UNIVERSIDADE FEDERAL DO PARÁ
NÚCLEO DE PESQUISAS EM ONCOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ONCOLOGIA E CIÊNCIAS MÉDICAS

REDUÇÃO DE MIR-218 NO SORO COMO BIOMARCADOR DE PIOR PROGNÓSTICO EM PACIENTES COM CÂNCER GÁSTRICO

Nina Nayara Ferreira Martins

BELÉM - PA

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Orientadora: Prof.ª Dr.ª Danielle Queiroz Calcagno

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Dedico este trabalho a Gabriel Martins e Lidia Amorim, por terem me apoiado e por renovarem minhas forças quando não pensei mais tê-las.

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Resumo

Recentemente, a biópsia líquida tem surgido como uma ferramenta promissora para identificação de potenciais biomarcadores de diagnóstico, prognóstico e preditivo no sangue de pacientes com diferentes tipos de doenças, incluindo o câncer. Dentre os biomarcadores, encontram-se os microRNAs que quando desregulados, contribuem para o surgimento de vários tipos de câncer, dentre eles, o câncer gástrico. Dados da literatura demonstram a correlação da diminuição da expressão de miR-218 como potencial supressor tumoral associado a progressão do câncer gástrico. Entretanto, apenas um estudo, em população oriental, avaliou a expressão do miR-218 circulante no soro de pacientes *vs* controle. Portanto, este trabalho tem por objetivo avaliar a expressão de miR-218 no soro de pacientes com câncer gástrico e a sua relação com as características clínico-patológicas. Foram coletadas amostras de 302 pacientes e 120 indivíduos saudáveis para análise da expressão de miR-218 por meio de *Real-Time Quantitative Polymerase Chain Reaction*. Foi possível observar a expressão diminuída do miR-218 no soro de pacientes com câncer gástrico em relação aos controles nos resultados encontrados. Além disso, a redução da expressão de miR-218 foi associada com maior invasão tumoral, presença de metástases de linfonodos, tipo difuso de Lauren, estágios mais avançados de câncer, indicando um pior prognóstico. Os resultados do presente estudo corroboraram com os achados da literatura descritos, que sugerem o potencial uso do miR-218 no soro como biomarcador de prognóstico em pacientes com câncer gástrico.

Palavras-chave:*miR-218-5p, adenocarcinoma gástrico, miRNA circulante, biópsia líquida, prognóstico*

Abstract

Recently, liquid biopsy has emerged as a promising tool for the identification of potential diagnosis, prognosis and/or predictive biomarkers in blood of patients with many different diseases, including cancer. MicroRNAs are among that potential biomarkers, and when deregulated, could contribute to the development of various types of cancer, such as gastric cancer. The literature demonstrates an association of miR-218 expression as a potential tumor suppressor associated with gastric cancer progression. However, only one previous study in Asiatic population evaluated the expression of circulating miR-218 in the serum of patients vs control. Therefore, the aim of this study was to evaluate the expression of miR-218 in the serum of patients with gastric cancer and its correlation with clinical-pathological characteristics. Samples were collected from 302 patients and 120 healthy subjects for analysis of mirR-218 expression by *Real-Time Quantitative Polymerase Chain Reaction*. The results demonstrated decreased expression of miR-218 in the serum of patients with gastric cancer in association with health subjects. In addition, the reduction of miR-218 expression was significantly associated with tumor invasion, presence of lymph node metastases, Lauren's diffuse type, advanced stages of cancer, indicating worse prognosis. Therefore, corroborating with findings from the literature, theses results suggest the potential use of miR-218 in serum as a prognostic biomarker in gastric cancer patients.

Key words: *miR-218-5p, gastric cancer, circulating miR, liquid biopsy, prognosis*

LISTA DE ILUSTRAÇÕES

Figura 1. Representação de biomarcadores em fluídos biológicos. **14**

Figura 2. Biogênse do miRNA parte I. **16**

Figura 3. Representação linear da estrutura da *Drosha.* **16**

Figura 4. Estrutura linear dos domínios da proteína *Dicer.* **17**

Figura 5. Biogênese completa dos miRNAs na célula. **18**

Figura 6. Desregulações em miRNAs no câncer. **20**

Figura 7. Representação da Biogênse e secreção de miRNAs pelas células tumorais. **22**

Figura 8. Ilustração da localização dos genes MIR-218-1 e do gene MIR-218-2. **34**

LISTA DE TABELA

Tabela 1. Expressão desregulada de miRNAs e sua implicação clínica em pacientes com CG. **25**

Tabela 2: Identificação dos *primers* utilização no método RTqPCR para análise de expressão no soro de pacientes com CG. **40**

Tabela 3: Expressão do miR-218 correlacionado aos dados clínico-patológicos de pacientes com CG. **41**

LISTA DE ABREVIATURAS

AGO	Argonauta
ANG 2	Angeopoietina 2
BL	Biópsia líquida
CA 19-9	*Cancer antigen 19-9*
CEA	*Carcinoembryonic antigen*
CED	*Conserved central domain*
CG	Câncer gástrico
CTC	Célula tumoral circulante
ctDNA	Circulating tumor DNA
DGCR8	*DiGeorge syndrome critical region gene 8*
dsRNA	RNA de dupla fita
MLN	Metástase de linfonodo
ncRNAs	RNAs não codificantes
oncomiR	miRNA oncogênico
PACT	*Protein Kinase Interferon-Inducible Double Stranded RNA Dependen tActivator*
qPCR	*Real-Time Quantitative Polymerase Chain Reaction*
qRT–PCR	*Reverse transcription polymerase chain reaction quantitative real time*
RISC	Complexo de indução do silenciamento do RNA
RNAm	RNA mensageiro
SNP	polimorfismo de nucleotídeo único
TRBP	*HIV-1 TAR RNA-bindingprotein*
tsmiR	miRNA supressor tumoral
XPO-5	Exportina 5

