent (Applied Biosystems, Grand Island, NY,USA) in a Plus One platform (Applied Biosystems) [42]. The primer sequences used for PCR are shown in Table I. Relative comparative CT method was applied to compare gene expression levels between groups using the equation $2^{-\Delta\Delta CT}$[43].

Statistical analysis

Statistical analyses were performed using SPSS® 18.0 (SPSS Inc., Illinois, USA) and Graph Pad Prism®5.0 software. Results were expressed as mean ± SE or as percentage. MTT assay, DNA fragmentation, and SIRT1 mRNA expression were compared between groups using one-way analysis of variance test and the Bonferroni posttests. p values ≤ 0.05 were considered statistically significant.

Results

Resveratrol and sirtinol decreases B16F10 cell viability

Resveratrol and sirtinol significantly reduced the viability of B16F10 cells. The IC50 values of resveratrol and sirtinol towards B16F10 cells were 75.09 μM and 62.68 μM, respectively, after 48 hours of treatment (Fig. 1A, B).Tumor cells respond to resveratrol at concentrations lower than the normal cell, which IC50 of the resveratrol and sirtinol treatments for Melan-A cell line were 80.15μM and 86.93μM, respectively (Fig. 1C, D). From these results, concentrations lower than the IC50 were adopted to perform the next experiments.

Resveratrol and sirtinol provoked DNA fragmentation of B16F10 cells.

The results of DNA fragmentation assay revealed that both resveratrol and sirtinol caused DNA fragmentation in tumor cells in the studied concentrations (Fig. 2 A,B). Comparatively, DNA fragmentation was not observed on melan-A cells after treatment with resveratrol, while treatment with sirtinol provoked DNA fragmentation in lower percentages than those observed in B16F10 cells (Fig. 2 C,D).

Resveratrol and Sirtinol does not alter the expression of SIRT1 at the Transcriptional Level

Analysis of the SIRT1 mRNA levels performed by qPCR demonstrated that resveratrol and sirtinol treatments did not alter the SIRT1 mRNA expression on B16F10 cells in the studied concentrations. However, in Melan-A cells, resveratrol treatment increased SIRT1 mRNA expression ($p = 0.024$) (Fig. 3).

Discussion

Since increasing evidence shows that sirtuins play a role in many biological processes, there has been sustained interest in developing small molecules that can regulate sirtuins. First, we evaluate cell viability and performed DNA fragmentation analysis on B16F10 cells treated with different concentrations of resveratrol, in order to evaluate if resveratrol has anti-tumor effects in these cell lines. The DNA fragmentation can be considered indicative of cell death [44].Consistent with our hypothesis there is also a report which resveratrol is capable of binding to DNA of cells and in the presence of Cu^{2+} to cleave DNA [45]. Thus, we suggest that the arrested replication machinery with stalled replication forks provides the primary

signal which in certain cell types results in apoptosis induction. Consistent with our studies, Nile and colleagues demonstrated that human melanoma cells are susceptible to resveratrol-induced proliferation inhibition. A major portion of the decreased growth appears to be due to an induction of apoptosis [46]. In other study, resveratrol decreased the viability of cell melanoma, induced cell cycle arrest and inhibited the proliferation of human melanoma cells [47].

Molecular screening of SIRT1 activators led to the identification of plant polyphenols as SIRT1 activators, among which is resveratrol [48]. Upregulation of SIRT1 mRNA expression was observed human umbilical vein endothelial cells (HUVECs) upon resveratrol treatment [49]. Many of the health-promoting effects of resveratrol have been shown to be mediated by the activation of SIRT1 [50]. Resveratrol shift the physiology of middle-aged mice on high calorie diet, promote changes associated with longer lifespan, and protect mice against diet-induced obesity and insulin resistance [51]. In this study, there was no detectable increase in SIRT1 protein expression on resveratrol-treated mice. However, the SIRT1 enzymatic activity, evaluated according to the acetylation status of target proteins, was enhanced by resveratrol [51]. Despite these findings, which appear to be consistent *in vitro* and *in vivo* results, the notion of resveratrol as a SIRT1 activator has been questioned in recent studies [52, 53]. Beher et al demonstrated that resveratrol is not a direct activator of SIRT1 enzyme activity and suggests its pharmacological action via AMPK activation in a SIRT1-independent manner [53]. It has been reported that AMPK enhances SIRT1 activity indirectly by increasing cellular NAD+ levels [54]. Our findings demonstrated that there was no change in mRNA SIRT1 expression after treatment with resveratrol on low concentrations. Based on these observations, aiming a better understanding of the effect of resveratrol on SIRT1 on melanoma cells, we see the need to evaluate the SIRT1 expressions in levels of mRNA and protein and its enzymatic activity. Furthermore, the SIRT1 mRNA expression on melanoma cells should be evaluated after resveratrol administrations in higher concentrations.

First described in 2001, sirtinol was found to inhibit sirtuins transcriptional activity directly without affecting the other classes of histone deacetylases [55].We used sirtinol, known a specific inhibitor of sirtuins, to check their effects on SIRT1 inhibition and viability of melanoma cells. Sirtinol significantly reduced the cell viability in a concentration-dependent manner, although the SIRT1 expression has not been significantly reduced on studied concentrations. Additional data has corroborated our results for cytotoxicity of sirtinol [21, 56]. Wilking et al, 2014 reported what human melanoma cells after treatment with sirtinol resulted in inhibition of cellular growth, viability, and/or metabolism [57]. Peck et al

demonstrated that the cytotoxic effect of sirtinol on breast carcinoma cell lines is mediated predominantly through the inactivation of both SIRT1 and SIRT2 and the subsequent acetylation of the tumor suppressor p53, and consequent increase of p53 transcription-dependent cell cycle arrest and viability cell decrease [58]. A possible mechanism to explain the cytotoxic effect of sirtinol in melanoma cells could be the inhibition of SIRT1protein translocation, in addition to inhibition of SIRT1 activity [59]. Particularly on melanoma cell lines, Ohanna et al, 2014 showed that different cell lines overexpressing the SIRT1 protein, however the transcription of SIRT1 mRNA after sirtinol treatment was not assessed [21, 56]. Our results of qRT-PCR showed what the SIRT1 mRNA expression was not affected by sirtinol treatment on studied concentrations. One possible explanation is that, the drug act in catalytic activity of the SIRT1 enzyme and not the mRNA molecule [59]. Another explanation is that sirtinol concentrations may not have been enough to alter the levels of SIRT1 primary transcript. More studies are needed to validate the use of the resveratrol and sirtinol as modulators of SIRT1on melanoma cells, including assessment of the levels of transcripts and protein.

