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Meta-análise para identificação de alterações na expressão de microRNAs e vias moleculares do desenvolvimento vascular reguladas por microRNAs em Angiossarcoma

Tese apresentada ao Programa de Pós-Graduação em Bases Gerais da Cirurgia da Faculdade de Medicina de Botucatu UNESP, para obtenção do título de Doutor.

Orientador: Prof. Dr. Titular Winston Bonetti Yoshida
Co-orientadora: Profa. Dra. Patrícia Pintor dos Reis
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Palavras-chave: Angiossarcoma; MicroRNAs; Tumor vascular; Tumorigênese; Vias moleculares.

“A única maneira de fazer um bom trabalho é amando o que você faz. Se você ainda não encontrou, continue procurando. Não se desespere. Assim como no amor, você saberá quando tiver encontrado.”

Steve Jobs

DEDICATÓRIA

À Deus,
pelo dom da vida que é
a maior viagem de todas.

Aos meus pais,
que tanto se dedicaram
e me fizeram a mulher que sou hoje.

Ao meu marido,
pela paciência infinita
pelas palavras de incentivo nas horas de fraqueza
e pelo amor incondicional .

Aos meus filhos Pedro e Gabriel,
que trouxeram outro sentido a minha vida
e a tornaram mais completa.

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SUMÁRIO

RESUMO:

INTRODUÇÃO: O Angiossarcoma (AS) é um tumor vascular maligno raro. As vias moleculares associadas ao desenvolvimento e progressão do AS ainda são pouco entendidas. miRNAs são moléculas reguladoras da expressão gênica com papel importante na tumorigênese e constituem biomarcadores em potencial, podendo definir prognóstico e tratamento de pacientes com câncer. A identificação de perfis de expressão de miRNAs e das vias moleculares reguladas por miRNAs pode contribuir para a elucidação dos mecanismos de tumorigênese em AS. **OBJETIVOS:** Identificação da expressão global de miRNAs e vias moleculares em AS. Identificar miRNAs alterados em AS; identificar genes-alvo regulados pelos miRNAs e mapear miRNAs e genes alvo relacionados ao desenvolvimento vascular. **MATERIAL E MÉTODOS:** Realizamos uma meta-análise segundo a Declaração de Prisma e utilizando as principais bases de dados, PubMed e EMBASE. Após a aplicação de critérios de inclusão e exclusão específicos, um estudo (incluindo 5 amostras de AS) foi considerado elegível e selecionado para extração dos dados. Deste, foram identificados os miRNAs significativamente desregulados (FC>=1,5 e p<0,05). A seguir, os dados de expressão de miRNAs foram analisados utilizando as ferramentas de bioinformática miRWalk v.2.0 para predição de genes-alvo regulados pelos miRNAs e STRING e Cytoscape v.3.1.1/BINGO para identificação de redes de interação (miRNAs-mRNAs-alvo) e funções biológicas, respectivamente. **RESULTADOS:** 59 miRNAs estavam com expressão significativamente aumentada (FC>=1,5 e p<0,05) em AS. Destes, 21 miRNAs interagem com 28 genes-alvo enriquecidos para funções associadas ao desenvolvimento vascular. Os genes-alvo identificados têm papel fundamental na tumorigênese, pois regulam o crescimento celular e estão envolvidos em mecanismos de invasão, metástase e resposta a quimioterápicos. **CONCLUSÕES:** Os miRNAs identificados, em particular miR-1323, miR-520h, miR-1283 e miR-144-5p regulam genes envolvidos em desenvolvimento vascular e mecanismos de tumorigênese. Estudos como este contribuem para o melhor entendimento do desenvolvimento e progressão do AS, bem como para a identificação de novos biomarcadores e tratamentos mais precisos, impactando a sobrevida dos pacientes.

ABSTRACT:

INTRODUCTION: Angiosarcoma (AS) is a rare malignant vascular tumor. The molecular pathways associated with AS development and progression are still poorly understood. MiRNAs are regulatory molecules of gene expression with important role in tumorigenesis and are potential biomarkers, which can define the prognosis and treatment of cancer patients. The identification of expression profiles of miRNAs and molecular pathways regulated by miRNAs may contribute to the elucidation of the mechanisms of tumorigenesis in AS. **OBJECTIVES:** Identificação da expressão global de miRNAs e vias moleculares em AS. Identificar miRNAs alterados em AS; identificar genes-alvo regulados pelos miRNAs e mapear miRNAs e genes alvo relacionados ao desenvolvimento vascular. **MATERIAL AND METHODS:** We performed a meta-analysis following the Prisma Statement and using the main databases PubMed and EMBASE. Following the application of specific inclusion and exclusion criteria, one study (including 5 AS samples) was eligible and selected for data extraction and analysis. Of this study, we identified significantly deregulated miRNAs (FC>=1.5 and p<0,05). Further, miRNA expression data was analyzed using the bioinformatic tools miRWalk v.2.0 for target gene prediction and STRING and Cytoscape v.3.1.1/BINGO for identification of miRNA-mRNA networks and biological functions, respectively. **RESULTS:** 59 miRNAs were significantly over-expressed (FC>=1,5 and p<0,05) in AS. Of these, 21 miRNAs interact with 28 target genes with functions enriched for vascular development. Target genes identified have an important role in tumorigenesis, since they regulate cell growth and are involved in mechanisms of invasion, metastasis and chemotherapy response. **CONCLUSIONS:** miRNAs identified herein, in particular miR-1323, miR-520h, miR-1283 and miR-144-5p regulate genes involved in vascular development and tumorigenesis mechanisms. Studies such as this contribute to better understand the development and progression of AS, as well as to identify novel biomarkers and more precise treatment strategies, impacting patient survival.

PALAVRAS-CHAVE: Angiossarcoma, tumor vascular, microRNA, mRNA, vias moleculares, tumorigênese, tratamento, meta-análise.

KEY-WORDS: Angiossarcoma, vascular tumor, microRNA, mRNA, molecular pathways, tumorigenesis, treatment, meta-analysis.

INTRODUÇÃO

Os tumores vasculares podem ser classificados, segundo a *International Society for the Study of Vascular Anomalies* (ISSVA), em benignos, intermediários e malignos (Tabela1)[1]. O Angiossarcoma (AS) é um tumor vascular maligno derivado de células endoteliais vasculares e linfáticas[2]. Pode ser classificado como visceral e periférico e seu comportamento é agressivo independe do local acometido[2, 3]. Pode acometer a cabeça e o pescoço (27%), mamas (19,7%), extremidades (15%), tronco (9,5%), fígado (6%), coração (4,7%), ossos (3,6%), baço (2,6%) e outros sítios (11,6%)[2]. O AS periférico representa um raro subgrupo dos sarcomas de partes moles (menos de 2%) caracterizado por um comportamento agressivo[3, 4]. Possui um prognóstico ruim, com sobrevida livre de doença em 5 anos de 35% nos casos não metastáticos e uma taxa de recidiva acima de 75% nos 2 primeiros anos após o tratamento[5, 6]. Ocorre igualmente em ambos os sexos, podendo aparecer em qualquer faixa etária, sendo mais comum em idosos[2].

TABELA 1-2014 ISSVA - Classificação das Anomalias Vasculares	
Tumores Vasculares Benignos	Hemangioma infantil Hemangioma congênito Hemangioma em tufo Hemangioma de células fusiformes Hemangioma epitelióide Granuloma piogênico Outros
Tumores Vasculares Intermediários ou de Agressividade Local	Hemangioendotelioma Kaposiforme Hemangioendotelioma Retiniforme Angioendotelioma papilar intralinfático Hemangioendotelioma composto Sarcoma de Kaposi Outros
Tumores Vasculares Malignos	Angiossarcoma Hemangioendotelioma epitelióide Outros

A maior parte dos casos de AS ocorre espontaneamente, porém há alguns relatos de lesões vasculares benignas com transformação maligna[2]. Apesar de raro, lesões benignas como hemangiomas que mudam o comportamento clínico e aumentam repentinamente de tamanho devem ser investigadas devido à possibilidade

de transformação maligna[7]. O Linfedema crônico, seja de qualquer origem, está associado à maior incidência de AS[2]. A Radioterapia também pode aumentar a freqüência deste tumor, com pico de incidência de 5 a 10 anos após o tratamento[2]. Há um aumento da incidência de transformação maligna de lesões vasculares benignas previamente irradiadas[2, 7].

O AS de partes moles se caracteriza por massas multinodulares, hemorrágicas com áreas císticas e necrose[8]. Este tumor pode apresentar vários padrões morfológicos, indo desde áreas de vasos bem formados a áreas aonde não se consegue definir um padrão vascular[8]. Possui áreas sólidas compostas de células epitelióides e fusiformes de alto grau, com citoplasma levemente eosinofílico, núcleos grandes e nucléolos proeminentes[8, 9]. São neoplasias de alto grau, com atividade mitótica intensa, necrose e atipia nuclear significativa, normalmente apresentando extensas áreas hemorrágicas[8].

Estudos de análise imunohistoquímica em AS mostraram que essa neoplasia normalmente expressa marcadores endoteliais como: CD34, CD31, Fli1, fator VIII, Ulex europaeus agglutinin 1, ERG e ocasionalmente a podoplanina (D2-40) um marcador linfático[2, 8-10]. Os tumores também podem apresentar positividade para vimentina, actina e mioglobulina[9].

Devido ao AS constituir um tumor de origem endotelial, há um grande interesse no papel dos fatores de angiogênese na sua patogênese e como podem ser usados como marcadores e terapias-alvo[2]. Os principais promotores da angiogênese são: fator de crescimento endotelial vascular (VEGF) e fator de crescimento básico de fibroblastos (bFGF)[11]. O VEGF e seus subtipos podem estar com expressão aumentada no AS[2, 12]. Alterações na expressão do *TP53* foram relatadas, assim como superexpressão do *WT1* e Galectina-3[2, 13, 14]. Comparando com outros sarcomas, o AS apresenta

expressão aumentada de receptores vasculares específicos incluindo *TIE1*, *KDR*, *TEK* e *FLT1*[8]. Alterações dos genes *MYC* e *FLT4* são fortemente relacionadas ao AS[4, 8]. Os mecanismos de tumorigênese do AS ainda não estão completamente elucidados. Portanto, a realização de estudos moleculares que contribuam para o entendimento de tais mecanismos moleculares em AS são justificados, visto que podem levar ao desenvolvimento de terapêuticas mais precisas e efetivas nesta doença[15].

Os miRNAs são RNAs pequenos (19-24 nucleotídeos) não codificadores de proteínas e que regulam uma proporção significativa dos genes. Os miRNAs regulam genes que participam de um grande número de processos biológicos e são fundamentais no controle da homeostase celular, desenvolvimento, diferenciação, reprogramação celular, oncogênese, entre outros[16].

De acordo com pesquisa recente (data de acesso em 02 de Dezembro de 2016) na base de dados miRBase (http://www.mirbase.org/), 2.588 sequências de miRNAs maduros foram identificadas e anotadas no genoma humano. Alterações na expressão do miRNAs induzem fenótipos alterados, tais como o que ocorre doenças cardiovasculares e o câncer. O entendimento dos mecanismos de expressão e desregulação dos miRNAs e os genes regulados por miRNAs contribuirá para o desenvolvimento de estratégias de diagnóstico e tratamento de doenças complexas[16-18]. Nos últimos anos, evidenciou-se que a desregulação dos miRNAs está fortemente ligada a processos celulares associados a doenças crônico-degenerativas e ao câncer[19, 20].

Uma aplicação clínica importante da identificação de perfis de expressão de miRNAs constitui a potencial utilização desses perfis como biomarcadores com valor diagnóstico, prognóstico e preditivo no câncer e em outras doenças[21, 22].

Considerando que os miRNAs existem de forma estável em flúidos corporais como soro e plasma, muitos estudos têm explorado sua aplicação como biomarcadores circulantes, em várias condições fisiológicas e patológicas[23-28].

Estudos recentes mostraram que células tumorais liberam, na circulação sanguínea, uma quantidade aumentada de microvesículas, as quais podem conter miRNAs, com papéis importantes na sinalização celular e na tumorigênese[29, 30].

No endotélio vascular, miRNAs endoteliais estão presentes e controlam a resposta endotelial a estímulos angiogênicos, indicando o seu papel como moduladores da resposta angiogênica[31]. Em particular, o miR-126 foi descrito como regulador positivo da sinalização angiogênica e da integridade endotelial vascular. Alterações na expressão do miR-126 foram associadas a defeitos no desenvolvimento vascular, onde células deficientes em miR-126 não responderam a fatores angiogênicos como VEGF e bFGF[31]. Outros miRNAs, como o miR-221 e o miR-222, atuam como inibidores do fator estimulador de células-tronco associado ao processo de angiogênese. Alguns miRNAs desempenham um papel pró-angiogênico como foi demonstrado na inibição da expressão dos miRNAs miR-27b e o miR-let-7f reduzindo a angiogênese[31].

Igualmente, a hipóxia, um fenômeno associado à tumorigênese, pode levar à ativação da atividade angiogênica modulada por miRNAs. Recentemente, foi demonstrado que a atividade pró-angiogênica do miR-210 é estimulada por eventos de hipóxia celular ou tecidual[31].

A expressão aumentada de miRNAs pró-angiogênicos em células endoteliais pode alterar a produção de fatores angiogênicos, contribuindo com o processo de tumorigênese. Por outro lado, alguns miRNAs quando inibidos, como os miRNAs miR-

126 e miR-296, levam à redução da vascularização tumoral, apresentando ação antitumoral[31].

O entendimento de mecanismos genéticos e epigenéticos, como alterações em miRNA e expressão de genes alvo, assim como as vias moleculares reguladas pelos miRNAs, podem contribuir para o desenvolvimento de novas estratégias de diagnóstico e tratamento de pacientes com AS[32].

São raros os estudos de meta-análise para o melhor entendimento da expressão global de miRNAs em AS. Uma pesquisa recente no PubMed (22 de Setembro de 2016), revelou poucos estudos sobre a expressão de miRNAs em AS. Entre estes, um estudo mostrou que o miR-222-3p estava com expressão aumentada e os miRNAs, miR-378-3p, miR-483-5p e miR-497-5p estavam com expressão diminuída em AS (n=5 AS e 5 hemangiomas capilar humano)[15]. Esse mesmo estudo demonstrou que o miR-497-5p regula positivamente os canais de cálcio da membrana celular (KCa3.1), atuando como inibidor do crescimento tumoral[15]. Outro estudo mostrou o papel do miR-17-92 como regulador positivo na amplificação do *MYC*, responsável pelo fenótipo angiogênico do AS e como regulador negativo do gene *THBS1*, um potente inibidor endógeno da angiogênese.

Em estudo prévio do nosso grupo, uma meta-análise de dados de expressão gênica em hemangioma identificou genes comumente alterados, bem como miRNAs como potenciais reguladores da expressão gênica, associados aos hemangiomas. Os dados gerados nesse estudo contribuíram para um melhor entendimento das vias moleculares desreguladas e potencialmente associadas ao desenvolvimento e progressão do hemangioma. Adicionalmente, esses dados são úteis para o delineamento de estudos de validação, objetivando o desenvolvimento de terapêuticas mais precisas com alvos moleculares.

