DANIELLE RIBEIRO LUCON

“PERFIL DE microRNAs DIFERENCIALMENTE EXPRESSOS EM MEDULOBLASTOMA E ANENCEFALIA”

CAMPINAS
2013

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE CIÊNCIAS MÉDICAS

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"PERFIL DE microRNAs DIFERENCIALMENTE EXPRESSOS EM MEDULOBLASTOMA E ANENCEFALIA"

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Médicas da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutora em Ciências Médicas, área de Concentração Ciências Biomédicas.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA DANIELLE RIBEIRO LUCON E ORIENTADO PELO PROF. DR.JOSE ANDRES YUNES.

_____
Assinatura do Orientador

CAMPINAS
2013

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Médicas
Maristella Soares dos Santos - CRB 8/8402

L964p

Lucon, Danielle Ribeiro, 1977-
Perfil de microRNAs diferencialmente expressos em meduloblastoma e anencefalia / Danielle Ribeiro Lucon. – Campinas, SP : [s.n.], 2013.

Orientador: Jose Andres Yunes.
Coorientadores: Denise Pontes Cavalcanti e Claudia Vianna Maurer Morelli.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. MicroRNAs. 2. Meduloblastoma. 3. Anencefalia. I. Yunes, Jose Andres. II. Cavalcanti, Denise Pontes, 1957-. III. Maurer-Morelli, Claudia Vianna, 1966-. IV. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. V. Título.

Informações para Biblioteca Digital

Título em outro idioma: Differential expression profile of microRNA in medulloblastoma and anencephaly
Palavras-chave em inglês:
MicroRNAs
Medulloblastoma
Anencephaly
Área de concentração: Genética Médica
Titulação: Doutora em Ciências Médicas
Banca examinadora:
Jose Andres Yunes [Orientador]
Carlos Alberto Scrideli
Jörge Kobarg
Vera Lúcia Gil da Silva Lopes
Gustavo Jacob Lourenço
Data de defesa: 31-07-2013
Programa de Pós-Graduação: Ciências Médicas

BANCA EXAMINADORA DA DEFESA DE DOUTORADO

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Data: 31 de julho de 2013

DEDICATÓRIA

Aos meus Pais Sérgio e Ermelinda,
pelo incentivo irrestrito e por
acreditarem na minha luta.

Aos meus irmãos Denise e Júnior,
pelo carinho e compreensão.

Para as pessoas que caminharam
comigo, meu Amor e o meu
Muito Obrigada.

A Deus, pela dádiva de tornar realidade um sonho que se tornou tanto meu quanto de minha família e amigos que trilharam comigo durante esse período.

Aos meus pais e irmãos, que sorriram e sofreram comigo nessa etapa da minha vida, a minha eterna gratidão e o meu amor incondicional.

Aos colegas de laboratório do Centro Infantil Boldrini e Biologia Molecular da FCM pelo apoio, carinho e ombro amigo. Com certeza vocês foram importantes na minha trajetória.

As Amigas Simoni e Priscila pelo apoio, carinho e palavra amiga. Com certeza vocês me ajudaram a enfrentar os obstáculos do desenvolvimento da tese e estarão presentes para sempre.

A equipe da Anatomia Patológica da FCM. Ao técnico Wanderley e residentes da Neurologia que estiveram presentes para que esse trabalho fosse realizado, o meu Muito Obrigado e Todo meu Carinho.

Aos colegas Tiago e Maria Eugenia do LNLS e a bioinformata Cristiane da FCM por me ajudarem com os dados de expressão do chip e as figuras do IPA. A Dra Izilda Cardinalli pela análise histológica e Dra. Silvia Brandalise do Centro Infantil Boldrini pelos dados clínicos que contribuíram para o conhecimento e enriquecimento do trabalho.

A equipe do laboratório da Universidade de Bonn, Alemanha. Dani, Anne, Barbara, Claudia e Kevin pelo carinho que me receberam, me acolheram e pela amizade que ficará guardada para sempre. Ao supervisor Rogério e a chefe Dillo por tornarem meu sonho realidade. Guardarei para sempre toda a lembrança de seis meses junto com vocês.

Ao orientador Andrés pela amizade, atenção e orientação. Caminhamos juntos por quatro anos e passamos por momentos doces e amargos, mas tudo foi e será um grande aprendizado para minha vida pessoal e carreira profissional.

As co-orientadoras, Claudia e Denise, pela amizade, carinho e co-orientação deste trabalho.

A CAPES pela bolsa concedida no Brasil e Alemanha.

A todos, que estiveram presentes nessa luta, deixo aqui um pouco de mim e levo muito de vocês comigo. Meu Amor e Muito Obrigada!

Epígrafe

DIANTE DE TUDO,

VOCE TAMBÉM

JÁ APRENDEU,

QUE SE DÁ SEMPRE UM

PASSO APÓS O OUTRO.

EMBORA SE SAIBA QUE

A VIDA É CURTA

E O TEMPO É LONGO...

Sérgio Lucon

AN	Anencefalia
CV	Cérebro-vascular
DTN	Defeito do Tubo Neural
MB	Meduloblastoma
miRNA	microRNA
mRNA	RNA mensageiro
nt	Nucleotídeo
OMS	Organização Mundial da Saúde
RN	Recém-nascido
RNA	Ácido ribonucléico
RT-qPCR	PCR quantitativo em tempo real
SHH	Sonic Hedgehog
SNC	Sistema nervoso central

Crianças com anomalias congênitas possuem um risco significativamente aumentado para desenvolver algum tipo de câncer. Anomalias do sistema nervoso central (SNC) estão associadas à maior incidência de tumores também do SNC. A comparação entre tecido 'anômalo', tecido tumoral e tecido normal pode ajudar na identificação dos genes mais importantes na carcinogênese. microRNAs (miRNAs) são pequenas moléculas que atuam negativamente na expressão gênica e têm papel importante no controle do desenvolvimento, diferenciação, apoptose e proliferação celular. Vários miRNAs são expressos no SNC e são conhecidos por serem dinamicamente regulados durante o neurodesenvolvimento. Recentemente, miRNAs foram associados com tumores e malformações do SNC, como o meduloblastoma (MB) e a anencefalia (AN), respectivamente. Ambos tecidos são de origem neuroectodérmica e embrionária. Neste projeto foram estudados os miRNAs diferencialmente expressos no tecido tumoral de MB desmoplástico de pacientes jovens (1-2 anos) *versus* cerebelo e no tecido cérebro-vascular de fetos com AN *versus* córtex frontal. Os controles foram obtidos de tecidos normais provenientes de autópsias de fetos e recém-nascidos. As vias gênica-metabólicas importantes na carcinogênese e morfogênese do perfil de miRNAs de MB e AN foram analisados *in silico*. No primeiro trabalho, apresentado no segundo capítulo, investigamos o perfil de miRNAs de MB que foi predominantemente baixo expresso (64/84 miRNAs) e regulam genes envolvidos com desenvolvimento e/ou câncer. Muitos dos miRNAs baixo expressos (32/64) foram localizados no lócus cromossômico 14q32 (miRNA 14q32). Possíveis mecanismos da baixa expressão de miRNA 14q32 foram investigados por bancos de dados públicos disponíveis. A expressão do gene receptor de estrógeno gama (*ESRRG*), um regulador transcricional positivo de alguns miRNAs 14q32, foi encontrada baixo expresso em MB desmoplástico. miR-129-5p (11p11.2/7q32.1), miR-206 (6p12.2) e miR-323-3p (14q32.2) foram escolhidos para estudos funcionais em células DAOY. A super expressão do miR-129-5p usando miRNA *mimics* diminuiu a proliferação das células DAOY. No segundo trabalho, apresentado no terceiro capítulo, analisamos o perfil de expressão de miRNAs em AN que foi predominantemente super expressos (34/52 miRNAs) e regulam genes envolvidos com defeito do tubo neural e/ou câncer. Dentre estes miRNAs estão os miR-21, 34a/c, 182, 500 cluster. miRNAs importantes no desenvolvimento do cérebro (miR-124, 128, 137, 139) foram encontrados baixo expressos nas amostras de AN. A

prospecção dos genes alvos destes miRNAs mostrou que eles desempenham um papel importante durante o desenvolvimento e a diferenciação neural. Por fim, nós comparamos os miRNAs diferencialmente expressos entre MB e AN e identificamos 19 miRNAs em comum (baixo expressos: miR-124, 128, 129*, 129-5p, 138, 138-1*, 138-2*, 139-3p, 490-5p, 650, 770-5p; super expressos: miR-199a-3p, 199b-3p, 199a-5p, 21, 214, 214*, 34a, 574-3p). A maioria destes miRNAs em comum encontrados nas duas patologias fazem parte dos miRNAs mais descritos em câncer e/ou são importantes no desenvolvimento do cérebro. O fato destes miRNAs estarem desregulados em duas condições diferentes (MB e AN) faz pensar que sejam funcionalmente relevantes nestas patologias. Nossos resultados indicam a correlação de assinatura de miRNAs com cada amostra destacando a heterogeneidade molecular e complexidade na sinalização celular regulada por miRNAs, e também revela que o câncer foi a via de sinalização predominante em MB e AN.

Palavras-chave: microRNA, meduloblastoma, anencefalia.

Children with birth defects have a significantly increased risk for developing some type of cancer. Anomalies of central nervous system (CNS) are associated with increased incidence of tumours also from CNS. The comparison between tissue 'anomalous', tumor tissue and normal tissue can help identify genes important in carcinogenesis. microRNAs (miRNAs) are small non-coding RNA molecules that act negatively on gene expression and play an important role in controlling development, differentiation, apoptosis and cell proliferation. Many miRNAs are expressed in CNS and are known to be dynamically regulated in neurodevelopment. Recently, miRNAs have been associated with CNS tumors and malformations, as meduloblastoma (MB) and anencephaly (AN), respectively. Both tissues are from neuroectodermal and embryonic origins. In this project, we studied the miRNAs differential expressed in tumor tissue of desmoplastic MB of young patients (1-2 years) versus cerebellum and cerebrovascular tissue of fetal with AN versus frontal cortex. The normal tissues were obtained from fetal and newborn autopsy. The gene-metabolic pathways important in carcinogenesis and morphogenesis of miRNAs profile of MB and AN were analyzed in silico. In second chapter, we investigated the MB miRNAs profile that were predominantly downregulated (64/84 miRNAs) and regulates genes involved in development and/or cancer. Most downregulated miRNAs (32/64) were found to belong at the 14q32 locus (14q32 miRNA). Possible mechanisms of 14q32 miRNAs downregulation were investigated by the analysis of publicly available gene expression data sets. The expression of estrogen-related receptor-g (*ESRRG*), a reported positive transcriptional regulator of some 14q32 miRNAs, was found downregulated in desmoplastic MB. miR-129-5p (11p11.2/7q32.1), miR-206 (6p12.2), and miR-323-3p (14q32.2), were chosen for functional studies in DAOY cells. Overexpression of miR-129-5p using mimics decreased DAOY proliferation. In third chapter we investigated the AN miRNAs profile that were predominantly upregulated (34/52 miRNAs) and regulates genes involved with tube neural defects (DTN) and/or cancer. Between these miRNAs are the miR-21, 34a/c, 182, 500 cluster. miRNAs important in brain development (miR-124, 128, 137, 139) were found downregulated in AN samples. Prospecting for target genes of these miRNAs showed that they play an important role during development

and neuronal differentiation. Finally, we compare the miRNAs differential expressed between MB and AN and identified 19 miRNAs in common (underexpression: miR-124, 128, 129 *, 129-5p, 138, 138-1 *, 138-2 *, 139 - 3p, 490-5p, 650, 770-5p; overexpression: miR-199a-3p, 3p-199b, 199a-5p, 21, 214, 214 *, 34a, 574-3p). Most common miRNAs found in MB and AN are known to be involved in cancer and/or are important in brain development. The fact that these miRNAs are deregulated in two different conditions (MB and AN) makes one think that they are functionally relevant in these pathologies. Our results indicate the correlation of miRNAs signature with each sample highlighting the molecular heterogeneity and cellular signaling complexity regulated by miRNAs, and also reveals that the cancer was the predominant signaling pathway in MB and AN.

Keywords: microRNA, meduloblastoma, anencephaly.

CAPÍTULO 1

Revisão Bibliográfica

1.1. Introdução

A relação entre câncer infantil e a presença de anomalias congênitas tem sido relatada por vários estudos (Altmann *et al*., 1998; Mehes *et al*., 1985; Merks *et al*., 2005; Agha *et al*., 2005). Crianças com anomalias congênitas possuem um risco seis vezes maior de desenvolver algum tipo de câncer no primeiro ano de vida, incluindo leucemia, tumor do sistema nervoso central (SNC), tumor do sistema nervoso periférico (SNP) e sarcomas de tecidos moles (Altmann *et al*., 1998; Agha *et al*., 2005). Em crianças com anomalias do sistema nervoso, o mais frequente tipo de câncer diagnosticado foi também do SNC (77,8%) (Agha *et al*., 2005; Mutafoglu-Uysal *et al*., 2009). O risco significativo encontrado nesses estudos fornece evidência de ligação entre anomalias congênitas e câncer, relação que pode ajudar a compreender os mecanismos moleculares subjacentes à tumorigênese.

O câncer é uma doença de base genética. Durante a progressão neoplásica, uma série de mutações somáticas é acumulada em genes críticos, afetando a regulação do ciclo celular, diferenciação, apoptose e interações célula-célula e célula-matriz. Estima-se que de 8 a 12 defeitos cumulativos em genes potencialmente oncogênicos seriam necessários para desencadear a doença. Dada à frequência relativamente baixa de ocorrência de mutações sequenciais, o câncer leva tempo para se manifestar e por isso é uma doença que acomete pessoas em idade avançada. Nesta linha de raciocínio seria matematicamente impossível que uma criança tivesse câncer. O fato delas terem a doença talvez seja um forte indício da existência de um mecanismo de carcinogênese típico para essa fase do desenvolvimento. Talvez por isso que os tumores da criança e do adolescente apresentam características próprias, diferentes daquelas observadas entre os adultos mais velhos.

O câncer mais comum nas crianças é a leucemia. A ocorrência de translocações cromossômicas envolvendo genes de imunoglobulinas sugere o mecanismo de recombinação VDJ como um dos prováveis defeitos subjacentes ao surgimento das leucemias (Aplan *et al*., 2006; Yin *et al*., 2007). O segundo câncer mais comum é do SNC, sendo o meduloblastoma o segundo tumor cerebral mais freqüente em crianças,

após o astrocitoma. MB são tumores cerebelares malignos e invasivos (OMS grau IV), classificados como tumores neuroectodérmicos (Chaves *et al.*, 2008). A incidência anual de meduloblastoma primário em crianças com menos de 15 anos é de 2,4 em 100.000. A causa do MB ainda não é clara, A criança não sofre interferência da maioria dos fatores que causam alterações nos adultos, como fumo, álcool ou alimentação. Especula-se sobre a origem exata do MB, uma das possibilidades é atribuída a neurônios imaturos remanescentes da camada granulosa externa que sofrem transformação neoplásica, pelo menos em parte dos casos (Katsetos *et al*., 1994).

Alguns miRNAs (conforme descrito adiante no item miRNAs em comum entre anencefalia e meduloblastoma) têm o mesmo perfil de expressão nos tecidos de AN e MB (ambos com tecido de origem neuroectodérmica) em comparação a tecidos normais sugerindo que o mecanismo molecular que origina a malformação do tubo neural pode compartilhar algum ponto com o mecanismo que leva ao câncer do SNC na criança.

O presente projeto visou investigar se o mecanismo entre malformações e tumorigênese do SNC poderia ser uma desregulação dos miRNAs, visto que estas pequenas moléculas têm sido também implicadas no controle do desenvolvimento do SNC.

1.2. Meduloblastoma

O MB está localizado na linha média do cerebelo (Figura 1; Roussel & Hatten, 2011). Nos últimos anos, foram feitos grandes progressos em relação a classificação molecular e estabelecimento de biomarcadores para prognose, mas os mecanismos de iniciação, manutenção e progressão do MB ainda não está claro e sugere vários pontos de discussão (Zhi *et al.,* 2013). Análise integrativa do perfil transcricional do MB sugere a existência de pelo menos quarto subgrupos distintos: Grupo Wingless (Wnt), Grupo Sonic Hedgehog (SHH), Grupo 3 e Grupo 4 (Kool *et al*, 2008; Northcott *et al*, 2010). Novas evidências indicam que as diferentes populações de células precursoras e as suas vias de sinalização celular que regulam o desenvolvimento do cerebelo provalvelmente representam compartimentos distintos do qual surge vários subtipos de MB (Ellison, 2002; Gibson *et al*, 2010; Gilbertson & Ellison, 2008; Schüller *et al*, 2008; Yang *et al*, 2008).

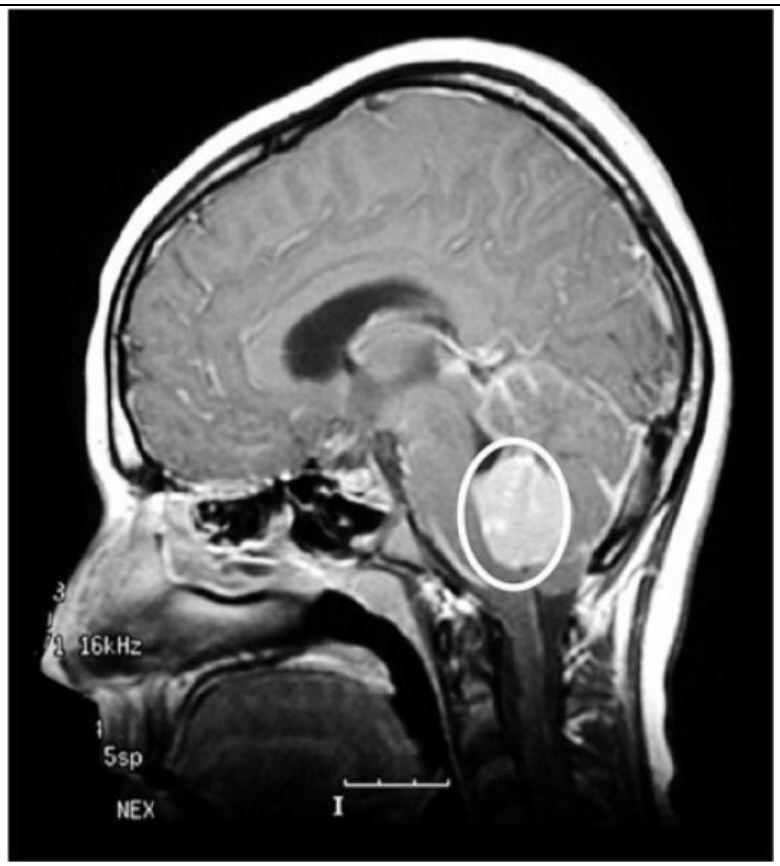


Figura 1. Localização do Meduloblastoma. Ressonância magnética após gadolínio. O círculo representa o tumor (Roussel & Hatten, 2011)

As células-tronco cancerosas do MB podem surgir a partir de células precursoras multipotentes do embrião em desenvolvimento, embora os tipos de mutações que estas células podem ser suscetíveis e as formas da doença para a qual estes tumores podem dar origem permanecem obscuros. Meduloblastomas que se desenvolvem com mutações ativadoras nas vias de WNT e SHH são mutualmente exclusivos e são predominantemente do tipo histológico clássico e desmoplástico, respectivamente. As células tronco da substância branca podem ser células de origem de alguns MB adulto (Figura 2; Gilbertson & Ellison, 2008).

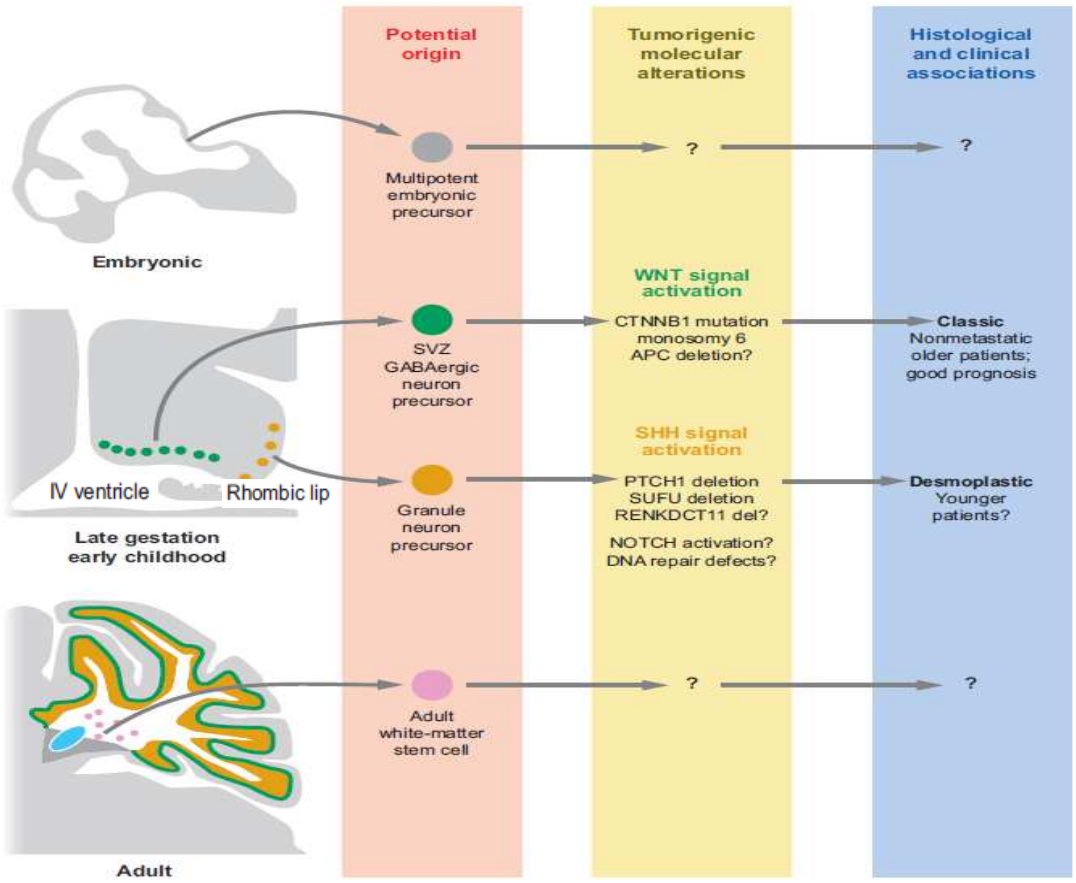


Figura 2. Potencial origem cellular e molecular dos subgrupos de meduloblastoma (Gilbertson & Ellison, 2008).