In conclusion, this study demonstrates that resveratrol and sirtinol can affect cell viability of B16F10 cells in the absence of SIRT1 mRNA expression change.

Declaration of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

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LEGEND OF FIGURES

Figure 1 - Assessment of the B16F10 cell viability induced by resveratrol. (A) The cells were treated with resveratrol at various concentrations (1.56 – 200 μM) for 48 h. (B) The cells were treated with sirtinol at various concentrations (60–200 μM) for 48 h. (C) Assessment of the Melan-A cell viability induced by resveratrol. The cells were treated at various concentrations (20–100 μM) for 48 h. (D) The cells Melan-A were treated at various concentrations of sirtinol (20–100 μM) for 48h. Viable cells were detected by MTT assay and viability was determined as the ratio between treated cells and untreated controls. The data are represented as the means ± SEM from three independent experiments.

Figure 2: Effect of resveratrol and sirtinol on DNA fragmentation of cells. Cells B16F10 were treated with increasing concentrations of resveratrol (A) and Sirtinol (B) for 48 h, respectively. Cells Melan-A were treated with increasing concentrations of resveratrol (C) and Sirtinol (D) for 48 h, respectively. Cells were fixed, stained and analyzed for DNA content. Data are presented as the means ± SD (n=3). *P<0.005 and ***P<0.001 between control and treated cells.

Figure 3: Analyses of mRNA expression of SIRT1 in B16F10 and Melan-A cells using qPCR method. *$p < 0.05$.

Tables

Table 1. Oligonucleotide sequences for RT-PCR.

Primer	Primer Sequence
SIRT1	F: 5’ GTG TTG GTG GCA ACT CTG AT 3’
	R: 5’ CCT TGG AGA CTG CGA TGT TA 3’
B- ACTIN	F: 5’ GCA AGT TCA ACG GCA CAG 3’
	R: 5’ CGC CAG TAG ACT CCA CGA 3’