OBJETIVOS

Geral: Identificar alterações na expressão de miRNAs para caracterização de vias moleculares potencialmente associadas ao desenvolvimento e progressão do AS.

Específicos:

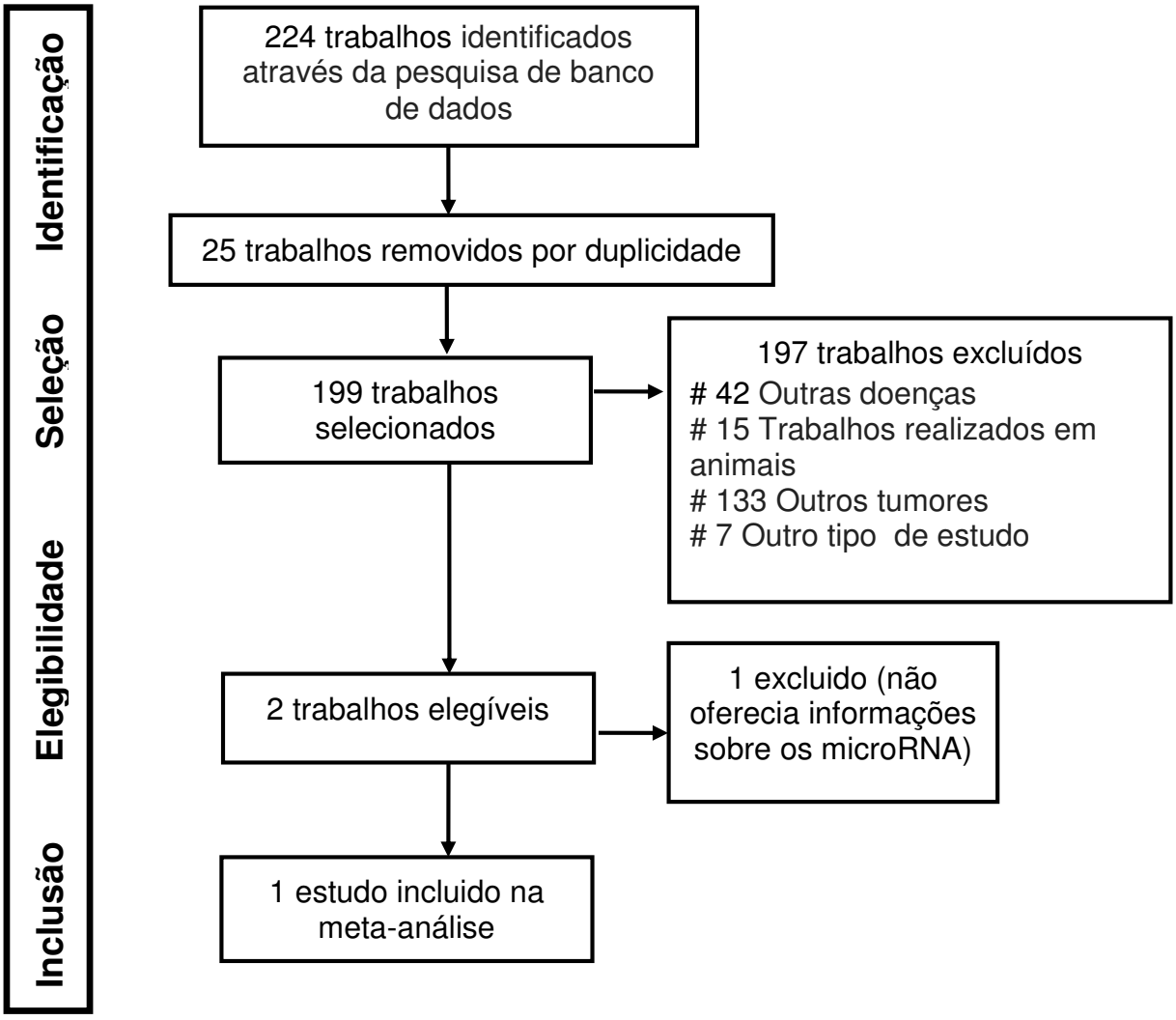
1. Identificar miRNAs significativamente alterados em AS, utilizando estratégia meta-análise
2. Identificar genes-alvo potencialmente regulados pelos miRNAs utilizando métodos de predição bioinformática
3. Mapear miRNAs e genes-alvo relacionados ao desenvolvimento vascular

METODOLOGIA

1. Meta-análise de expressão gênica em Angiossarcoma:

O estudo de meta-análise seguiu as etapas da Declaração de PRISMA[33, 34](Figura 1). A meta-análise dos dados foi realizada a partir do levantamento de dados de expressão de miRNAs em trabalhos publicados em periódicos indexados e disponíveis nas principais bases de dados: PubMed (http://www.ncbi.nlm.nih.gov/pubmed) e EMBASE (https://www.elsevier.com). Adicionalmente, foram pesquisadas teses nas bases Teses UNESP (http://www.unesp.br), Teses USP (http://www.teses.usp.br) e Teses UNICAMP (http://www.bibliotecadigital.unicamp.br). As palavras-chave utilizadas na pesquisa foram: “microRNAs e angiosarcoma”, “microRNAs e sarcoma vascular” e microRNAs e doença proliferativa endotelial maligna”. Os filtros ativados para seleção dos trabalhos foram: artigos publicados nos últimos 10 anos (2006-2016). Foram incluídos estudos considerados elegíveis, os quais atendiam aos critérios de inclusão: estudos de expressão de miRNAs em amostras de AS periférico humano, infantil ou adulto, estudos de modelos experimentais, *in vivo* ou *in vitro*, com amostras controle e com dados brutos disponíveis publicamente ou como material suplementar do artigo. Os critérios de exclusão foram: estudos em outros sarcomas e que não contemplam o AS, artigos de revisão da literatura, relatos de caso, cartas ou editoriais.

Figura 1: Fluxograma do processo de meta-análise segundo declaração de PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).



2. Extração dos dados:

Os dados foram obtidos do texto completo ou dados suplementares de cada estudo selecionado. Cada estudo foi descrito conforme as suas características, tais como: nome do primeiro autor, ano da publicação, tipo de amostras e controles utilizados e método de quantificação de miRNAs. Após a aplicação dos critérios de inclusão e exclusão, apenas 1 estudo foi incluído: S-MED: sarcoma microRNA expression database[35]. Esse trabalho analisou 310 amostras de sarcomas de 22 tipos diferentes e amostras de tecido normal, formando uma base de dados. sendo 5 de AS periférico. Os autores compararam a expressão global de miRNAs em amostras de AS com tecido vascular normal e outros sarcomas. Os dados de expressão de miRNAs foram organizados em uma base de dados, a qual utilizamos para identificar miRNAs desregulados exclusivamente em AS e não em outros tipos de sarcoma.

Os demais estudos identificados na literatura, mas que não foram incluídos por não atenderam aos critérios de inclusão e exclusão, foram considerados na interpretação/discussão dos resultados.

3. Metodologia de predição de genes regulados por miRNAs e integração de dados:

Identificamos os RNAs mensageiros (mRNAs) preditos a serem regulados pelos miRNAs; tais mRNAs serão denominados “mRNAs-alvo”. Para tal, utilizamos a ferramenta “microRNA Data Integration Portal” – mirDIP (http://ophid.utoronto.ca/mirDIP/), a qual integra conjuntos de dados de diferentes fontes públicas[36]. Os resultados da análise de predição foram posteriormente

filtrados para considerarmos apenas os mRNAs-alvo com maiores *scores*, conforme obtido na predição.

Os dados obtidos foram integrados utilizando métodos de bioinformática para o mapeamento dos genes (mRNAs-alvo) em proteínas, as quais por sua vez foram utilizadas para a construção de redes de interação proteica (PPI). As redes PPI foram geradas utilizando a ferramenta Metasearch STRING platform v9[37, 38]. A visualização e anotação dos dados foi realizada utilizando a ferramenta Cytoscape v3.1.1[39, 40]. As redes miRNA-mRNA e PPI foram ilustradas como gráficos onde os nós representarão os genes, miRNAs e proteínas e as linhas conectoras as suas interações (figura 2). Os dados de interação foram analisados utilizando o programa Cytoscape. As proteínas com expressão alterada foram identificadas por meio de pesquisa no banco de dados dbDEPC (http://lifecenter.sgst.cn/dbdepc/index.do) e no *The Human Protein Atlas* (http://www.proteinatlas.org/).

4. Análise de Enriquecimento:

Realizamos essa análise para a identificação de vias biológicas importantes em angiossarcoma. Para tal, utilizamos a ferramenta BiNGO disponível no Cytoscape[41]. A ferramenta BiNGO permite a identificação de quais categorias funcionais do Gene Ontology (GO) estão mais representadas para um conjunto determinado de genes.

RESULTADOS

MicroRNAs com expressão alterada em Angiossarcoma

Segundo o delineamento experimental e após aplicação dos critérios de inclusão e exclusão estabelecidos, conforme mostrado na Figura 1, foi selecionado 1 estudo para realização da meta-análise: S-MED: sarcoma microRNA expression database[35]. De acordo com dados desse estudo, 59 miRNAs foram identificados com expressão significativamente aumentada nas 5 amostras de AS. Estes 59 miRNAs desempenham papéis na regulação de genes associados a processos de apoptose, diferenciação celular, controle de expressão gênica e desenvolvimento vascular.

Considerando que o AS é um tumor de origem vascular e que a rede de interação entre miRNA e genes é apresenta alta complexidade (Figura 2), selecionamos os miRNAs e genes-alvo associados ao desenvolvimento vascular e identificamos 21 miRNAs com expressão aumentada, os quais regulam 28 genes, como mostra a Figura 3. Na Tabela 1 temos os 21 microRNAs com expressão aumentada, quais genes regulam e quais as principais atuações do gene no desenvolvimento vascular.

Observamos que 7 dos 21 microRNAs estão mapeados no cromossomo 19. O cromossomo 19 é o mais denso do genoma humano, contendo cerca de 1500 genes. Dentre eles genes que codificam para doenças como diabetes insulino-dependente, distrofia miotônica, enxaquecas e hipercolesterolemia familiar. Também já foi observado um cluster de microRNAs no cromossomo 19, encontrado em vários tipos de câncer, e que é responsável por regular a apoptose e controlar a tumorigênese, podendo ser possível alvo para novas terapias no câncer[42].

Notamos que vários dos genes citados na Tabela 1 estão associados a pior, ou melhor, prognóstico em vários tipos de câncer. São genes que podem regular

processos como a angiogênese e sua inibição pode atuar como fator antitumoral. Outras vezes atuam facilitando a disseminação de células neoplásicas.

Por fim, observamos inúmeros genes com possibilidades de se tornarem marcadores tumorais. Alguns definindo pior, ou melhor, prognóstico da doença; outros possíveis marcadores periféricos de controle da doença; e outros servindo como terapia alvo.

Figura 2: Rede de Interação miRNA-miRNA-alvo em Angiossarcoma

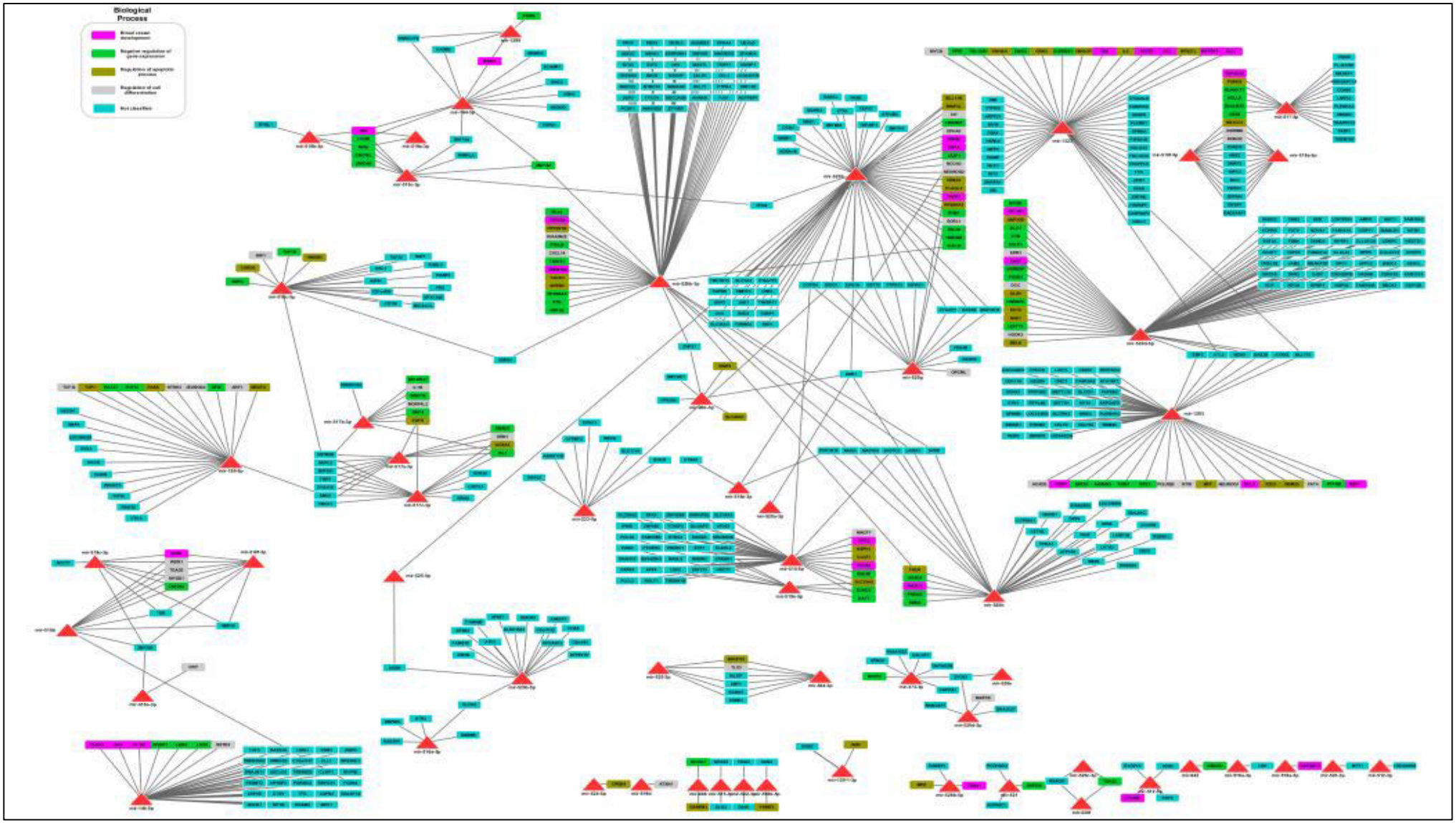
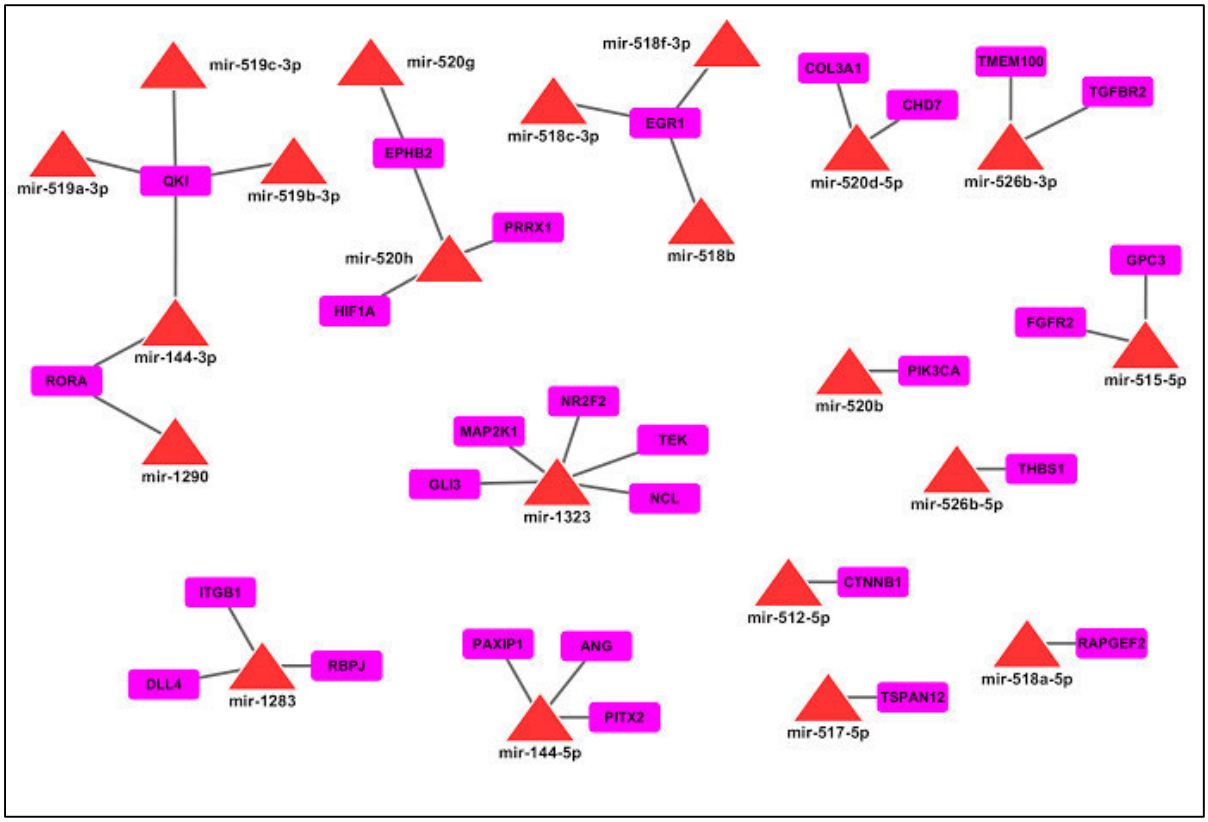