SUMÁRIO

1. INTRODUÇÃO 12
1.1 Considerações Gerais 12
1.2 miRNAs 14
1.3 miRNAs no câncer 19
1.3.1 miRNA circulante no câncer 21
1.4 Papel do miR-218 no câncer 34
2. APLICABILIDADE CLÍNICA 37
3. OBJETIVOS 38
3.1 Objetivo Geral 38
3.2 Objetivos Específicos 38
4. METODOLOGIA 39
4.1 Pacientes e Processamento do Sangue 39
4.2 Extração do RNA Total 39
4.3 RT-PCR qPCR 39
4.4 Testes Estatísticos 40
5. RESULTADOS 41
6. DISCUSSÃO 43
7. CONCLUSÃO 46
REFERÊNCIAS 47
ANEXO I 57
ANEXO II 73

1.INTRODUÇÃO

1.1 Considerações Gerais

O câncer gástrico (CG) é o quinto câncer de maior incidência global e a terceira maior causa de mortalidade relacionada ao câncer (FERLAY *et al.* 2015). Embora o número de novos casos tenham diminuído, a detecção de lesões malignas acontece usualmente em estágio avançado, o que dificulta o tratamento e compromete o prognóstico e sobrevida do paciente (CALCAGNO; DE ARRUDA CARDOSO SMITH; BURBANO, 2015).

A definição de CG é de qualquer neoplasia maligna compreendida entre a junção gastroesofágica e o piloro, capaz de alcançar diferentes camadas de tecido (mucosa, submucosa, muscular, subserosa e serosa). Aproximadamente 90% dos casos de CG são de adenocarcinomas, que se desenvolvem das glândulas da mucosa do estomago (KARIMI et al., 2014).

A classificação histopatológica comumente utilizada é classificação de Lauren, que divide adenocarcinoma em dois maiores tipos: difuso e intestinal, que apresentam diferenças epidemiológicas como incidência em gênero, idade e diagnóstico (JIMÉNEZ FONSECA et al., 2017; LAUREN, 1965) A divisão do estômago inclui cárdia (porção superior), fundo, corpo, piloro e antro, que são demarcações anatômicas com diferenças microscópicas (KARIMI et al., 2014).

A bactéria *H. pylori*. é considerada um carcinógeno tipo 1 pela Organização Mundial da Saúde (OMS)– e principal fator de risco etiológico para o desenvolvimento do CG (WANG *et al*. 2014a). Além da bactéria, o vírus Epstein-Barr (EBV) é apontado como outro possível agente etiológico do tumor (CHANG; KIM, 2005; CHEN *et al.*, 2015). Geralmente, os pacientes com CG já são diagnosticados em estágios avançados contribuindo para uma taxa de sobrevida reduzida de aproximadamente 20% após 5 anos da detecção do CG, que não manifesta sintomas específicos nos estágios iniciais da doença (NAGINI, 2012). Atualmente, o diagnóstico do CG é realizado por endoscopia do trato gastrointestinal alto e biópsia do tecido, procedimento invasivo, de alto custo e que pode ter efeitos adversos raros, porém graves (HAMASHIMA, 2016). Os biomarcadores tumorais utilizados para acompanhar os pacientes de CG, como CEA (do inglês, *carcinoembryonic antigen*) e CA 19-9 (do inglês, *cancer antigen 19-9*), podem ter suas concentrações elevadas em diversos tipos de câncer e em algumas doenças

benignas (falso-positivo) permitindo uma baixa especificidade e sensibilidade para alcançarem objetivo de um marcador ideal para o diagnóstico precoce, rastreamento determinação do prognóstico de pacientes com câncer (ZHENG; ZHAO; GULENG, 2015). Além disso, existem casos raros em que os tumores não produzem CEA e, uma pequena parte da população (3-7%) pode não expressar o CA 19-9 (BALLEHANINNA; CHAMBERLAIN, 2011; HAMADA *et al.* 2012; LUO *et al.* 2017), demonstrando a necessidade da busca de novos biomarcadores com maior sensibilidade e especificidade no diagnóstico e de monitoramento da resposta terapêutica em pacientes com CG. Esses fatos reforçam a gravidade do CG e a necessidade do desenvolvimento de novos estudos que possam ajudar na identificação de características biológicas peculiares ao tumor, ampliando a capacidade de prever o comportamento dessa neoplasia e permitindo o estabelecimento de conduta terapêutica mais precisa.

Recentemente, surgiu o termo “Biópsia Líquida” (BL) que consiste em uma potencial ferramenta com diversas aplicações na prática clínica capaz analisar biomarcadores em diferentes fluidos biológicos, como sangue (soro e/ou plasma), saliva e urina, tornando-se um método minimamente invasivo cada vez mais investigado (SIRAVEGNA *et al.* 2017). Despontando cada vez mais como ferramenta promissora para diagnóstico precoce, monitoramento de recidivas e preditor de prognóstico (UCHÔA GUIMARÃES *et al.* 2018)

Originalmente o termo biópsia líquida foi utilizado somente para detecção de células tumorais circulantes (CTCs) porém, nos últimos anos, passou a ser empregado para moléculas como DNA tumoral circulante (ctDNA) e RNAs não codificantes (ncRNAs). Como exemplo do grupo de ncRNAs, temos os microRNAs (miRNAs) circulantes livres ou em exossomas na corrente sanguínea (Figura 1)(KALNINA *et al.* 2015).