Figures

Figure 1

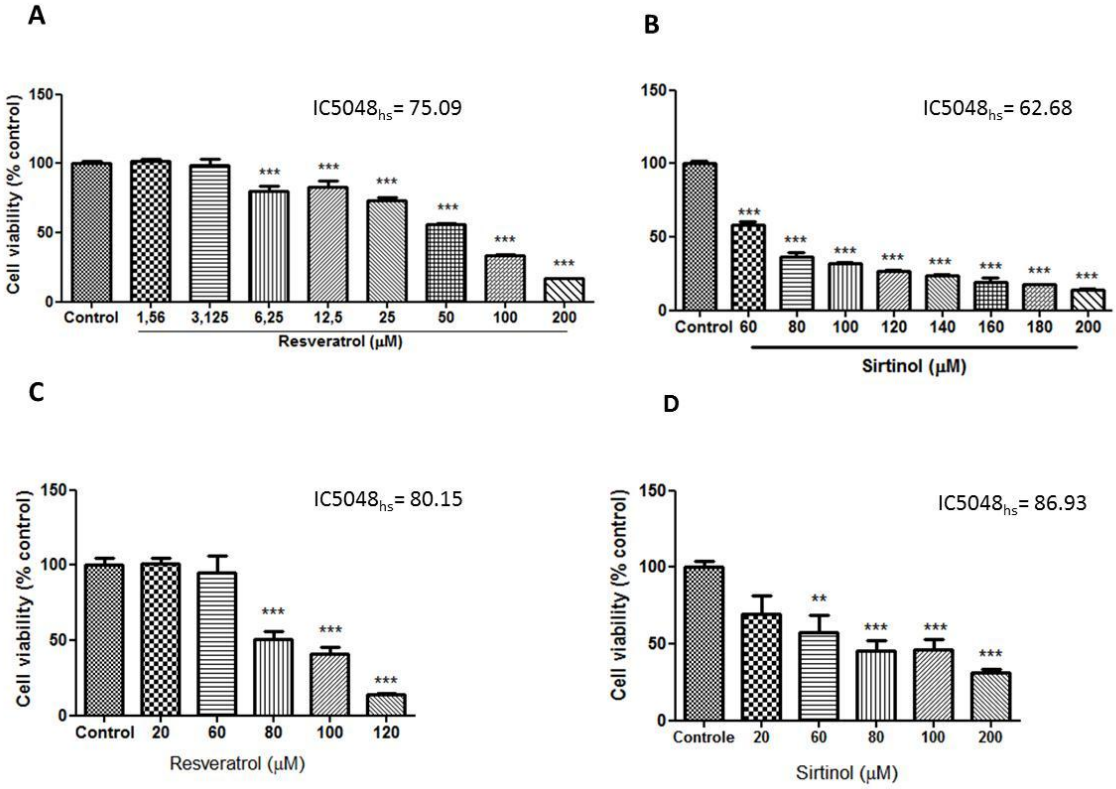


Figure 2

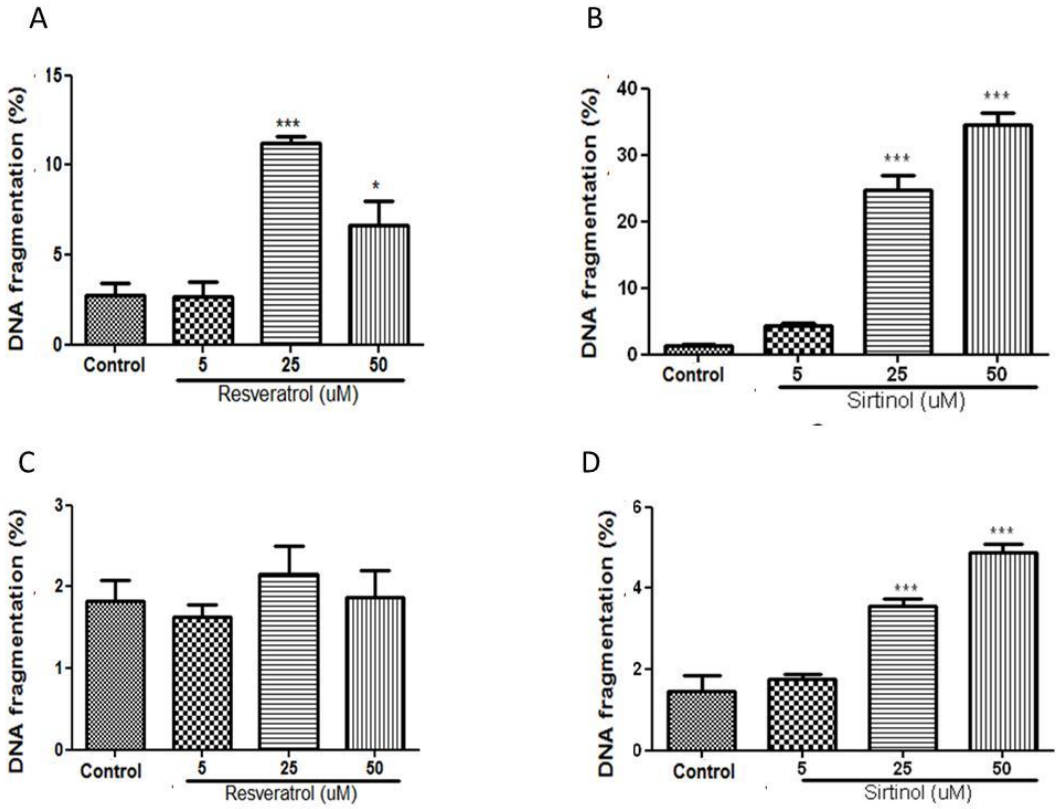
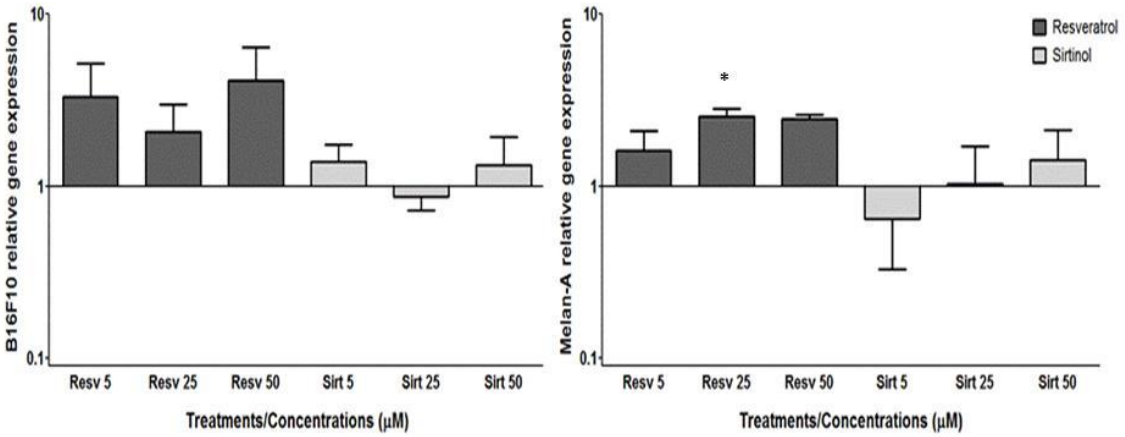

A
DNA fragmentation (%)
15
10
5
0
***
*
Control
5
25
50
Resveratrol (uM)
B
DNA fragmentation (%)
40
30
20
10
0
***
***
Control
5
25
50
Sirtinol (uM)
C
DNA fragmentation (%)
3
2
1
0
Control
5
25
50
Resveratrol (uM)
D
DNA fragmentation (%)
6
4
2
0
***
***
Control
5
25
50
Sirtinol (uM)

Figure 3


B16F10 relative gene expression
10
1
0.1
Resv 5
Resv 25
Resv 50
Sirt 5
Sirt 25
Sirt 50
Treatments/Concentrations (μM)
Melan-A relative gene expression
10
1
0.1
*
Resveratrol
Sirtinol
Resv 5
Resv 25
Resv 50
Sirt 5
Sirt 25
Sirt 50
Treatments/Concentrations (μM)

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3.3 PRODUTO

Analysis of immunohistochemical expression of PECAM-1 and endoglin in normal skin, benign melanocytic nevi, and cutaneous malignant melanoma

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ABSTRACT

We analyzed expression of PECAM-1 and endoglin for a correlation with clinicopathological behavior in CMM. Control (n = 12), CMN (n = 48), and CMM (n = 44) samples were submitted for immunohistochemistry. PECAM-1 and endoglin expression were counted in the stroma (*hot spots*) of all samples in order to calculate the MVD. Data analyses were performed using univariate statistical tests, with significance set at $p<0.05$. Our findings showed that CMM exhibited higher MVD estimates for both PECAM-1 and endoglin compared to control and CMN samples ($p<0.001$, for all associations). Moreover, CMN samples exhibited higher MVD compared to control samples ($p<0.001$ for all associations). CMM from subjects with metastatic disease showed higher MVD by PECAM-1 ($p=0.036$) and endoglin ($p=0.015$) compared to non-metastatic CMM. In conclusion, increasing MVD from normal skin to benign and malignant melanocytic tumors suggests the importance of a rich vascular network in the peritumoral stroma to support greater metabolic and energetic demands, which favors the dissemination of melanocytic tumor cells.