Figura 3: Rede de Interação miRNA-miRNA-alvo no desenvolvimento vascular



miRNA

Gene associado ao desenvolvimento vascular

TABELA 1: MicroRNAs e Genes associados a desenvolvimento vascular em Angiossarcoma.

miRNAs	Genes regulado pelo MicroRNA	Identificação do gene	Função do gene
miR-519a-3p	*QKI*		
miR-519b-3p	*QKI*	ENSP00000355094	A proteína codificada por este gene regula o pré-mRNA, a exportação de mRNAs a partir do núcleo, a tradução de proteínas e estabilidade mRNA.Está envolvida na mielinização e na diferenciação de oligodendrócitos.
miR-519c-3p	*QKI*		
miR-144-3p	*QKI*		
miR-144-3p	*RORA*	ENSP00000261523	A proteína codificada por este gene é um membro da subfamília NR1 de receptores hormonais nucleares. Pode ligar-se a elementos de resposta hormonal a montante de vários genes para aumentar a expressão desses genes. A proteína codificada mostrou interagir com NM23-2, uma nucleosídeo difosfato quinase envolvida na organogénese e diferenciação, bem como com NM23-1, o produto de um gene candidato supressor de metástase tumoral. Além disso, tem sido demonstrado que ajuda na regulação transcricional de alguns genes envolvidos no ritmo circadiano.
miR-1290	*RORA*		
miR-520h	*HIF1A*	ENSP00000338018	Funciona como um regulador principal da resposta homeostática celular e sistêmica à hipóxia, ativando a transcrição de muitos genes, incluindo aqueles envolvidos no metabolismo energético, angiogênese, apoptose e outros genes cujos produtos proteicos aumentam o fornecimento de oxigênio ou facilitam a adaptação metabólica à hipóxia. HIF-1 desempenha assim um papel essencial na vascularização embrionária, angiogénese tumoral e fisiopatologia da doença isquémica.
miR-520h	*PRRX1*	ENSP00000239461	A proteína funciona como um co-ativador de transcrição, aumentando a actividade de ligação ao DNA do factor de resposta ao soro, uma proteína necessária para a indução de genes por factores de crescimento e diferenciação. A proteína regula a creatina- quinase muscular, indicando um papel no estabelecimento de diversos tipos de músculo mesodermal.
miR-520h	*EPHB2*	ENSP00000363763	Codifica um membro da família de receptores Eph de glicoproteínas transmembranares de receptores tirosina quinase. Estes receptores estão envolvidos em diversos processos celulares, incluindo motilidade, divisão e diferenciação. As variantes alélicas estão associadas com a susceptibilidade ao cancer de próstata e cerebral.
miR-520g	*EPHB2*		
miR-518b	*EGR1*		A proteína codificada por este gene funciona como um regulador transcricional. Os produtos dos genes-alvo que ele ativa são necessários para diferenciação e mitogênese.Estudos sugerem que este é um gene supressor do câncer.
miR-518c-3p	*EGR1*	ENSP00000239938	
miR-518f-3p	*EGR1*		
miR-520d-5p	*COL3A1*	ENSP00000304408	Este gene codifica as cadeias pro-alfa1 do colagénio tipo III, um colagénio fibrilar que se encontra nos tecidos conjuntivos extensíveis como pele, pulmão, útero, intestino e sistema vascular, frequentemente associado ao colagénio tipo I. As mutações neste gene estão associadas com a síndrome de Ehlers-Danlos, tipos IV, e com aneurismas aórticos e arteriais. Quando superexpresso associado a fibrose pulmonar e câncer colorrectal. Envolvido em mecanismos de resistência a quimioterápicos
miR-520d-5p	*CHD7*	ENSP00000392028	Este gene codifica uma proteína que contém vários domínios da família helicase. Mutações neste gene foram encontradas em alguns pacientes com a síndrome CHARGE. Pode ser usado como biomarcador em Ca Pancreas e regula sensibilidade da Gencitabina.
miR-526b-3p	*TMEM100*	ENSP00000395328	Apoptose celular, angiogênese e altura corporal. Marcador de inervação entérica. Associado a estadiamento clínico de adenocarcinoma de pulmão e hepatocarcinoma..
miR-526b-3p	*TGFBR2*	ENSP00000351905	A proteína codificada forma um complexo transmembranar que fosforila as proteínas, que então entram no núcleo e regulam a transcrição de um subconjunto de genes relacionados com a proliferação celular. As mutações neste gene foram associadas à Síndrome de Marfan, à Síndrome do Aneurisma Aórtico de Loeys-Deitz e ao desenvolvimento de vários tipos de tumores.
miR-515-5p	*GPC3*	ENSP00000377836	A proteína codificada por este gene pode induzir apoptose em certos tipos de células. As mutações de deleção neste gene estão associadas à síndrome de Simpson-Golabi-Behmel, também conhecida como síndrome de dismorfia de Simpson.
miR-515-5p	*FGFR2*	ENSP00000410294	A proteína codificada por este gene é um membro da família de receptores de factor de crescimento de fibroblastos As mutações neste gene estão associadas à síndrome de Crouzon, síndrome de Pfeiffer, craniossinostose, síndrome de Apert, síndrome de Jackson-Weiss, síndrome de Beare-Stevenson cutis gyrata, síndrome de Saethre-Chotzen e craniossinostose sindrômica.
miR-520b	*PIK3CA*	ENSP00000263967	Este gene foi encontrado para ser oncogênico e tem sido implicado em canceres cervicais. Um pseudogene deste gene foi definido no cromossomo 22.Importante via de sinalização para ajuste de funções celulares (proliferação, sobrevida, atividade, adesão, diferenciação).Associado a mecanismos de resistência a quimioterápicos(ex:Transtuzumab)
miR-526b-5p	*THBS1*	ENSP00000260356	A proteína codificada por este gene é uma glicoproteína adesiva que medeia interações de célula para célula e de célula para matriz. Esta proteína pode ligar-se a fibrinogénio, fibronectina, laminina, colagénio do tipo V e integrinas alfa-V / beta-1. Esta proteína demonstrou desempenhar papéis na agregação plaquetária, angiogénese e tumorigénese.

miR-512-5p	*CTNNB1*	ENSP00000344456	Mutações neste gene são uma causa de câncer colorretal (CRC), pilomatrixoma (PTR), meduloblastoma (MDB) e câncer de ovário. Intermediário de várias vias de regulação celular. Estudos mostram que pode interferir no comportamento da célula tumoral e causar resistência a quimioterápicos (ex:Carboplatina)
miR-518a-5p	*RAPGEF2*	ENSP00000264431	Os factores de troca de nucleótidos de guanina (GEF), tais como RAPGEF2, servem como ativadores do RAS promovendo a aquisição de GTP para manter o GTP ativo e são a ligação chave entre os receptores de superfície celular e a activação de RAS. Participa de vias responsáveis pela migração celular e metástases em câncer de mama.
miR-517-5p	*TSPAN12*	ENSP00000222747	A proteína codificada por este gene é uma proteína de superfície celular que medeiam eventos de transdução de sinal que desempenham um papel na regulação do desenvolvimento celular, ativação, crescimento e motilidade. Age no crescimento, invasão, metástases e angiogênese das células neoplásicas, além de inibidor do p53
miR-144-5p	*PITX2*	ENSP00000304169	A proteína codificada atua como um factor de transcrição e regula a expressão do gene procolágeno lisil hidroxilase. Esta proteína desempenha um papel na diferenciação terminal dos fenótipos das células somatotróficas e lactotróficas, está envolvida no desenvolvimento dos órgãos dos olhos, dos dentes e do abdome e age como um regulador transcricional envolvido na atividade basal e regulada pelas hormonios como a prolactina. Mutações neste gene estão associadas com síndrome de Axenfeld-Rieger, síndrome de iridogoniodysgenesis e casos esporádicos de anomalia de Peters.
miR-144-5p	*ANG*	ENSP00000336762	A proteína codificada por este gene é um mediador extremamente potente da formação de novos vasos sanguíneos. Além disso, tem actividade antimicrobiana contra algumas bactérias e fungos, incluindo S. pneumoniae e C. albicans.
miR-144-5p	*PAXIP1*	ENSP00000384048	Este gene codifica uma proteína nuclear BRCT (Breast Cancer Carboxy-Terminal). Esta proteína desempenha um papel crítico na manutenção da estabilidade do genoma, condensação da cromatina e progressão através da mitose.
miR-1283	*DLL4*	ENSP00000249749	A família de genes delta codifica ligandos Notch. Colabora com o VEGF no controle da angiogênese e progressão tumoral em vários tipos de câncer
miR-1283	*ITGB1*	ENSP00000303351	As integrinas são proteínas receptoras de membrana envolvidos na adesão celular e reconhecimento numa variedade de processos incluindo embriogénese, hemostasia, reparação de tecidos, resposta imunitária e difusão metastática de células tumorais. Responsável pela colonização, disseminação e metástases ósseas em vários tipos de cânceres.
miR-1283	*RBPJ*	ENSP00000345206	A proteína codificada por este gene é um regulador transcricional importante na via de sinalização Notch. Atua como um repressor quando não ligado a proteínas Notch e um activador quando ligado a proteínas Notch.
miR-1323	*NCL*	ENSP00000318195	A nucleolina (NCL), uma fosfoproteína nucleolar eucariótica, está envolvida na síntese e maturação dos ribossomas.
	MAP2K1	ENSP00000302486	A proteína codificada por este gene é um membro da família de proteína quinase e está envolvida em muitos processos celulares tais como proliferação, diferenciação, regulação da transcrição e desenvolvimento.
	GLI3	ENSP00000379258	A proteína codificada por este gene localiza-se no citoplasma e activa a expressão do gene homologado de Drosophila (PTCH). Também responsável por desempenhar papel durante a embriogênese. As mutações neste gene têm sido associadas com várias doenças, incluindo a síndrome de Cephalopolisyndactyly Greig, síndrome de Pallister-Hall, polidactilia pre-axial tipo IV e polidactilia pos-axial de tipos A1 e B.
	TEK	ENSP00000369375	Este gene codifica um receptor que pertence à família de proteína tirosina quinase Tie2. As mutações neste gene estão associadas com malformações venosas hereditárias da pele e das membranas mucosas. O splicing alternativo resulta em variantes de transcrito múltiplas.
	NR2F2	ENSP00000377721	A proteína codificada é um factor de transcrição indutível que está envolvido na regulação de muitos genes diferentes.

Fonte: Sarver AL, Phalak R, Thayanithy V, Subramanian S.S-MED: Sarcoma microRNA Expression Database. *Laboratory Investigation (2010) 90, 753-761.*

DISCUSSÃO

O AS é um tumor maligno originado da linhagem vascular, raro, de prognóstico ruim e ainda muito pouco entendido. As análises genéticas são extremamente valiosas para identificar os mecanismos de formação deste câncer, bem como biomarcadores e vias moleculares potencialmente úteis para o desenvolvimento de terapias mais eficazes.

Nossa meta-análise identificou 21 miRNAs preditos para regularem 28 genes relacionados ao desenvolvimento vascular. Notavelmente, sete miRNAs (miR-512, miR-515, miR-517, miR-518, miR-519, miR-520 e miR-526) estão mapeados no cromossomo 19, o qual apresenta a maior densidade de genes no genoma humano[43]. Dentre esses genes existem alguns que codificam doenças como diabetes insulino-dependente, distrofia miotônica, enxaquecas e hipercolesterolemia familiar. Foi observado um cluster de microRNAs no cromossomo 19, encontrado em vários tipos de câncer, e que é responsável por regular a apoptose e controlar a tumorigênese, podendo ser possível alvo para novas terapias no câncer[42]

Os miRNAs da família miR-519 (a, b, c) e o miR-144-3p preditos para regularem, entre outros, o gene *QKI*, inibindo sua expressão. O *QKI* é um gene responsável por conservar o sinal de tradução e ativação do RNA, também regula a estabilidade da molécula de miRNA e os mecanismos de retenção nuclear, transporte e modulação de RNAs[44, 45]. O gene *QKI* atua no controle de proliferação celular e sinal de estresse celular[45]. Estudos em carcinoma de cavidade oral e de pulmão relataram o aumento da expressão de *QKI* associado ao pior prognóstico dos pacientes[44, 45]. Recentemente foi demonstrado que o QKI regula as propriedades de células tronco do sistema nervoso central. Esse estudo demonstrou que a diminuição dos níveis de

endolisossomos pela perda de QKI contribuiu para a manutenção das propriedades de células tronco em gliomas[46].