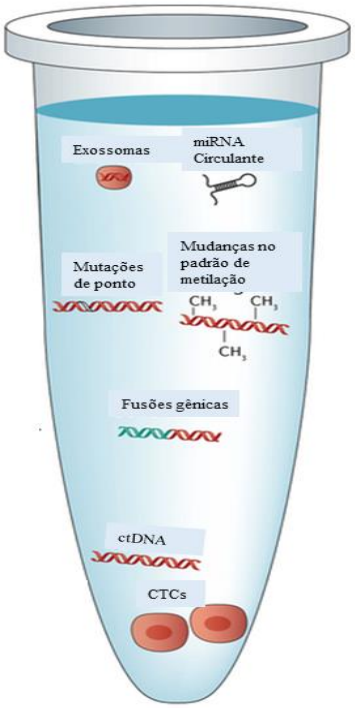

Figura 1. Representação da presença de alguns biomarcadores em fluídos biológicos, como CTCs, ctDNA e miRNAs (Adaptado de SIRAVEGNA et al., 2017).

É notável a relevância das análises de ncRNAs na avaliação do prognóstico e o monitoramento clínico do paciente com CG, embora ainda sejam considerados como evidência moderada-fraca na utilização da biópsia líquida na prática clínica pela falta de padronização dos protocolos (GUIMARÃES *et al.* 2018). Portanto, faz-se necessário novos estudos para melhor compreender os seus mecanismos de ação e a aplicação dos miRNAs na medicina de precisão.

1.2 miRNAs

Os miRNAs são pequenos RNAs não-codificantes, formados por 18-25 nucleotídeos (nt), capazes de regular negativamente funções do organismo a nível pós-transcricional, regulando assim a expressão gênica (FERREIRA MARTINS *et al.* 2016; OLIVEIRA *et al.* 2017). Na célula, os miRNAs estão envolvidos em diversos processos fisiológicos, incluindo a diferenciação, apoptose, hematopoiese, crescimento e proliferação celular (WAHID *et al.* 2010). Portanto, quando os miRNAs estão

desregulados, implicam em quebra da homeostase, podendo contribuir para o surgimento de várias doenças, como o câncer (DA SILVA OLIVEIRA *et al.* 2016; FERREIRA MARTINS *et al.* 2016)

Os mecanismos de ação dos miRNAs não são completamente compreendidos, entretanto a repressão gênica a nível de tradução de RNA mensageiro (RNAm) em proteína é o mecanismo mais descrito na literatura. Porém, outras funções também já foram mencionada, como repressão gênica a nível de estabilidade do RNAm, ou seja, antes do processo de tradução como decréscimo da expressão do RNAm alvo, e ainda a repressão a nível transcricional, onde os miRNAs podem mediar a organização da cromatina (MOROZOVA *et al.* 2012).

A biogênese dos miRNAs tem início no núcleo, quando a enzima RNA polimerase II (pol II) gera um transcrito de miRNA primário (pri-RNA) que pode chegar até 1000 nt de comprimento, formando estruturas em grampo com fita dupla não complementar (LIN; GREGORY, 2015). Ainda no núcleo, os pri-miRNAs passam pela primeira clivagem, por meio de um complexo protéico composto pela endonucleaseRNase III Drosha e a proteína de ligação dupla fita DGCR8 (*DiGeorge syndrome critical region gene 8*) (MACFARLANE; MURPHY, 2010), originando o o precursor do miRNA (pre-miRNA). Esta molécula é composta de 60-120 nt e compreende a estrutura da cabeça do grampo “hairpin”, que será exportada ao citoplasma com auxílio da Exportina-5 (XPO-5) e o cofator RanGTP (Figura 2) (WANG *et al.* 2011).

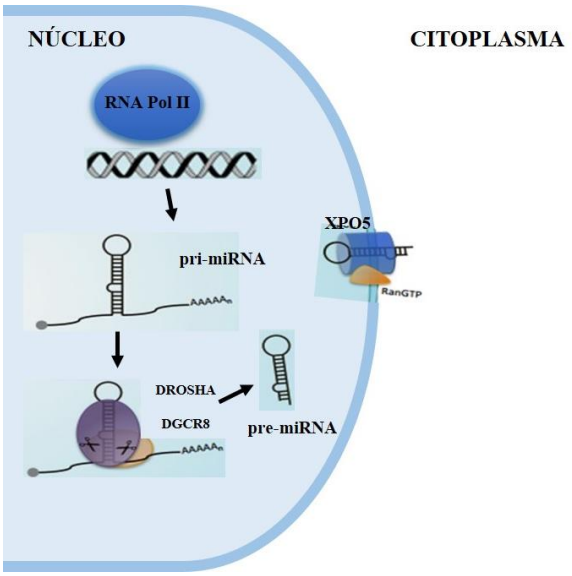

Figura 2. Biogênse do miRNA parte I. Formação da molécula de miRNA a partir do material genético não-codificante pela RNA polimerase II (RNA Pol II), formando o pri-miRNA que será clivado pela Drosha e seus cofatores (DGR8) em uma estrutura chamada microprocessador, transformando-se em pre-miRNA. Este será exportado para o citoplasma por meio da Exportina-5 (XPO-5) e RanGTP (Adaptado de DAUGAARD; HANSEN, 2017).