O perfil de expressão de miRNAs de camundongos e humanos com MB levou à identificação de assinaturas correlacionando com os subgrupos moleculares de MB, o diagnóstico do tumor e da resposta ao tratamento, assim como novos alvos de relevância clínica (Ferreti *et al*, 2009; Northcott *et al*, 2009; Cho *et al*, 2011; Fernandez *et al*, 2009).

1.3. Anencefalia

No transcurso do desenvolvimento, a placa neural se invagina formando uma calha neural que acaba por se fechar completamente originando o tubo neural. O tubo neural dará origem ao encéfalo e a medula espinhal, componentes do SNC. O neuroectoderma contribui para este processo de dobramento alterando ativamente a forma das células que o compõem. A intensa proliferação que ocorre no tubo neural vai, aos poucos, restringindo os destinos fenotípicos de cada uma das células-filhas, e elas se

transformam de células-tronco a progenitores neurais e, finalmente, precursores neuronais ou gliais (Carvalho & Collares-Buzato, 2005*;* Kleihues *et al*., 2002). Transtornos na proliferação, migração e maturação neuronal do SNC resultante de causas genéticas e/ou ambientais podem levar a anomalias congênitas letais e não letais. Por exemplo, a falha do fechamento do tubo neural durante o desenvolvimento fetal é uma das mais comuns anomalias congênitas com a prevalência de 1:1000 recém-nascidos (Castilla & Orioli, 1985; Brunoni, 1986). Embora a maioria dos DTNs apareça de forma isolada, esses defeitos podem fazer parte de quadros sindrômicos ou estarem associados a outros defeitos sem constituir síndrome conhecida. A anencefalia é a forma mais letal e grave de DTN, caracterizada pela ausência completa ou parcial da calota craniana com exposição e degeneração do tecido nervoso.

Segundo Van Allen *et al.* (1993), há fortes evidências que o tubo neural se feche a partir de cinco pontos iniciais e a conclusão do fechamento dar-se-ia também de modo semelhante ao de um zíper. O primeiro sítio de fechamento se iniciaria na região cervical e progrediria nas direções cranial e caudal, formando caudalmente o neurotubo torácico e prossegue rostralmente até abaixo das fossetas ópticas. O segundo sítio de fechamento teria início na junção entre o prosencéfalo e o mesencéfalo e também seguiria em ambas direções, caudal e cranial. O terceiro sítio de fechamento se inicia na parte mais rostral do término das pregas neurais, adjacente ao estomodeu, prosseguindo caudalmente até encontrar o segundo sítio de fechamento, formando o verdadeiro neuróporo anterior. O quarto sítio de fechamento começa na extremidade caudal do rombencéfalo e é unidirecional, prosseguindo rostralmente em direção ao sítio dois. O fechamento do quinto sítio, descrito somente em humanos, tem início na extremidade caudal do tubo neural prosseguindo da região da segunda vértebra sacral à região da segunda vértebra lombar unidirecionalmente (Figura 3). Uma falha do fechamento no sítio 2 daria origem ao merocrânio; nos sítios 2 e 4 levaria ao holocrânio; nos sítios 2, 4 e 1 resultaria em cranioraquisquise; e no sítio 3 corresponderia à faciocranioraquisquize (Van Allen *et al.*, 1993; Urioste & Rosa, 1998). A deficiência do ácido fólico, que é associada a uma maior incidência de DTN, afeta sítios específicos de fechamento (sítios 2, 4, e 1 caudal) (Van Allen *et al.*, 1993).

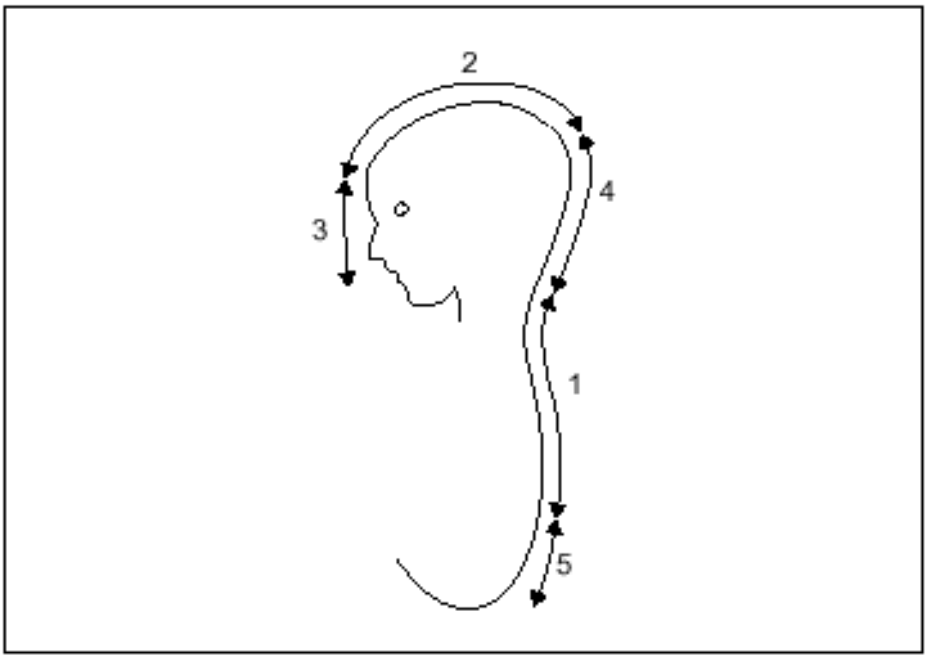


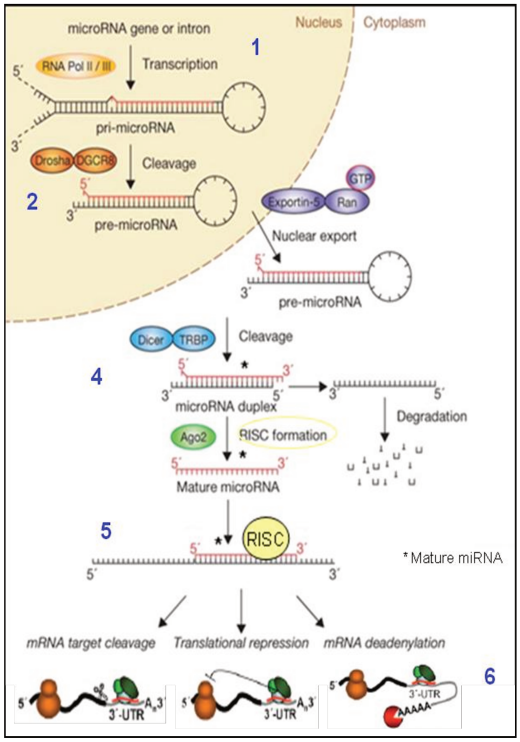
Figura 3. Esquema dos sítios de fechamento de acordo com Van Allen *et al.* (1993).

Uma série de estudos de perfis de miRNA têm mostrado a expressão de miRNAs durante a diferenciação de células-tronco neurais e no desenvolvimento morfológico do cérebro de mamíferos (Croce & Calin, 2005; Miska *et al*., 2004). Estas pesquisas sugerem que os miRNAs tem um papel importante nesses processos. No entanto, há somente um estudo do perfil de miRNAs de anencefalia humana (Zhang *et al.,* 2010), sendo necessário estudos complementares para um melhor conhecimento do papel de miRNAs em defeito do tubo neural.

1.4. microRNAs

Os miRNAs são moléculas de RNA de fita simples com aproximadamente 22 nucleotídeos, não codificadores de proteínas e que regulam de modo sequência-específica a expressão gênica por inibir a tradução ou ainda por degradar o RNA mensageiro (mRNA) do gene alvo (Sood *et al*., 2006; Guo *et al*., 2010).

O processamento do miRNA começa com a transcrição do gene miRNA pela RNA polymerase II ou III resultando em miRNA primário ou pri-miRNA. O pri-miRNA é clivado pelo complex DROSHA resultando em miRNA precursor ou pre-miRNA no núcleo. O pre-miRNA é exportado do núcleo para o citoplasma pela expostina-5. Este pre-miRNA é clivado pelo complex RISC que facilita a interação entre miRNA e mRNA e, entretanto, regula a expressão de gene pela clivagem, repressão traducional ou deadenilação do mRNA (Figura 4; Winter *et al.*, 2009).



1. Transcrição
2. Clivagem por Drosha
3. Exporte para o citoplasma
4. Processo por Dicer
5. Seleção da fita por RISC (RNA-induced silencing complex)
6. Expressão do gene

Figura 4. Biogênese do miRNA (Winter et al., 2009 – adaptado)

O controle pós-transcricional do mRNA pelo miRNA é um mecanismo importante, uma parte da regulação da expressão gênica pode ser influenciada por uma diferença na taxa de transcrição como consequência do encurtamento da cauda poli-A ou ainda pela clivagem através da RISC, inclusive em células humanas (Lau e Hudson, 2010; Pillai *et al.*, 2004; Zhang *et al.*, 2007;). miRNAs também podem se ligar a proteínas Argonauta (Ago) e inibir a tradução ou promover a degradação de mRNA alvos, por exemplo, o miRNA let-7 humano é conhecido por inibir a tradução do mRNA

alvo (Kiriakidou *et al*., 2007), e encontrado inclusive em linhagem celular neuronal de humanos (Nelson *et al.*, 2004).

Presume-se que os miRNAs humanos regulam numerosos mRNA alvos (Guo *et al*., 2010; John *et al*. 2004; Krek *et al*. 2005; Kiriakidou *et al*. 2004; Lewis *et al*. 2003, 2005; Lim *et al*. 2005). Estimativas recentes sugerem que um terço de mRNA humanos é regulado por miRNA (Lewis *et al*. 2005). Alguns estudos sugerem um importante papel dos miRNAs no SNC. De fato, foram encontrados aproximadamente 1000 miRNAs atuando no SNC em diversas vias regulatórias, incluindo o controle do desenvolvimento, diferenciação e proliferação celular e apoptose (Cohen *et al*., 2010).

Análise do perfil de miRNAs tem mostrado a dinâmica da expressão dessas moléculas durante a diferenciação neural de células tronco (Krichevsky *et al*., 2003; Smirnova *et al*., 2005; Lau e Hudson, 2010; Hirabayashi e Gotoh, 2010), em anomalia congênita que resulta da falha do fechamento do tubo neural durante o desenvolvimento fetal (Yoo *et al*., 2009; Zhang *et al*., 2010) e na regulação de genes envolvidos com tumor do SNC, como meduloblastoma (Zhang *et al*., 2007; Ferreti *et al*., 2009; Wei *et al*., 2009) e glioblastoma (Kim *et al*., 2009; Malzkorn *et al*., 2009; Ujifuku *et al*., 2010, (Ciafre et al., 2005) e em outros cânceres (Schulte *et al*., 2008; Hayashita *et al*., 2005, He *et al*., 2005).

1.5. miRNAs em comum entre anencefalia e meduloblastoma

No desenvolvimento do córtex embrionário, a sinalização Sonic Hedgehog (Shh) aumenta o crescimento de células com propriedade de célula-tronco (Gulino *et al*., 2007). Na diferenciação celular granular normal, a sinalização Shh é limitada por ações combinadas de miR-125b, miR-324-5p e miR-326, através da inibição de componentes regulatórios da sinalização celular, Smo e Gli1. A perda da expressão de miR324-5p causada pela deleção 17p, uma alteração estrutural encontrada em mais da metade das células de meduloblastoma (Fruhwald 2001; De Chiara *et al*., 2002; De Smaele *et al*., 2004), reduz os níveis de miR-125b e miR-326 e leva a superexpressão de Smo, uma segunda proteína transmembrana, e do fator de transcrição Gli1, que resulta na inibição

da repressão da sinalização Shh, assim sustentando o desenvolvimento do câncer (De Smaele *et al*., 2004; Ferreti *et al*., 2008).

Algumas evidências suportam uma associação entre a localização cromossômica de miRNAs e alguns fatores de transcrição, como os genes Homeobox (HOX; Wynter, 2006), que desempenham um papel crucial no desenvolvimento animal e na oncogênese. Os genes *HOXB4*, *HOXB5*, *HOXC9*, *HOXC10*, *HOXD4*, e *HOXD8*, todos com miRNAs em regiões vizinhas, são desregulados em determinados tipos de câncer hematopoiético e sólidos (Cillo *et al*., 1999; Owen & Hawley, 2002). MiRNAs, como miR-196 e miR-10a, são localizados nos clusters HOX, regulando negativamente a sua expressão por clivagem do mRNA ou repressão da tradução gênica (Calin *et al*., 2004; Chopra & Mishra, 2006; Kosik 2006).

Um único miRNA pode potencialmente regular um grande conjunto de genes, sugerindo que os miRNAs estão provavelmente envolvidos em redes de vias gênicas mais complexas (Bartel & Chen, 2004). Por exemplo, o miR-9 é especificamente expresso em áreas neurogênicas, onde suprime a expressão do receptor nuclear TLX para regular negativamente a proliferação e a diferenciação de células tronco neurais (Denli *et al*., 2009; Zhao *et al*., 2009). Leucht *et al.* (2008), evidenciaram que o miR-9 é expresso no desenvolvimento tardio do SNC em peixe paulistinha (*zebrafish*) em um perfil que evita o mecanismo de manutenção do limite do mesencéfalo e rombencéfalo (MHB), um centro de organização do tubo neural que coordena eventos celulares como diferenciação, proliferação, sobrevivência, migração e destino celular.

Ferreti *et al.* (2009) relataram que os miRNAs em meduloblastoma estão predominantemente em baixos níveis em comparação com tecido normal, o que sugere uma função de crescimento tumoral inibitória. Esta propriedade foi validada para o miR-9 e o miR-125a. A baixa expressão de miR-9 também foi encontrada em tecidos malformados de anencefalia (Zhang *et al*., 2010). Em contraposição, o miR-9 é encontrado super expresso em tumores derivados de célula glial, como glioblastoma (Malzkorn *et al*., 2010) e oligodendriglioma (Lau *et al*., 2008). Esses dados sugerem uma assinatura específica de miRNA para cada tumor, destacando a heterogeneidade molecular e complexidade da sinalização celular regulada pelos miRNAs (Ferreti *et al*., 2009; Zhou *et al.*, 2007).

Outro miRNA com baixa expressão em meduloblastoma e anencefalia é o miR-124, um dos mais abundantes miRNAs expressos no sistema nervoso normal, sendo amplamente expresso na diferenciação de neurônios e persistindo nos neurônios maduros. Análise da expressão quantitativa de meduloblastoma demonstrou significativa baixa expressão de miR-124 em 72% (21 de 29) dos tumores comparado com cerebelo normal (Wai *et al*., 2009). Wai e colaboradores (2009) mostraram que a expressão ectópica de miR-124 na linhagem celular, ONS-76 e DAOY, de meduloblastoma inibiu a proliferação celular. A baixa expressão deste miRNA também foi encontrado em fetos com anencefalia (Zhang *et al*., 2010) sugerindo que miR-124 desempenha um papel importante durante o desenvolvimento e a diferenciação neural, possivelmente na regulação do citoesqueleto (Visvanathan *et al*., 2007; Yoo *et al*., 2009 Yu *et al*., 2008). Vários estudos indicam que a super expressão deste miR pode promover a diferenciação neural via inibição do fator antineural Ctdsp1, que age junto com um complexo de repressão transcricional REST nas células não-neuronais e célula-tronco neural. O complexo REST mantém genes relacionados com linhagem neuronal em um estado equilibrado, pois quando presente reprime miR-9 e miR-124, promovendo a proliferação de progenitores neuronais (Singh *et al*., 2008; Wu *et al*., 2006; Yoo *et al*., 2009).

Diante do exposto, a análise de miRNA surge como uma poderosa ferramenta para identificar genes que possam desempenhar um papel importante nas neoplasias. A análise do perfil de expressão de miRNAs poderá trazer importantes informações sobre a relação entre malformações e câncer, além de revelar marcadores para diagnóstico de tumores do SNC, bem como evidenciar alvos terapêuticos.

Além disso, este estudo abre perspectivas para estudo futuros onde manipulações nos miRNAs identificados, como as técnicas de silenciamento (Wai *et al*., 2009), poderão ser realizados em modelos animais, contribuindo assim para um maior conhecimento do potencial oncogênico destas moléculas.

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Dada à baixa frequência de ocorrência das mutações sequenciais, seria matematicamente impossível que crianças desenvolvessem câncer. O fato delas terem a doença sugere a existência de um mecanismo de carcinogênese típico para essa fase do desenvolvimento. Diversas anomalias congênitas têm sido associadas à maior incidência de câncer, o que sugere haver um componente genético alterado no processo normal de desenvolvimento e na etiologia do câncer. Os miRNAs são importantes reguladores do desenvolvimento do SNC e tem sido associados tanto a malformações quanto ao câncer no SNC. Portanto, a identificação de alterações de miRNAs comuns aos defeitos do tubo neural e meduloblastoma (ambos tecidos de origem neuroectodérmica) pode ajudar a compreender os mecanismos moleculares da carcinogênese e da morfogênese do SNC em crianças.

No presente projeto analisamos o perfil de expressão de miRNAs no tecido tumoral de meduloblastoma e tecido cérebro-vascular de anencefalia, visando à identificação de mecanismos moleculares que expliquem a ocorrência da carcinogênese infantil e sua possível associação com o desenvolvimento do sistema nervoso.

Mais especificamente:

1. Avaliamos o perfil da expressão de miRNAs nos tecidos de anencefalia (AN), meduloblastoma (MB) comparados com tecido normal por meio do *chip* de microarranjos a fim de identificar miRNAs diferencialmente;

2. Dentre os miRNAs diferencialmente expressos, identificamos os genes alvos que atuem em vias gênica-metabólicas importantes na carcinogênese e morfogênese através de análises *in silico*;

3. Uma vez selecionados as vias de interesse, validamos os miRNAs candidatos por RT-qPCR, nos mesmos tecidos usados nos *chips*, bem como em um número maior de amostras de MB, AN e controles;

4. Comparamos o perfil de miRNAs de MB e AN a fim de identificar miRNAs que sejam comuns nas duas patologias.

CAPÍTULO 2

Manuscrito 1:

Downregulation of chromosome 14q32 microRNA cluster in primary human desmoplastic medulloblastoma

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Artigo publicado no
Frontier Oncology (2013) **3**:254. doi: 10.3389/fonc.2013.00254

frontiers in ONCOLOGY

ORIGINAL RESEARCH ARTICLE
published: 25 September 2013
doi: 10.3389/fonc.2013.00254

Downregulation of 14q32 microRNAs in primary human desmoplastic medulloblastoma

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Medulloblastoma (MB) is one of the most common pediatric cancers, likely originating from abnormal development of cerebellar progenitor neurons. MicroRNA (miRNA) has been shown to play an important role in the development of the central nervous system. Microarray analysis was used to investigate miRNA expression in desmoplastic MB from patients diagnosed at a young age (1 or 2 years old). Normal fetal or newborn cerebellum was used as control. A total of 84 differentially expressed miRNAs (64 downregulated and 20 upregulated) were found. Most downregulated miRNAs (32/64) were found to belong to the cluster of miRNAs at the 14q32 locus, suggesting that this miRNA locus is regulated as a module in MB. Possible mechanisms of 14q32 miRNAs downregulation were investigated by the analysis of publicly available gene expression data sets. First, expression of estrogen-related receptor-γ (*ESRRG*), a reported positive transcriptional regulator of some 14q32 miRNAs, was found downregulated in desmoplastic MB. Second, expression of the parentally imprinted gene *MEG3* was lower in MB in comparison to normal cerebellum, suggesting a possible epigenetic silencing of the 14q32 locus. miR-129-5p (11p11.2/7q32.1), miR-206 (6p12.2), and miR-323-3p (14q32.2), were chosen for functional studies in DAOY cells. Overexpression of miR-129-5p using mimics decreased DAOY proliferation. No effect was found with miR-206 or miR-323 mimics.

Keywords: 14q32 miRNA cluster, desmoplastic medulloblastoma, *ESRRG*, miR-129-5p, miRNA profile

INTRODUCTION

Medulloblastoma (MB) is an embryonic tumor of the cerebellum and the most common malignant brain tumor in childhood, likely originating from abnormal development of cerebellar progenitor neurons (1, 2). Transcriptional profiling of large number of MB samples unraveled the existence of at least four distinct molecular subgroups: (i) Wingless (Wnt) group, (ii) Sonic Hedgehog (SHH) group, (iii) Group 3 and (iv) Group 4 (3, 4). These various subtypes of MB are suggested to arise from different populations of precursor or stem cells which form the cerebellum (5–8). This transcriptome-based classification has opened new avenues for the understanding of the molecular mechanism contributing to MB.