O gene *RORA* é provavelmente regulado no AS pelos miRNAs miR-144-3p e miR-1290. *RORA* regula outros genes envolvidos no metabolismo lipídico e na tumorigênese[47]. É interessante notar que vários tipos de câncer têm um aumento da atividade lipogênica, relacionado ao rápido crescimento e divisão celular. Os metabólitos do lipídio são fundamentais para manter íntegra a membrana celular das células neoplásicas. A diminuição de expressão do gene *RORA* é observada em vários tipos de câncer (colorretal, próstata e mama) e estão associados à progressão tumoral[47-49].

O miR-520h potencialmente regula os genes *HIF1A*, *PRRX1* e *EPHB2*. *HIF1A* é um regulador importante da adaptação e sobrevida de células e tecidos à hipóxia. O aumento na expressão do gene *HIF1A* tem um papel importante na progressão de vários tipos de cânceres em humanos[50, 51]. *PRRX1* tem papel importante na regulação e desenvolvimento de processos morfogenéticos. Quando com expressão aumentada está associado a metástases e pior prognóstico em câncer colorectal e glioblastoma, no entanto, quando tem expressão aumentada em câncer de mama e pulmão está associado a um melhor prognóstico[52, 53]. *EPHB2* está à invasão e progressão de vários tipos de cânceres associado. É essencial para o desenvolvimento neural, dinâmica do citoesqueleto, migração guiada, proliferação celular e angiogênese. Tem efeito direto na proliferação e migração de células neoplásicas, além de ativar diretamente o fator de crescimento endotelial vascular (VEGF2 e VEGF3)[54-57]. O *EPHB2* é provavelmente regulado por dois miRNAs, miR-520g e miR-520h.

O gene *EGR1* é potencialmente regulado por 3 miRNAs: miR-518b, miR-518c-3p e miR-518f-3p. Possui papel importante no controle do crescimento celular,

proliferação, diferenciação e apoptose. Pode agir como supressor de tumor ou promotor do crescimento tumoral, dependendo do tipo de célula e do estímulo externo. A atividade de supressão tumoral do *EGR1* foi demonstrada em alguns tipos de cânceres, tais como o fibrossarcoma, glioblastoma, câncer de pulmão e de mama. Entretanto, promove crescimento tumoral em cânceres de pele, próstata e rim[58, 59].

O miR-520d-5p é predito a regula dois genes: *COL3A1* e *CHD7*. O *COL3A1* é um gene da família do colágeno, responsável pela produção de colágeno III, encontrado em vários tecidos como pulmão, pele, útero, intestino e tecidos vasculares. Pode associar-se a doenças vasculares quando mutado e quando tem expressão aumentada pode causar fibrose pulmonar. *COL3A1* foi relatado com expressão aumentada no câncer colorrectal[60] e foi associado a mecanismos de resistência celular a quimioterápicos[61]. Estudos mostraram que o gene *CHD7* pode servir como biomarcador no acompanhamento de pacientes com câncer de pâncreas ressecados em estágio inicial e tratados com Gencitabina. A expressão diminuída de *CHD7* aumenta a ação da Gencitabina no tumor[62].

O miR-526b-3p provavelmente regula os genes *TMEM100* e *TGFBR2*. O gene *TMEM100* desempenha um papel importante na apoptose e na angiogênese[63]. Alterações em *TMEM100* foram associadas ao estadiamento clínico de adenocarcinoma de pulmão e hepatocarcinoma[63, 64]. Alterações em *TGFBR2* foram associadas ao prognóstico e resposta a quimioterapia em vários tipos de tumores[65, 66]. Esse gene foi sugerido como biomarcador de pior prognóstico e resistência à quimioterapia em câncer de pulmão[65] e pior prognóstico em câncer de mama com receptor de estrógeno negativo[66]. Interessantemente, pode atuar como supressor ou promotor de tumor dependendo do microambiente celular[66].

O miR-515-5p potencialmente regula os genes *GPC3* e *FGFR2*. *GPC3* regula a proliferação, diferenciação, adesão e metástase de células tumorais[67]. Foi associado com o grau de diferenciação tumoral, ocorrência de metástase e recidiva em vários tipos de neoplasias incluindo o câncer de pulmão, hepatoblastoma e câncer de endométrio[67-69]. *FGFR2* atua na angiogênese, diretamente na formação de capilares e vasos linfáticos, processo fundamental para o desenvolvimento e progressão tumoral[70]. Responsável pela ativação de algumas proteínas que regulam múltiplos processos celulares como: angiogênese tumoral, crescimento celular, diferenciação celular, migração celular e metabolismo celular[70]. Tem importante papel como supressor de angiogênese tumoral inibindo a proliferação de tumores como câncer de mama, mieloma múltiplo, câncer de colon, próstata e endométrio[71].

O miR-520b é predito a regular o gene *PIK3CA*, o qual participa de uma importante via de sinalização que regula diferentes funções celulares como proliferação, sobrevida, atividade, adesão, diferenciação, reestruturação celular e transporte intracelular[72, 73]. *PIK3CA* tem expressão aumentada, sendo amplificado em câncer de ovário e apresentando altos índices de mutação (25-30%) em câncer colorretal, cerebral, de mama, estômago e de fígado[72]. Está associado a mecanismos de resistência a quimioterápicos como o Trastuzumab[73].

O miR-526b-5p provavelmente regula o gene *THBS1*, com função de inibição de crescimento tumoral, migração celular e neovascularização[74, 75]. A expressão diminuida desse gene está associada ao pior prognóstico de pacientes com carcinoma pulmonar; a expressão diminuída de *THBS1* foi demonstrada inibir o desenvolvimento tumoral e metástase em carcinoma de células escamosas de laringe[74, 75].

O miR512-5p potencialmente regula o gene *CTNNB1*, um importante intermediário de várias vias de regulação celular e vários estudos mostram que pode

interferir no comportamento da célula tumoral[76, 77]. A expressão aumentada de *CTNNB1* foi associada ao pior prognóstico de pacientes com câncer de mama[76] e à resistência ao tratamento com carboplatina em tumores de ovário[77].

O miR-518a-5p provavelmente regula o gene *RAPGEF2* o qual desempenha um papel na migração neuronal e integração do córtex cerebral[78] e foi demonstrado atuar na migração celular e metástase em câncer de mama[79].

O miR-517-5p é predito em regular o gene *TSPAN12* que atua diretamente na tumorigênese[80, 81]. Age diretamente no crescimento, invasão, metástases e angiogênese das células neoplásicas, além de agir como inibidor do *p53*[80, 81]. O gene *p53* é um importante supressor de tumores e quando supresso ou mutado propicia o crescimento tumoral[80].

O miR-144-5p possivelmente atua em 3 genes: *PAXIP1*, *ANG* e *PITX2*. O *PAXIP1* é um gene responsável pela resposta ao dano no DNA, mantendo o genoma estável[82]. O gene *ANG* é um dos mais potentes indutores de neovascularização[83]. É um gene que induz a angiogênese sob a influência de outros fatores angiogênicos como fator de crescimento endotelial vascular (VEGF) e fator de crescimento de fibroblastos (FGF-1 e FGF-2)[83]. O gene *PITX2* é um marcador de metilação do DNA e age como fator de transcrição[84-86]. Tem papel importante no desenvolvimento e manutenção de olhos, dentes e órgãos abdominais; além de atuar na atividade da prolactina[85]. Quando com expressão aumentada está relacionado a pior prognóstico em carcinoma escamoso esofágico. Já no câncer de mama, principalmente os com receptores de progesterona, mostrou-se um marcador de bom prognóstico[85, 86].

O miR-1323 preditamente regula 5 genes: *NCL*, *TEK*, *NR2F2*, *MAP2K1* e *GLI3*. A *NCL* é multifuncional, agindo no DNA, RNA e miRNA[87-89]. Atua em vários genes de progressão tumoral, podendo ser um poderoso biomarcador [87-89]. O gene *TEK* atua

em inúmeros processos celulares e sua desrregulação é observada em vários tipos de câncer como na Leucemia Mieloblastica Aguda (LMA)[90, 91]. Mutações do exon 17 no gene *TEK* foram e[88]ncontradas em pacientes com tumores vasculares e malformações vasculares[91]. O gene *NR2F2* regula a angiogênese e tem papel fundamental no desenvolvimento de metástases em câncer. Alterações em sua expressão são encontradas em vários tipos de câncer como: mama, próstata, colon, pâncreas, pulmão e ovário[92].

O gene *MAP2K1* é responsável pela regulação de atividades celulares como proliferação, regulação transcripsional, diferenciação e sobrevida[93-95]. É fator de pior prognóstico em vários tipos de câncer (pulmão, ovário) e atua na resistência a quimioterápicos como Carboplatina[94, 95]. O gene *GLI3* tem importante papel na angiogênese e neovascularização, principalmente em tecidos isquêmicos[96]. Quando com sua expressão aumentada estimula a expressão de outros fatores pró-angiogênicos, promovendo a neovascularização[96, 97]. Associado a mal prognóstico em alguns tipos de câncer como pulmão[97].

O miR-1283 potencialmente regula 3 genes: *DLL4*, *ITGB1* e *RBPJ*. O gene *RBPJ* pode impedir ou promover o desenvolvimento tumoral, dependendo do tipo histológico do câncer[98, 99]. A inibição deste gene tem efeito supressor na tumorigênese de vários tipos de câncer como de próstata e rabdomiossarcoma[98, 99]. O gene *ITGB1* é responsável pela colonização, disseminação e principalmente pelas metástases ósseas em vários tipos de cânceres como no câncer de mama[100]. É um alvo promissor no desenvolvimento de novos tratamentos[100]. O gene *DLL4* é essencial para o desenvolvimento vascular[101]. É um fator vascular específico que colabora com o VEGF no controle da angiogênese e progressão tumoral em vários tipos de câncer[101, 102].

CONCLUSÕES E DIREÇÕES FUTURAS

Identificamos a expressão significativamente aumentada (FC>= 1,5 e p<= 0,05) de 59 miRNAs, sendo 21 relacionados ao desenvolvimento vascular. Esses 21 miRNAs potencialmente regulam 28 genes associados ao processo de desenvolvimento vascular e a mecanismos importantes no crescimento tumoral, invasão e metástase. Também atuam na resposta a vários quimioterápicos como: Gencitabina, Carboplatina e Transtuzumabe. Apresenta comportamento diferenciado dependendo do tipo histológico, podendo influenciar o prognóstico em diferentes tipos de tumor. É um caminho promissor para novas terapias alvo, entretanto estudos adicionais são necessários para entender melhor seus mecanismos no AS.

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ABREVIATURAS

ISSVA - International Society for the Study of Vascular Anomalies

AS – Angiossarcoma

D2-40 – Podoplanina

VEGF - Fator de Crescimento Endotelial Vascular

bFGF - Fator de Crescimento Básico de Fibroblastos

miRNAs – microRNAs

mRNA-alvo – RNA mensageiro alvo

PRISMA - Preferred Reporting Items for Systematic Reviews and Meta-Analyses

mirDIP - microRNA Data Integration Portal

PPI - Redes de Interação Proteica

GO - Gene Ontology

FGF - Fator de Crescimento de Fibroblastos

LMA – Leucemia Mieloblástica Aguda

ANEXO – I: ARTIGO PARA PUBLICAÇÃO

Identification of microRNA-regulated networks associated with vascular development in angiosarcoma.

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ABSTRACT

Angiosarcoma (AS) is a rare malignant tumor derived from endothelial vascular and lymphatic cells. Although the previous studies in AS, molecular pathways associated with disease development and progression are not fully understood. MicroRNAs (miRNAs) are gene expression regulators with an important role in molecular pathways of tumorigenesis and are considered biomarkers with potential clinical value in the diagnosis, prognosis and treatment of patients with cancer. Therefore, the identification of miRNA expression profiles and miRNA-regulated pathways may significantly contribute to elucidate the mechanisms of tumorigenesis in AS. Our aims were to identify global miRNA expression and molecular pathways in AS. We performed a meta-analysis following the Prisma Statement and using the main databases PubMed and EMBASE. Following the application of specific inclusion and exclusion criteria, one study (including 5 AS samples) was eligible and selected for data extraction and analysis. Of this study, we identified significantly deregulated miRNAs (FC>=1.5 and p<0,05). Further, miRNA expression data was analyzed using the bioinformatic tools miRWalk v.2.0 for target gene prediction and STRING and Cytoscape v.3.1.1/BINGO for identification of miRNA-mRNA networks and biological functions, respectively. We identified 59 miRNAs as significantly over-expressed (FC>=1.5 and p<0,05) in AS. Of these, 21 miRNAs were identified to interact with 23 target genes with functions enriched for vascular development. Target genes identified have important roles in tumorigenesis, since they regulate cell growth, invasion, metastasis and have been associated with chemotherapy response. miRNAs identified herein, in particular miR-1322, miR-520h, miR-1283 and miR-144-5p regulate genes involved in mechanisms of vascular development and tumorigenesis. Studies such as this may contribute to better understand the development and progression of AS, as well as to identify novel biomarkers and more precise treatment strategies, impacting patient survival.

KEY-WORDS: Angiossarcoma, vascular tumor, microRNA, mRNA, molecular pathways, tumorigenesis, treatment, meta-analysis.

INTRODUCTION

Angiosarcoma (AS) is a malignant vascular tumor derived from vascular endothelial and lymphatic cells[1] and classified as visceral or pheripheral[1, 2]. AS may arise in the head and neck (27%), breast (19,7%), extremities (15%), chest (9,5%), liver (6%), heart (4,7%), bones (3,6%), spleen (2,6%) and other sites (11,6%)[1]. Pheripheral AS is rare subtype of soft tissue sarcomas (<2% of cases) with very aggressive tumor behavior[2, 3] and poor prognosis, with a low 5-year disease-free survival of less than 35% in non-metastatic cases and a recurrence rate greater than 75% in the first 2 years of treatment[4, 5].

A few AS tumors may develop due to malignant transformation of previous bening vascular lesions[1]. Although a rare finding, bening tumors such as hemangioma may be further investigated to exclude malignancy if there is a sudden change in clinical behavior and growth of lesion[6].