Keywords: Cutaneous melanoma, benign melanocytic nevi, microvascular density, immunohistochemistry, PECAM-1, endoglin, metastasis.

Introduction

Cutaneous malignant melanoma (CMM) represents a relevant public health issue in many countries worldwide. Although CMM represent less than 10% of all skin cancers, it is responsible for over 90% of deaths associated to these types of cancer. Currently, CMM patients with metastatic disease exhibit a 5-year overall survival of about 5-10% [1, 2].

During early stages of CMM progression, endogenous factors, such as pale-skin, red or blonde hair, occurrence of many freckles on the upper back, use of oral contraceptives for ≥ 5 years, a previous history of blistering sunburns and actinic keratosis, a number of cutaneous melanocytic nevi, and family history of melanoma, and exogenous factors such as intermittent high intensity of ultraviolet radiation exposure, promote a series of genetic and epigenetic disturbances that affects cell differentiation, proliferation, and cell death control [3, 4]. These disturbances might promote the development of benign cutaneous melanocytic nevi (CMN), the common or dysplastic subtypes, which might progress to CMM eventually. However, CMM also can directly arise from a normal melanocyte without necessarily going through the benign neoplasm phase [5, 6]. Typically, CMM cells exhibit a highly efficient, precocious metastatic capacity to invade local and distant organs. In early stages, CMM is morphologically characterized by initial radial growth phase, with a typical spreading of melanoma cells in the superficial layers of the skin. Later, disseminating melanoma cells promote the development of vertical growth phase of the malignancy, morphologically characterized as invasion of disseminating melanoma cells into the peritumoral stroma. Invading CMM cells use both lymphatic and haematogenous vascular networks to disseminate to local or distant organs during the metastatic process [7, 8].

Tumor angiogenesis involves a series of molecular disturbances that promote extracellular matrix degradation, recruitment of circulating endothelial progenitor cells, survival, migration and proliferation of endothelial cells (ECs), and ultimately, the formation of neovasculature from pre-existing blood vessels. This complex process ensures the supply of nutrients and oxygen to metabolically active cancer cells, removal of catabolic wastes from cancer cells and the tumor microenvironment, and provides a hematogenous route for disseminating cancer cells [9-11]. Tumor angiogenesis results in increasing activity of ECs induced by a spectrum of angiogenic signaling factors. Endoglin (CD105) is a proliferation-associated and hypoxia-inducible transmembrane phosphorylated glycoprotein component of the receptor complex of transforming growth factor-beta (TGF-β). Notably, endoglin is preferentially expressed in active, proliferative ECs and has a pivotal role for vascular development. Endoglin promotes migration and endothelial cell turnover by stimulating the

TGF-β/ALK-1/Smad5 phosphorylation pathway while inhibiting the TGF-β/ALK-5/Smad2-3 signaling pathway [12-14]. Platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31), is a vascular-associated adhesion and signaling membrane glycoprotein expressed on filopodia of leukocytes, platelets, and ECs. PECAM-1 plays important roles in regulation of leukocyte trans-endothelial migration and motility and adhesion mechanisms of ECs [15-18].

The quantification of tumor microvessel density (MVD) is a good indicator of tumor angiogenesis and the total vascular network of a malignancy. Additionally, MVD plays a pivotal role in tumor progression, as well as, acting as a reliable prognostic indicator for human solid cancers [19-21]. However, it is important to highlight that, due to diversity of immunomarkers used to quantify MVD and lack of standardization on the method, the association between MVD and progression of cutaneous melanocytic neoplasms [22-24], as well as, their influence on metastasis of melanoma cells still remains inconclusive [25-28].

In this study, we aimed to analyze the MVD, as determined by both endoglin and PECAM-1 expression, in the stroma of normal skin, CMN, and CMM human samples. Moreover, we investigated if MVD is associated with sociodemographic and clinicopathological factors related to CMM patients.

Material and Methods

Ethical aspects

Ethical approval for this study was obtained from a relevant local ethic committee (Committee on ethic in research - Unimontes: protocol no: 691.408/2014).

Samples

This retrospective, cross-sectional study was performed on archived tissue blocks of normal human skin (n = 12 women, mean age: 37.2 ± 9.4 years old) and resected primary CMN (n = 48, mean age: 33.8 ± 11.8 years old, male:female ratio: 1:2.7, white skin color: 53.8%), and CMM (n = 44, mean age: 55 ± 14.9 years old, male:female ratio: 1:1.93, white skin color: 87.5%), with confirmed histopathological diagnosis. Sociodemographic and clinicopathological data were obtained from clinical charts from patients attended at public health centers for Oncology treatment at Montes Claros city, Minas Gerais state, Brazil.

Clinicopathological analyses

Samples of sunlight-exposed (n = 1) and non-exposed (n = 11) normal skin from 12 healthy individuals were used as controls. These samples were obtained from patients who underwent esthetic or corrective surgical procedures. Clinically, CMN were acquired melanocytic nevi exclusively and were found in both sunlight-non-exposed (n = 17) and sunlight-exposed (n = 31) cutaneous sites. All CMM were classified according to the *American Joint Committee on Cancer* (AJCC) melanoma staging [29]. TNM clinical staging IA-IB and IIA-IIB-IIC (localized melanoma) was observed in 9 CMM samples (20.5%), while stages III and IV (regional and distant metastatic disease, respectively) were noted in 35 (79.5%) of CMM patients. All CMM samples were obtained from patients prior to the initiation of most common anticancer therapies (surgical resection, radiotherapy, and chemotherapy). CMM presented the clinical forms as follow: superficial spreading (n = 18, 40.9%), nodular (n = 7, 15.9%), lentigo malignant (n = 10, 22.7%), and acral lentiginous melanomas (n = 9, 20.5%). Primary tumor ulceration was detected in 6 cases (13.6%) and recurrence was noted in 10 (14.3%) of CMM lesions. According to risk of death in CMM individuals related to cutaneous anatomical site where melanoma occurred, we classified CMM samples as *low-risk* (lower trunk, thigh, lower leg, foot, lower arms, hands, and face) and *high-risk sites* (back and breast/thorax, upper arm, neck, and scalp) [30-32]. According to primary tumor thickness, CMM samples were categorized as T1 (≤ 1 mm thickness, n = 13), T2 (1.01 to 2 mm thickness, n = 10), T3 (2.01 to 4 thickness, n = 13), and T4 (> 4 mm thickness, n = 8). Both distant and locoregional metastatic diseases were diagnosed in 29 (66%) of CMM patients. According to therapeutic approach, CMM patients underwent to surgery (n = 17, 38.7%), radiotherapy (n = 11, 25%), chemotherapy (n = 10, 22.7%), or therapeutic combination (n = 6, 13.6%).