MicroRNAs (miRNAs) are suggested to play an important role in controlling the development of the central nervous system (CNS) by regulating cell proliferation and differentiation, as well as apoptosis (9). miRNAs are small non-coding RNA molecules of ~22–25 nucleotides that post-transcriptionally downregulate gene expression by binding the 3′-untranslated region (UTR) of protein coding transcripts, resulting in either mRNA cleavage or translational repression (10, 11). miRNA expression profiling of both mouse and human MB has led to the identification of signatures associated with the molecular subgroups of MB, tumor diagnosis, and response to treatment, as well as novel targets of potential clinical relevance (12–15). Previous studies, however, interrogated limited number of miRNAs and included adult cerebellum in the normal control group. We investigated the expression profile of 847 miRNA in primary human desmoplastic MB of younger children in comparison to normal fetuses or newborn cerebellum. Eighty-four miRNAs were found to be differential expressed in MB, most of them belonging to the cluster 14q32. Possible mechanisms of 14q32 locus downregulation were investigated by the analysis of publicly available gene expression data set. Functional studies using mimic miR-129-5p (11p11.2/7q32.1), miR-206 (6p12.2), and miR-323-3p (14q32.2) and the DAOY cell line, suggested a suppressive role for miR-129-5p in MB proliferation.

MATERIALS AND METHODS

PRIMARY MEDULLOBLASTOMA TISSUE SAMPLES

Surgical specimens were obtained from 1 to 5 years old children ($n = 10$), with desmoplastic MB (**Table 1**). Of note, microarray analyses were performed with MB samples from children with 1–2 years old. Desmoplastic MBs belong, with rare exceptions, to the SHH molecular subgroup (13–16). All MB samples used in the present study had high mRNA levels of *PTCH1* and low levels of *OTX2* (**Figure A1** in Appendix), in comparison to normal cerebellum, which is in keeping with the differential transcriptional profile of SHH tumors (3). Normal cerebellum tissues were

Table 1 | Summary of the medulloblastoma samples included in the study.

Medulloblastoma	Age at diagnosis	Gender	Histology
MB 1[a]	1	M	N/D
MB 2[a]	1	F	N/D
MB 3[a]	2	M	D
MB 4[a]	2	M	N/D
MB 5[a]	1	M	N/D
MB 6[a]	2	M	N/D
MB 7	5	M	N/D
MB 8	5	M	D
MB 9	4	M	D
MB 10	5	M	N/D

[a]*Samples used in Affymetrix miRNA microarray analysis; F= female; M= male; D= desmoplastic; N/D= nodular/desmoplastic.*

Table 2 | Summary of the normal cerebellum tissues.

Normal cerebellum	Gestational age	Gender	Diagnosis
C1	37	M	Bilateral renal agenesis
C2[a]	39	M	Hydropsy
C3[a]	22	–	NM
C4[a]	31	M	NM
C5[a]	36	–	NM
C6[a]	24	M	NM
C7	30	–	Cardiopathy
C8[a]	26	M	NM

[a]*Samples used in Affymetrix miRNA microarray analysis; F= female; M= male; NM= no malformation and no aneuploidy.*

obtained from 22 to 39 weeks old fetal and newborn (NW) autopsy ($n = 8$) (**Table 2**). Ethical approvals were obtained from the Ethical Research Committee of the Faculdade de Ciências Médicas (n°656/2009), CAISM (n°064/2010), the Ethical Research Committee of Centro Infantil Boldrini (n°1.90-030710), and National Committee of Ethics in Research (CONEP) n°0005.0.144.146-09. Subtyping of MB was obtained by histological analysis.

TOTAL RNA ISOLATION AND ANALYSIS OF GLOBAL miRNA EXPRESSION

Total RNA was extracted by Trizol™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with an additional overnight precipitation step at −20°C with isopropanol (Merck). RNA quantification was carried out in a *Qubit Quantitation Platform* (Invitrogen) and RNA quality was analyzed via gel electrophoresis. Five hundred nanograms of RNA from 12 samples (6 MB and 6 fetal cerebellum) were labeled with the 3′-DNA FlashTag Biotin HSR kit (Genisphere, Hatfield, PA, USA) and hybridized to GeneChip miRNA Array 1.0 (Affymetrix Inc., Santa Clara, CA, USA), which comprises 847 human miRNAs. Data was acquired using a GeneChip Scanner 3000 7G (Affymetrix).

VALIDATION OF miRNA DEREGULATION BY QUANTITATIVE REAL-TIME PCR

Reverse transcription (RT) and quantitative real-time RT-PCR (RT-qPCR) analysis were carried out using commercially available TaqMan microRNA assays (Applied Biosystems, Foster City, CA, USA) and a 7500 Real-time PCR System (Applied Biosystems). RT reactions (50 ng of total RNA) were performed in a 15 μl final volume containing specific stem-loop primers for each miRNA (129-5p, 206, 323-3p, 495, and internal control small RNA, RNU6B), 10× RT Buffer, dNTPs, reverse transcriptase, RNase inhibitor, and water in 96-well plates. Thermal cycling included 30 min at 16°C, 30 min at 42°C, and a final step of RT inactivation for 5 min at 85°C. PCR reactions were performed in a 10 μl final volume containing 5 μl TaqMan Universal Master Mix II, without UNG (Applied Biosystems), 3.5 μl water, 0.5 μl TaqMan microRNA assay, and 1 μl cDNA. Thermal cycling included an initial step of 10 min at 95°C for Taq activation followed by 40 cycles of 15 s denaturation at 95°C and 1 min of annealing/extension at 60°C. Each reaction was performed in triplicate and the miRNAs expression levels were normalized against RNU6B. The threshold cycle numbers (Ct) were calculated by relative quantification using the $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen (17). One of the control samples was chosen as calibrator.

CELL LINES

Four human MB cell lines were utilized: DAOY (HTB 186), D283 Med (HTB185), and D431 Med (HTB-187) were obtained from American Type Culture Collection (ATCC). The MB cell line, MEB-Med-8A, was kindly provided by Prof. T. Pietsch (18). The MB cell lines DAOY, D283 Med, and MEB-Med-8A were maintained in High Glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM sodium pyruvate (PAA), L-glutamine, 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10% fetal bovine serum (FBS, Invitrogen). The MB cell line D341 Med was maintained in DMEM with L-glutamine supplemented with 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% Human Serum (HS, PAA, UK).

TRANSIENT TRANSFECTION OF miRNAs

DAOY cells (1.5×10^5) were seeded in six-well plates in 2 ml of RPMI-1640 medium (Cultilab, Campinas, Brazil) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and penicillin/streptavidin (Cultilab). Transfection of miRVana miRNA mimics (Invitrogen Ambion, Austin, TX, USA) of miR-206, miR-129-5p, miR-323-3p, or miRVana miRNA mimic negative control #1 (referred to as scrambled) was carried out 24 h after seeding, in a final concentration of 3 nM, using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's recommendation. Efficiency of transfection was evaluated 24 post-transfection by RT-qPCR using total RNA.

CELL VIABILITY: MTS ASSAY

Cell survival/proliferation after the transfection with mimic-miRNAs was evaluated by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Wallisellen, Switzerland), a colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium inner salt (MTS) assay. Briefly, mimic-miR-206, mimic-miR-129-5p, and mimic-miR-323-3p or mimic-negative control #1 transfected

Table 3 | Deregulated miRNA in desmoplastic medulloblastoma compared to normal cerebellum.

Our miRNA profile (84)	Chromosomal localization	Fold change	Reference
DOWNREGULATED			
hsa-miR-206	6p12.2	−7.53	(29)
hsa-miR-219-2-3p	9q33.3	−6.64	(52)
hsa-miR-383	8p22	−6.56	(12, 55, 56)
hsa-miR-138	16q13.3/3p21.32	−5.16	(12, 14)
hsa-miR-323-3p	14q32.2	−4.96	(12, 52)
hsa-miR-122	18q21.31	−4.82	
hsa-miR-105	Xq28	−4.66	
hsa-miR-129-5p	11p11.2/7q32.1	−4.56	(23)
hsa-miR-935	19q13.43	−4.53	(52)
hsa-miR-329	14q32.2	−4.48	
hsa-miR-129-3p	11p11.2/7q32.1	−4.43	
hsa-miR-650	22q11.21	−4.19	
hsa-miR-184	15q24.3	−4.14	
hsa-miR-370	14q32.2	−3.99	(12)
hsa-miR-433	14q32.2	−3.96	(29)
hsa-miR-138-2*	16q13.3/3p21.32	−3.91	
hsa-miR-487b	14q32.2	−3.82	(29)
hsa-miR-487a	14q32.2	−3.78	
hsa-miR-758	14q32.2	−3.65	
hsa-miR-485-5p	14q32.2	−3.60	
hsa-miR-138-1*	16q13.3/3p21.32	−3.55	
hsa-miR-382	14q32.2	−3.53	(12, 29)
hsa-miR-504	Xq26.3	−3.45	(52)
hsa-miR-128	2q21.3/3p22.3	−3.43	(12, 14, 51, 59)
hsa-miR-490-5p	7q33	−3.42	
hsa-miR-770-5p	14q32.2	−3.35	
hsa-miR-410	14q32.2	−3.30	(29)
hsa-miR-432	14q32.2	−3.29	
hsa-miR-485-3p	14q32.2	−3.02	
hsa-miR-490-3p	7q33	−2.88	
hsa-miR-381	14q32.2	−2.73	(12)
hsa-miR-377*	14q32.2	−2.72	
hsa-miR-7	15q25.3/19p13.3/9q21.32	−2.72	(12, 14)
hsa-miR-124	20p23.1/8q12.3/8p23.1	−2.71	(12, 14, 29, 48, 49)
hsa-miR-323-5p	14q32.31	−2.69	(12)
hsa-miR-873	9p21.1	−2.65	
hsa-miR-129*	11p11.2/7q32.1	−2.63	
hsa-miR-338-5p	17q25.3	−2.61	(14)
hsa-miR-409-5p	14q32.2	−2.61	
hsa-miR-874	5q31.2	−2.46	
hsa-miR-495	14q32.2	−2.46	(52)
hsa-miR-885-5p	3p25.3	−2.45	
hsa-miR-376c	14q32.2	−2.43	(52)
hsa-miR-299-5p	14q32.2	−2.41	
hsa-miR-539	14q32.2	−2.40	(52)
hsa-miR-127-5p	14q32.2	−2.35	(12, 29)
hsa-miR-127-3p	14q32.2	−2.35	(52, 59)
hsa-miR-411*	14q32.2	−2.30	
hsa-miR-125b-1*	11q24.1/21q21.1	−2.27	
hsa-miR-411	14q32.2	−2.23	(29)
hsa-miR-379	14q32.2	−2.22	(29, 52)
hsa-miR-431*	14q32.2	−2.22	
hsa-miR-767-5p	Xq28	−2.20	
hsa-miR-139-3p	11q13.4	−2.17	
hsa-miR-154	14q32.2	−2.16	(12)
hsa-miR-1224-5p	3q27.2	−2.15	
hsa-miR-187	18q12.1	−2.14	(12)
hsa-miR-95	4p16.1	−2.10	(14)
hsa-miR-369-5p	14q32.2	−2.05	
hsa-miR-665	14q32.2	−2.05	
hsa-miR-494	14q32.2	−2.03	(52)
hsa-miR-134	14q32.2	−2.03	(12, 29)
hsa-miR-346	10q23.2	−2.01	(12, 13)
hsa-miR-324-5p	17p13.1	−2.00	(12, 50)
UPREGULATED			
hsa-miR-199b-3p	9q33.3	4.56	(12)
hsa-miR-199a-3p	19p13.2/1q24.1	4.49	
hsa-miR-199a-5p	19p13.2/1q24.1	4.14	(28)
hsa-miR-21	17q22	3.70	(12–14, 29, 31)
hsa-miR-214	1q24.2	3.59	
hsa-miR-19a	13q31.3	3.11	(12–14)
hsa-miR-92a-1*	13q31.3/Xq26.2	3.06	
hsa-miR-214*	1q24.2	2.93	
hsa-miR-34a	1p36.23	2.78	(13, 30, 53)
hsa-miR-18b	Xq26.2	2.74	
hsa-miR-422a	15q22.2	2.72	(14)
hsa-miR-34a*	1p36.23	2.58	(14)
hsa-miR-574-3p	4p14	2.49	(14)
hsa-miR-378	5q32	2.39	(14)
hsa-miR-1244	12p13.2/12p13.31/2q37.1/5q23.1	2.39	
hsa-miR-18a	13q31.3	2.39	(12–14)
hsa-miR-93*	7q22.1	2.26	
hsa-miR-497	17p13.1	2.17	(13)
hsa-miR-195*	17p13.1	2.14	
hsa-miR-216a	2p16.1	2.07	(14)

Deregulated miRNAs previously described in human primary medulloblastoma compared with normal cerebellum or cell lines: (i) miRNAs found downregulated in Ref. (12–14, 23, 26, 29, 48–52, 55, 56, 59); (ii) miRNAs found upregulated in Ref. (12–14, 28–31, 53).

cells were harvested 20 h after transfection and seeded in triplicate in 96-well plate (1,500 cells/well) in serum-free RPMI-1640 (Cultilab). At 24, 48, or 72 h post-transfection (i.e., 4, 28, or 52 h after passage to the 96-well plate) cells were incubated for 1 h with MTS reagent and absorbance read at 492 nm (reference wavelength 620 nm) using an ASYS Expert Plus Microplate Reader (Biochrom, Holliston, MA, USA). Three independent experiments were performed.

APOPTOSIS ASSAY

DAOY cells transfected with miR-206, 129-5p, 323-3p, or scramble mimics were cultured for 24 h in serum-free RPMI-1640 (Cultilab), harvested and part of it was resuspended in the appropriate binding buffer, stained with FITC-conjugated Annexin V (BD Biosciences, San Jose, CA, USA) and propidium iodide at room temperature for 15 min, and subsequently analyzed by flow cytometry in a FACS Canto II (Becton Dickinson). The remaining cells were replated in six-well plates for another 24 h culture period in serum-free RPMI-1640 (Cultilab) and harvested 48 h post-transfection for Annexin V labeling.

STATISTICAL ANALYSIS AND BIOINFORMATICS METHODS TO SIGNALING PATHWAY PREDICTION

MicroRNA expression was analyzed in R environment[1] using the packages Affy and RankProd from Bioconductor (19–21). The MB miRNA profile was compared to the cerebellum profile. Differentially expressed miRNAs were selected according to the fold change ≥ 2.00 and p-value ≤ 0.05. Heat maps were created using tools of the MetaboAnalyst 2.0[2]. Signaling pathways were prospected by DIANA-miRPath (microT-v4.0, beta version)[3]. The input dataset enrichment analysis was performed by Pearson's chi-squared test and each pathway was represented by the negative natural logarithm of the P-value ($-\ln P$). The Ingenuity Pathway Analysis (IPA) software[4] was used to identify possible pathways associated to differentially expressed miRNAs.

Comparisons of RT-qPCR values from MB versus normal cerebellum were performed by the Mann–Whitney test. Cell proliferation results, at each time point, from mimic miRNA transfections versus mimic-negative control #1 were analyzed by the two-tailed unpaired t-test. Alpha error of $P = 0.05$ was tolerated. The GraphPad Prism 5 software was used throughout.

RESULTS

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNAs IN DESMOPLASTIC MBs OF 1–2 YEARS OLD CHILDREN

Global miRNA profiles were generated for primary MB of the desmoplastic subtype and most likely SHH molecular subgroup ($n = 6$), and normal fetal/NW cerebellum ($n = 6$). Eighty-four miRNAs (64 miRNAs downregulated and 20 miRNAs upregulated) were considered to be differentially expressed (fold change ≥ 2.0, $p \leq 0.05$) in MB in comparison to normal fetal/NW cerebellum (**Table 3**; **Figure 1**). Among these 84 miRNAs, 46 had been previously described as deregulated in human primary MB (**Table 3**), and only 8 were previously validated by functional assays (**Table 4**). Upregulation of miRNAs from the miR-17 ~ 92 cluster (in this work miR-18a, 19a, and 92a-1) and downregulation of miR-324-5p were previously described in human MB of the SHH subgroup (12, 13). Of especial note, 32 of the 64 downregulated miRNAs belong to a large cluster on human chromosome 14q32 (**Figure 1**; **Table 3**).

[1] www.r-project.org
[2] www.metaboanalyst.ca
[3] http://diana.cslab.ece.ntua.gr/pathways/index_multiple.php
[4] http://www.ingenuity.com/

SIGNALING PATHWAYS ANALYSIS BY DIANA

Signaling pathways putatively altered by MB deregulated miRNA were depicted by DIANA-miRPath. The list of the top 20 pathways is shown in **Table 5**. The Ribosome pathway was only pointed by the list of downregulated miRNAs. Adherens junction, oxidative phosphorylation, and TGF-beta signaling pathways showed higher enrichment when the list of downregulated miRNAs was used in the analysis. On the other hand, the MAPK pathway and genes associated to cancer showed higher enrichment when upregulated miRNAs were used in the analysis.

Interestingly, oxidative phosphorylation, TGF-beta signaling pathway, and ubiquitin mediated proteolysis were enriched in the list of 14q32 miRNAs.

INGENUITY PATHWAY ANALYSIS

Network analysis by IPA identified two networks as putative targets for 73 out of the 84 MB miRNAs. Networks were prospected considering only relationships that were experimentally observed. Interestingly, both networks were enriched with miRNAs belonging to the 14q32 cluster.

Network 1 (**Figure 2A**) included 13 miRNAs of the 14q32 cluster (also known as miR-154 cluster), which were all downregulated in MB samples (miR-154, 323-3p, 323-5p, 369-5p, 377*, 381, 382, 409-5p, 410, 485-3p, 487a, 487b, 539) and were depicted by IPA as having direct interactions with *BCL2L11*, *JUN*, *BIRC5*, *MAP2K4*, and *NR0B2*. *BIRC5* and *BCL-2* have anti-apoptotic roles, and are expected to be at increasing levels in MB as all miRNAs connecting to these genes were found downregulated (**Figure 2A**). *NR0B2* and *JUN* were suggested in this network as candidate genes controlling the expression of the 14q32 miRNA cluster.

Again, most miRNAs shown in Network 2 (**Figure 2B**) belong to the 14q32 cluster (miR-127-3p, 127-5p, 134, 379, 411, 432, 433, 495, and 758). In this case, *NR0B2* and estrogen-related receptor-γ (*ESRRG*) were suggested as candidate genes controlling the expression of the 14q32 miRNA cluster. Insulin appeared in Network 2 as indirectly controlling the expression of miR-206, 324-5p, 432, and 95.

RT-qPCR VALIDATION OF SOME DEREGULATED miRNAs

miR-323-3p and 495, both belonging to cluster 14q32, were chosen for validation by RT-qPCR. In addition, miR-206 and miR-129-5p were chosen for analysis because of their high fold change (see **Table 3**), lack of previous functional studies and possible oncogenic role. miR-206 expression was reported to inhibit cell proliferation in breast cancer cells (22). miR-129 is reported to be significantly downregulated in pediatric brain tumors compared to normal tissues (23). Most importantly, miR-129 downregulation is associated to SOX4 overexpression in endometrial and gastric cancers (24, 25). SOX4 is upregulated and has prognostic impact in MB (26, 27).

Real-time RT-qPCR analysis were performed with all samples used in the microarray analysis ($n = 6$ MB and $n = 6$ cerebellum) plus four other samples of MB (**Table 1**) and two new fetal/NW cerebellum controls (**Table 2**). As expected, miR-206 ($p = 0.0001$; Mann–Whitney test), miR-129-5p ($p = 0.002$), miR-323-3p ($p = 0.014$), and miR-495 ($p = 0{,}054$), had lower expression in MB in comparison to normal cerebellum (**Figure 3**), thus confirming our microarray findings. Expression of miR-206,

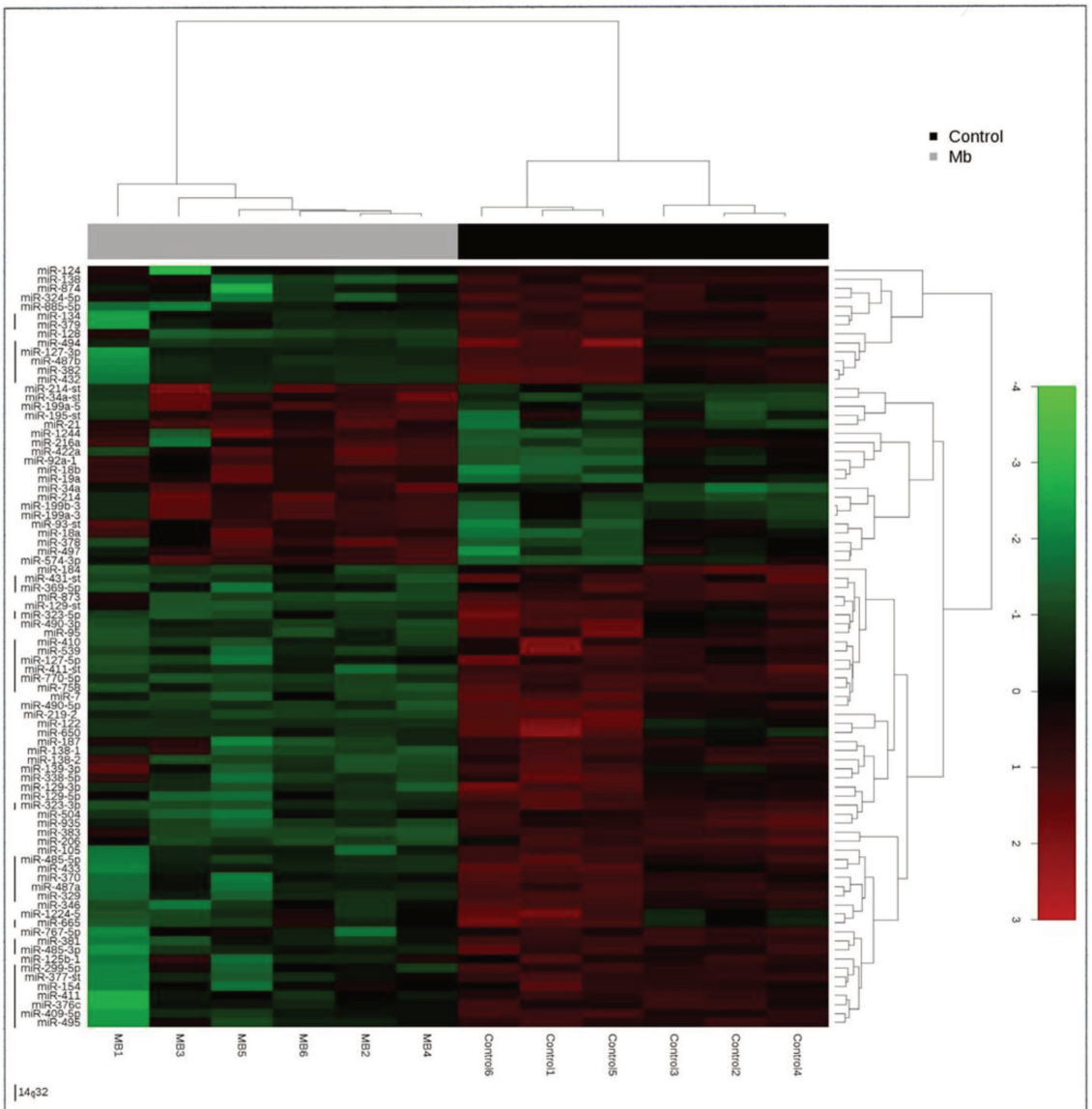

FIGURE 1 | Hierarchical clustering analysis of medulloblastoma and normal cerebellum. Unsupervised hierarchical cluster analysis representing the 84 miRNAs expressed in medulloblastoma samples versus normal cerebellum. 14q32 miRNAs are depicted by a left vertical line. -st, star.