Drosha é uma endonuclease, pertencente à família RNase III que age especificamente sobre RNAs de dupla fita (dsRNA). É composta por um domínio central extremamente conservado (do inglês, *conserved central domain*- CED) importante para atividade de segmentação e a parte carboxi-terminal (C-terminal), que contém dois domínios RNase III (RIIIDa e RIIIDb) e um domínio de ligação dsRBD (Figura 3). Sendo que RIIIDa corta a fita 3´ do pri-miRNA e RIIIDb corta a fita 5´ para produzir uma extensão terminal 2-nt 3'protuberante (KWON *et al.* 2016; LI; PATEL, 2016).

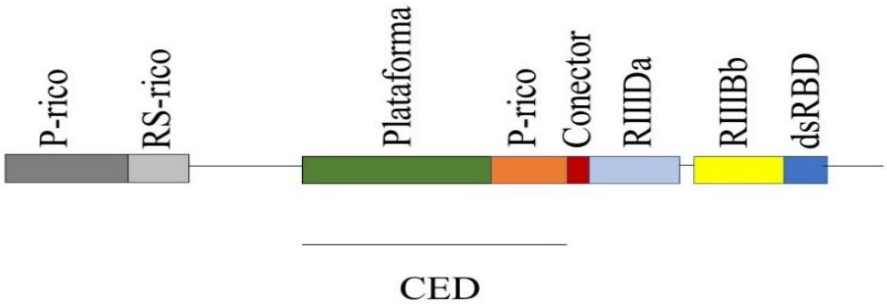

Figura 3. Representação linear da estrutura da Drosha. CED: domínio central; P-rico: rico em prolina;RS-rico: rico em Arginina/Serina; PAZ: Piwi-Argonauta-Zwille; dsRBD: domínio de ligação de RNA de fita dupla (Adaptado de KWON et al., 2016).

Já a XPO-5 é uma proteína nuclear transportadora de miRNA que também age como proteção contra ação digestiva das enzimas nucleases (WANG *et al.* 2011). Sua estrutura é em formato de U, extremante compactada, com aproximadamente 20 *tandem* HEAT *repeat*. Além disso, sua interação com o pré-miRNA acontece pela superfície interna da proteína que reconhece o grupo fosfodiéster externo do pré-miRNA, enquanto o túnel da XPO-5 vai interagir com 2-nt 3'protuberante, através de pontes de hidrogênio e ligações iônicas, indicativo que o reconhecimento do pré-miRNA acontece pela assinatura do terminal 2-nt 3'protuberante (CONNERTY; AHADI; HUTVAGNER, 2015; WANG *et al.* 2011).

No citoplasma, o pré-miRNA é clivado pela endonucleaseRNase III Dicer com auxílio de dois cofatores PACT (*Protein Kinase Interferon-Inducible Double Stranded RNA Dependent Activator*) e TRBP (*HIV-1 TAR RNA-bindingprotein*) para uma forma madura de miRNA de dupla fita, denominado miRNA duplex (ACHKAR; CAMBIAGNO; MANAVELLA, 2016; CHAN *et al.* 2015; HAMMOND, 2015).

Dicer é uma larga proteína com domínios ATPase/RNA helicase (DExD/H, HELICc), um domínio de dimerização (TRBP-BD), um domínio Piwi-Argonauta-Zwille (PAZ), dois domínios RNase III, um domínio de função desconhecida (DUF283), além do domínio dsRBD (Figura 4).

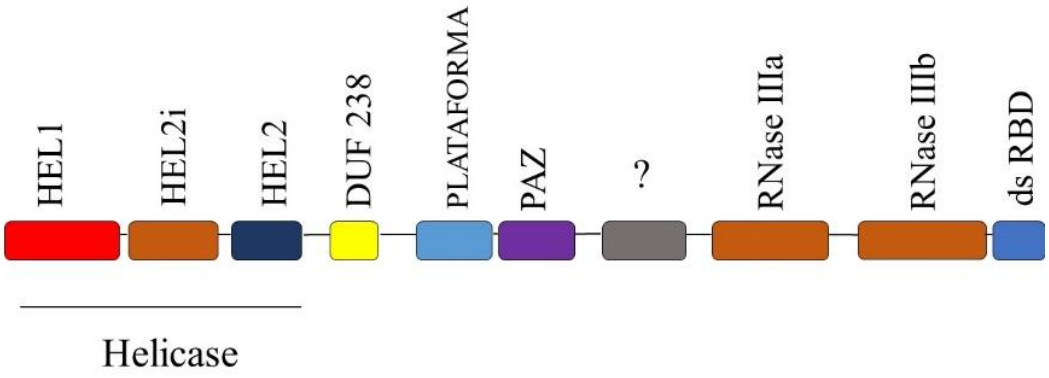

Figura 4. Estrutura linear dos domínios da proteína Dicer(Adaptado de(LAU et al., 2012).

Embora *Dicer* possa fazer a clivagem do pré-miRNA sozinha, sofre a modulação de sua atividade pela interação com as duas proteínas, PACT e TRBP; pela proteína de ligação TAR RNA (TRPB), capazes de estabilizar a função de *Dicer*, afetando o processamento do miRNA e a escolha da fita que será o miRNA maduro final (CONNERTY; AHADI; HUTVAGNER, 2015).

Posteriormente, o miRNA duplex se liga à proteínas da família Argonauta (AGO) formando o complexo de indução do silenciamento do RNA (RISC), onde uma fita poderá permanecer no complexo como o miRNA expresso e a outra será degradada para iniciar a regulação pós-transcricional do RNAm (Figura 5) (KIM; HAN; SIOMI, 2009).