Previous studies showed expression of endothelial markers CD34, CD31, Fli1, factor VIII, Ulex europaeus agglutinin 1, ERG and podoplanin in AS[1,7-9] , as well as vimentin, actin and mioglobulin[8]. Genes associated with angiogenesis, such as VEGF, have been demonstrated as over-expressed in AS[1,10]. In addition, under-expression of *TP53* and over-expression of *WT1* and Galectina-3 have been reported[1,11, 12]. Compared to other sarcomas, AS has increased expression of vascular receptors including *TIE1*, *KDR*, *TEK* and *FLT1*[7] and *MYC* and *FLT4* changes have been correlated with disease development[3, 7]. Although these several studies, the mechanisms of AS tumorigenesis are still not fully understood. Therefore, additional studies are required to elucidate the molecular regulatory changes associated with AS. Such studies may contribute for future development of novel therapeutic strategies for patients with this deadly disease[13].

microRNAs (miRNAs) are small (~19-24 nucleotide length) non-coding RNAs that act as post-transcriptional regulators of gene expression. miRNAs regulate genes that participate in a large

number of biological processes in development, cellular homeostasis, differentiation, reprogramming and have been shown to play key roles in tumorigenesis[14].

miRNAs expressed in the vascular endothelium may control response to angiogenic stimuli, indicating their role as angiogenic response modulators[15]. miR-126 was reported as a positive regulator of angiogenesis signaling and vascular endothelial integrity; miR-126 expression changes were associated with vascular development defects, with miR-126 deficient cells being no responsive to VEGF e bFGF angiogenic factors[15]. Other miRNAs, such as miR-221 and miR-222 act as inhibitors of stemm cell stimulating factor associated with angiogenesis. Some miRNAs play pro-angiogenic roles as demonstrated by inhibition of expression of miR-27b and miR-let-7f, which reduced angiogenesis[15]. Therefore, increased expression of pro-angiogenic miRNAs in endothelial cells may alter production of angiogenic factors, contributing with tumorigenesis[15].

A comparison between miRNA changes in AS and hemangioma identified that four miRNAs were differentially expressed, being miR-222-3p over- and miR-378-3p, miR-483-5p and miR-497-5p under-expressed in five AS compared to five hemangioma samples[13]. In addition, this study showed that miR-497-5p positively regulates membrane calcium channels, supressing tumor growth[13]. Another study showed that miR-17-92 is associated with AS in the presence of *MYC* amplification and also acts as a negative regulator of *THBS1*, a potent endogenous inhibitor of angiogenesis.

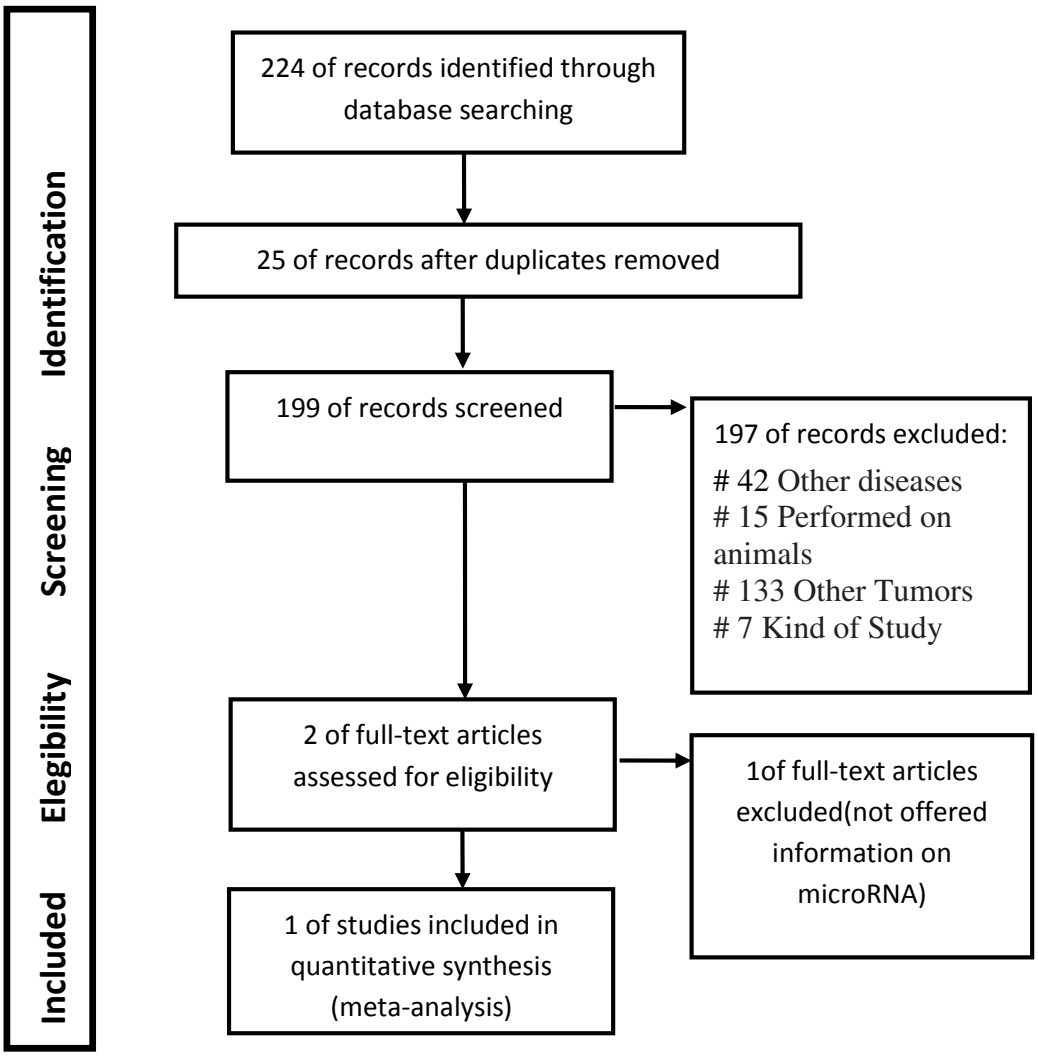
In this meta-analysis study, we identififed miRNAs and genes regulated by miRNAs that are commonly altered and associated with vascular development pathways in AS.

META-ANALYSIS METHODS:

Search Strategy

This meta-analysis followed the PRISMA Statement[16, 17] (Figure 1) and was performed by searching published studies containing miRNA expression data available in main databases; PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and EMBASE (https://www.elsevier.com). Key-words used were: "microRNAs and angiosarcoma", "microRNAs and vascular sarcoma" and microRNAs and malignant endothelial proliferative disease". We searched for articles published in the past 10 years (2006-2016). Studies were considered eligible based on inclusion and exclusion criteria, as follows: Inclusion criteria were: miRNA expression data in human pheripheral AS, from patients of any age, *in vivo* and/or *in vitro* experimental studies, including control samples and with raw data publicly available. Exclusion criteria were: studies in other sarcomas, review articles, case reports, letters or editorials.

Figure 1: Flowchart of the meta-analysis process according to PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).



Data Extraction

Data were obtained from supplementary material or from databases reported in the study. Studies were screened and data was recorded including: first author's name, sample type and controls used, miRNA quantification method or platform used. Following application of inclusion and exclusion criteria, 1 study was selected: S-MED: sarcoma microRNA expression database[18]. This study included 310 sarcoma samples (from 22 different types of sarcoma, being 5 of AS) as well as normal controls. The authors compared miRNA expression profiles in AS with normal vascular tissue and other sarcomas. miRNA expression data retrieved from this study has been organized in a database, which we used to identify miRNAs exclusively deregulated in AS and to apply further downstream bioinformatic analyses, as outlined below. Other studies that were identified through literature searches but were not included as they did not meet all criteria, were used in data interpretation and discussion of study results.

Target mRNA prediction and miRNA-mRNA interaction networks in AS

Significantly deregulated miRNAs (FC >=1.5 and p<0,05) were used for bioinformatic analysis, in order to identify predicted mRNA targets of miRNAs. This analysis was performed using microRNA Data Integration Portal (mirDIP) (http://ophid.utoronto.ca/mirDIP/), which integrates data from different sources[19]. Results were filtered to consider target mRNAs with the highest scores. Target genes were then mapped into proteins, which were used to construct protein-protein interaction (PPI) networks. We also performed searches for the identified target genes in the European Bioinformatics Institute (EBI) database (https://www.ebi.ac.uk/gxa/home), which provides information on gene expression patterns, allowing to perform searches by gene or protein name, as well as gene and protein expression in specific tissues. Proteins identified as having expression changes were also verified by searches using the database dbDEPC (http://lifecenter.sgst.cn/dbdepc/index.do) and The Human Protein Atlas (http://www.proteinatlas.org/).

Additionally, enrichment analysis was carried out to identify the main biological functions of miRNAs and target genes, using STRING 10.0 (http://string-db.org/). STRING is a comprehensive biological and protein-protein interaction database, which allows to identify known, validated or predicted interactions[20, 21]. In addition, Cytoscape v3.1.1 (http://www.cytoscape.org/) was used for data visualization[22, 23]. Enrichment analysis for biological function identification used Gene Ontology (GO) tool following the statistical structure: evaluation of over-represented GO categories, hypergeometric for over-represented functions, multiple correction Benjamini & Hochberg (FDR) test with p<=0,05 and complete genome as reference.

RESULTS

Analysis of the data provided in the S-MED: sarcoma microRNA expression database[24] showed 59 significantly deregulated miRNAs exclusively in AS. These 59 miRNAs were identified to regulate genes with roles in apoptosis, cell differentiation, gene expression regulation and vascular development. Considering that AS is a vascular tumor, and that all miRNAs identified participate in a complex network (Figure 2), we chose to focus on miRNA-mRNA targets with roles in vascular development. We identified 21 miRNAs, all over-expressed in AS and that regulate 23 genes, as shown in Figure 3.

Figure 2: Network miRNA-miRNA target interaction in Angiosarcoma

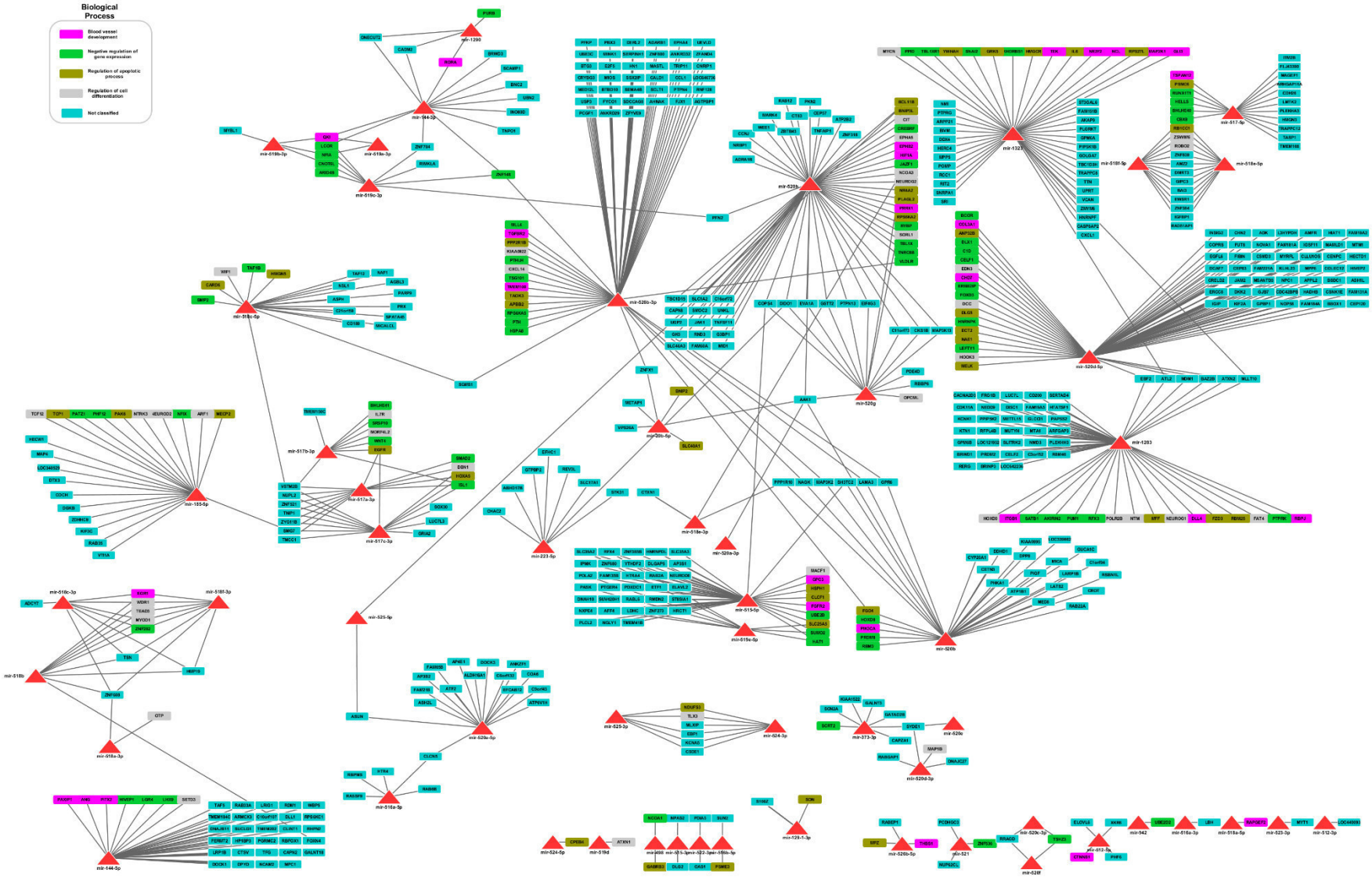
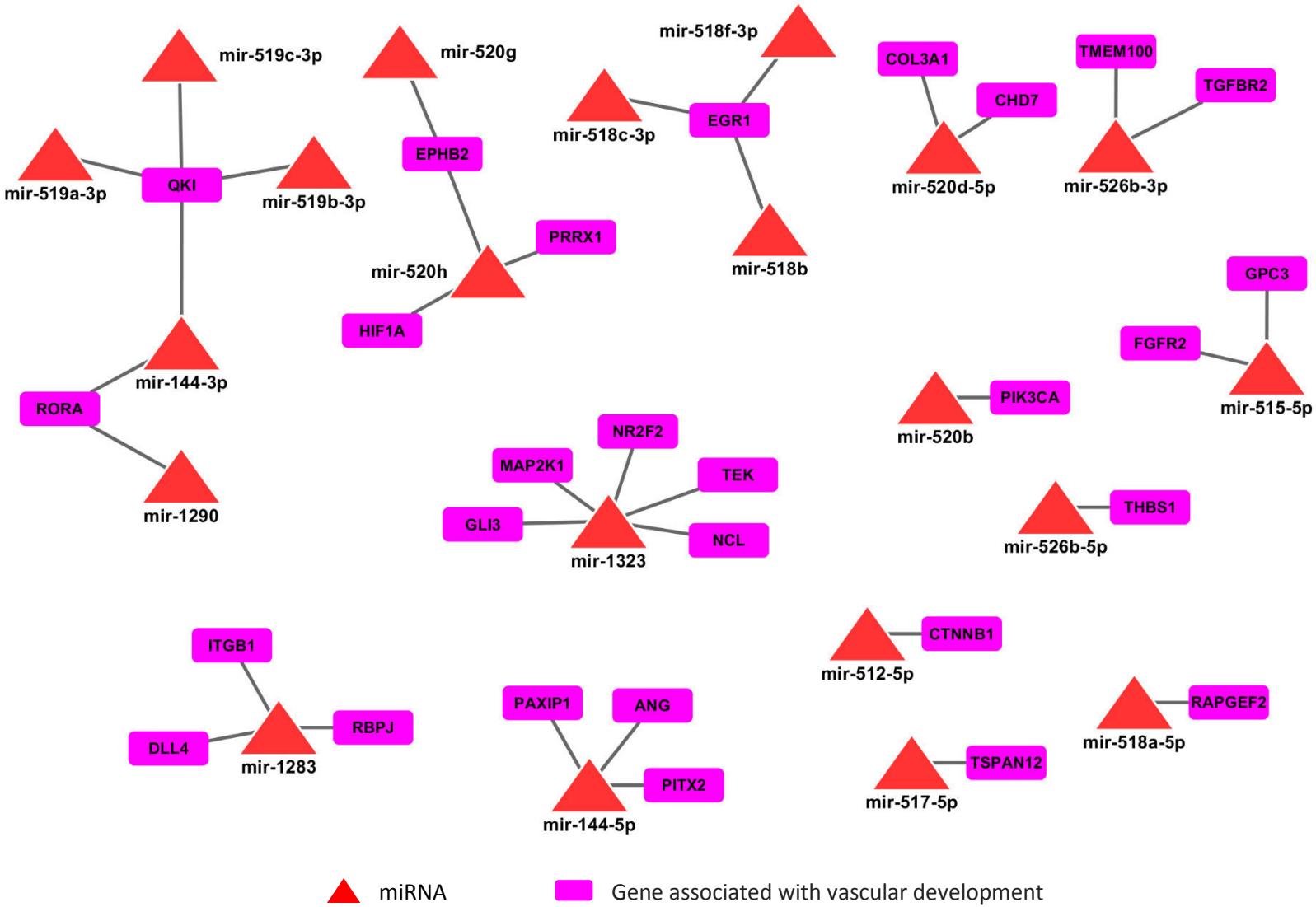