129-5p, 323-3p, and 495 were also investigated in a representative panel of MB cell lines. Compared with normal human cerebellum, miR-206, 129-5p, and 323-3p expression were found to be downregulated in all MB cell line tested (**Figure 3**).

UPREGULATION OF miR-206, 129-5p, AND 323-3p IN DAOY CELLS

As a first approach to investigate the functional significance of miRNAs downregulation in MB, DAOY cells were transiently transfected with mimics of miR-206, 129-5p, 323-3p, or negative control #1. Transfection efficiency was confirmed by RT-qPCR (**Figure A2** in Appendix). Twenty hours post-transfection, cells were collected and seeded in 96-well plates in serum-free medium. DAOY cells survive and even proliferate in serum-free medium for a short period of time. As shown in **Figure 4**, no consistent differences in proliferation were found in transfections with miR-206 and miR-323-3p. On the contrary, transfections with miR-129-5p

Table 4 | miRNAs validated by functional assay in human and mouse medulloblastoma.

miRNA	Deregulation	Cells	Functional assay[a]	Target genes	Reference
124	Down	Primary human MB, cell lines	↑ Cell cycle progression at G1 ↓ Cell proliferation	CDK6 SLC16A1	(48, 49)
324-5p 326 125p	Down	Primary human and mouse MB, cell lines	↓ Cell proliferation	SMO GLI1	(50)
9 125a	Down	Primary human MB, cell lines	↑ Apoptosis ↓ Cell proliferation	Trkc	(12)
199b-5p	Up	Primary human and mouse MB, cell lines	↑ Cell cycle progression at G1 ↓ Cell proliferation	HES1	(28)
128	Down	Primary human and mouse MB	↓ Cell proliferation ↑ Cell senescence	BMI-1	(51)
21	Up	Primary human MB, cell lines	↓ Cell migration	PDCD4	(31)
935	Down	Primary human MB, cell lines	–	KIAA0232 SLC5A3 TBC1D9 ZFAND6	(52)
34a	Down	MB cell lines	↑ Apoptosis ↑ Cell cycle progression at S/phase and G2/M ↓ Cell proliferation ↑ Cell senescence	MAGE-A Dll1 Notch1 Notch2	(30, 53)
512-5p	Down	Primary human MB, cell lines	–	MYCC	(54)
383	Down	Primary human MB, cell lines	↑ Apoptosis ↑ Cell cycle progression at G1 ↓ Cell proliferation	PRDX3	(55, 56)
183~96~ 182	Down	MB cell lines	↑ Cell cycle progression at G0/G1 and G2 ↓ Cell migration ↓ Cell proliferation	AKT	(57)
218	Down	MB cell lines	↓ Cell migration ↓ Cell proliferation	CDK6 REST	(58)

Underlined miRNAs are miRNAs also found deregulated in the present study;
[a]results of ectopic expression or knockdown assays; MB = medulloblastoma.

resulted in a significant decrease in DAOY cell proliferation, as evaluated by the MTS assay (**Figure 4**). Similar experiments were conducted to evaluate cell survival and apoptosis by the Annexin V and propidium iodide staining methodology. No significant differences were found on cell viability or apoptosis after miR-206, miR-129-5p, and miR-323-3p transfections in comparison to control (**Figure 4**; **Figure A3** in Appendix, respectively).

DISCUSSION

We investigated the expression profile of miRNAs in primary human MB of desmoplastic histology and SHH molecular subgroup, in comparison to normal fetal/newborn cerebellum. Eighty-four miRNAs were found to be differentially expressed in MB. The majority of these differentially expressed miRNAs were downregulated in comparison to normal cerebellum, corroborating previous studies. Most upregulated miRNAs identified in our study (12 out of 20) had been previously described in MB (12–14, 23, 28–32). On the contrary, 31 out of the 64 downregulated miRNAs are here described for the first time in association to MB (**Table 3**). Differences may be explained by the fact that a more comprehensive version of Affymetrix miRNA microarray was used in the present study. Moreover, previous studies included different subtypes of MB and a mix of children and adults cerebellum samples in their analysis (12, 13, 29). We believe that analysis on more uniform groups of both cancer and control samples may have helped us in detecting some smaller but consistent differences between groups.

Table 5 | Top 20 pathways predicted by DIANA-miRPath analysis.

Pathway signaling	All deregulated miRNAs	Downregulated miRNAs	Upregulated miRNAs	14q32 miRNAs
P-value[a]				
Ribosome	30.03	25.34	–	17.78
Axon guidance	24.96	19.98	17.95	17.7
Wnt signaling pathway	18.6	19.34	17.71	16.99
Focal adhesion	16.65	17.02	15.13	14.73
Adherens junction	16.23	20.37	12.18	16.6
Oxidative phosphorylation	14.95	14.76	7.34	14.45
ErbB signaling pathway	14.8	11.03	9.14	9.02
Metabolism of xenobiotics by cytochrome P450	14.06	15.33	3.15	10.01
Renal cell carcinoma	13.48	13.21	9.35	11.64
TGF-beta signaling pathway	12.4	14.11	5.64	17.56
Regulation of actin cytoskeleton	12.25	11.52	7.86	10.63
Chronic myeloid leukemia	12.06	11.02	8.14	10.54
MAPK signaling pathway	11.86	9.81	17.06	12.4
Colorectal cancer	11.72	13.24	9.79	16.92
Glioma	10	8.47	16.21	7.23
Pancreatic cancer	9.69	9.61	13.18	5.83
Melanogenesis	9.4	9.32	9.07	10.59
Ubiquitin mediated proteolysis	9.28	10.09	6.63	11.11
Prostate cancer	9.1	7	18.2	6.06
Insulin signaling pathway	9	8.54	5.86	4.65

[a]*The negative natural logarithm of the enrichment P-value calculated for the specific pathway. Underlined shows higher enrichment in downregulated, upregulated, or 14q32 miRNAs lists.*

A computational analysis was performed to predict the network and signaling pathways collectively targeted by the 64 downregulated and 20 upregulated miRNAs. Downregulated miRNAs in MB were predicted to target genes related to the ribosome, adherens junction, oxidative phosphorylation, metabolism of xenobiotics by cytochrome P450, and transforming growth factor-beta (*TGF-β*) signaling pathways. Axon guidance, *TGF-β*, *WNT*, insulin signaling pathways are known to play an important role in neurulation, CNS developmental, and/or MB pathogenesis (3, 33, 34). Since miRNA act as negative regulators of gene expression, a simpler interpretation of these findings is that MB has increased activation of these pathways in comparison to normal cerebellum.

Most importantly, half (32/64) of downregulated miRNAs reported in our study were found to belong to the cluster at 14q32 locus (also known as miR-154 cluster). This is in keeping with a previous study in a mouse model of MB, reporting that activation of SHH signaling leads to downregulation of the miR-154 cluster (35). Moreover, previous publications with primary MB found downregulation of some 14q32 miRNAs in MBs of the molecular subgroups WNT, SHH, and C as compared to normal cerebellum and MBs of subgroup D (13, 29). However, this is the first time that so many 14q32 miRNAs are shown to be downregulated in MB, thus suggesting a co-regulatory control of this cluster's expression.

Deletions at locus 14q32 would be one possible explanation to the decreased 14q32 miRNAs expression. The recent analysis of somatic copy number aberrations in 1,087 MB samples report significant losses of chromosome arm 14q in the SHH subgroup of MBs, though not restricted to 14q32 (36). Alternative explanations are discussed below. Our Ingenuity pathways analysis pointed to nuclear orphan receptor *NR0B2* (also known as Small Heterodimer Partner, *SHP*) and (*ESRRG*) as possible controllers of 14q32 miRNA cluster expression. There is indeed experimental evidence in mouse showing that *NR0B2* is a repressor while *ESRRG* is and activator of a miRNA cluster in chromosome 12, which is ortholog to the 14q32 cluster in humans (37). Our analysis of microarray mRNA expression data for 64 primary human MB samples, accessible through GEO Series accession number GSE28245 (38) in NCBI's Gene Expression Omnibus (39) revealed that *NR0B2* is not expressed in MB. Interestingly, *ESRRG* expression was found to be relatively high in MBs of the molecular subgroup D, intermediate in MBs of the WNT and C subgroups, and very low or absent in MBs of the SHH subgroup (**Figure A4A** in Appendix), thus reflecting 14q32 miRNAs abundance in each of the MB subgroup. These findings were confirmed by the analysis of gene expression data of an independent cohort of 90 primary MB samples (accession number GSE21166) deposited by Northcott et al. (13) (data not shown). *ESRRG* suppress cell proliferation in prostate cancer cells (40) and the estrogen receptor beta agonist diarylpropionitrile (*DPN*) exhibit a pro-apoptotic and anti-proliferative effect on MB (41). Experiments are warranted to investigate a possible causal connection between *ESRRG* and 14q32 miRNA cluster expression in MB.

The miRNA cluster at 14q32 lies within a parentally imprinted chromosomal area spanning genes *Dlk1*, *Meg3*, *Rtl1*, *Meg8*, and *Dio3* (42). *Dlk1*, *Rtl1*, and *Dio3* are paternally-, whereas Meg3 and Meg8 are maternally expressed transcripts (43). Imprinting of 14q32 is regulated, to some extent, by two intergenic differentially methylated regions known as IG-DMR and MEG3-DMR (44, 45).

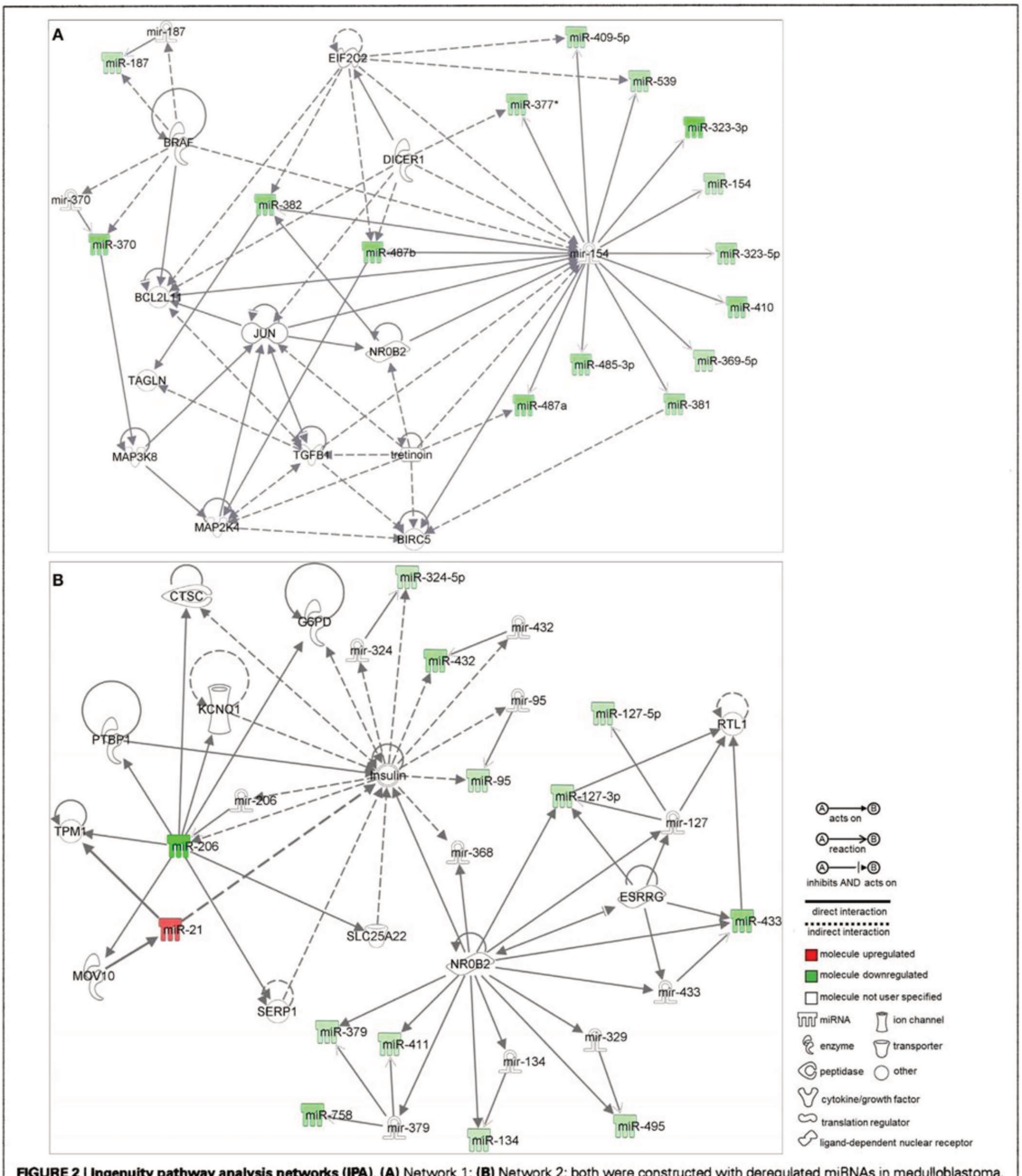

FIGURE 2 | Ingenuity pathway analysis networks (IPA). **(A)** Network 1; **(B)** Network 2; both were constructed with deregulated miRNAs in medulloblastoma.

Deletions of the regulatory regions and/or epigenetic modifications may in theory cause aberrant 14q32 silencing in cancer. The recent 1,000 genome study of somatic copy number aberrations shows no recurrent focal deletions at locus 14q32 in MB (36). However, our analysis of public mRNA microarray expression data GSE28245 (38) revealed that *MEG3* is downregulated in MB in comparison to normal cerebellum. MBs of the molecular group C and WNT have the lowest expression, SHH has intermediate

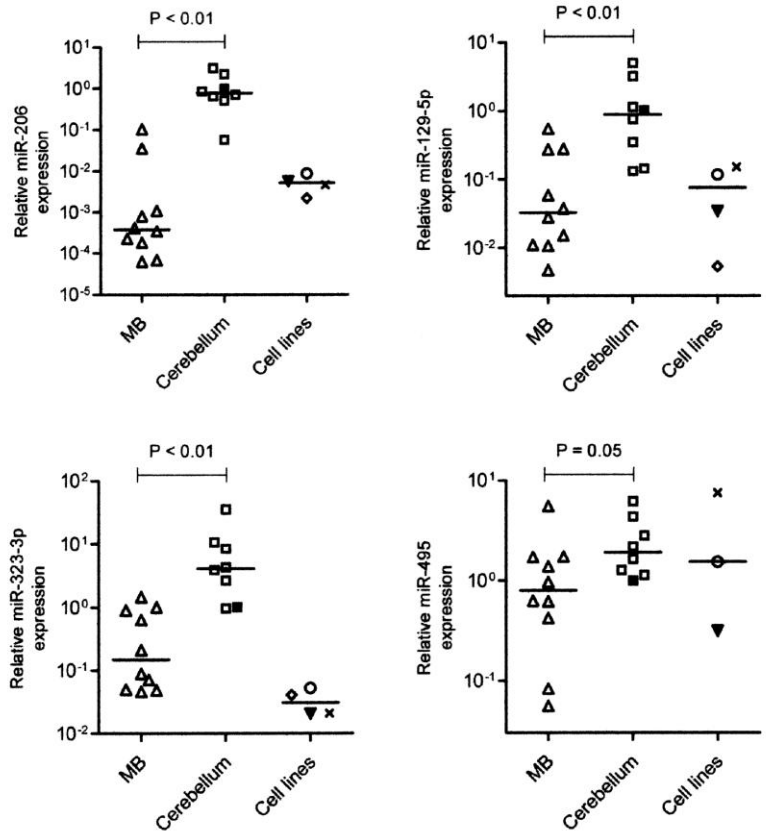

FIGURE 3 | Validation of miR-206, 129-5p, 323-3p, and 495 downregulation by reverse transcriptase-quantitative PCR. Expression levels of miR-206, 129-5p, 323-3p, and 495 were performed in MB, normal fetal/newborn cerebellum and four different MB cell lines. Expression of miR-495 was not performed in D341 cell line. ○, DAOY; ▼, Meb-Med-8A; x, D283Med; ◇, D341. Comparisons of MB versus normal cerebellum were done by the Mann–Whitney test.

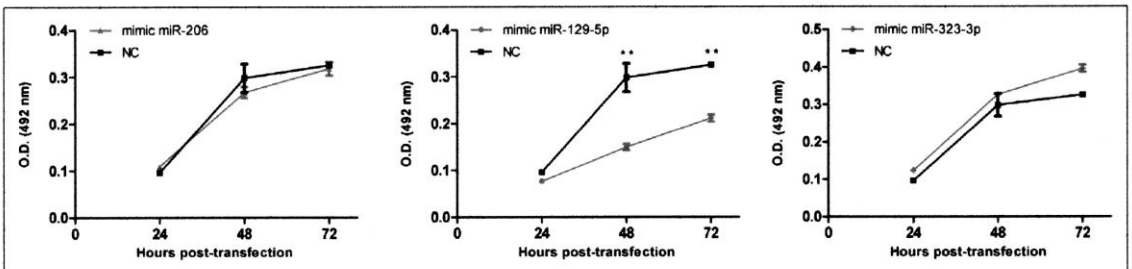

FIGURE 4 | Effect of miR-206, 129-5p, and 323-3p overexpression in DAOY cells proliferation. DAOY cells were transiently transfected with miRNAs mimic and replated after 20 h at a 1,500 cells/well density. Cell proliferation was evaluated by the MTS assay at 4, 28, and 52 h after re-plating, thus after 24, 48, and 72 h post-transfection. Results are representative of three independent experiments. Mean ± SE are shown; $^{**}P < 0.01$ according to two-tailed *t*-test; NC, negative control miRNA mimic (scrambled).

levels while group D have *MEG3* levels closer to normal cerebellum (**Figure A4B** in Appendix). Thus *MEG3* expression seems to correlate with the expression of 14q32 miRNAs among the different MB molecular groups, suggesting that the 14q32 miRNA locus may be under epigenetic regulation in MB. However, a genome wide analysis of promoter methylation on four primary MB samples showed no consistent methylation of 14q32 gene promoters (46). Although higher number of samples should be analyzed, this result corroborates findings in osteosarcoma, a tumor also presenting with downregulated 14q32 miRNAs expression and with no consistent changes in the methylation patterns at 14q32. Instead, silencing of 14q32 miRNA in osteosarcoma seems to be mediated by histone modification(s) (47).

Preliminary functional studies were performed in DAOY cells by ectopic expression of miR-129-5p, 206, and 323-3p mimics. Mimics for miR-206 and 323-3p had no significant effect on DAOY cells. miR-129-5p overexpression resulted in decreased cell proliferation, which may suggest a tumor suppressor role in MB.

ACKNOWLEDGMENTS

We thank Dr. Carlos A Scrideli for helpful discussion and for the cell line DAOY and Dr. Priscila P. Zenatti and Dr. Angelo B. A. Laranjeira for technical assistance with FACS analysis. We also acknowledge the Microarray Laboratory at Brazilian Biosciences National Laboratory, CNPEM-ABTLuS, Campinas, Brazil for their support with the use of Fluidics station and Scanner GeneChip equipments. This work was supported by grant from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2010/07020-9). DRL have Coordenação de Aperfeiçoamento de Pessoal de Nível Superior PhD scholarship (2012/890912-1).

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

Received: 01 July 2013; accepted: 10 September 2013; published online: 25 September 2013.