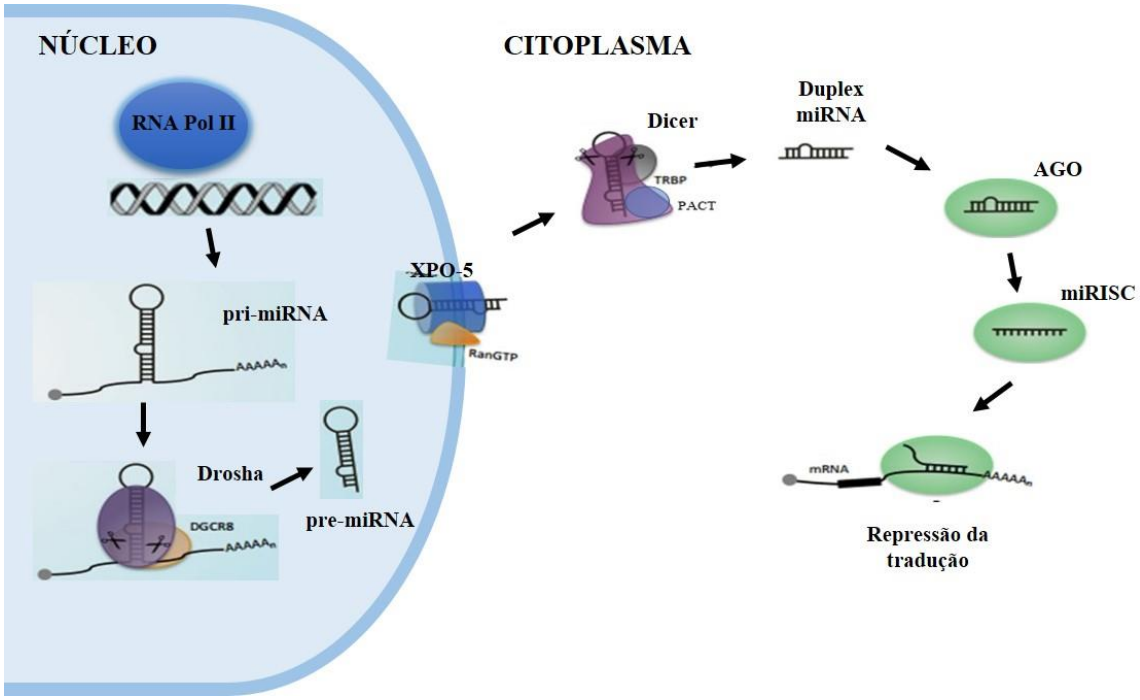

Figura 5. Biogênese completa dos miRNAs na célula. No citoplasma o pré-miRNA será clivado novamente, agora pela enzima Dicer e TRBP e PACT, em miRNA duplex; que se juntará a uma proteína Argonauta para formar o complexo RISC, responsável pela regulação pós-transcricional. DGR8: *DiGeorgesyndromecriticalregion gene 8;* XPO5: Exportina 5;TRBP: *HIV-1 TAR RNA-bindingprotein;* PACT: *ProteinKinase, Interferon-Inducible Double Stranded RNA DependentActivator* (Adaptado de DAUGAARD; HANSEN, 2017).

Os miRNAs ligada à sua capacidade de regular a expressão gênica por meio de pareamento de bases com a região 3'UTR do terminal 5' do RNAm alvo. A complementariedade entre miRNA presente no complexo RISC e RNAm alvo pode ser

completa ou parcial, o que torna um miRNA capaz de se ligara vários RNAm como alvo, causando repressão da tradução ou mesmo a degradação do alvo (OLIVETO *et al.* 2017).

1.3miRNAs no câncer

A desregulação de miRNAs está relacionada ao desenvolvimento do câncer por essas moléculas possuírem diversas funções de regulação em vários processos do metabolismo como controle do ciclo celular, transição epitélio mesenquimal (TEM), resistência a drogas, angiogênese e metástase (MANASA; KANNAN, 2017).

Apesar de vários estudos demonstrarem os efeitos da desregulação dos miRNAs e o surgimento do câncer, os processos que causam essa desregulação ainda não são bem compreendidos, incluindo falha na biogênese e transcrição causado por alterações em proteínas, alterações epigenéticas e amplificação ou perdas das regiões genômicas referentes aos genes de miRNAs (SONG *et al.* 2012a). A Figura 6 ilustra os possíveis mecanismos de desregulação da expressão de miRNAs.