Figure 3: Network miRNA-mRNA-target interaction in vascular development



DISCUSSION:

Based on a meta-analysis study, we identified 21 miRNAs that regulate 23 genes with roles in vascular development. Notably, seven miRNAs (miR-512, miR-515, miR-517, miR-518, miR-519, miR-520 and miR-526) are all mapped on chromosome 19, which is the chromosome with the highest gene density on the human genome[25].

The miRNA family miR-519 (a, b, c) and miR-144-3p regulate *QKI* gene, which is responsible for conserving translational signaling and RNA activation mechanisms, also regulating RNA stability, nuclear retention and transport[26, 27]. *QKI* plays a role in cellular proliferation control and stress signaling[27]. Over-expression of *QKI* was associated with poor prognosis of patients with oral cavity and lung cancer[26, 27]. It has been recently demonstrated that QKI regulates stemness in the central nervous system, as it was shown that decreased endolysosomal levels due to QKI loss contributed to stemness phenotype maintenance in gliomas[28].

miR-520h regulates *HIF1A*, *PRRX1* and *EPHB2*. *HIF1A* is an important regulator of cell survival following hypoxia and its decreased expression is detected during cancer progression[29, 30]. *PRRX1* over-expression is associated with metastasis and worse prognosis of patients with colon cancer and glioblastoma and better prognosis in breast and lung cancer[31, 32], suggesting that this gene may play different roles in distinct diseases. *EPHB2*, which is regulated by miR-520g and miR-520h, has been associated with invasion and tumor progression, plays an essential role in neural development, cytoskeleton dynamics, guided migration, cell proliferation and angiogenesis. EPHB2 is able to directly activate VEGF2 and VEGF3[33-36].

Over-expression of miR-520d-5p may down-regulate *COL3A1* and *CHD7*. *COL3A1* syntesizes collagen III, a protein expressed in several tissues such as skin, lung, uterus, intestine and vascular tissue. Mutations on this gene were associated with vascular diseases and its over-expression leads to lung fibrosis. *COL3A1* is over-expressed in colorectal cancer[37] and correlated with cellular resistance to chemotherapy drugs[38]. *CHD7* may be useful as a biomarker of treatment response in patients with

early stage resected pancreatic cancer and treated with Gencitabin, as decreased *CHD7* expression increased Gencitabin effects on the tumor[39].

Interestingly, miR-526b-3p regulates *TMEM100*, which plays an important role in apoptosis and angiogenesis[40]. *TMEM100* changes were correlated with clinical stage in lung adenocarcinoma and hepatocellular carcinoma[40, 41]. It has been shown that miR-526b-5p regulates *THBS1*, which is able to inhibit tumor cell growth, cell migration and neovascularization[42, 43]. Decreased *THBS1* expression was able to inhibit tumor development and metastasis in laryngeal squamous cell carcinoma[42, 43]. Other angiogenesis-related genes, such as *FGFR2* and *TSPAN12* were identified. *FGFR2* is regulated by miR-515-5p and directly acts on capillary and lymphatic vessel formation, which are essential processes for tumor progression, invasion and metastasis[44]. *TSPAN12* is regulated by miR-517-5p and it has been shown that this gene has important roles in cell growth, invasion and metastasis, also playing a role in *TP53* inactivation[45, 46].

miR-144-5p was identified as a regulator of *PAXIP1* and *ANG*. *PAXIP1* is responsible for DNA damage response and genome stability maintenance[47]. *ANG* is one of the main genes that induce neovascularization and angiogenesis upon angiogenic factor stimuli by VEGF, FGF1 and FGF2[48]. Among other genes, *TEK*, which is regulated by miR-1323, is deregulated in acute mieloblastic leukemia[49, 50] and harbors exon 17 mutations in vascular tumors and vascular malformations[50]. *NR2F2* and *GLI3*, also regulated by miR-1323, regulate angiogenesis and may play a role in metastasis. *NR2F2* expression is altered in several cancer types, such as breast, prostate, colon, pancreas, lung and ovarian carcinomas[51]. In addition, *GLI3* has important roles in angiogenesis and neovascularization, mainly in ischemic tissues[52] and its over-expression stimulates expression of other pro-angiogenic factors, promoting neovascularization[52, 53]. In addition, miR-1283 regulates *DLL4*, which is essential for vascular development[54] consisting a specific vascular factor that cooperates with VEGF in angiogenesis control and tumor progression[54, 55].

Target genes identified have important roles in tumorigenesis, since they regulate cell growth, invasion, metastasis and have been associated with chemotherapy response. miRNAs identified herein, in particular miR-1322, miR-520h, miR-1283 and miR-144-5p regulate genes involved in mechanisms of vascular development and tumorigenesis. Studies such as this may contribute to better understand the development and progression of AS, as well as to identify novel biomarkers and more precise treatment strategies, impacting patient survival.

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ANEXO – II: Trabalho publicado pelo grupo de tumores vasculares

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RESEARCH ARTICLE Open Access

Integrative meta-analysis identifies microRNA-regulated networks in infantile hemangioma

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Abstract

Background: Hemangioma is a common benign tumor in the childhood; however our knowledge about the molecular mechanisms of hemangioma development and progression are still limited. Currently, microRNAs (miRNAs) have been shown as gene expression regulators with an important role in disease pathogenesis. Our goals were to identify miRNA-mRNA expression networks associated with infantile hemangioma.

Methods: We performed a meta-analysis of previously published gene expression datasets including 98 hemangioma samples. Deregulated genes were further used to identify microRNAs as potential regulators of gene expression in infantile hemangioma. Data were integrated using bioinformatics methods, and genes were mapped in proteins, which were then used to construct protein-protein interaction networks.

Results: Deregulated genes play roles in cell growth and differentiation, cell signaling, angiogenesis and vasculogenesis. Regulatory networks identified included microRNAs miR-9, miR-939 and let-7 family; these microRNAs showed the most number of interactions with deregulated genes in infantile hemangioma, suggesting that they may have an important role in the molecular mechanisms of disease. Additionally, results were used to identify drug-gene interactions and druggable gene categories using Drug-Gene Interaction Database. We show that microRNAs and microRNA-target genes may be useful biomarkers for the development of novel therapeutic strategies for patients with infantile hemangioma.

Conclusions: microRNA-regulated pathways may play a role in infantile hemangioma development and progression and may be potentially useful for future development of novel therapeutic strategies for patients with infantile hemangioma.

Keywords: Infantile hemangioma, MicroRNAs, Gene expression, Protein-protein interaction networks, Molecular pathogenesis, Treatment

Background

Infantile Hemangioma, a common benign tumor in childhood, occurs in 10 % of children, more frequently in prematures and females [1]. It shows a cycle with three phases: an initial proliferative phase (rapid growth during the first year), a plateau and an involution phase (spontaneous regression over 1–8 years) [2, 3]. In a study by Chang et al. [4], growth characteristics were examined in a large number (n = 526) of infantile hemangiomas and the results showed that infantile hemangioma growth occurred mainly in infancy, at a mean age of 3 months. Infantile hemangiomas may be deep or superficial, classified based on the depth of lesions. Deep infantile hemangiomas usually appear later in life and may be associated with a longer growth phase compared to the superficial form. Superficial infantile hemangiomas may be focal or segmental [4]. Segmental lesions are associated with a longer proliferative phase and could require a longer period of treatment [5].

The body area more frequently affected by hemangioma is the head and neck, mainly the face, with an association

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with the embryological development of the face [6]. Available treatment for patients with infantile hemangioma includes the use of corticosteroids and/or surgical resection of the tumor. The standard of care treatment strategy is the use of propranolol hydrochloride, a β-blocker entered as a safer form of treatment for proliferating infantile hemangioma [7, 8]. Although the advances in therapeutic strategies for infantile hemangioma, the main clinical problems are still the lack of reliable parameters able to distinguish proliferative from involuting IH lesions and the diverse response rates of patients to treatment.

Therefore, the identification of genetic and epigenetic alterations in proliferating and involuting infantile hemangioma lesions will likely contribute to better understand the underlying molecular mechanisms of development and progression of this disease, which is a leading cause of morbidity in affected children. Indeed, differences in the expression of genomic biomarkers have been reported in infantile hemangioma; e.g., insulin-like growth factor 2 (IGF-2) was found as highly expressed in proliferative lesions compared to involuting lesions [9].

In infantile hemangioma, neural crest markers (NG2 and nestin), pericytes markers (δ-like kinase, smooth muscle actin, calponin and CD90) and stem cell markers (OCT4, NANOG and SOX2) are frequently overexpressed both at mRNA and protein levels. In addition, pericytes (perivascular cells surrounding microvessels and that are related to the development and regulation of angiogenesis) and the derm of the face are derived from neural crest, suggesting that the neural crest may be involved in disease pathogenesis [10]. Importantly, expression of lymphatic endothelial hyaluronan receptor-1 (LYVE-1) has been reported in kaposiform hemangioendothelioma and tufted angioma [11]. LYVE-1 was detected as strongly expressed in proliferative infantile hemangiomas but not in pyogenic granulomas or intramuscular hemangioma lesions, suggesting an important role of these markers in the biology of infantile hemangioma [12]. microRNAs (miRNAs) play an important role in gene expression regulation and have been demonstrated to play a role in the pathogenesis of several human diseases [13]. miRNAs are small, non-coding RNAs containing ~18–24 nucleotides. They can bind to the 3' and 5'ends of the mRNA, leading, in most cases, to translation inhibition or mRNA degradation [14, 15]. Furthermore, miRNAs are related to important biological processes, such as embryonic development, differentiation, apoptosis, cell proliferation [16–18] and oncogenesis [19–21]. To date, 2588 miRNAs were identified and characterized as to their sequence and function in the human genome (http://www.mirbase.org/cgi-bin/browse.pl?org=hsa) [22–26].

Different mechanisms can lead to deregulated miRNA expression, including genomic alterations, such as DNA gains or amplifications and mutations, epigenomic changes including DNA methylation and defects in miRNA biogenesis, including transcription and processing of miRNAs [27, 28]. miRNAs that are altered by these mechanisms may lead to deregulated gene expression.

The understanding of genetic and epigenetic mechanisms, such as deregulated miRNA and target gene expression, as well as molecular pathways regulated by miRNAs, may contribute for the development of new strategies for diagnosis and treatment of complex human diseases [13, 14, 16]. Currently, it is known that miRNAs and miRNA-target genes may represent useful biomarkers to help improve diagnosis, prognosis and treatment of human diseases, such as cancer [29, 30]. Although some gene expression studies have been previously published [31–37], there are no current data on miRNAs or deregulated protein-protein interaction networks in hemangioma. Such data integration strategy is important to understand the functional significance of deregulated genes, miRNAs and molecular pathways involved in hemangioma development and progression. In addition, miRNAs and their target genes may be clinically applicable as therapeutic targets. Indeed, a systematic integration of data derived from multiple sources may achieve the appropriate statistical power and lead to robust, reproducible and accurate predictions [38].

To the best of our knowledge, there are no studies on global miRNA expression in infantile hemangioma. A recent PubMed search (August 19, 2015) showed only one published study on the involvement of miRNAs in senile hemangioma [39], which reported decreased miR-424 expression and increased levels of CCNE1 and MEK1 proteins, which are targeted by miR-424, in patient samples. This study suggested that abnormal proliferation in senile hemangioma may be regulated, at least in part, by miR-424 [39].

Herein, we performed a comprehensive meta-analysis of gene expression data in infantile hemangioma and identified miRNAs as potential regulators of target genes in these tumors. Gene expression datasets were integrated with miRNAs for the identification of molecular pathways potentially involved in infantile hemangioma development and progression. These data may be clinically valuable to predict which infantile hemangioma lesions may respond and which lesions will be resistant to currently available treatment modalities. Furthermore, these data are useful for the identification of robust biomarkers applicable in the development of novel and better molecularly-targeted treatment strategies in infantile hemangioma.

Methods

Meta-analysis of gene expression data in infantile hemangioma

Meta-analysis study design followed the stages of the PRISMA Statement [40] (Fig. 1). Herein, we performed a meta-analysis of previously published gene expression data in infantile hemangioma, by searching PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Key words used were: *"infantile hemangioma* AND *global gene expression"*, *"infantile hemangioma* AND *gene signature"*, *"infantile hemangioma* AND *microRNAs"*, *"microRNA in infantile hemangioma"*, *"infantile hemangioma* AND *microarray"*, *infantile hemangioma* AND *mRNA expression"*. Meta-analysis searches comprised studies published between the years of 2000–2015. Considering that our searches did not retrieve any records on miRNA studies in infantile hemangioma, we included only gene expression studies in this meta-analysis. Deregulated genes reported in selected studies were further used for bioinformatics prediction of miRNAs as potential regulators of gene expression, as described below.

Inclusion criteria were: gene expression data in primary patient samples of infantile hemangioma or pure cell populations of infantile hemangioma, any subtype of disease, inclusion of normal tissues for comparison, data subjected to independent validation. Exclusion criteria were: non- infantile hemangioma, patients treated before molecular genetic analysis and in vivo model studies.