Formalin fixed and paraffin embedded normal skin, CMN, and CMM tissues sample were submitted to histopathological analysis. Tissue sections were cut at a thickness of 3–5μm and stained with hematoxylin and eosin (H&E). Two experienced examiners (De-Paula, AMB and Santos, EP) morphologically reviewed all control, CNM, and CMM samples investigated in this study. Histopathological 5-μm-thick sections of archived formalin fixed-paraffin embedded samples were subjected to morphometrical analysis of tissue invasion by melanoma cells using Breslow's thickness [33] and Clark's level [34] criteria. According to Breslow's thickness grade, CMM samples were categorized as follows: TI (up to 0.75mm, n = 3, 6.8%), TII (from 0.75 to 1.5mm, n = 7, 15.9%), TIII (1.5 to 3mm, n = 27, 61.4%), and TIV

(3 to 4mm, n = 7, 15.9%). According to Clark's level (degree of invasion), CMM samples were categorized as follows: level I (intraepidermal and epithelium adnexal lesion, n = 3, 6.8%), level II (invasion up to the papillary dermis, n = 7, 15.9%), level III (invasion fills the entire reticular dermis, though without invading it, n = 18, 40.9%), level IV (invasion of the reticular dermis, n = 13, 29.5%), and level V (invasion of the hypodermis, n = 3, 6.8%).

Immunohistochemical reactions

PECAM-1 and endoglin expression were performed using immunohistochemical (IHC) method with streptavidin–biotin-peroxidase detection system. 4μm-thick CMN and CMM sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Sections were submitted to antigen retrieval combined with pressure cooking. Afterwards, endogenous peroxidase, biotin, and streptavidin were blocked by using specific reagents prior to incubation with each primary antibody (mouse monoclonal anti-PECAM-1, clone 1A10, 1:100; Novocastra, Newcastle, United Kingdom; and rabbit polyclonal anti-endoglin, 1:100; Abcam, Cambridge, United Kingdom), overnight at 4°C. The sections were thereafter incubated with LSAB^{TM}-Kit Plus Peroxidase$^{®}$ (DakoCytomation, Glostrup, Denmark) for 1h. Tissues were stained with a chromogen (3,3′-diaminobenzidine tetrahydrochloride, DAB), counterstained with Mayer's hematoxylin, cover slipped, and visualized under an optical microscope. Positive and negative controls were applied according to the manufacturer's instructions (DakoCytomation, Glostrup, Denmark).

Counting of immunostained microvessels and MVD estimates

All control, CMN, and CMM samples were morphologically evaluated by one independent observer without knowledge of the clinical factors (EP, Santos). Photomicrographs were taken at 100X and 400X magnification using an optical Olympus$^{®}$ BH2 microscope (model: CX31; RTSF, Miami, USA). The counts of immunostained cells for PECAM-1 and endoglin were manually performed using the Image J software, version 1.44 for Windows$^{®}$. For MVD analyses, all samples of each group investigated were initially inspected at microscopic magnification of 100X in order to identify microscopic fields containing the greatest number of distinctly immunostained microvessels in normal or peritumoral stroma (referred to as *hot spots*). Microvessels were noted as isolated stained endothelial cells or transversally sectioned vascular tubes with a single layer of endothelial cells, either with or without a thin basement membrane. If two or more positive foci appeared

to belong to a single continuous vessel, this was counted as only one microvessel. MVD estimated by PECAM-1 and endoglin expression were performed in each sample in three *hot spots*, at final 40X microscopic magnification [21, 35]. In each *hot spot* five microscopic areas were randomly selected for manual microvessel counting (total of 15 microscopic fields, with area of 2.69 mm^2). The MVD was then determined dividing the number of microvessels by the total microscopic area for each marker. MVD score of each sample was expressed as mean percentage of microvessels count/mm^2 and respective standard deviation (SD).

Statistical analyses

All data were transferred and statistically tested using SPSS® 18.0 software (SPSS Inc., Illinois, USA). Scores of immunohistochemical expression of PECAM-1 and endoglin were exhibited as mean percentage of protein expression and error bars represent standard deviation (± SD). Association between PECAM-1 and endoglin expression and control, CMN, and CMM groups were compared using analysis of variance (ANOVA) after post-hoc test with Bonferroni correction. Association between clinicopathological variables and MVD in CMM samples were compared using Student's t test. Differences between groups were considered as statistically significant when $p < 0.05$.

Results

PECAM-1 and endoglin immunohistochemical expression showed selective reactivity for isolated endothelial cells or immature/mature vascular structures located in normal or peritumoral stroma areas of skin, benign CMN, and CMM samples, respectively (Figure 1). PECAM-1 and endoglin expression was detected in controls (9.7 ± 1.4 and 5.5 ± 2.3, respectively), CMN (27.6 ± 15.8 and 15.8 ± 9.5, respectively), and CMM (44.7 ± 10.5 and 35.6 ± 12.6, respectively). Our findings showed CMM samples with higher PECAM-1 and endoglin expression compared to controls ($p < 0.001$ and $p < 0.001$, respectively) and CMN ($p < 0.001$ and $p < 0.001$, respectively) (Figure 2).

Table 1 shows the association between clinicopathological variables related to CMM and MVD analyses. A significant association was noted between the occurrence of metastatic disease (both local and distant) and higher MVD performed for PECAM-1 ($p = 0.036$) and endoglin ($p = 0.015$) (Figure 3).