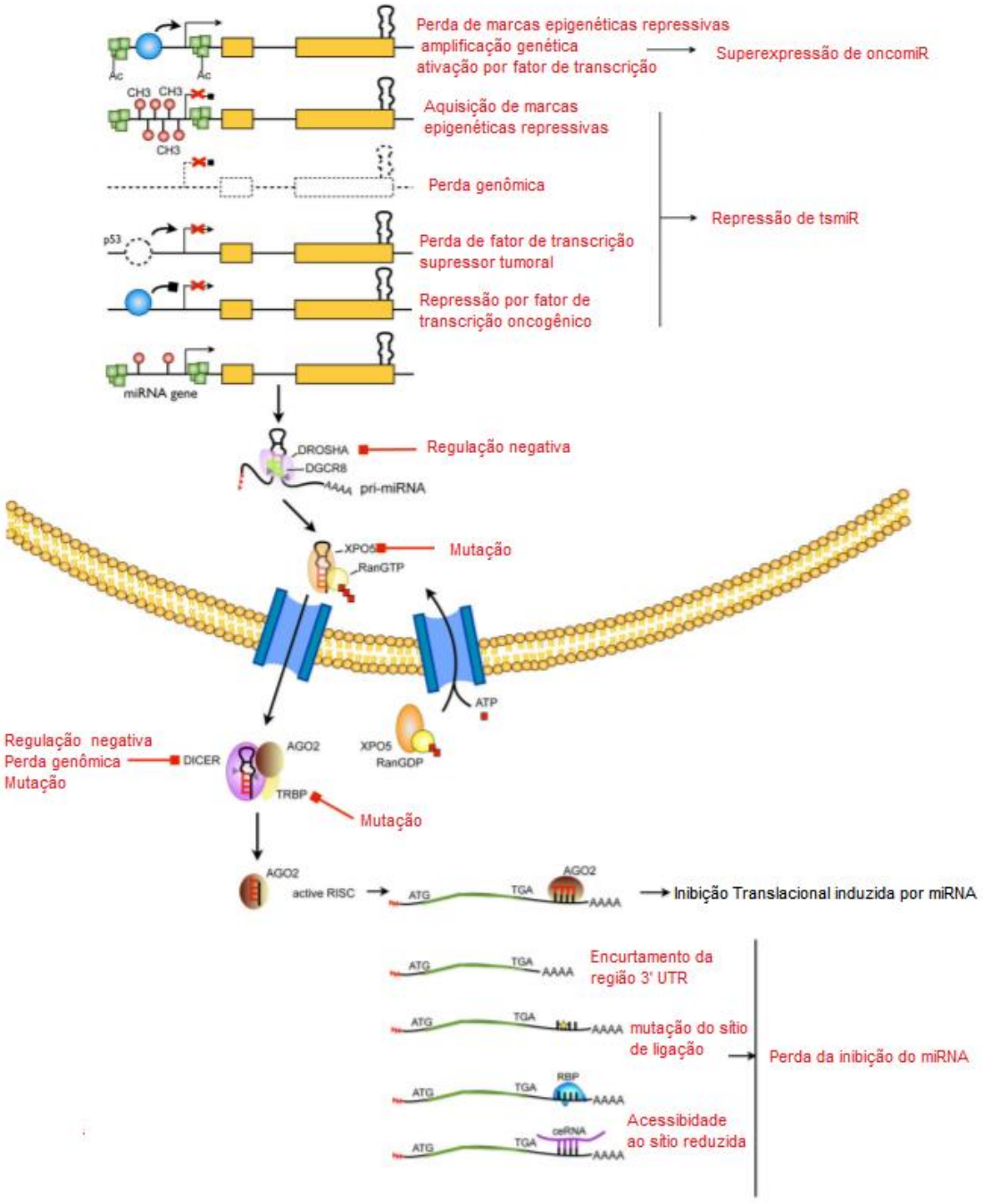

Figura 6. Desregulações em miRNAs no câncer. Representação da biogênese do miRNA e atividade pós-transcricional do complexo RISC. Possíveis alterações estão descritas em vermelho e associadas com eventuais alterações no câncer. Proteína de ligação do RNA (*RNA binding protein*): RBP; CH3, metilação; Ac, acetilação. (Adaptado de DI LEVA; GAROFALO; CROCE, 2014)

Particularmente, modificações epigenéticas são alterações que modulam a expressão sem alterar a sequência dos genes, principalmente metilação do DNA e a modificações de histonas (CALCAGNO; DE ARRUDA CARDOSO SMITH;

BURBANO, 2015). Na metilação do DNA ocorre a inclusão do radical metil no carbono 5’ da base nitrogenada citosina no dinucleotídeo CpG (ilhas CpG) pela DNA metiltransferases (DNMTs). A hipermetilação, metilação em sítios que não deveriam estar metilados, está relacionada ao silenciamento da região promotora de genes, inibindo a expressão de genes de reparo do DNA e supressores tumoral. Já a hipometilação, a perda de metilação em sítios que deveriam estar metilados, pode levar à instabilidade cromossômica e/ou superexpressão ou ativação de proto-oncogenes (BAYLIN; JONES, 2016; CALCAGNO; OLIVEIRA; MARTINS, 2017; DA SILVA OLIVEIRA *et al.* 2016). Desta forma, a expressão de genes de miRNAs podem ser reguladas por metilação da região promotora como acontece nos genes codificantes.

No câncer, os miRNAs desregulados podem ser classificados em dois grupos de acordo com o RNA mensageiro alvo: oncogênicos (oncomiRs) ou supressores tumorais (tsmiRs). Entretanto, tal classificação varia de acordo com o tecido alvo (CHOI; LEE, 2013; OLIVEIRA *et al.* 2017).

Os oncomiRs regulam negativamente a expressão de genes que estão relacionados ao reparo do DNA, controle do ciclo celular, adesão/invasão, proliferação e apoptose. Quando há aumento da expressão de oncomiRs, estes podem promover a progressão da doença, resistência a apoptose, invasão e metástase (LIU; MELTZER, 2017). Enquanto que os tsmiRs estão normalmente diminuídos no câncer e regulam negativamente as proteínas responsáveis por controlar a proliferação celular, estimulação angiogênica, invasão e metástase (SVORONOS; ENGELMAN; SLACK, 2016). Adicionalmente, um miRNA pode exercer um papel controverso, funcionando como oncomiR e/ou como tsmiRs (OLIVEIRA *et al.* 2017).

1.3.1 miRNA circulante no câncer

Os miRNAs são potenciais biomarcadores de diagnóstico, prognóstico ou preditivos por serem abundantes e estáveis na corrente sanguínea e nos outros fluidos corporais, o que estimula sua utilização em métodos minimamente invasivos de detecção e monitoramento do câncer (ANFOSSI *et al.* 2018).