Identification of miRNAs as potential modulators of deregulated genes in infantile hemangioma

Deregulated genes identified in the meta-analysis were used for bioinformatics prediction of miRNAs as regulators of gene expression. We used microRNA Data Integration Portal, mirDIP [41], a computational tool that integrates several predicted and validated miRNA databases. mirDIP allows searching for genes that are targeted by miRNAs as well as for miRNAs predicted to regulate genes. Additionally, relevant biological pathways for differentially expressed genes were identified using Biological Networks Gene Ontology (BiNGO) tool, application available in Cytoscape v3.1.1 [42]. BiNGO allows recognizing which of Gene Ontology (GO) categories are statistically more represented in a specific set of genes. Protein-protein interaction (PPI) networks were then generated using Metasearch STRING v9.1 [43, 44] and visualization and annotation data of PPI and miRNA-gene interaction networks were generated using Cytoscape v3.1.1 [45, 46]. Furthermore, we identified drug-gene interactions using Drug-Gene Interaction Database (DGIdb), a database and web-interface for identifying known and potential drug-gene relationships. Genes were defined by Entrez Gene and Ensembl and matched with genes from drug-gene interactions and druggable gene categories. Drugs were defined by searching PubChem and then matched with drugs from drug-gene interaction data. Drug-gene interactions were

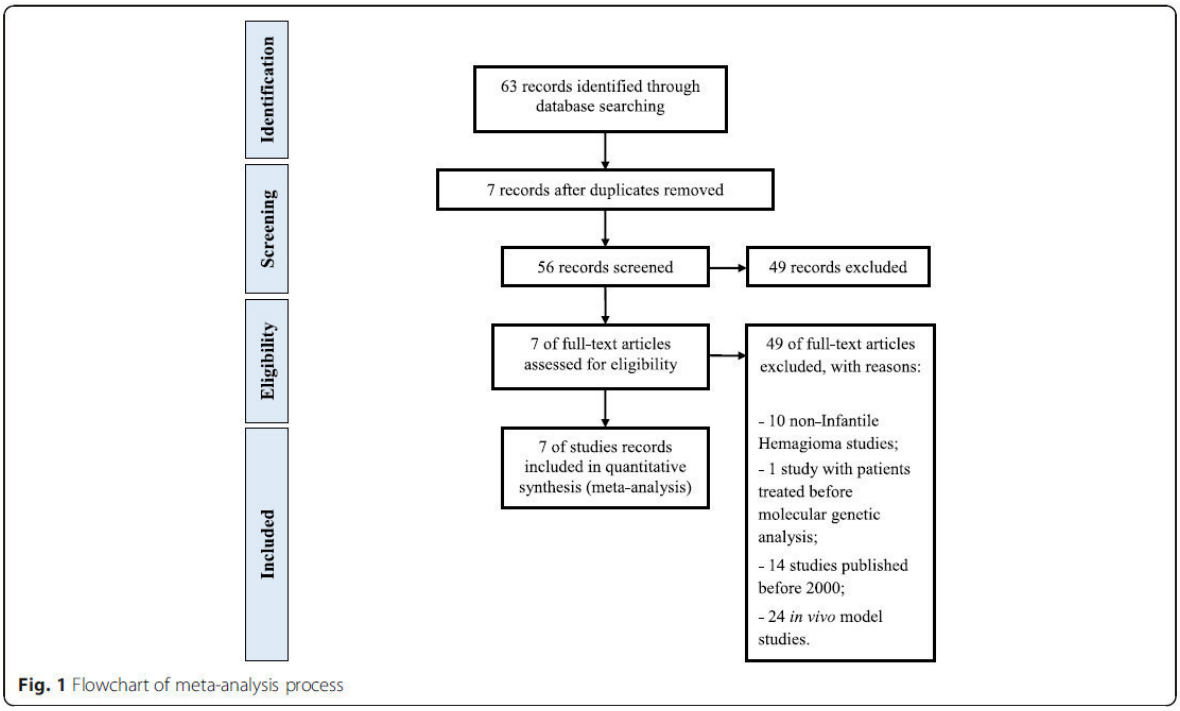

Fig. 1 Flowchart of meta-analysis process

obtained from multiple sources, including DrugBank, Therapeutic Target Database (TTD) and Pharmacogenomics Knowledge Base (PharmGKB) [47].

Results

Protein-protein interaction networks Identified in infantile hemangioma

According to the meta-analysis study design and the inclusion and exclusion criteria (Fig. 1), we selected 7 studies reporting gene expression data in infantile hemangioma [31–37] (Table 1). Altogether, these studies reported a total of 54 differentially expressed genes (36 over- and 18 under-expressed) in 98 patient samples (Table 2).

Enrichment pathways analysis showed information on the biological role of differentially expressed genes in infantile hemangioma. Gene Ontology (GO) categories were divided into 3 hierarchically structured groups, in order to identify proteins encoded by deregulated genes in infantile hemangioma, and associated with biological processes, molecular functions and cellular components. The top 10 statistically significant enriched GO terms are shown in Fig. 2. Integrated, complex interactome analysis for deregulated genes in infantile hemangioma and functional annotations are shown in Fig. 3. A higher number of interactions was identified between genes with roles in vascular and matrix remodeling, hematopoiesis, cell growth and differentiation and transcriptional control. An interaction network between genes and miRNAs predicted to regulate the expression of these specific genes is shown in Fig. 4. The red and green triangles represent up-regulated and down-regulated genes, respectively. Notably, DGIdb data showed that 5 genes were predicted to interact with drugs that have been demonstrated as clinically useful in other tumor types (Table 3).

Discussion

Molecular pathways deregulated in hemangioma

Molecular pathogenesis of infantile hemangioma is not well understood. Advances in methods of global genetic and epigenetic analyses represent an extremely valuable approach for the identification of disease development mechanisms and have the potential to identify biomarkers and/or pathways that may be useful for the development of better treatment approaches, including molecularly-targeted therapies.

Our meta-analysis approach allowed us to integrate mRNA expression data in infantile hemangioma and to predict which miRNAs are potential regulators of gene expression. Among the different mechanisms that can lead to gene expression alterations; miRNA alteration is an important mechanism of over- or under-expression of target genes [48]. Herein, we aimed to utilize data on deregulated genes in infantile hemangioma, in order to predict which miRNAs could potentially regulate these genes, and to construct interaction networks between genes and miRNAs.

Gene enrichment analysis showed that deregulated genes previously reported in infantile hemangioma [31–37] are mainly involved in cell signaling and angiogenesis, functioning in vascular and matrix remodeling, hematopoiesis, cell growth and differentiation and transcriptional control.

Table 1 Description of publicly available studies used in the meta-analysis

Reference ID	Sample size	Gene expression analysis and validation analysis platforms
[31]	6 hemangiomas and 7 normal term placental tissues	U95Av2 GeneChip oligonucleotide microarrays (Affymetrix)
[32]	7 hemangiomas (3 proliferating, 4 involuting) and 3 normal term placental tissues	Human Genome U133 Plus 2.0 (Affymetrix)
[33]	4 pairs of early proliferative stage and spontaneously early involution stage of the same hemangiomas, 11 hemangiomas (6 proliferative and 5 involuting), 5 controls (normal skin) Serum from 69 patients with hemangioma (46 proliferative and 23 involuting), 20 patients with venous malformations and 31 negative controls (children with cheilopalatognathus)	Illumina Human-6 bead chip, QRT-PCR
[34] GSE43742	HEMECs, HDMVECs, 16 infantile hemangioma, 4 normal controls (neonatal foreskin)	Illumina HumanHT-12 V4.0 expression beadchip Immunohistochemistry
[35]	hemSCs, bm-MPCs, HDMECs, cbEPCs and abEPCs	RQ-PCR, Functional assays, Immunofluorescence
[36]	HemSCs, HemECs, HDMECs and MSCs	RQ-PCR, Immunofluorescence
[37]	48 hemangiomas, 9 vascular malformations and vascular tumor specimens, 11 neonatal foreskin controls and HemECs from proliferating hemangioma	GeneFilter GF211 (Invitrogen)

QRT-PCR quantitative reverse-transcription polymerase chain reaction, *HEMECs* infantile hemangioma endothelial cells, *HDMVECs* dermal microvascular endothelial cells, *HemECs* hemangioma-derived endothelial cells, *hemSCs* proliferating hemangioma-derived CD133+ cells, *HDMECs* human dermal microvascular endothelial cells, *cbEPCs* cord blood endothelial progenitor cells, *abEPCs* adult blood endothelial progenitor cells, *bm-MPCs* bone marrow-mesenchymal progenitor cells, *HemSCs* hemangioma-derived stem cells, *MSCs* mesenchymal stem cells

Table 2 List of 54 deregulated genes identified in infantile hemangioma, as reported by the seven studies included in the meta-analysis

Gene symbol	Gene name	Gene function	Gene ID
Over-expressed			
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	chromatin remodelling	6605
RGS5	Regulator of G-protein signaling 5	cell signaling	8490
CTAG2	Cancer/testis antigen 2	autoimmunogenic tumor antigen	30848
LTBP2	Latent transforming growth factor beta binding protein 2	cell growth and differentiation	4053
ANG	Angiogenin, ribonuclease, RNase A family, 5	cell growth and differentiation	283
IGF2	Insulin-like growth factor 2	cell growth and differentiation	3481
TBX2	T-box 2	transcription factor	6909
NOTCH3	Notch 3	cell fate and signalling	4854
HSD17B2	Hydroxysteroid (17-beta) dehydrogenase 2	uncharacterized	3294
TFPI2	Tissue factor pathway inhibitor 2	tumor supressor	7980
GNG11	Guanine nucleotide binding protein (G protein), gamma 11	cell signaling	2791
NID1	Nidogen 1	cell interactions	4811
COL4A1	Collagen, type IV, alpha 1	basement membrane/metabolism	1282
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	cell growth and differentiation	3791
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32)	immunocomplex phagocytosis/antibody production regulation	2213
PLAGL1	Pleiomorphic adenoma gene-like 1	tumor supressor	5325
DLK1	Delta-like 1 homolog (Drosophila)	cell growth and differentiation	8788
JAM3	Junctional adhesion molecule 3	cell adhesion	83700
NID2	Nidogen 2 (osteonidogen)	cell adhesion	22795
MEOX2	Mesenchyme homeobox 2	myogenesis regulation	4223
GABRE	Gamma-aminobutyric acid (GABA) A receptor, epsilon	synaptic transmission	2564
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	cell adhesion	634
BET1	Bet1 golgi vesicular membrane trafficking protein	vesicular transport	10282
MXRA5	Matrix-remodelling associated 5	matrix remodelling	25878
IGFBP7	Insulin-like growth factor binding protein 7	cell growth and differentiation	3490
NETO2	Neuropilin (NRP) and tolloid (TLL)-like 2	cell signaling	81831
BAI3	Brain-specific angiogenesis inhibitor 3	angiogenesis	577
PLXDC1	Plexin domain containing 1	uncharacterized	57125
JAG1	Jagged 1	hematopoiesis	182
EDNRA	Endothelin receptor type A	cell signaling	1909
ICAM2	Intercellular adhesion molecule 2	cell adhesion	3384
NOTCH4	Notch 4	cell fate	4855
STAB1	Stabilin 1	cell growth and differentiation	23166
EPHB3	EPH receptor B3	cell signaling	2049
LPHN1	Latrophilin 1	cell adhesion/signal transduction	22859
NPR1	Natriuretic peptide receptor 1	cell signaling	4881
Under-expressed			
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	cell signaling	2861
IGFBP3	Insulin-like growth factor binding protein 3	cell growth and differentiation	3486
FLT1	Fms-related tyrosine kinase 1	cell growth and differentiation	2321

Table 2 List of 54 deregulated genes identified in infantile hemangioma, as reported by the seven studies included in the meta-analysis *(Continued)*

PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	cell growth and differentiation	5156
TGFBR3	Transforming growth factor, beta receptor III	cell growth and differentiation	7049
LPAR1	Lysophosphatidic acid receptor 1	cell growth and differentiation	1902
IGFBP5	Insulin-like growth factor binding protein 5	cell growth and differentiation	3488
EDNRB	Endothelin receptor type B	cell signaling	1910
PDGFC	Platelet derived growth factor C	cell growth and differentiation	56034
BMP4	Bone morphogenetic protein 4	cell growth and differentiation	652
ANGPTL1	Angiopoietin-like 1	cell growth and differentiation	9068
VCAM1	Vascular cell adhesion molecule 1	cell adhesion	7412
BMP5	bone morphogenetic protein 5	cell growth and differentiation	653
IGF1R	Insulin-like growth factor 1 receptor	cell growth and differentiation	3480
ANGPT2	Angiopoietin 2	vascular remodeling	285
ANTXR1	Anthrax toxin receptor 1	cell signaling	84168
CLDN11	Claudin 11	cell adhesion	5010
KISS1	KiSS-1 metastasis-suppressor	cell adhesion	3814

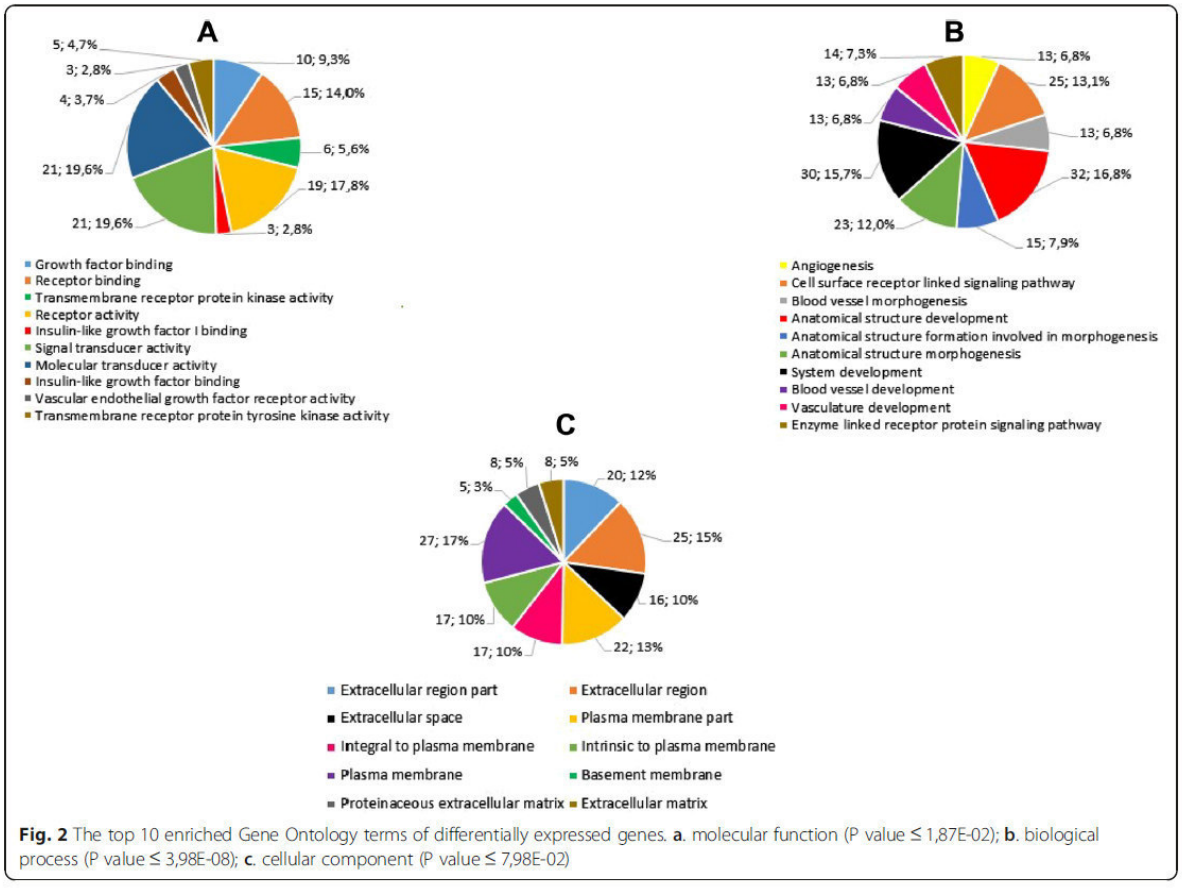

Fig. 2 The top 10 enriched Gene Ontology terms of differentially expressed genes. **a**. molecular function (P value ≤ 1,87E-02); **b**. biological process (P value ≤ 3,98E-08); **c**. cellular component (P value ≤ 7,98E-02)