Citation: Lucon DR, Rocha CdS, Craveiro RB, Dilloo D, Cardinalli IA, Cavalcanti DP, Aguiar SdS, Maurer-Morelli C and Yunes JA (2013) Downregulation of 14q32 microRNAs in primary human desmoplastic medulloblastoma. Front. Oncol. 3:254. doi: 10.3389/fonc.2013.00254

This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology.

APPENDIX

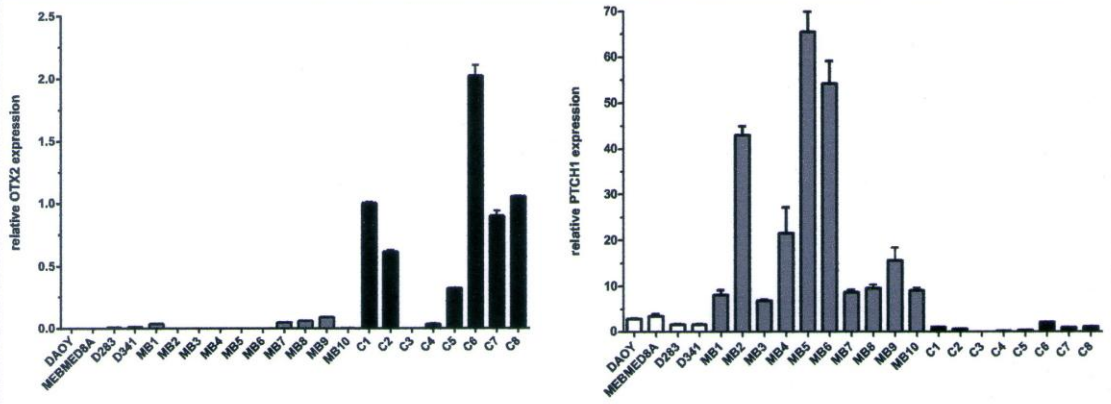


FIGURE A1 | Expression of OTX2 and PTCH1 in the different primary medulloblastomas (MB), medulloblastoma cell lines (named), and normal cerebellum (C). Total RNA (100 ng) was reverse transcribed and gene expression was measured by qPCR, in triplicates, using the SYBR method. Primers are available upon request to the authors. Values were normalized to the HPRT endogenous control gene. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method.

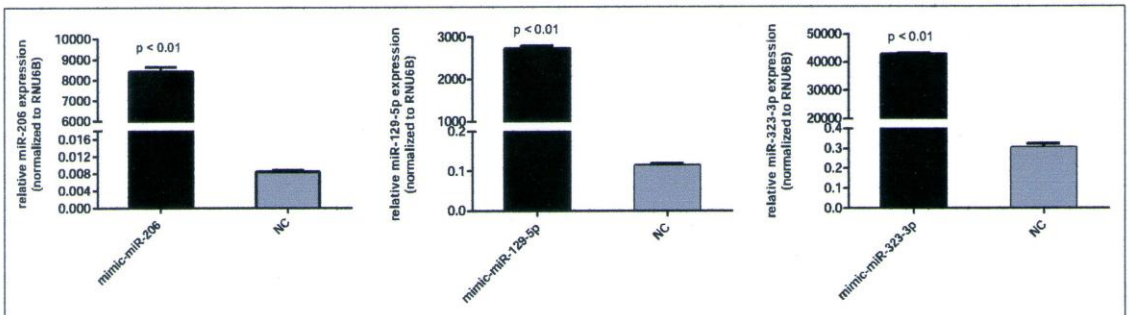


FIGURE A2 | Transfection efficiency of miR-206, miR-129-5p, and miR-323-3p in DAOY cells as evaluated by RT-qPCR. Cells transfected with the scrambled mimic were included as negative control (NC). Values were normalized to the RUN6B endogenous control RNA. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent mean ± SD. Comparisons were performed by the Mann–Whitney test.

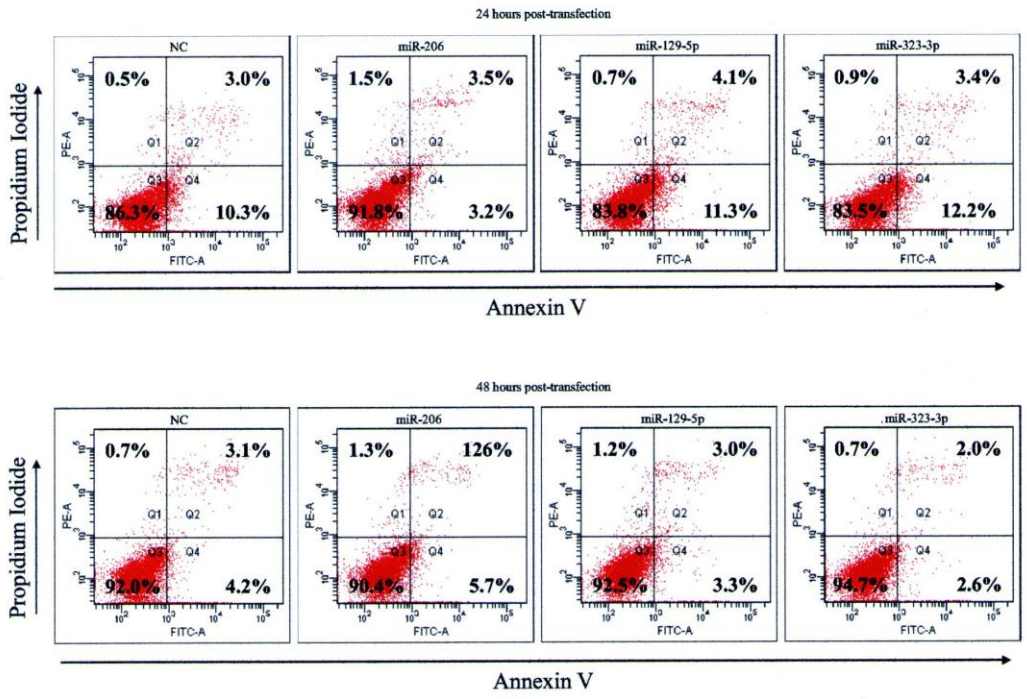


FIGURE A3 | Apoptosis analysis of DAOY cells at 24 and 48 h post-transfection with miR-206, 129-5p, 323-3p, or negative control mimics, as evaluated by Annexin V and propidium iodide staining and FACS analysis. DAOY cells grown in serum-free RPMI-1640 medium. The percentage of necrotic (Q1), late apoptotic (Q2), viable (Q3), and early apoptotic (Q4) cells are shown in the corresponding quadrants. NC, negative control (scrambled mimic).

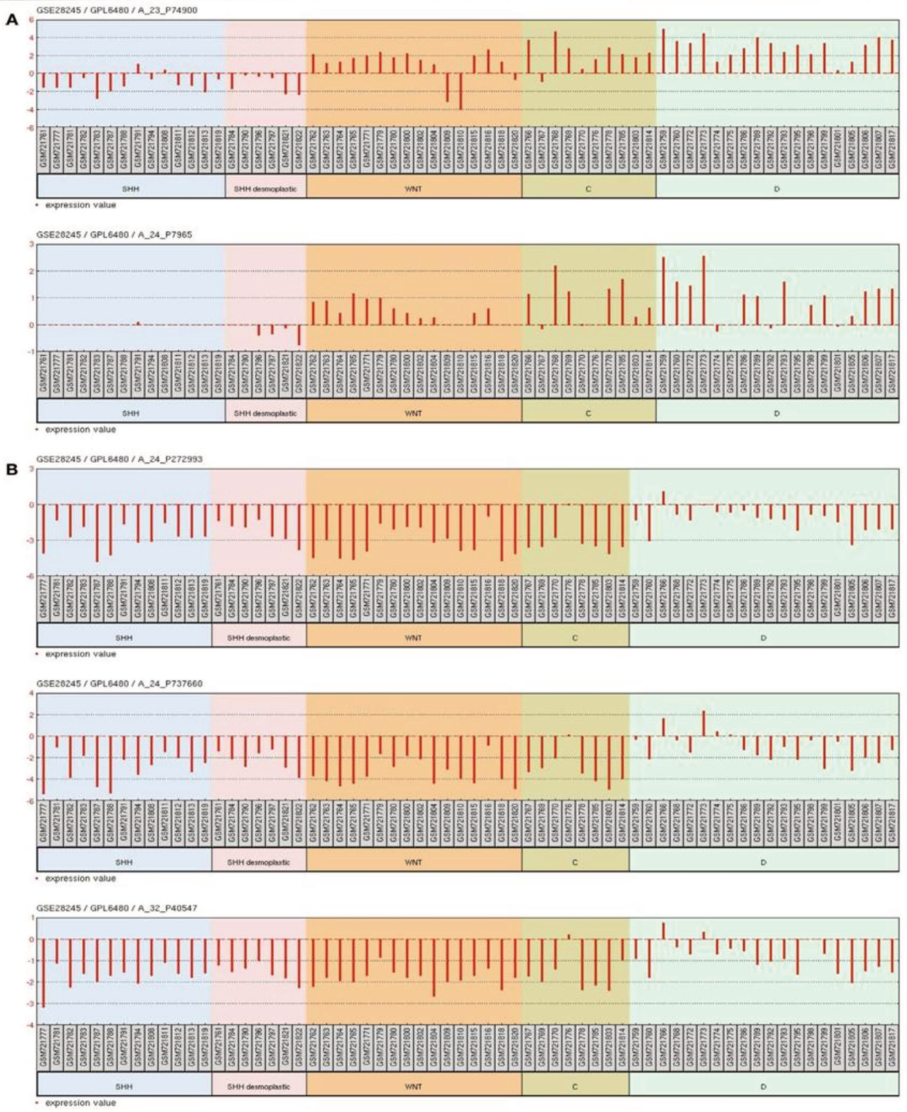


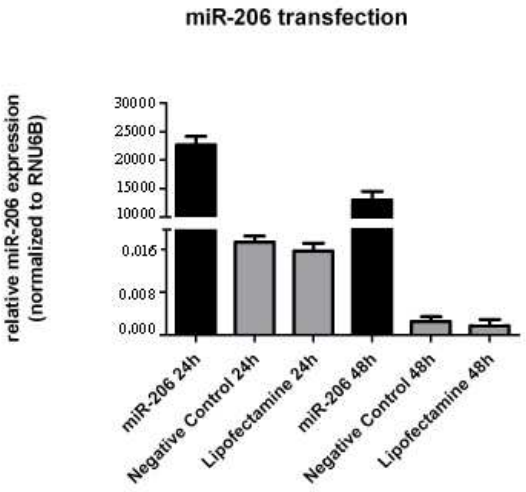
FIGURE A4 | Gene expression graph for different microarray probes of interest. Data from 64 primary human MB samples accessible through GEO Series accession number GSE28245 (38) in NCBI's Gene Expression Omnibus. **(A)** probe sets for *ESRRG*. **(B)** Probe sets for *MEG3*. Samples were grouped according to the molecular MB subgroups into WNT, SHH, C, and D. SHH desmoplastic was separated from SHH classic.

DC1. Teste com diferentes concentrações de miRNA mimic

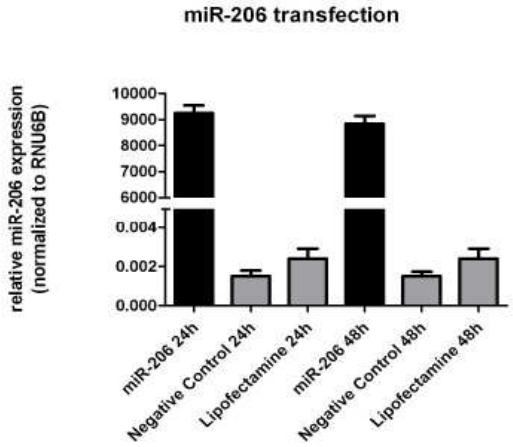
Para saber qual a melhor concentração de miRNA mimic a ser utilizada na transfecção de células DAOY, nós testamos três diferentes concentrações, 30nM, 3nM e 0,03nM, do miR-206 mimic e miR-1 mimic (controle negativo). Os testes foram realizados em placa de 6-well. Nós avaliamos a eficiência da transfecção e a expressão do miRNA mimic em 24 e 48 horas pós transfecção por RT-qPCR. O controle negativo (miR-1) e células DAOY sem transfecção com miRNA mimic (chamado de lipofectamine na Figura Complementar 1) foram utilizados como controle do miR-206. Como controle endógeno foi utilizado o RNU6B.

A transfecção do miR-206 mimic em células DAOY mostrou super expressão do miR-206 comparada com os dois controles (controle negativo e *lipofectamine*), confirmando a eficiência da transfecção. A concentração de 30nM de miR-206 mimic (Figura Complementar 1A) mostrou diferença nos níveis de expressão do miR- 206 entre 24 e 48 horas pós-transfecção. Também observamos que os níveis de expressão do miR-206 em 24 horas foi maior 10000 vezes na concentração de 30nM do que a concentração de 3nM (Figura Complementar 1B). Em contrapartida, a expressão do miR-206 foi menor que 8000 vezes na concentração de 0,03nM (Figura Complementar 1C) do que 3nM. Entretanto, para a análise funcional, escolhemos a concentração de 3nM de miRNA mimic.

A



B



C

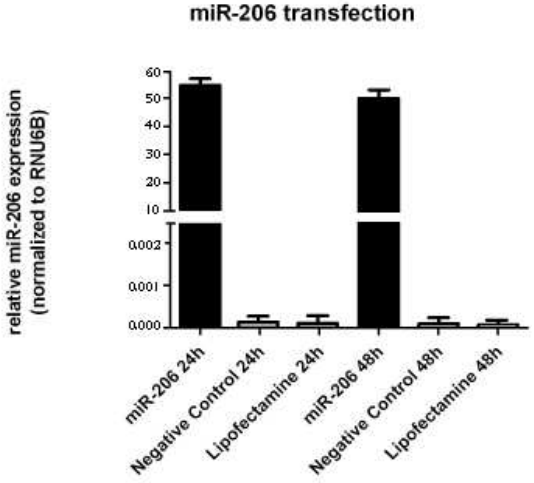


Figura Complementar 1. Teste com diferentes concentrações de miRNA mimic para a análise funcional. (A) 30 nM; (B) 3 nM; e (C) 0,03 nM. O valor da expressão relativa foi calculado usando o método $2^{-\Delta\Delta Ct}$.

DC2. Análise dos possíveis genes alvos do miR-206 por RT-qPCR

Genes alvos do miR-206 (Tabela Complementar 1) foram prospectados utilizando os softwares DIANA-miRPath (versão microT v4.0, beta) e miR-Ontology Database (miRò).

O RNA total foi extraído por trizol 24 e 48 horas pós transfecção com miR-206 mimic nas células DAOY. Cem nanogramas de RNA total foi usado para a síntese de cDNA usando o kit SuperScript vilo cDNA (Invitrogen) no volume final de 20µl, de acordo com instruções do fabricante. Análises de RT-qPCR dos genes GLI1, PTCH1, CSNK1A1, MEIS1, APC, PAX3, PDGFA and BNDF foram realizadas no Sistema LightCycler 480 PCR Systems (Roche) usando o SYBR Green I Master Mix (Roche), de acordo com instruções do fabricante. Cada reação foi realizada em duplicata. Os valores de CT dos genes alvo foram normalizados com o gene HPRT. O valor da expressão relativa foi calculado usando o método $2^{-\Delta\Delta Ct}$. A amostra DAOY foi escolhida como calibrador.

Tabela Complementar 1. Genes alvos do miR-206 e primers usados na reação de RT-qPCR.

		PRIMERS	
ID	**GENE**	**forward**	**reverse**
GLI1	GLI family zinc finger 1	ccagccagagagaccaacag	cccgcttcttggtcaactt
PTCH1	patched 1	aacacctggactcggcact	tctgtgataagctctcctgatttg
CSNK1A1	casein kinase 1	cttcggggacatctatttgg	agcttcactgccacttcctc
MEIS1	meis homeobox 1	gcatgaatatgggcatgga	catactcccctggcatactttg
APC	adenomatosis polyposis coli	catttccaagaagagggtttgt	gatcagcaagaagcaatgacc
PAX3	paired box 3	ttggcaatggcctctcac	aggggagagcgcgtaatc
PDGFA	platelet-derived growth factor alpha polypeptide	acacgagcagtgtcaagtgc	attccaccttggccacct
BNDF	Brainderived neurotrophic factor	gtaacggcggcagacaaa	gacctttcaaggactgtgacc
HPRT1	hypoxanthine phosphoribosyltransferase 1	tgaccttgatttattttgcatacc	cgagcaagacgttcagtcct

Não foram encontradas diferenças estatisticamente significativas entre os genes alvos estudados e o miR-206 por RT-qPCR no presente estudo. Sugerimos duas possíveis explicações: (i) Sequência *seed* inviável. Várias abordagens computacionais foram recentemente desenvolvidos para a predição de alvos miRNA incluindo, entre os mais populares, os softwares miRanda, TargetScan, and PicTar (John *et al.,* 2004; Krek *et al.,* 2005; Kuhn *et al*., 2008; Lewis *et al*., 2005; Rajewsky *et al.,* 2006), que se baseiam principalmente na identificação da sequência *seed* entre o miRNA e o genes-alvo correspondente. A sequência *seed* é uma região de 6-8nt na extremidade 5' do miRNA, que determina a qual RNA mensageiro (mRNA) o miRNA se ligará (Lewis *et al*., 2005). Infelizmente, a presença da sequência *seed*, embora conservado ao longo da evolução, não é uma forma viável para identificar miRNAs alvos funcionais. Estudos mostram que uma proporção significativa dos miRNA alvos preditos são falsos positivos (Didiano & Hobert 2006; Lewis et ai 2005), tornando assim a pré-seleção *in silico* de miRNA alvos muito complexo e laborioso; (ii) Controle pós-transcricional do miRNA. Recentemente, tem sido sugerido que o perfil de expressão simultâneo de miRNAs e mRNAs pode ser uma estratégia eficaz para a identificação de miRNA alvos (Huang *et al*., 2007). Isso ocorre porque, ao contrário da ideia original que miRNAs principalmente agem a nível traducional em células animais, há cada vez mais evidências de que muitos miRNAs podem causar degradação de seus alvos (Bagga *et al*., 2005; Lim *et al.,* 2005; Wu & Belasco, 2008). É importante notar que os genes, os quais são apenas pós-transcricionalmente regulados, não podem ser identificados através de métodos padrão, como RT-qPCR, para a investigação de mRNA.