No meio extracelular, os miRNAs podem ser encontrados ligados a lipoproteínas ou a algumas moléculas da família argonauta (AGO2) para torna-se mais estáveis, ou envolvidos por moléculas secretórias que são corpos apoptóticos, exossomas e

microvesículas conferindo uma proteção contra ribonucleases e garantindo uma boa utilização dos miRNAs como biomarcadores de diagnóstico e prognóstico (DA SILVA OLIVEIRA *et al.* 2016; MARTINS *et al.* 2016). A secreção de miRNAs na corrente sanguínea pode ocorrer de forma livre, associada a proteínas ou dentro de microvesículas ou exossomas. A figura 7 esquematiza a biogênse e liberação de miRNAs pelas células tumorais e processo de formação de exossomas e microvesículas.

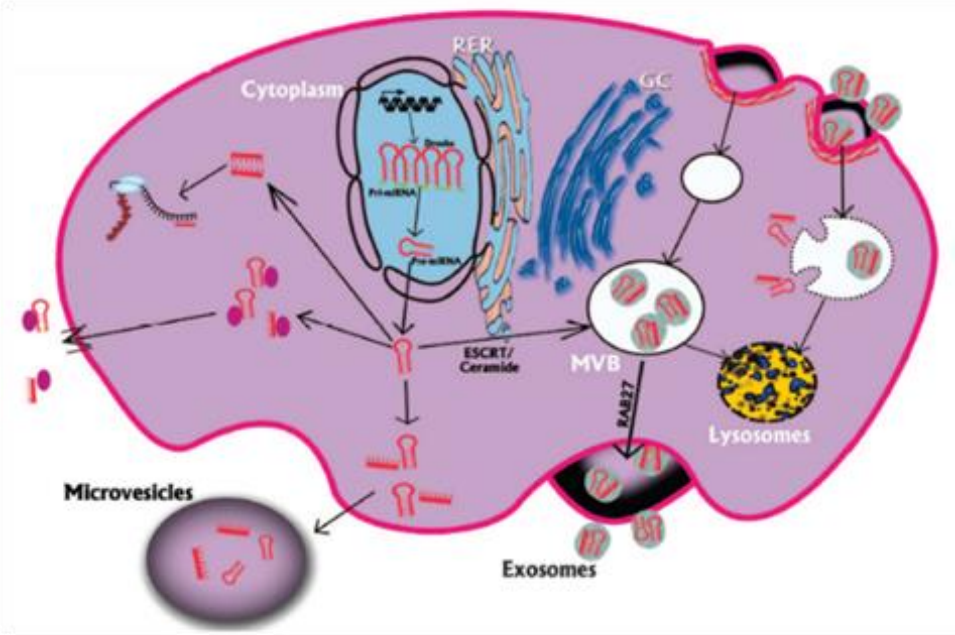

Figura 7. Representação esquemática da Biogênese e liberação de miRNAs pelas células tumorais e processo de formação de exossomas, microvesículas e endossomas. Exossomas são microvesículas secretadas pelas células (40 a 100 nm) formadas a partir da endocitose e da via de formação de endossomas que são liberados após a fusão de corpos multivesiculares com a membrana. Enquanto que amicrovesículas (200 e 1000 nm) tem origem por brotamento da membrana plasmática e semelhança funcional aos exossomas no transporte e comunicação celular. Microvesículas (MVB); Retículo endoplasmático rugoso (RER); Complexo para separação do endossoma requerido para transporte (endosomal sorting complex required for transport)(ESCRT)(KIM et al., 2017a; NEDAEINIA et al., 2017; TRAN, 2016).

Exossomas são vesículas extracelulares secretadas pelas células, variam entre 40 a 100nm de diâmetro, com funções de transporte e comunicação celular, podendo

carrear DNA, ncRNA e proteínas. A formação dos exossomas acontece a partir da endocitose e da via de formação de endossomas a partir de corpos multivesiculares que são liberados após fusão com a membrana. A função de transporte de substância pode ser tanto em células próximas, quanto em locais distantes do corpo (TRAN, 2016).

As microvesículas são maiores que os exossomas em diâmetro, variando entre 200 e 1000 nm, têm origem por brotamento da membrana plasmática e semelhantemente aos exossomas, podem carrear DNA, ncRNA e proteínas tanto para locais próximos quanto distantes da origem (KIM et al., 2017).

Os miRNAs envoltos em vesículas extracelulares secretórias, como exossomas e microvesículas, e estão protegidos contra a ação de degradação das RNAses. Tokuhisa et al. (2015) descreveram que uma maior concentração de miRNAs desregulados no lavado peritoneal pode ser um fator preditivo de metástase no peritônio de indivíduos com CG. Ademais, a expressão desregulada de miRNAs no sangue, plasma, saliva e urina em pacientes com câncer em comparação com indivíduos sadios demonstra que a secreção de exossomas contendo miRNAs encapsulados está relacionado ao processo de carcinogênese em vários tipos de câncer (RABINOWITS et al., 2009)**.**

Assim, moléculas secretadas para a corrente sanguínea são, geralmente, resultado de morte celular (programada ou não), ou, produtos ativos secretados do ambiente celular e por isso, podem refletir o perfil do ambiente tumoral primário no tecido correspondente (SCHWARZENBACH et al., 2016).

1.3.2 miRNAs circulantes no câncer gástrico

Diversos estudos relacionam a expressão de miRNAs como possível ferramenta de rastreamento na identificação de diferentes estágios do CG de acordo com a classificação TNM utilizada pela União Internacional de Combate ao Câncer (UICC). (GORUR *et al.* 2013; HUANG *et al.* 2017; LIU *et al.* 2012; QIU *et al.* 2016; TSUJIURA *et al.* 2010; YEGIN; DUMAN, 2014).