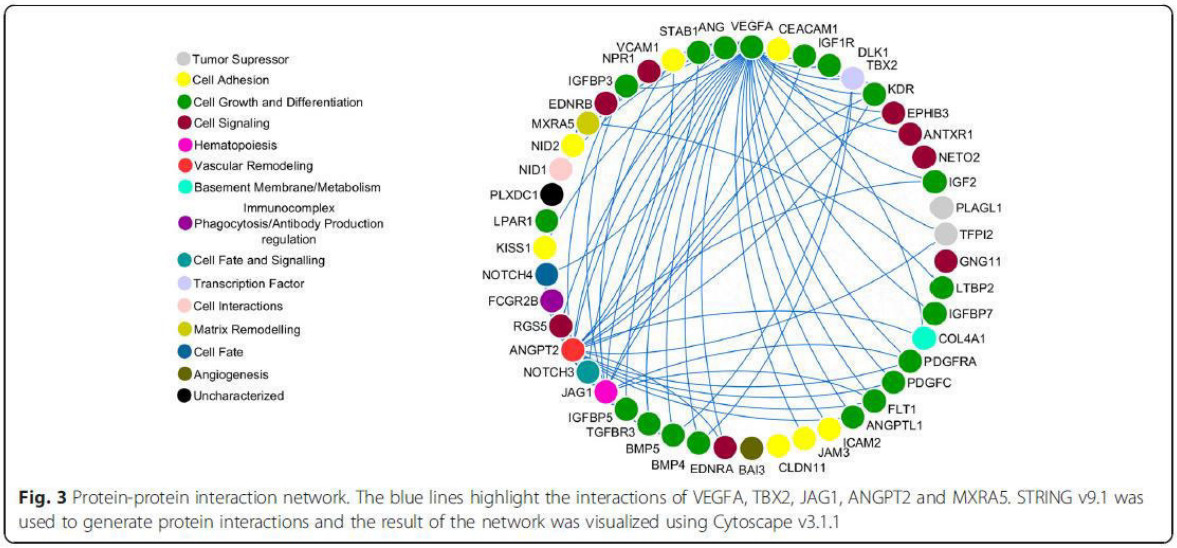

Fig. 3 Protein-protein interaction network. The blue lines highlight the interactions of VEGFA, TBX2, JAG1, ANGPT2 and MXRA5. STRING v9.1 was used to generate protein interactions and the result of the network was visualized using Cytoscape v3.1.1

It is known that the formation of vascular tumors including infantile hemangioma is partly related to increased expression of angiogenic growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which lead to the development of a disorganized blood vessel mass [49]. Indeed, angiogenesis is mainly regulated by the vascular endothelium [50].

miRNAs control and modulate cell response of vascular endothelium to angiogenic stimuli; for example, miR-126 is a positive regulator of angiogenic signaling and vascular endothelial integrity. Vascular development defects were demonstrated in an in vitro model of miR-126-depleted cells, which did not respond to bFGF and VEGF angiogenic factors [51]. Angiogenic response is also controlled by miRNAs, such as miR-221 and miR-222, which play a role as inhibitors of stem cell factors. Other miRNAs, such as miR-27b and miR-let-7f, play a pro-angiogenic role, since their expression promotes angiogenesis [51]. Notably, miRNA expression in vascular endothelial cells can be modified in response to cellular stimuli or to the microenvironment. For example,

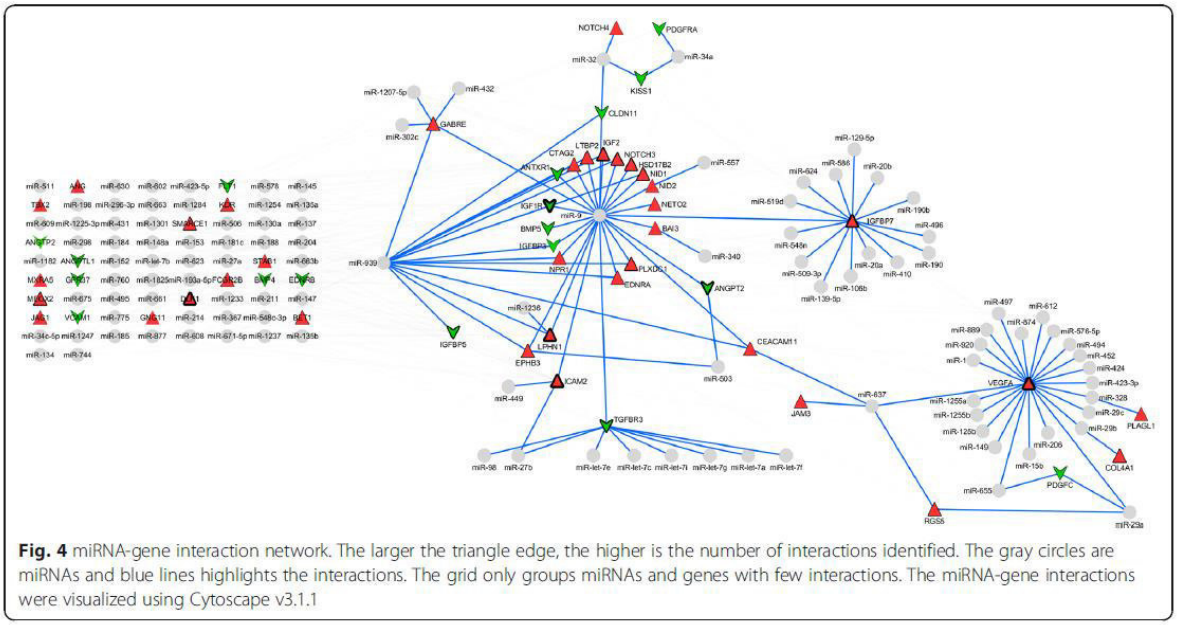

Fig. 4 miRNA-gene interaction network. The larger the triangle edge, the higher is the number of interactions identified. The gray circles are miRNAs and blue lines highlights the interactions. The grid only groups miRNAs and genes with few interactions. The miRNA-gene interactions were visualized using Cytoscape v3.1.1

Table 3 Potential target agents identified based on protein-protein interaction networks of deregulated genes in infantile hemangioma

Gene symbol	Gene name	Selected target agent	Clinical relevance
EDNRA	endothelin receptor type A	Zibotentan, Atrasentan	Colorectal [62], prostate [63] and renal cell [64] carcinomas
IGF1R	insulin-like growth factor 1 receptor	Linsitinib, Ganitumab, Figitumumab, Dalotuzumab, Cixutumumab, Robatumumab	Adrenocortical [65], ovarian [66], non-small cell lung [67], colorectal [68, 69] carcinomas and soft tissue sarcoma [70]
PDGFC	platelet derived growth factor C	Sunitinib	Renal cell carcinoma [71] and breast carcinomas [72]
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	Motesanib, Ramucirumab, Midostaurin, Amuvatinib, Nintedanib, Pazopanib, Tandutinib, Crenolanib, Nilotinib, Masitinib, Sorafenib, Sunitinib, Regorafenib, Dovitinib, Telatinib, Vatalanib, Axitinib, Lenvatinib, Imatinib	Colorectal [73, 74], hepatocellular [75–78], kidney [79], non-small [80] and small cell lung [81], pancreatic [82–84], colon [85], gastrointestinal [86], renal cell [71, 87], breast [72, 88], melanoma [89], thyroid [90] carcinomas and soft tissue sarcoma [91]
VEGFA	vascular endothelial growth factor A	Ziv-aflibercept, Bevacizumab, Sorafenib tosylate, Lenalidomide, Thalidomide, Aflibercept	Colorectal [92, 93] ovarian [94], non-small cell lung [95], hepatocellular [96] carcinomas and multiple myeloma [97]

a hypoxic environment promotes the production of miR-210, which has pro-angiogenic activity. Therefore, increase in pro-angiogenic miRNA expression in endothelial cells may stimulate the production of angiogenic factors, contributing to the process of tumorigenesis [51].

VEGFA plays an important role in vascular development and in pathological angiogenesis and its protein is highly expressed in vessels of proliferating infantile hemangioma [52]. Interestingly, angiogenin protein (ANG), which is required for cell proliferation and is an important mediator of blood vessel formation, regulates VEGFA expression [53]. Although VEGFA was not identified among the deregulated genes reported in the studies used for meta-analysis, VEGFA is shown in the PPI and miRNA-gene interaction networks, likely due to its important role in angiogenesis and to its indirect interaction with other proteins in the network.

To our knowledge, the only available previously published study on miRNAs in hemangioma identified miR-424 under-expression in senile hemangioma [39]. miR-424 is shown interacting with VEGFA in our miRNA-gene network analysis. miR-424 overexpression has been associated with greater cell motility, decreased cell adhesion and other alterations associated with epithelial-to-mesenchymal transition (EMT) [54]. Notably, this study showed that miR-424 expression levels are increased in primary tumors and decreased in metastasis compared to primary breast tumors and additional functional data suggested that miR-424 may play different roles in the different stages of tumor development and progression [54].

Several genes with roles in vascular and matrix remodeling, hematopoiesis, cell growth and differentiation and transcriptional regulation were shown in the PPI and miRNA-gene interation network. Among these, ANGPT2, MXRA5, JAG1, VEGFA and TBX2 showed a large number of interactions. Interestingly, some of these genes also play roles in cell signaling pathways that have been linked to the pathogenesis of infantile hemangioma [55]; namely, VEGFA in the VEGF/VEGFR pathway, ANGPT2 and ANGPTL1 in the Tie2/Angiopoietin signaling pathway and NOTCH3, NOTCH4 and JAG1 in the Notch pathway. Growth factors and angiopoietins have roles in embryonic development and angiogenesis-dependent diseases and Notch components are involved in modulation of cell fate and differentiation [55].

Herein, miRNA-gene interaction networks generated by integrative meta-analysis showed miRNAs with a large number of interactions (miR-9, miR-939, and let-7 family of miRNAs); these miRNAs are likely acting as main regulators in the network. Notably, miR-9 has been demonstrated as pro-metastatic and suppressor of E-cadherin in breast cancer cells, promoting cell motility and increasing invasive potential of carcinoma cells, besides activating β-catenin signaling, which in turn contributes to high VEGFA expression and consequently to induction of angiogenesis [56]. Increased miR-9 expression levels were also associated with EMT in breast cancer cells and with poor prognosis of patients with breast cancer [57]. In ovarian cancer, miR-939 plays an important role in the progression and regulation of cell growth and cell cycle; it has been demonstrated that ES-2 cells transfected with miR-939 mimic show APC2 decreased expression, suggesting that APC2 may be a target of miR-939 [58].

A recent meta-analysis suggested that the let-7 family of miRNAs are potential biomarkers for tumor grade prediction in breast cancer [59] as well as in other cancers, since let-7 family is highly conserved across species [60].

Among other miRNAs with a significant number of interactions, miR-637 links to main networks through

direct interactions with VEGFA and CEACAM; the latter interacting with miR-9 and miR-939. Functional data has shown that miR-637 is one of the effective regulators of HER2 signaling; in HER2-positive trastuzumab non-responsive cell lines, miR-637 was efficient to inhibit breast cancer cell growth [61].

Conclusion

Herein, we identified several interconnected genes and miRNAs as potential regulators of gene expression. Such miRNAs and genes may play important roles in the development and progression of infantile hemangioma. Additionally, these molecules show potential to be targets for drugs that may be clinically useful in the development of new therapies for infants and children affected by this tumor. Data generated herein may be used for validation of expression of miRNAs and genes regulated by miRNAs in infantile hemangioma. Validation analysis in a large representative cohort of primary untreated patient samples is necessary in order to establish robust biomarkers for prediction of treatment response and for the development of better treatment modalities.

Abbreviations

miRNA or miR: microRNA; miRDIP: microRNA data integration portal; BiNGO: biological networks gene ontology; GO: gene ontology; PPI: protein-protein interaction; DGIdb: drug-gene interaction database; TTD: therapeutic target database; PharmaGKB: pharmacogenomics knowledge base; bFGF: basic fibroblast growth factor; VEGF: vascular endothelial growth factor; ANG: angiogenin protein; EMT: epithelial-to-mesenchymal transition; QRT-PCR: quantitative reverse-transcription polymerase chain reaction; HEMECs: Infantile hemangioma endothelial cells; HDMVECs: dermal microvascular endothelial cells; HemECs: hemangioma-derived endothelial cells; hemSCs: proliferating hemangioma-derived CD133+ cells; HDMECs: human dermal microvascular endothelial cells; cbEPCs: cord blood endothelial progenitor cells; abEPCs: adult blood endothelial progenitor cells; bm-MPCs: bone marrow-mesenchymal progenitor cells; HemSCs: hemangioma-derived stem cells; MSCs: mesenchymal stem cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PPR designed the study, helped analyze the data and wrote the manuscript. NB helped with study design, performed the meta-analysis and wrote the manuscript. FES performed bioinformatics data analyses and generated figures. LMSP, RM and WBY helped with study design and with writing of the manuscript. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Authors' information

Since 2002, WBY and RM coordinate the Vascular Malformations laboratory at a university-based, teaching hospital at the Faculty of Medicine, São Paulo State University, Botucatu, SP, Brazil. The oncopediatric service of our institution (LMSP) work closely with the vascular malformations laboratory. The clinical group (WBY, RM, LMSP) develops multiple quality of life projects for patients with vascular malformations and tumors. Our research group (NB, FES and PPR) is investigating the molecular basis of infantile hemangioma with the main goal of identifying and validating genetic and epigenetic pathways involved in disease development and progression. Such pathways may represent clinically useful biomarkers for prediction of treatment response, or as a basis for the development of novel therapeutic targets for treatment of children affected by hemangioma.

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