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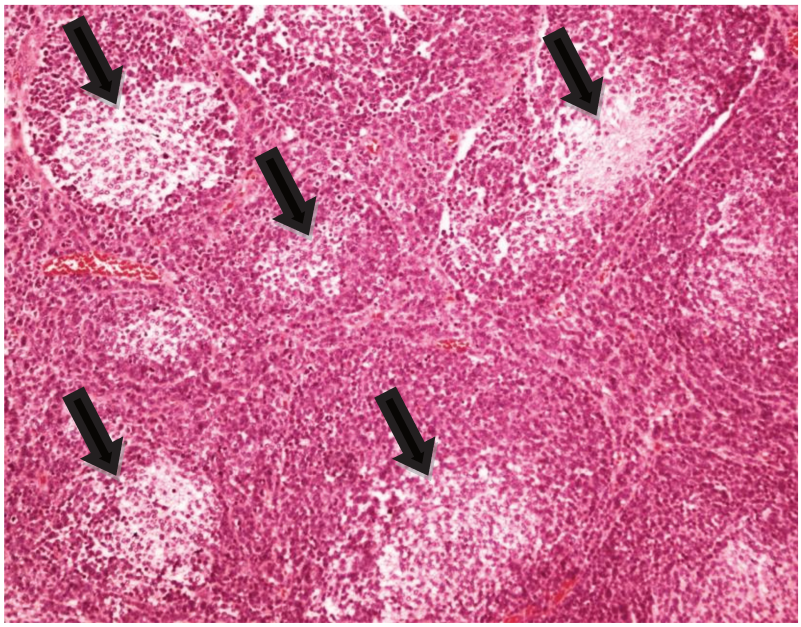
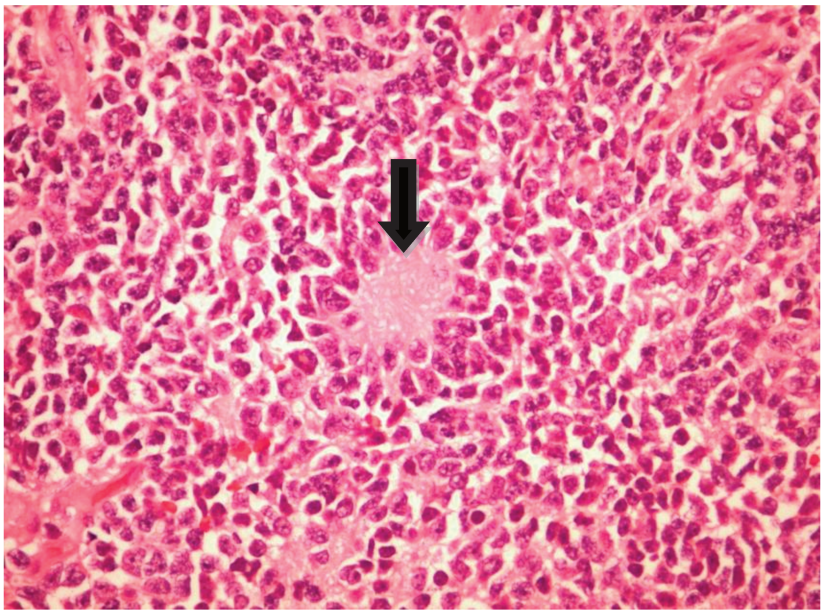
DC3. Fotos da análise histológica do tecido tumoral de meduloblastoma (MB7)

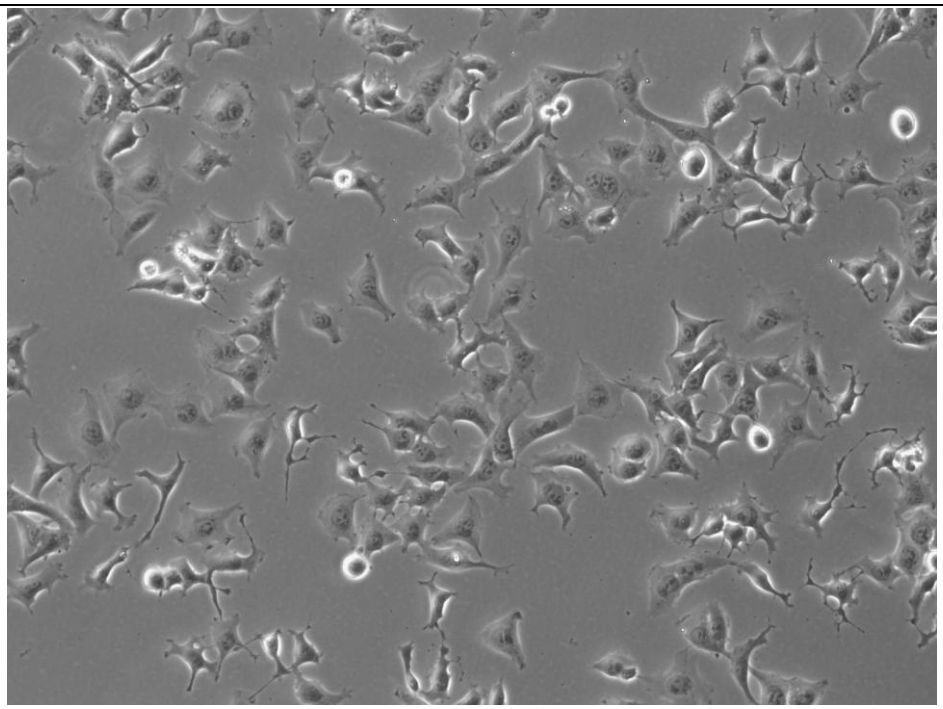


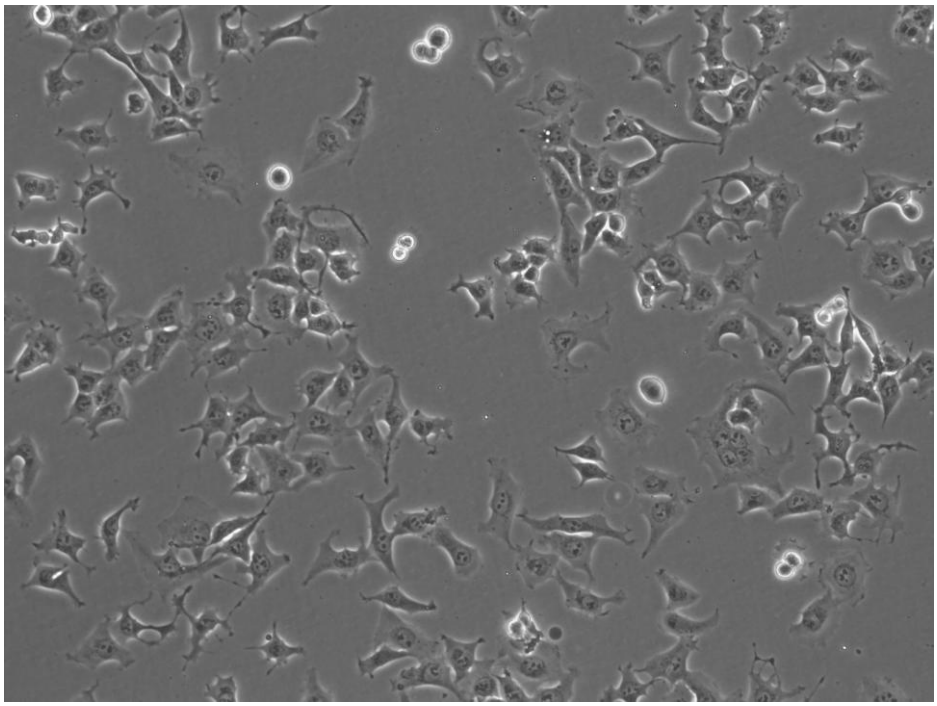
(MB7 sample) – Medulloblastoma Desmoplastic/Nodular: Histological features show embryonal tumor highly cellular composed of small round cells with scanty cytoplasma and hyperchromatic nuclei. Tumor demonstrates focal nodular architecture (arrows). (H&E, 100x).



(MB7 sample) – Medulloblastoma Desmoplastic/Nodular. Microscopic findings show small round tumor cells forming Homer-Wright (neuroblastic) rosettes whose center is composed of neurofibrillary matrix (arrow). (H&E, 400x).

DC4. Fotos do cultivo da linhagem celular de meduloblastoma DAOY





CAPÍTULO 3

Manuscrito 2:

Differential expression profile of microRNA in anencephaly is associated with cancer pathways

Lucon DR, Rocha CdS, Cardinalli IA, Maurer-Morelli C, Cavalcanti DP and Yunes JA

Artigo a ser submetido na Revista *Frontiers in Genetics*

Differential expression profile of microRNA in anencephaly is associated with cancer pathways

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ABSTRACT

Anencephaly (AN) is one of the most severe forms of neural tube defects (NTD). MicroRNA (miRNA) has been shown to play an important role in the development of the central nervous system. By using miRNA microarray approach, we found 52 miRNAs differentially expressed in AN compared to fetal or newborn frontal cortex, most of them overexpression. In this study, we report for the first time that AN miRNA target genes and functional gene networks were associated with cancer pathways. We also found the miRNAs significantly upregulated in human AN, like miR-34 family, in connection to *TP53.* Activation of *TP53* and/or indirect mechanisms may increase the expression of miR-34 family, as well as others miRNAs, ables to control the expression of developmental genes that have a role in body plan formation during embryogenesis and also involved in cancer development.

Keywords: Anencephaly, microRNAs, miR-34c-5p, *TP53*, cancer

i. Introduction

Anencephaly (AN) is one of the most severe forms of neural tube defects (NTD), and is characterized by full or partial absence of skull, brain and scalp. However, some rudimentary cerebrovascular tissue is usually identifiable. Exencephaly, absence of cranium with exposure and posterior degeneration of nerve tissue, is not clinically differentiable from AN in the older fetuses. Enviromental and genetic factors can contribute to such defects and NTD approximately occur in 1 out of every 1000 live births in Brazil (Castilla and Orioli, 1985; Brunoni, 1996).

microRNAs (miRNAs) are suggested to play an important role in controlling the development of the CNS by regulating neural stem cell differentiation and apoptosis (Cohen, 2010; Croce and Calin, 2005; Miska et al., 2004). miRNAs are small non-coding RNA molecules of ~22-25 nucleotides that post-transcriptionally down-regulate gene expression by binding of the 3´untranslated region (UTR) of protein coding transcripts, resulting in either mRNA cleavage or translational repression (Sood et al, 2006; Guo et al, 2010).

We report expression differentially profile of 52 miRNAs in AN in comparison to normal fetal or newborn frontal cortex by microarray analysis. Although miRNA expression profiling have been related in human and mouse with NTD (Zhang et al, 2010; Mukhopadhyay et al, 2011), this is the first time that was identify AN miRNA target genes and functional gene networks associated with cancer pathways. Associations have been reported between birth defects and increased risk of cancer (Agha *et al,* 2005; Fisher *et al,* 2012; Carozza *et al*, 2012). The significant risk found in these studies provides evidence of a link between birth defects and cancer, but this association is unclear.

We found the miR-34 family and others miRNAs significantly upregulated in human AN in connection to *TP53*. Activation of *TP53* leads to the coordinated induction of multiple downstream effectors, many of which act in a partly or fully redundant manner, indicating that such increases in expression might be secondary effects of miR-34. These findings suggest that TP53 activation increases the expression of miR-34 family, as well as others miRNAs, able to control the expression of developmental genes that have a role in body plan formation during embryogenesis and also involved in cancer development.

ii. Material and Methods

Human anencephaly tissue samples

Surgical specimens were obtained from 17 to 28 weeks-old fetuses with AN (n=8) (Table 1). Normal frontal cortex (NFC) tissues were obtained from 22 to 39 weeks-old and newborn (NW) autopsy (n=9) (Table 2). Ethical approvals were obtained from the Ethical Research Committee of the Faculty of Medical Science (n° 656/2009), CAISM (n° 064/2010), the Ethical Research Committee of Centro Infantil Boldrini (n° 1.90-030710) and National Committee of Ethics in Research (CONEP) n° 0005.0.144.146-09.

Total RNA isolation and analysis of global miRNA expression

Total RNA was extracted by Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, with an additional overnight precipitation step at -20°C with isopropanol (Merck). RNA quantification was done in a *Qubit® Quantitation Platform* (Invitrogen) and RNA quality was analysed via gel electrophoresis. Five hundred nanograms (ng) of RNA from twelve samples (six MB and six fetal cerebellum) were labeled with the 3'DNA FlashTag Biotin HSR kit (Genisphere, Hatfield, PA, USA) and hybridized to GeneChip miRNA Array 1.0 (Affymetrix Inc., Santa Clara, CA), which comprises 847 human miRNAs. Data was acquired using a GeneChip Scanner 3000 7G (Affymetrix).

Statistical analysis and bioinformatics methods to signaling pathway prediction

miRNA expression was computed in R environment (www.r-project.org) and the packages Affy and RankProd from Bioconductor. The MB miRNA profile was compared to that of cerebellum. Differentially expressed miRNAs were selected according to fold change ≥ 2.00 and p-value ≤ 0.05. Heat maps were created using tools of MetaboAnalyst 2.0 (www.metaboanalyst.ca). Signalling pathways were prospected by DIANA-miRPath (microT-v4.0, beta version) (http://diana.cslab.ece.ntua.gr/pathways/index_multiple.php). The input dataset enrichment analysis was performed by Pearson´s chi-squared test and each pathway was represented by the negative natural logarithm of the P-value (-In *P*). The Ingenuity Pathway Analysis software (IPA; http://www.ingenuity.com/) was used to identify possible pathways associated to differentially expressed miRNAs.

Validation of miRNA deregulation by quantitative real-time PCR

Reverse transcription (RT) and quantitative real time RT-PCR (RT-qPCR) analysis were carried out using commercially available TaqMan microRNA assays (Applied Biosystems, Foster City, CA) and a 7500 Real-time PCR System (Applied Biosystems). RT reactions (50 ng of total RNA) were performed in a 15 µl final volume containing specific stem-loop primers for miR-34c-5p and internal control small RNA, RNU6B), 10xRT Buffer, dNTPs, reverse transcriptase, RNase inhibitor and water in 96-well plates. Thermal cycling included 30 min at 16°C, 30 min at 42°C, and a final step of RT inactivation for 5 min at 85°C. PCR reactions were performed in a 10 µl final volume containing 5 µl TaqMan Universal Master Mix II, without UNG (Applied Biosystems), 3.5 µl water, 0.5 µl TaqMan microRNA Assay and 1 µl cDNA. Thermal cycling included a initial step of 10 min at 95°C for Taq activation followed by 40 cycles of 15 sec denaturation at 95°C and 1 min of annealing/extension at 60°C. Each reaction was performed in triplicate. miRNA expression level was normalized against RNU6B. The threshold cycle numbers (Ct) were calculated by relative quantification using the $2^{-\Delta\Delta Ct}$ Method, as described by Livak and Schmittgen (17). One of the control samples was chosen as calibrator.

iii. Results

Identification of differentially expressed miRNA in AN

Global miRNA profiles were generated for human AN (n=6) and NFC (n=6). Fifty two miRNAs (34 miRNAs upregulated and 18 miRNAs downregulated) were considered to be differentially expressed (fold change $\geq$ 2.0, $p \leq 0.05$) in AN in comparison to NFC (Table 3 and Figure 1). Among these, many miRNAs were related in human anencephaly for the first time.

Depict signaling pathway of AN miRNA profile

Signaling pathways putatively altered by AN deregulated miRNA were depicted by DIANA-miRPath. The list of the top 20 pathways is shown in Table 4. Axon guidance represents a key stage in the formation of neural network, such as cell migration during development. The Insulin signaling pathway showed higher enrichment when downregulated miRNAs were used in the analysis. Interestingly, three cancer pathways (prostate cancer, colorectal cancer, glioma) showed higher enrichment when the list of upregulated miRNAs was used in the analysis. WNT pathway also was more enrichment in this upregulated miRNAs list. WNT and Insulin signaling pathways are known play an important role in neurulation, central nervous system (CNS) developmental and/or CNS cancer (Chedotal *et al*, 2005; Aref *et al*, 2013; Kool et al., 2008).

Ingenuity pathway analysis (IPA)

Network analysis by IPA identified two networks as putative targets for 39 out of the 52 AN miRNAs. Networks were prospected considering only relationships that were experimentally observed. Interestingly, the all networks prospected in this study have been associated network functions with cancer. Two networks were enriched with miR-34 family and miR-21.

Network 1 (Figure 2A) included 9 miRNAs upregulated in AN samples (miR-21, 182, 486-5p, 489, 500a*, 501-3p, 501-5p, 574-3p, 574-5p) depicted by IPA as having direct interactions with tumor protein P53 (*TP53*) and drosha, ribonuclease type III (*DROSHA*). The *DROSHA* is the core nuclease that executes the initiation step of microRNA (miRNA) processing in the nucleus and have key roles in miRNA-mediated gene regulation in processes such as development and differentiation (Lee et al., 2003). *TP53* is involved in implantation and normal neural tube development (Pangilinan et al, 2008) and in animal study, deletion of *p53* leads to a significant increase in embryos that exhibit exencephaly (Hosako et al., 2009).

Intriguingly, all miRNAs connected to *TP53* in the Network 1 were found upregulated suggesting that this gene is activated in AN. *TP53* acts as a transcription factor to increase expression of a sets of miRNAs that include miR-34 (34a, 34c) and miR-500 (500a*, 500-3p, 500-5p). miR-500 which is expressed in the embriogenic mouse brain, is asymmetrically expressed in limb buds and might have a similar role related to asymmetry of brain structures (Wheeler et al, 2006). miR-34 dysregulation is

involved in the development of some cancer (Wetmore et al, 2001) and also highly predicted to be involved in neuronal processes and functions (Zovoilis et al, 2011). Among the experimentally confirmed targets of the miR-34 family is the *SIRT1* mRNA in neurons (Zovoilis et al, 2011). SIRT1 regulates p53 dependent apoptosis through deacetylating and stabilizing p53 suggesting a positive feedback loop, in which p53 induces expression of miR-34a which suppresses SIRT1 (Yamakuchi and Lowenstein, 2009). Besides miR-34 family (Yamakuchi and Lowenstein, 2009) only a few miRNAs, as miR-26a and anti-apoptotic miR-182, were consistently induced after p53 activation and may therefore represent direct p53 targets (Chang et al, 2007, Raver-Shapira et al., 2007; Tarasov et al, 2007). Therefore, p53-induced miRNAs may have tumor suppressive activity.

In the network 2 (Figure 2B), transforming growth factor-beta *TGF-β1* appeared as indirectly controlling the expression of four miRNAs overexpression (miR-21, 31, 34c-5p, 155). *TGF-β1* are expressed in neurons and glial cells and may play important roles in both brain development and neural stem cells maintenance and differentiation (Aigner and Bogdahn, 2008; Bottner et al, 2000; Miller et al, 2003). Previous studies have revealed strong evidence that folic acid supplementation during the critical period of organ formation (the first trimester of pregnancy) will decrease by 70% the incidence and prevalence of NTD (Fleming and Copp, 1988; Hall and Solehdin, 1998; Martinasevic et al, 1999). A possible mechanism of how folic acid prevents NTD might be due to the ability of folic acid to recover TGF-β1 expression, which, in turn, may induce normal functioning of TGF-β1 as growth factor (Santoso and Rohman, 2006).

RT-qPCR validation of miR-34c-5p

Among the miRNAs in the networks prospected, miR-34c-5p was chosen for validation by RT-qPCR. miR-34c-5p has been associated with TP53 and targeting many development genes, like *SIRT1, WNT1*, *NOTCH1*, *MYCN* and *SOX3* genes (Lewis et al, 2003; Yamakuchi and Lowenstein, 2009; Zovoilis et al, 2011), but has never been reported in human AN.

Real time RT-qPCR analysis were performed with the samples used in the microarray analysis (n=5 AN and n=5 cortex frontal) plus 2 other samples of AN (Table 1) and 2 new fetal/NW NFC (Table 2). As expected, miR-34c-5p (p=0,007; Man Whitney test) had higher expression in AN in comparison to NFC (Figure 3A), thus confirming our microarray findings.

iv. Discussion

Many miRNAs are expressed in the CNS during development (Bak et al, 2008; Kim et al, 2009) and found deregulated in human and murine NTD (Zhang et al, 2010; Mukhopadhyay, 2011). We investigated the expression profile of miRNAs in human AN in comparison to normal fetal/NW cortex frontal. Fifty two miRNAs were found to be differentially expressed in AN. Among these, four miRNAs were from a set of brain-enriched miRNAs: miR-124, miR-128, miR-137 and miR-139 (Sempere, 2004). The majority of differentially expressed miRNAs in AN were upregulated in comparison to NFC, corroborating previous study (Zhang et al, 2010). Most miRNAs were identified in this study for the first time in human AN, fact that can be explained by use a more

comprehensive version of Affymetrix miRNA microarray and the possibility of collecting cerebrovascular tissue.

Computational integration of AN miRNA profile (34 upregulated and 18 downregulated) and target genes were commonly enriched in cancer-related pathways, mainly the miRNAs upregulated. miRNAs may function as a novel class of oncogenes or tumor suppressor genes. Those miRNAs whose expression is increased in tumors may be considered as oncogenes. These oncogene miRNAs usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control cell differentiation or apoptosis (Zhang et al, 2007). Many miRNA genes have been found that are significantly over-expressed in different cancers (Ciafre *et al*, 2005; He *et al*, 2005; Malzkorn *et al*., 2009).

By comparing the list of differentially expressed miRNA reported for in MB (Lucon - article submitted in 2013) and AN, both originate from ectodermic tissue and during embriogenenic stage, we found 19 miRNAs deregulated in common, among these miR-34a, miR-21 and miR-124. miR-124 inhibits expression of CDK6 and prevents proliferation of medulloblastoma cells in vitro (Silber et al, 2013) and play an important role in neural development and differentiation, possibly in regulating the cytoskeleton (Yu *et al*., 2008; Visvanathan *et al*., 2007; Yoo *et al*., 2009). Developmental genes that have a role in body plan formation during embryogenesis are also involved in cancer development (Caroza et al, 2012).

Dysregulation of early human development likely plays an important role in the etiology of childhood cancer (fisher 2012). Associations have been reported between birth defects and increased risk of cancer, such as children with Down´s syndrome that display an increased risk of leukaemia and children with central nervous system (CNS) malformations with increased risk of development of brain tumours (Fisher *et al*, 2012; Altmann *et al*, 1998; Windham *et al* 1985; Bjorge *et al*, 2008, Mutafoglu-Uysal *et al*, 2009). Children with birth defects have a significantly increased risk, 2 to 3 fold higher risk, for developing cancer in comparison to children without birth defects (Agha *et al,* 2005; Fisher *et al,* 2012; Carozza *et al*, 2012). Based on cohort-studies the risk of developing cancer during the first year of life is approximately 5-fold greater in children with birth defects for those without birth defects (Agha *et al,* 2005; Bjorge *et al,* 2008). The significant risk found in these studies provides evidence of a link between birth defects and cancer.

Overexpressions of the mir-34a and mir-34c have recently been shown to be downstream effectors of p53-mediated senescence (Kumamoto et al., 2008). Recent studies have established miR-34a as a key effector of the p53 signaling pathway and have implicated its role in multiple cancer types. miR-34 and 182 were also found upregulated in colon cancer cell line (wt-p53) (Chang et al, 2007). wt-p53 regulates a number of noncoding miRNAs at the transcriptional level thereby influences certain cellular mRNAs translation through its mediated miRNAs (Xi et al,). miR-34a appears as a *TP53* target gene that mediates some of the biological effects elicited by *TP53*. Restoration of functional miR-34 inhibits cell growth and induces chemosensitization and apoptosis in p53-deficient human gastric cancer cells, indicating that miR-34 may restore p53 function (Ji et al, 2008). miR-34 also confers chemosensitivity through

modulation of MAGE-A and p53 in meduloblastoma (Weeraratne et al, 2011). In addition to p53-related expression, recent reports have shown that miR-34 (mir-34a, mir-34b, and mir-34c) are downstream effectors of p53-dependent senescent and apoptotic pathways (He et al, 2007; Raver-Shapira et al, 2007; Chang et al, 2007; Tarasov et al, 2007).

miR-34a is localized at chromosome 1p36, a region that is found the methylenetetrahydrofolate reductase (*MTHFR*) gene. *MTHFR* have been associated with an increased risk of neural tube defects. Thus, 1p36 genomic lesions might affect the p53 pathway at multiple levels, both upstream and downstream of p53 activation (He et al, 2007). Activation of p53 leads to the coordinated induction of multiple downstream effectors, many of which act in a partly or fully redundant manner, indicating that such increases in expression might be secondary effects of miR-34. Recent evidence suggests that the activation of oncogenes in the early phases of tumorigenesis may cause replication-stress which induces DNA damage (Menssen ate al, 2007; Tarasov et al, 2007). DNA damage is therefore presumably a common inducer of p53's tumor suppressive activity. Whether other miRNAs up-regulated after p53 activation, besides *miR-34a*, are direct p53 target genes is currently unknown. It is conceivable that indirect mechanisms also contribute to their up-regulation. E.g., changes in the cell cycle distribution which occur after p53 activation may result in changes in miRNA processing. Similar to miRNAs induced by the c-MYC transcription factor p53-induced miRNAs may serve to fine tune the response to p53 activation (O'Donnell et al, 2005). A small molecule was identified that specifically activates p53 without obvious genotoxicity (Lyubomir et al, 2004). Nutlin-3 disrupts the interaction between p53 and its principal antagonist Mdm2. Consequently, Mdm2-mediated ubiquitination and degradation of p53 is abolished, and active p53 accumulates to induce its target genes. Further experiments are necessary to underling the associations between miRNA upregulated and *TP53* activated in AN.