Discussion

Dynamic and sequential transformations occur from potentially malignant melanocytic cutaneous lesion to melanoma in susceptible individuals. Among these transformations, the stroma acquires an active and emergent vascular network that plays pivotal roles for both CMM tumorigenesis and progression [22, 28, 36].

In this study, we investigate the MVD in normal, benign, and malignant melanocytic cutaneous lesion established by PECAM-1 and endoglin immunohistochemical expression. Notably, it has been reported the use of several markers for MVD analysis in benign and malignant melanocytic cutaneous lesions. In this study, we evaluated PECAM-1 once it is considered a reliable marker of endothelial cells of mature blood vessels [37, 38] while endoglin is predominantly expressed on normal or tumoral proliferating endothelial [12-15]. According to our findings, we noted a significant, gradual increase of MVD from control to CMN to CMM samples. Additionally, our findings showed that CMM samples of individuals with metastatic disease exhibited a higher MVD. It has been reported that melanoma cells with high catabolic/metabolic and energetic demands need to induce a rich vascular network in the peritumoral stroma to sustain tumor progression [23, 26]. Notably, CMM exhibits a rapid progression towards widespread metastatic dissemination even when the primary tumor presents with small clinical size or microscopic thickness [33, 39, 40]. Premature metastatic behavior from melanoma cells has been associated with the developmental origins of melanocytes (from neural crest cells that have remarkable migratory behavior) [41] and the presence of lymphatic and blood vascular networks in the stroma, which gradually increases with CMM progression [22]. Malignant melanoma cells release various angiogenic growth factors that promote tumor angiogenesis from early stages of CMM progression [24, 42, 43]. A higher MVD identified by endoglin expression in our CMM samples suggests that melanoma cells might contribute to stimulation of EC proliferative activity. In turn, infiltrative melanoma cells that engage in a vascular metastatic route are capable of disseminating and establishing metastatic niches in target organs. However, it is pivotal the investigation of some angiogenic growth factors in our samples in order to strengthen such molecular mechanisms.

The association between high MVD expression and metastatic CMM has been noted in some studies [25, 27, 44, 45] but not in others [46, 47]. These controversial findings are related to some factors such as the diversity of angiogenic antigens (e.g. factor VIII-related antigen, vascular endothelial growth factors, kinase insert domain receptor, ulex europaeus

lectin-1, PECAM-1, CD34, endoglin, collagens types IV and XVIII, laminin, and neuropilin-1) that have been used to detect blood vessels and quantify the MVD of various human solid malignancies, including CMM. Moreover, the vascular density analysis performed with non-standardized microscopic assessment might contribute to the conflicting findings between MVD neoangiogenesis and metastasis, along with other clinicopathological factors [35]. Recent studies have investigated the presence of other vascular abnormalities that also result in a gain of vascularity for CMM, such as augmentation of the angiogenic response by recruitment of circulating progenitor ECs [48], vessel cooption [49], and vasculogenic mimicry [50].

Our study has some limitations that should be highlighted. Initially, the sample size of each group investigated is not large. Moreover, its cross-sectional design results in difficulty in establishing causal inferences. Additionally, as different anticancer therapeutical modalities might influence on tumor vascular pathology of CMM, further studies with a higher casuistic and higher follow-up periods will be necessary to clarify the importance of that association and its impact on metastasis occurrence and overall survival of CMM patients. On the other hand, our findings add more evidence that MVD identified by immunostaining of PECAM-1 and endoglin might constitute a potential target in the selection of high-risk CMM patients for complementary antiangiogenic therapeutic strategies. However, further studies are necessary to clarify these possibilities.

In conclusion, our findings suggest that higher MVD from normal skin to benign and malignant melanocytic tumors play important roles in supporting the growth of melanocytic neoplastic cells and favoring the dissemination of infiltrating melanoma cells.

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LEGEND OF FIGURES

Figure 1. Morphological aspects of normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples (Figures A, B, and C, respectively. H&E staining; higher magnification of 400x). Immunohistochemical expression of PECAM-1 (Figures D, E, and F) and endoglin (Figures G, H, and I) proteins in samples of control, CMN, and CMM (immunostaining: DAB; counterstaining: Mayer's hematoxylin; higher magnification of 400X).

Figure 2. PECAM-1 and endoglin expression in normal skin (control), cutaneous benign melanocytic nevi (CMN), and malignant melanoma (CMM) samples. Our findings showed a significant increase of microvsascular density from control to CMN and CMM samples. Scores of immunohistochemical expression of PECAM-1 and endoglin exhibited as mean percentage of protein expression and error bars represent standard deviation. Statistical analysiss was performed using ANOVA, with level of significance set at $\alpha = 5\%$ ($p < 0.05$).

Figure 3. PECAM-1 and endoglin expressions in resected primary metastatic and non-metastatic CMM samples. Our findings showed a significant increase of mean percentage expression of PECAM-1 and endoglin in resected CMM lesions of individuals with metastatic disease. Scores of immunohistochemical expression of PECAM-1 and endoglin exhibited as mean percentage of protein expression and error bars represent standard deviation. Statistical analysis was performed using Student´s t test, with level of significance set at $\alpha = 5\%$ ($p < 0.05$).

Tables

Table 1**.** Analysis between the microvessel density determined by both PECAM-1 and endoglin expression and clinicopathological factors related to CMM.

Variables	Microvascular Density			
	PECAM-1 (mean ±SD)	**p**	**Endoglin (mean ±SD)**	**p**
Anatomical site				
Low risk (n = 28)	45.4 (± 11.9)	0.586	34.8 (± 14.4)	0.594
High risk (n = 16)	43.5 (± 7.8)		37.0 (± 8.7)	
Clinical size				
Small (n = 9)	42.8 (± 9.0)	0.516	31.1 (± 13.3)	0.197
Large (n = 35)	45.3 (± 11.0)		36.9 (± 12.2)	
Ulceration				
Absent (n = 38)	45.2 (± 11.1)	0.477	35.4 (± 13.3)	0.775
Present (n = 6)	41.8 (± 5.5)		37.0 (± 6.4)	
Recurrence				
Absent (n = 10)	40.1 (± 7.1)	0.113	32.2 (± 9.5)	0.341
Present (n = 34)	46.1 (± 11.0)		36.6 (± 13.3)	
Level of invasion				
I/II/III (n = 28)	45.2 (± 10.5)	0.687	35.6 (± 13.4)	0.986
IV-V (n = 16)	43.8 (± 10.2)		35.6 (± 11.3)	
Tumor thickness				
< 2 mm (n = 23)	42.7 (± 8.1)	0.183	33.0 (± 11.2)	0.147
≥ 2 mm (n = 21)	46.9 (± 12.5)		38.5 (± 13.5)	

Figures

Figure 1

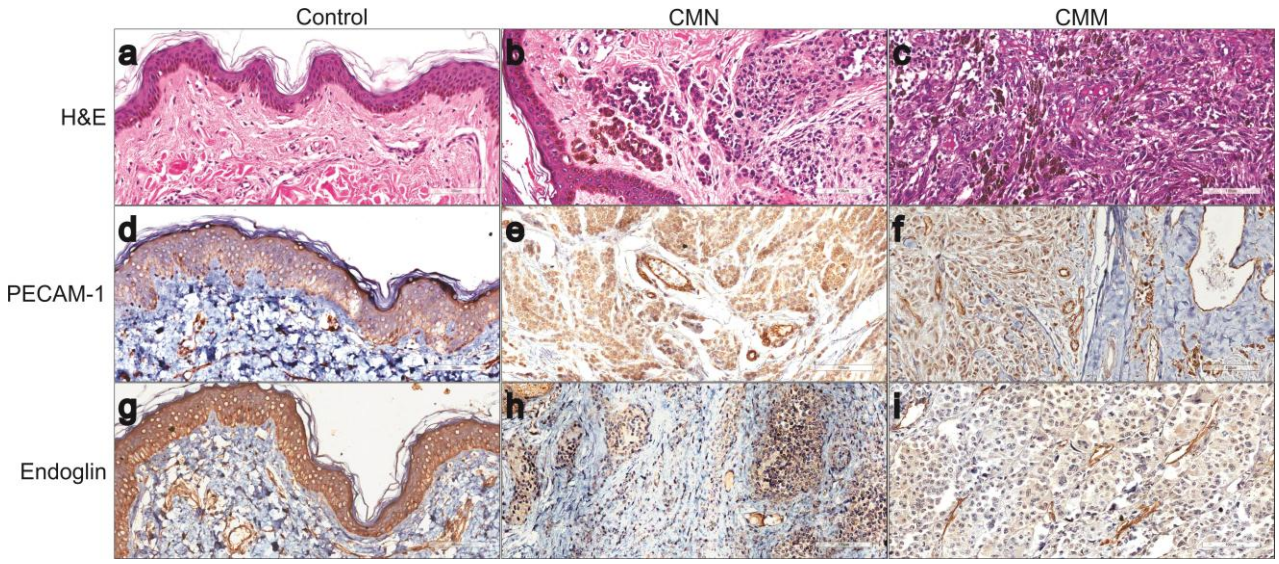


Figure 2

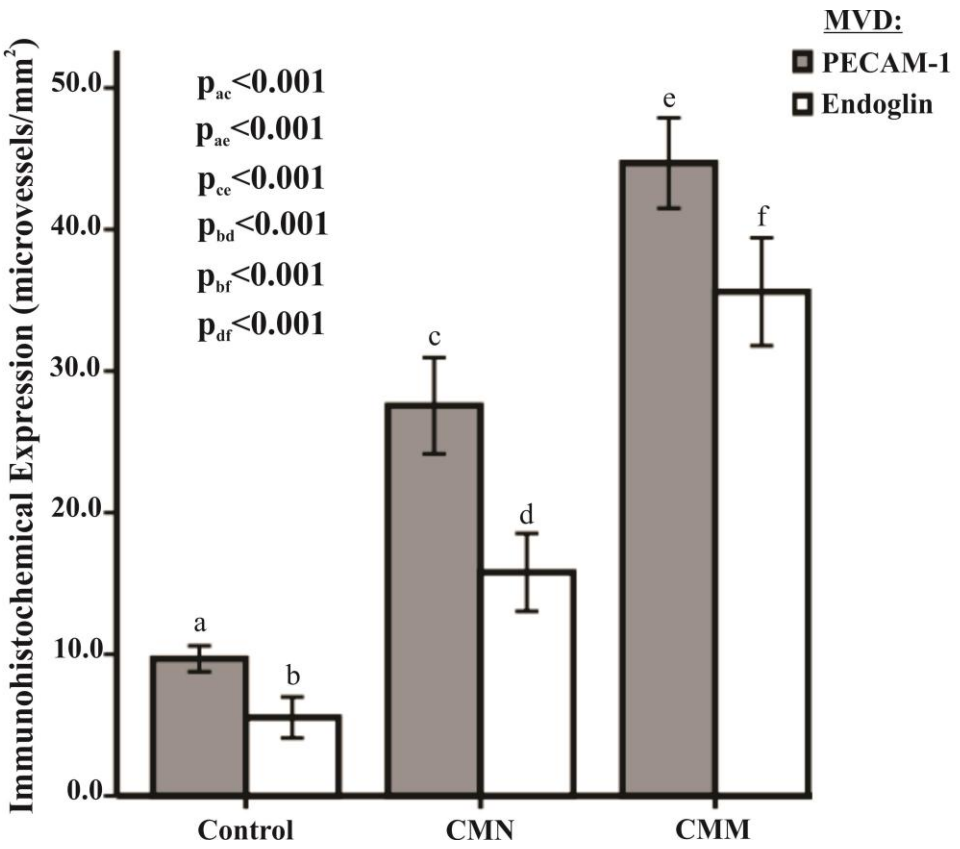
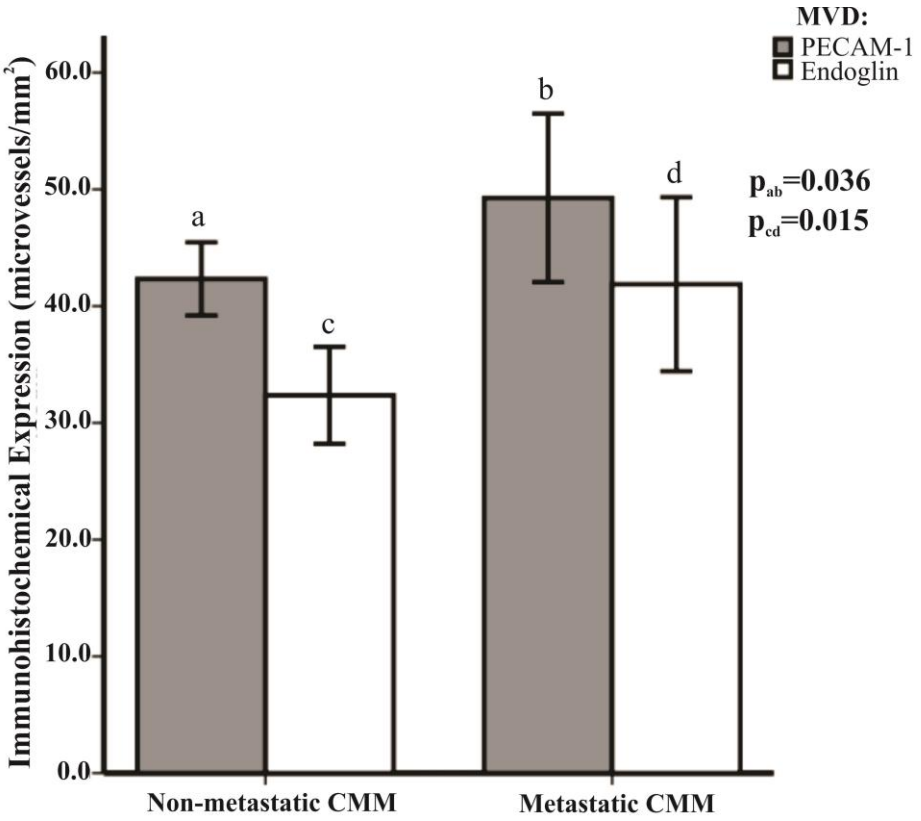