In conclusion, we report that the miR-34 family and others miRNAs are significantly upregulated in human AN, might be of *TP53* activation and/or indirect mechanisms that leads to neural tube defects by controlling the expression of development genes. These findings suggest that TP53 activation increases the expression of miR-34 family, as well as others miRNAs, able to control the expression of developmental genes that have a role in body plan formation during embryogenesis and also involved in cancer development.

Research support

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Acknowledgments

We thank Microarray Laboratory at Brazilian Biosciences National Laboratory, CNPEM-ABTLuS, Campinas, Brazil for their support with the use of equipaments Fluidics station and Scanner GeneChip.

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Tables and Figures

Table 1. Summary of the anencephaly samples included in the study.

Anencephaly	Gestational Age	Gender	Diagnosis
AN 1*	21	F	anencephaly
AN 2*	25	F	anencephaly - merocrania
AN 3	23	F	anencephaly
AN 4*	16	M	anencephaly - exencephaly
AN 5*	16	M	anencephaly - exencephaly - merocrania
AN 6*	28	M	anencephaly
AN 7	OF	-	anencephaly
AN 8*	17	F	anencephaly - exencephaly

* Samples used in Affymetrix miRNA microarray analysis; F=female; M=male

Table 2. Summary of the normal frontal cortex tissues.

Normal frontal cortex	Gestational Age	Gender	Diagnosis
Control 1*	31	M	NM
Control 2*	31	M	Disesq. esqueletica
Control 3*	OF	-	NM
Control 4*	24	M	NM
Control 5	39	M	hydropsy
Control 6	30	-	cardiopathy
Control 7	30	F	Renal dysplasia
Control 8*	26	M	NM
Control 9*	22	-	NM

* Samples used in Affymetrix miRNA microarray analysis; F=female; M=male; NM = no malformation and no aneuploidy.

Table 3. Deregulated miRNA in anencephaly compared to normal frontal cortex. List of 52 miRNAs found significantly deregulated (fold change ≥ 2.00 and p-value ≤ 0.05) in anencephaly.

miRNA	Chromosomal localization	Fold change	Reference
Downregulated			
hsa-miR-124*	20p23.1/8q12.3/8p23.1	-4,37	
hsa-miR-138	16q13.3/3p21.32	-3,76	*
hsa-miR-128	2q21.3/3p22.3	-3,66	
hsa-miR-650	22q11.21	-3,60	
hsa-miR-139-3p	11q13.4	-3,34	
hsa-miR-129*	11p11.2/7q32.1	-3,22	
hsa-miR-138-2*	16q13.3/3p21.32	-3,12	
hsa-miR-181a*	1q31.3/9q33.3	-2,96	
hsa-miR-137	1p21.3	-2,93	
hsa-miR-181c*	19p13.2	-2,81	
hsa-miR-720	3q26.1	-2,73	
hsa-miR-139-5p	11q13.4	-2,61	
hsa-miR-138-1*	16q13.3/3p21.32	-2,53	
hsa-miR-129-5p	11p11.2/7q32.1	-2,23	
hsa-miR-124	20p23.1/8q12.3/8p23.1	-2,23	*
hsa-miR-770-5p	14q32.2	-2,23	
hsa-miR-181d	19p13.2	-2,22	
hsa-miR-490-5p	7q33	-2,09	

(*Continued*)

Upregulated			
hsa-miR-886-3p	5q31.1	4,50	
hsa-miR-199b-3p	9q33.3	4,47	
hsa-miR-199a-3p	19p13.2/1q24.1	4,20	
hsa-miR-455-3p	9q32	4,08	
hsa-miR-489	7q321.3	3,99	
hsa-miR-34c-3p	11q23.1	3,96	
hsa-miR-886-5p	5q31.1	3,89	
hsa-miR-34c-5p	11q23.1	3,68	
hsa-miR-199a-5p	19p13.2/1q24.1	3,60	
hsa-miR-31	9p21.3	3,46	
hsa-miR-1298	Xq23	3,44	
hsa-miR-574-3p	4p14	3,34	
hsa-miR-449a	5q11.2	3,27	
hsa-miR-182	7q32.1	3,17	
hsa-miR-34a	1p36.23	3,15	
hsa-miR-451	17	3,06	*
hsa-miR-204	9q21.12	3,03	
hsa-miR-375	2q35	2,98	
hsa-miR-214	1q24.2	2,92	
hsa-miR-217	2p16.1	2,71	
hsa-miR-21	17q22	2,58	
hsa-miR-10b	2q31.1	2,55	
hsa-miR-214*	1q24.2	2,54	
hsa-miR-301a	17q22	2,48	
hsa-miR-216b	2p16.1	2,45	
hsa-miR-501-5p	Xp11.23	2,34	
hsa-miR-574-5p	4p14	2,27	
hsa-miR-501-3p	Xp11.23	2,26	
hsa-miR-122	18q21.31	2,24	
hsa-miR-486-5p	8p11.21	2,23	
hsa-miR-500*	Xp11.23	2,10	
hsa-miR-155	21q21.2	2,08	
hsa-miR-500	Xp11.23	2,06	
hsa-miR-455-5p	9q32	2,03	

* miRNAs previous related in human anencephaly by Zhang et al, 2010. AN, anencephaly.

Table 5. Enrichment analysis of top 20 pathways altered in anencephaly miRNA profile.

Pathway signalling	All deregulated miRNAs	Downregulated miRNAs	Upregulated miRNAs
		P value*	
Axon guidance	34.64	27.13	32.73
Wnt signaling pathway	24.86	11.09	30.66
MAPK signaling pathway	22.18	11.78	22.77
ErbB signaling pathway	21.8	16.5	19.35
Ribosome	21.53	12	14.6
Adherens junction	20.53	18.63	14.51
TGF-beta signaling pathway	20.3	12.88	16.77
Melanogenesis	18.09	10.65	18.29
Ubiquitin mediated proteolysis	16.38	8.84	13.82
Insulin signaling pathway	15.49	18.92	11.81
Prostate cancer	14.59	6.74	20.16
Focal adhesion	14.57	12.76	14.08
Renal cell carcinoma	14.28	12.54	16.38
Metabolism of xenobiotics by cytochrome P450	13.15	8.47	9.69
Colorectal cancer	12.89	6.64	17.76
Oxidative phosphorylation	12.54	10.57	8.29
Regulation of actin cytoskeleton	12	8.13	10.83
Long-term potentiation	10.66	11.22	13
mTOR signaling pathway	10.29	10.08	9.57
Glioma	10.08	7.1	15.23

*The negative natural logarithm of the enrichment *P*-value calculated for the specific pathway. Underline shows higher enrichment in downregulated or upregulated miRNAs lits.

Figure 1. Hierarchical clustering analysis of anencephaly and normal frontral cortex. Unsupervised hierarchical cluster analysis representing the 52 miRNAs expressed in anencephaly samples *versus* normal frontal cortex.

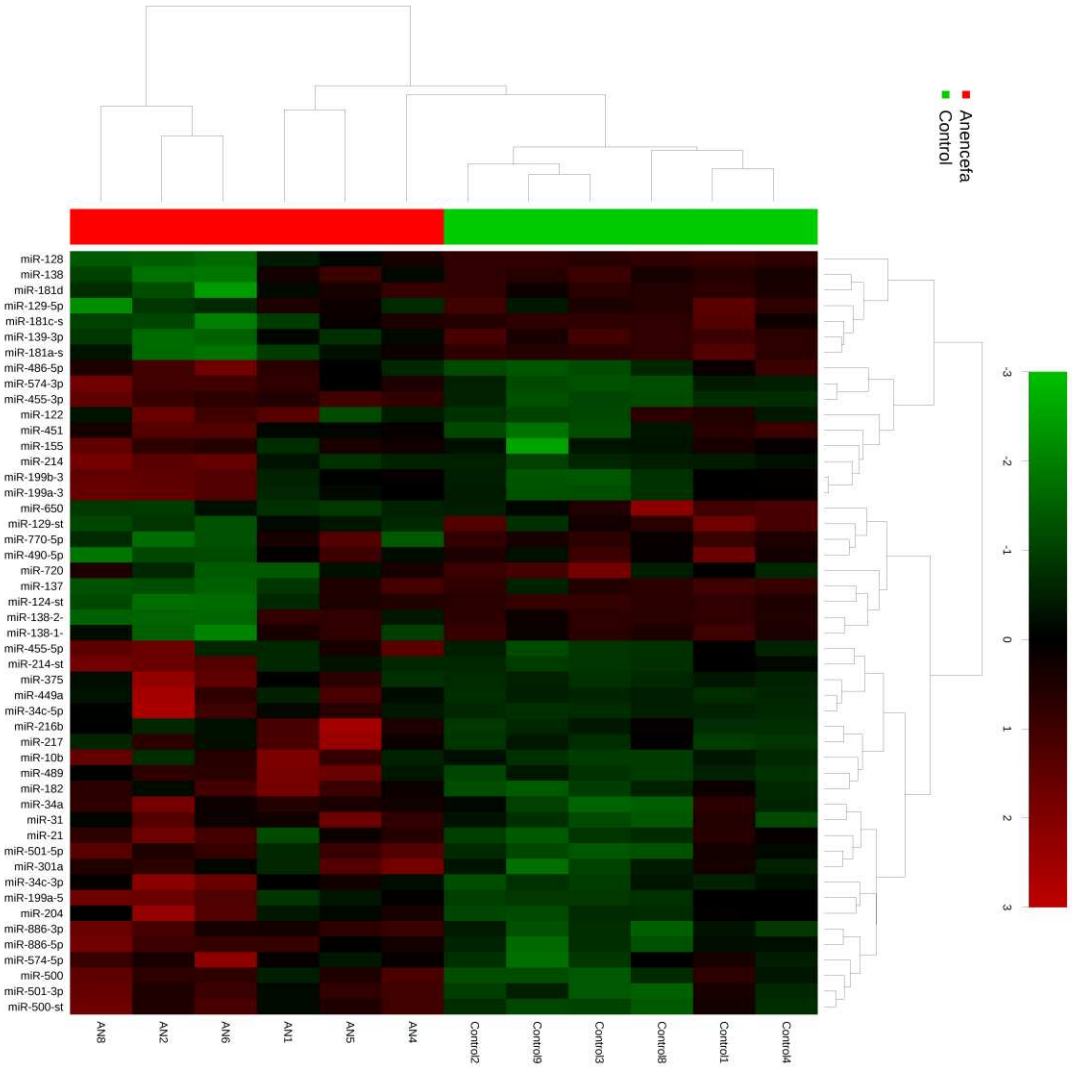
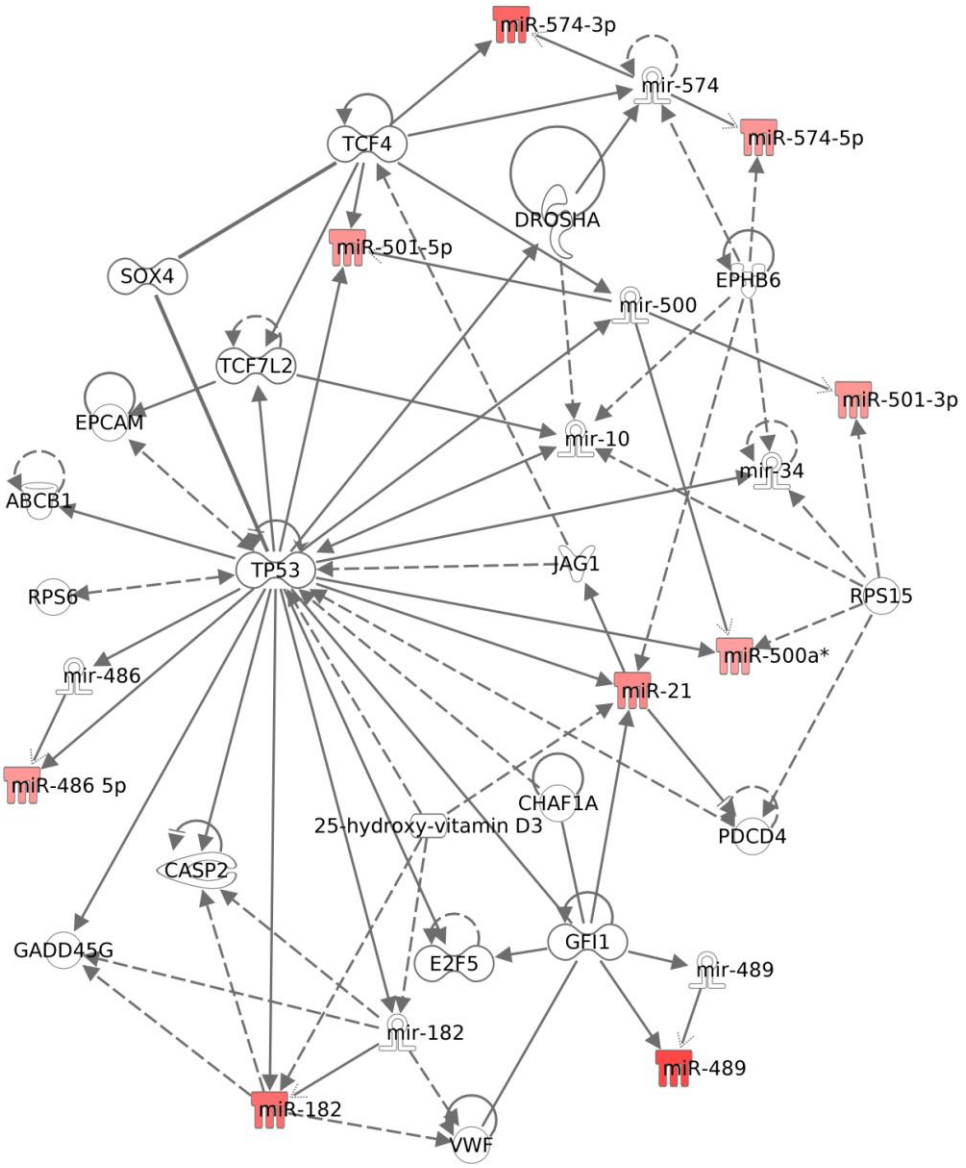


Figure 2. Ingenuity pathway analysis networks (IPA) constructed with the anencephaly deregulated miRNAs. (A) Network 1: miRNAs overexpression connecting to TP53 gene; (B) Network 2: TGF-β regulates miRNAs overexpression, like miR-21, 31, 34c, 155; (C) miR-34c as possible controller of development genes like *SIRT1, WNT1*, *NOTCH1*, *MYCN* and *SOX3* genes. Note that the miR-34c is present in both networks.

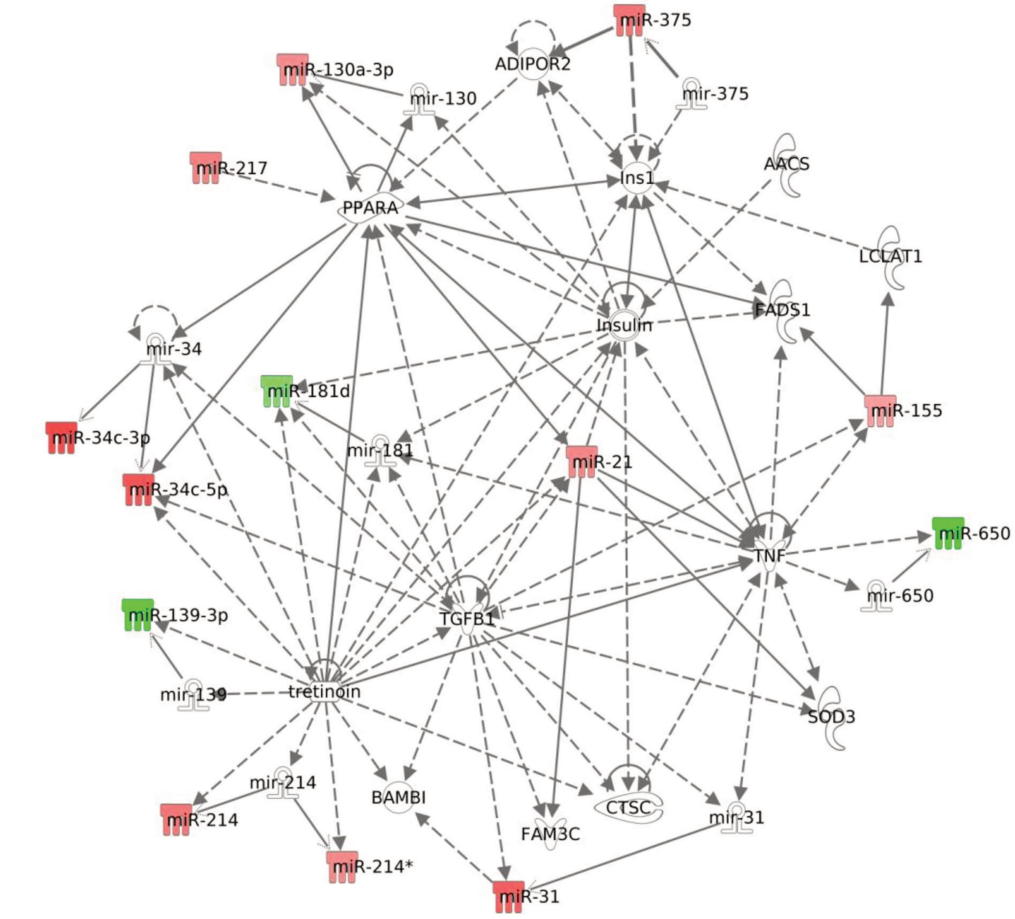
(A)

Path Designer Network an



(B)

Path Designer Network insul



Legend

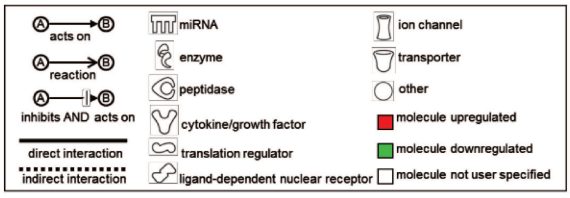
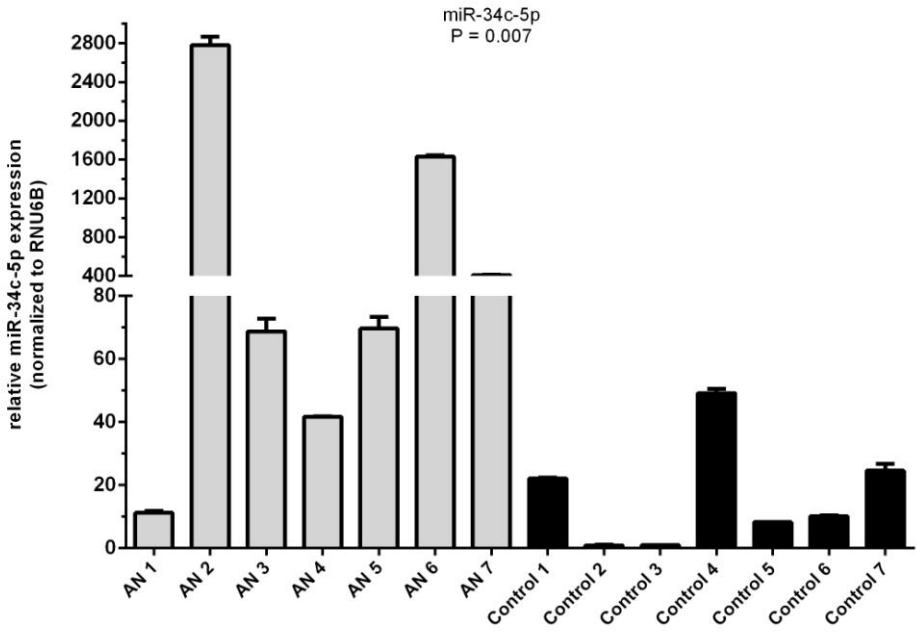


Figure 3. Expression levels of miR-34c-5p investigated in anencephaly (AN) and normal cortex frontal (NCF) by reverse transcriptase-quantitative PCR. Expression values were normalized with respect to the RNU6B endogenous control. Comparisons of AN *versus* NCF were performed by the Mann-Whitney test.