MVD:
PECAM-1
Endoglin
p_{ac}<0.001
p_{ae}<0.001
p_{ce}<0.001
p_{bd}<0.001
p_{bf}<0.001
p_{df}<0.001
Immunohistochemical Expression (microvessels/mm^2)
50.0
40.0
30.0
20.0
10.0
0.0
a
b
c
d
e
f
Control
CMN
CMM

Figure 3


MVD:
PECAM-1
Endoglin
Immunohistochemical Expression (microvessels/mm²)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
b
c
d
p_ab=0.036
p_cd=0.015
Non-metastatic CMM
Metastatic CMM

4 CONCLUSÕES E/ OU CONSIDERAÇÕES FINAIS

Os resultados desse estudo demonstram que resveratrol e sirtinol afetam a viabilidade das células B16F10 sem provocar alterações na expressão do RNA mensageiro da SIRT1. Há necessidade de mais estudos para conhecer a ação dessas drogas como possíveis alvos terapêuticos no melanoma maligno cutâneo. Também foi possível observar uma diminuição da expressão de SIRT1 no melanoma maligno cutâneo quando comparado com o nevo melanocítico benigno. As alterações quanto à expressão de SIRT1 podem revelar um comportamento diferenciado dessa enzima na progressão do melanoma maligno cutâneo e fornecer conhecimento a cerca de seu papel no câncer. Os resultados desse estudo sugerem que Ki67 pode ser usado para auxiliar no diagnóstico de lesões benignas e malignas cutâneas. Além disso, alta densidade microvascular no melanoma maligno cutâneo quando comparada com o nevo melanocítico benigno suportam o crescimento e disseminação das células neoplásicas e predizem sobre o comportamento clínico da lesão.

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ANEXO

ANEXO A – Parecer do Comitê de Ética e Pesquisa

FACULDADES INTEGRADAS PITÁGORAS DE MONTES CLAROS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise da expressão de sirtuínas 1 e 2, S100A4 e genes envolvidos no processo metastático em melanoma cutâneo humano

Pesquisador: Marcos Vinícius Macedo de Oliveira

Área Temática:

Versão: 2

CAAE: 31086114.6.0000.5109

Instituição Proponente: Faculdades Integradas Pitágoras de Montes Claros

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 714.865

Data da Relatoria: 10/07/2014

Apresentação do Projeto:

Trata-se de investigação científica para análise de manifestações genotípicas (SIRT1 e SIRT2, S100A4, ANXA2, MMP9, CD31 e CD105, e Ki67) presentes em melanoma cutâneo humano, nevo melanocítico e melanócitos; cuja expressão imunohistoquímica será avaliada em relação a parâmetros clinicomorfológicos e epidemiológicos dos nevos melanocíticos e melanomas cutâneos, e de amostras de pele normal cedidas a partir de cirurgias plásticas. Será desenvolvido um estudo "in vitro", com linhagens celulares humanas de melanoma metastático e não metastático, nevo melanocítico e melanócitos primários.

Objetivo da Pesquisa:

Correlacionar a expressão de genes e proteínas com o desenvolvimento tumoral, podendo revelar achados importantes sobre o comportamento clínico e biológico da doença. Espera-se que o estudo desses genes e proteínas envolvidos na progressão tumoral possa melhorar o entendimento do processo de desenvolvimento neoplásico e aspectos clínicopatológicos do melanoma cutâneo e,destarte, contribuir para terapias mais eficazes, menos invasivas e onerosas, resultando em prevenção e/ou regressão tumoral; além do desenvolvimento de possíveis marcadores de prognóstico.

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