Neste projeto foram estudados os miRNAs diferencialmente expressos no tecido tumoral de MB *versus* cerebelo normal (84 miRNAs) e tecido cérebro-vascular de NA *versus* cortex frontal (52 miRNAs), ambos tecidos de origem neuroectodérmica. O perfil de expressão de miRNAs foi predominantemente baixo expresso nos MB e super expressos na AN. Dentre os miRNAs diferencialmente expressos encontrados em ambos perfis, 19 miRNAs (baixo-expressos: miR-124, 128, 129*, 129-5p, 138, 138-1*, 138-2*, 139-3p, 490-5p, 650, 770-5p; super-expressos: miR-199a-3p, 199b-3p, 199a-5p, 21, 214, 214*, 34a, 574-3p) apresentaram desregulação da expressão em ambas patologias. Chama a atenção que este grupo de miRNAs comuns são parte dos miRNAs mais descritos em câncer (miR-21, miR-34a, miR-129, miR-138, miR-199, miR-214) (Antonellis *et al.,* 2011 ; Bandres *et al.,* 2009 ; Birks *et al.,* 2011; de Bont *et al.,* 2008 ; Chang *et al.,* 2007; Garzia *et al.,* 2009; Grunder *et al.,* 2011 ; Huang *et al.,* 2009 ; Ji *et al.,* 2008; Jim *et al.,* 2013; Lu *et al.,* 2013 ; Malzkorn *et al.,* 2010; Neben *et al.,* 2004 ; Shen *et al.,* 2010; Venkataraman *et al.,* 2010; Whang *et al.,* 2013; Weeraratne *et al.,* 2011;Wetmore *et al.,* 2001; Yang *et al.,* 2013; Zhang *et al.,* 2013) e especificamente expressos no cérebro em comparação com outros orgãos (miR-124, miR-128, miR-139) (Sempere *et al.,* 2004).

Os três miRNAs que são enriquecidos no cérebro também foram descritos como funcionalmente importantes no câncer: (i) miR-124, um dos mais abundantes miRNAs expressos no sistema nervoso normal, desempenha um papel importante durante o desenvolvimento e a diferenciação neural (Yu *et al.,* 2008; Visvanathan *et al*., 2007; Yoo *et al*., 2009). Além disso, estudos mostram que a expressão ectópica de miR-124 na linhagem celular de meduloblastoma inibe a proliferação celular (Wei et al., 2009; Silber et al., 2013). Estudos funcionais com o miR-124 também foram descritos em glioblastoma, câncer de mama e próstata (Lv e Yang, 2013; Han et al., 2013; Shi et al., 2012). Estes estudos sugerem que o miR-124 tem uma função de supressor tumoral; (ii) Estudo funcional com miR-128 mostrou que este miRNA inibe o crescimento do tumor e a angiogênese (Shi et al., 2012); (iii) A expressão ectópica do miR-139 diminui o crescimento celular e tumorigenicidade em carcinoma coloretal (Guo et al., 2012).

O fato destes miRNAs estarem deregulados em duas condições diferentes (MB e AN) fazem pensar que sejam funcionalmente relevantes nestas patologias.

Análises de enriquecimento das vias de sinalização possivelmente alteradas pelo perfil de miRNAs diferencialmenete expressos em MB e AN mostram também algumas vias de sinalização em comum (ver capítulo 2 e 3), como *WNT*, *Adherens junction, TGF-β, Regulation of actin cytoskeleton, Focal adhesion,* Colorectal cancer, Glioma, entre outras. No entanto, algumas vias são mais enriquecidas em MB, como *Ribossome*, enquanto que outras são mais enriquecidas em AN, como *Axon guidance, Insulin, MAPK*. Diferenças eram esperadas e destacam a heterogeneidade molecular e complexidade na sinalização celular regulada por miRNAs. Dois cluster de miRNA identificados em MB exemplificam claramente estas diferenças, são eles: miRNA 17~92 cluster e miRNA 14q32 cluster. Estes miRNAs não foram encontrados nos miRNAs deregulados em AN. A expressão do miR-17~92 cluster é conhecida por estar envolvida com MB (Northcott *et al*., 2009, Uziel *et al*., 2009, Wei *et al*., 2009) e outros cânceres, como linfoma (He *et al*., 2005), câncer de pulmão (Hayashita *et al*., 2005), neuroblastoma (Schulte *et al*., 2008) e glioblastoma (Malzkorn *et al*., 2009). O miRNA-14q32 locus foi descrito neste estudo pela primeira vez em MB desmoplástico (ver capítulo 2) e recentemente em fibrose pulmonar, glioblastoma e osteosarcoma (Milosevic *et al*., 2012; Laddha *et al*., 2012; Sarcer *et al*., 2013).

Alterações na expressão de miRNAs podem constituir um achado secundário ao próprio fenótipo patológico, uma vez que não se sabe se a desregulação dos miRNAs é causa ou conseqüência da malformação/transformação tumoral. Futuras investigações são necessárias para entender o papel principal dos miRNAs aqui descritos em meduloblastoma e na anencefalia.

Embora alguns miRNAs tenha o mesmo perfil de expressão nos tecidos de AN e MB (ambos com tecido de origem neuroectodérmica), os métodos aqui empregados não permitiram averiguar se o mecanismo molecular que origina a malformação do tubo neural pode compartilhar algum ponto com o mecanismo que leva ao câncer do SNC na criança.

Conclusões Capítulo 2:

- miRNAs localizados no lócus cromossômico 14q32 (miR-127-3p, 127-5p, 134, 154, 299-5p, 323-3p, 323-5p, 369-5p, 370, 376c, 377*, 379, 381, 382, 409-5p, 410, 411, 411*, 431*, 432, 433, 485-3p, 487a, 487b, 494, 495, 539, 665, 770-5p) são significantemente baixo expressos em MB desmoplástico.

- A expressão ectópica do miR-129-5p (não presente dentro do lócus 14q32) diminui a proliferação de células de meduloblastoma.

Conclusões Capítulo 3:

- O perfil de miRNAs diferencialmente expressos em AN foi predominantemente super-expressos e pelo menos 12 deles (miR-21, 34a, 34c-5p, 155, 182, 486-5p, 489, 500a*, 501-3p, 501-5p, 574-3p, 574-5p) estão, possivelmente, regulando genes envolvidos com defeito do tubo neural e/ou câncer.

- Dentre os miRNAs baixo expressos em AN, quatro (miR-124, 128, 137, 139) são especificamente expressos no cérebro em comparação com outros órgãos. Os possíveis genes alvos destes miRNAs desempenham um papel importante durante o desenvolvimento e a diferenciação neural.

Conclusões Gerais:

- A comparação dos miRNAs diferencialmente expressos entre AN *versus* córtex frontal e MB *versus* cerebelo identificou 19 miRNAs em comum (baixo-expressos: miR-124, 128, 129*, 129-5p, 138, 138-1*, 138-2*, 139-3p, 490-5p, 650, 770-5p; super-expressos: miR-199a-3p, 199b-3p, 199a-5p, 21, 214, 214*, 34a, 574-3p). A maioria destes miRNAs em comum encontrados nas duas patologias fazem parte dos miRNAs mais descritos em câncer e/ou são especificamente expressos no cérebro em comparação com outros órgãos. O fato destes miRNAs estarem desregulados em duas condições diferentes (MB e AN) faz pensar que sejam funcionalmente relevantes nestas patologias.

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Anexo 1- Termo de Consentimento Livre e Esclarecido – Pacientes com Meduloblastoma

Título da pesquisa: **Análise do perfil de miRNAs nos tecidos de origem neuroectodérmica de anencefalia e meduloblastoma comparados com tecido cerebral normal.**

Pesquisadora responsável: Danielle Ribeiro Lucon
Orientador: Jose Andrés Yunes Co-Orientadoras: Claúdia V. Maurer Morelli e Denise Pontes Cavalcanti

Para obter um maior conhecimento clínico e científico sobre o câncer e doenças hematológicas, os pesquisadores e corpo clínico deste hospital desenvolvem pesquisa científica, através de projetos analisados e aprovados pelo Comitê de Ética em Pesquisa (CEP) e, se for o caso, pela Comissão Nacional de Ética em Pesquisa (CONEP). Através desta pesquisa, que lida com busca de genes e proteínas associadas ao câncer, será possível conhecer melhor os mecanismos de resistência da doença e, portanto, oferecer novas possibilidades de diagnóstico e tratamento aos pacientes.

Seu filho(a) foi submetido a tratamento para tumor do sistema nervoso central. O tratamento inclui coletas de sangue e medula óssea para o diagnostico e acompanhamento da doença. Parte da amostra colhida, não usada nos diagnósticos, representa material muito útil para pesquisa científica.

Por isso, você está sendo convidado a colaborar com a pesquisa científica, autorizando a utilização de parte das amostras de tumor já coletadas nos procedimentos rotineiros de diagnóstico. Apenas o restante do material colhido, não usado para diagnóstico, será aproveitado na pesquisa. Assim sendo, não será feita nenhuma coleta adicional de material àquela que já foi feita para o diagnóstico, portanto, a sua colaboração na pesquisa não acarreta risco nem desconforto adicional algum.

Solicitamos também sua autorização para a consulta do prontuário clínico, a fim de estudar a ocorrência de eventuais associações entre os dados laboratoriais encontrados e a evolução clínica da doença. Os dados individuais, resultados de exames e testes, bem como do prontuário, somente serão acessíveis aos pesquisadores envolvidos e não será permitido o acesso a terceiros (seguradoras, empregadores, supervisores hierárquicos, etc).

Além disso, solicitamos autorização para armazenar a parte da amostra não utilizada para a consecução deste projeto de pesquisa. Na eventualidade de novo projeto de pesquisa, o material só será utilizado mediante aprovação do novo projeto pelo CEP e, se for o caso, pela CONEP.

Todo o material utilizado nesta pesquisa será identificado no laboratório por código formado por números e letras e, portanto, a privacidade e identidade do seu filho(a) serão preservadas. A eventual inclusão dos resultados em publicação científica será feita de modo a manter o anonimato do paciente.

Concordando com o uso deste material, conforme descrito, é necessário esclarecer que você não terá benefícios ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa. Se você não concordar em permitir o uso deste material para pesquisa, ou se futuramente, em qualquer fase da pesquisa, vier a retirar seu consentimento, sua decisão não influenciará, de nenhum modo, no tratamento de seu filho(a), nem incorrerá em discriminação ou penalização alguma.

Você receberá uma cópia deste documento e o original será arquivado no prontuário do paciente.

Caso você tenha questões a fazer sobre este termo de consentimento ou alguma dúvida que não tenha sido esclarecida pelo seu médico, bem como se desejar a qualquer momento retirar seu consentimento, por gentileza, entre em contato com o pesquisador responsável pelo projeto, Dr. José Andrés Yunes, R. Gabriel Porto, 1270, Campinas, SP - 13083-210, F. (19)3787-5070, andres@boldrini.org.br. O Comitê de Ética em Pesquisa está situado na rua Tessália Vieira de Camargo, 126; Campinas, SP - 13083-887, F.(19)3521-8936, Fax (19)3521-7187, cep@fcm.unicamp.br.

Autorizo que o material coletado seja armazenado para uso em outras pesquisas após sua aprovação como novo projeto, mediante nova avaliação pelo CEP?	SIM ☐
	NÃO ☐

Assinatura do doador(a) ou Representante Legal:_____
Nome do doador(a_____
RG do Prontuário Médico:_____
Médico Responsável:_____
Presidente do Centro Infantil Boldrini:_____

Anexo 2- Termo de Consentimento Livre e Esclarecido – Pacientes com Defeito do Tubo Neural

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS
PROGRAMA DE GENÉTICA PERINATAL

Título da pesquisa: **Análise do perfil de miRNAs nos tecidos de origem neuroectodérmica de anencefalia e meduloblastoma comparados com tecido cerebral normal.**

Pesquisadora responsável: Danielle Ribeiro Lucon
Orientador: Jose Andrés Yunes Co-Orientadoras: Claúdia V. Maurer Morelli e Denise Pontes Cavalcanti

A presente pesquisa tem por objetivo avaliar e comparar o perfil da expressão de miRNAs nos tecidos de defeito do tubo neural (TDTN), meduloblastoma (TM) do tecido cerebral normal (TCN), pela tecnologia de *chip* de miRNA *microarray.* Para tanto, será necessário coletar uma amostra do tecido envolvido na lesão de anencefalia. A amostra de anencefalia será retirada pelo geneticista que examinar a criança após o óbito da mesma. Os resultados da análise das amostras de tecidos poderão ser divididos com outros pesquisadores futuramente.

A participação nessa pesquisa pode gerar um maior conhecimento dos mecanismos moleculares que expliquem a ocorrência da carcinogênese infantil e sua possível associação com o desenvolvimento do sistema nervoso. A participação na pesquisa não resultará em nenhuma vantagem direta para os participantes.

Todas as informações médicas, assim como os resultados das análises moleculares realizadas nesta pesquisa, farão parte do prontuário médico do paciente e serão submetidos aos regulamentos do HC/UNICAMP referentes ao sigilo da informação médica. O sigilo também será mantido em todos os estudos colaborativos que possam advir desta pesquisa por meio da utilização de um número de código para a identificação dos indivíduos participantes. Se os resultados ou informações fornecidas forem utilizados para fim de publicação científica nenhum nome será utilizado.

A qualquer momento o paciente (no caso o responsável pelo paciente) pode solicitar informações adicionais relativas a presente pesquisa bem como se recusar a participar da mesma, sem qualquer discriminação ou penalização alguma. O Dra. Denise Pontes Cavalcanti estará disponível para responder às questões ou preocupações (tel. 19-35219395). Em caso de recurso, dúvida ou reclamação é possível contactar a secretaria do Comitê de Ética da FCM- UNICAMP, Rua Tessália Vieira de Camargo, 126, 13083-887 – Campinas - SP, Fone: 19-35218936, email: cep@fcm.unicamp.br.

A não participação na presente pesquisa não compromete qualquer cuidado médico que o paciente ou a sua família recebe ou receberá futuramente no HC/UNICAMP.

Você receberá uma cópia deste documento e o original será arquivado no prontuário do paciente.

Como responsável (responsáveis) pelo(a) meu/minha filho(a) estou/estamos ciente(s) do presente termo e assumo/assumimos que sua participação na presente pesquisa é voluntária. Assim, se a qualquer momento mudar (mudarmos) de idéia, posso (poderemos) recusar a participação ou retirar o consentimento e interromper a minha (nossa) não participação na presente pesquisa sem comprometimento algum aos cuidados médicos que eu e minha família recebemos hoje ou futuramente no HC/UNICAMP.

Autorizo que o material coletado seja armazenado para uso em outras pesquisas após sua aprovação como novo projeto, mediante nova avaliação pelo CEP?	SIM ☐
	NÃO ☐

_____ _____
Nome do pai ou Responsável Nome da mãe ou responsável

_____
Pesquisador responsável

Anexo 3- Termo de Consentimento para a Coleta de Tecidos Cerebrais Normais.

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE CIÊNCIAS MÉDICAS

Título da pesquisa: **Análise do perfil de miRNAs nos tecidos de origem neuroectodérmica de anencefalia e meduloblastoma comparados com tecido cerebral normal.**

Pesquisadora responsável: Danielle Ribeiro Lucon
Orientador: Jose Andrés Yunes Co-Orientadoras: Claúdia Maurer Morelli e Denise Pontes Cavalcanti

A presente pesquisa tem por objetivo avaliar e comparar o perfil da expressão de miRNAs nos tecidos de defeito do tubo neural (TDTN), meduloblastoma (TM) do tecido cerebral normal (TCN), pela tecnologia de *chip* de miRNA *microarray*. Os resultados da análise das amostras de tecidos poderão ser divididos com outros pesquisadores futuramente. Para tanto amostras de tecidos serão coletadas por profissional, devidamente treinado, durante a autópsia. Visto que tais procedimentos são rotineiros e realizados por profissional treinado e competente os riscos são praticamente inexistentes. Além disso, solicitamos autorização para armazenar a parte da amostra não utilizada para a consecução deste projeto de pesquisa. Na eventualidade de novo projeto de pesquisa, o material só será utilizado mediante aprovação do novo projeto pelo Comitê de Ética em Pesquisa (CEP).

A participação nessa pesquisa pode gerar um maior conhecimento dos mecanismos moleculares que expliquem a ocorrência da carcinogênese infantil e sua possível associação com o desenvolvimento do sistema nervoso. A participação na pesquisa não resultará em nenhuma vantagem direta para os participantes.

Todas as informações médicas, assim como os resultados das análises moleculares realizadas nesta pesquisa, farão parte do prontuário médico do paciente e serão submetidos aos regulamentos do HC/UNICAMP referentes ao sigilo da informação médica. O sigilo também será mantido em todos os estudos colaborativos que possam advir desta pesquisa por meio da utilização de um número de código para a identificação dos indivíduos participantes. Se os resultados ou informações fornecidas forem utilizados para fim de publicação científica nenhum nome será utilizado.

A qualquer momento o paciente (no caso o responsável pelo paciente) pode solicitar informações adicionais relativas a presente pesquisa bem como se recusar a participar da mesma, sem qualquer discriminação ou penalização alguma. O Dr. Jose Andrés Yunes estará disponível para responder às questões ou preocupações (tel. 19-37875070). Em caso de recurso, dúvida ou reclamação é possível contactar a secretaria do Comitê de Ética da FCM- UNICAMP, Rua Tessália Vieira de Camargo, 126, 13083-887 – Campinas - SP, Fone: 19-35218936, email: cep@fcm.unicamp.br.

A não participação na presente pesquisa não compromete qualquer cuidado médico que o paciente ou a sua família recebe ou receberá futuramente no HC/UNICAMP.

Você receberá uma cópia deste documento e o original será arquivado no prontuário do paciente.

Como responsável (responsáveis) pelo(a) meu/minha filho(a) estou/estamos ciente(s) do presente termo e assumo/assumimos que sua participação na presente pesquisa é voluntária. Assim, se a qualquer momento mudar (mudarmos) de idéia, posso (poderemos) recusar a participação ou retirar o consentimento e interromper a minha (nossa) não participação na presente pesquisa sem comprometimento algum aos cuidados médicos que eu e minha família recebemos hoje ou futuramente no HC/UNICAMP.

Autorizo que o material coletado seja armazenado para uso em outras pesquisas após sua aprovação como novo projeto, mediante nova avaliação pelo CEP?	SIM ☐
	NÃO ☐

_____ _____
Nome do pai ou Responsável Nome da mãe ou responsável

_____
Pesquisador responsável

Anexo 4- Aprovação do Comitê de Ética em Pesquisa da FCM/UNICAMP

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/pesquisa/etica/index.html

CEP, 28/09/10
(PARECER CEP: N° 656/2009)

PARECER

I – IDENTIFICAÇÃO:

PROJETO: **"ANÁLISE DE PERFIL DE MIRNAS NOS TECIDOS DE ORIGEM NEUROECTODÉRMICO DE ANENCEFALIA, ESPINHA BÍFIDA, ENCEFALOCELE E MEDULOBLASTOMA COMPARADOS COM TECIDO CEREBRAL NORMAL".**

PESQUISADOR RESPONSÁVEL: Danielle Ribeiro Lucon

II – PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP aprovou o Relatório Parcial, apresentado em agosto de 2010, do protocolo de pesquisa supracitado.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

III – DATA DA REUNIÃO.

Homologado na IX Reunião Ordinária do CEP/FCM, em 28 de setembro de 2010.

Prof. Dr. Carlos Eduardo Steiner
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

Comitê de Ética em Pesquisa - UNICAMP
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Anexo 5- Aprovação da Comissão de Pesquisa do DTF/CAISM

UNICAMP

Comissão de Pesquisa do DTG / CAISM

Campinas, 17 de novembro de 2010.

Protocolo nº: 064/2010

O protocolo de pesquisa ***"Análise do perfil de miRNAs nos tecidos de origem neuroectodérmica de anencefalia e meduloblastoma comparados com tecido cerebral normal"*** da pesquisadora Danielle Ribeiro Lucon, orientada pelo Prof. Dr. José Andres Yunes, foi aprovado pela Comissão de Pesquisa do DTG/CAISM em 16/11/2010.

Atenciosamente,

PROF. DR. JOSÉ GUILHERME CECATTI
Presidente da Comissão de Pesquisa do DTG/CAISM

Rua Alexander Fleming, n.º101 – Cidade Universitária Zeferino Vaz – Campinas-SP
Fone: (19) 3521-9400
comissaopesquisa@caism.unicamp.br

Anexo 6- Aprovação do Comitê de Ética em Pesquisa do Centro Infantil Boldrini

Centro Infantil Boldrini

Campinas, 17 de dezembro de 2010.

Ilma. Sra.
Danielle Ribeiro Lucon

Prezada Senhora,

Informamos que o projeto intitulado "**ANÁLISE DO PERFIL DE MIRNAS NOS TECIDOS DE ORIGEM NEUROECTODÉRMICA DE ANENCEFALIA E MEDULOBLASTOMA COMPARADOS COM TECIDO CEREBRAL NORMAL**" foi aprovado pelo Comitê de Ética em Pesquisa do Centro Infantil Boldrini (CEP/Boldrini) em reunião de 17/12/2010.

Informamos que a cada 6 meses, deverá ser apresentado um relatório parcial sobre o andamento da pesquisa em questão (formulário: acompanhamento das pesquisas; disponível no site: www.boldrini.org.br ou no L: público/ CEC/ CEP Formulários) ao Comitê de Ética em Pesquisa do Centro Infantil Boldrini (CEP/Boldrini).

Solicitamos que, após a conclusão do estudo, o mesmo seja encaminhado à Secretaria do CEP/Boldrini e apresentado aos profissionais do Centro Infantil Boldrini.

Colocamo-nos à disposição para maiores esclarecimentos.

Cordialmente,

Prof. Dr. Flávio César de Sá
Vice-Coordenador do CEP/Boldrini

P.S.: Pedimos a gentileza de acusar o recebimento desta.

Util. Pub. Municipal Lei nº 466
Util. Pub. Estadual Dec. nº 33.018
Util. Pub. Federal Dec. nº 80.747
Registro no CNSS nº 33.002.080.581/84-0
Ens. Gratuito + Bolsas
CNPJ: 50.048.687/0001-27

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