Outros estudos têm demonstrado a relevância clínica dos miRNAs como marcadores de prognóstico e progressão do CG associando os níveis de invasão, infiltração e/ou tamanho do tumor, avaliação de sobrevida e a presença de metástase em linfonodo ou metástase distantes (KOMATSU *et al.* 2013; SU *et al.* 2014; WANG *et al.*

2014b, 2012; ZHANG *et al.* 2014).A Tabela 1 descreve miRNAs circulantes e sua implicação clínica em pacientes com CG.

O estudo de Tsai et al. (2016) comparou a expressão de miR-196a/b com a especificidade e sensibilidade do CEA e CA19-9 no plasma de pacientes com CG versus controles, permitindo verificar que os miRNAs possuem melhor potencial de diagnóstico do que os marcadores clássicos.

Em 2014, Wang et al. demonstraram elevada expressão de miR-16 quando comparado grupo de pacientes e controles, além de observar o aumento dos níveis plasmático deste miRNA com a evolução da doença, associando com o crescimento tumoral e surgimento de metástases. Assim, os autores consideraram o miR-16 como um potencial marcador de diagnóstico e prognóstico.

Alguns autores associaram expressão de miRNAs com presença da *Helicobacter pylori*, uma causa comumente conhecida no processo de carcinogênese gástrica. Li et al. (2012) encontraram níveis elevados de miR-223 no sangue de pacientes com infecção por *H.pylori*, além de elevada expressão também em controles saudáveis com a presença da bactéria, sugerindo que este miRNA poderia ser um biomarcador de diagnóstico tanto em indivíduos sadios quanto em pacientes para presença de *H.pylori.*

Utilizando o sequenciamento MiSeq, Jiang et al. (2017) realizaram uma triagem inicial no soro de dez pacientes com CG positivos para metástase de linfonodo (MLN+), dez pacientes negativos para metástase de linfonodo (MLN-) e dez controles, posteriormente validou os miRNAs candidatos (miR-501-3p, miR-143-3p, miR-451, e miR-146a) no soro de 73 controles, 103 MLN+ e 103 MLN- de pacientes com CG por qRT-PCR (*Reverse transcription polymerase chain reaction quantitative real time*). Como a previsão de MLN+ em CG é restrito a cirurgia de mucosa com a criação deum painel de miRNAs circulantes seria possível discriminar a necessidade de ressecção do linfonodo ou não, permitindo assim, determinar casos em que a utilização de um tratamento menos invasivo como uma ressecção endoscópica da mucosa para tumores iniciais do estômago ou para casos em que o melhor tratamento seria uma ressecção cirúrgica com linfonodectomia, adequando a escolha do tratamento para cada caso específico.

Tabela 1. Expressão desregulada de miRNAs e sua implicação clínica em pacientes com CG.

miRNAs	Expressão	Amostras	Classificação	Método	Implicação Clínica	Referência
let-a	↓	69 CG/30 C Plasma	Diagnóstico	qRT-PCR	-	TSUJIURA et al., 2010
miR-1	↑	164 CG/127 C Soro	Diagnóstico	Solexa sequencing	-	LIU et al., 2011
miR-10b-5p	↑	441 CG /233C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-16	↑	40 AGNC/40 C Plasma	Diagnóstico	Taqman low-density array/ qRT-PCR	-	ZHU et al., 2014
	↑	50 CG/47 C Plasma	Diagnóstico	qRT-PCR	TNM Metástase	WANG et al., 2014
miR-17	↑	90 CG/27 C Soro	Diagnóstico Prognóstico	qRT-PCR	Detecção CTCs	ZHOU et al., 2010
miR-17-5p	↑	69 CG/30 C Plasma	Diagnóstico	qRT-PCR	-	TSUJIURA et al., 2010
	↑	79 pré-GAS/ 30 pós-GAS/ 6 recidiva Plasma	Prognóstico	qRT-PCR	TNM Sobrevida	WANG et al., 2012

miR-18	↑	82 CG/65 C Plasma	Diagnóstico Prognóstico	qRT-PCR	TNM Metástase	SU et al., 2014
miR-18a	↑	104 CG/65 C Plasma	Diagnóstico Prognóstico	qRT-PCR	-	TSUJIURA et al., 2015
miR-20a	↑	164 CG/127 C Soro	Diagnóstico	Solexa sequencing qRT-PCR	-	LIU et al., 2011
	↑	90 CG/90 C Plasma	Diagnóstico Prognóstico	qRT-PCR	TNM	CAI et al., 2013
	↑	79 pré-GAS/ 30 pós-GAS/ 6 recidiva Plasma	Prognóstico	qRT-PCR	TNM Pior prognóstico	WANG et al., 2012
miR-20a-3p	↑	441 CG /233 C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-20a-5p	↑↓	67 CG/12 C Soro	Diagnóstico Prognóstico	miRNA microarray qRT-PCR	TNM Metástase	YANG et al., 2017
miR-21	↑	60 CG/60 C Plasma	Diagnóstico	qRT-PCR	-	LI et al., 2012

	↑	87 AGNC/ 114C Soro	Diagnóstico	qRT-PCR	*H. pylori* Pior prognóstico	SHIOTANI et al., 2013
	↑	69 CG/30 C Plasma	Diagnóstico	qRT-PCR	-	TSUJIURA et al., 2010
	↑	69 CG Plasma	Prognóstico	qRT-PCR	Invasão Pior prognóstico	KOMATSU et al., 2013
	↑	16 MLN+/15 MLN-/15 C Soro	Prognóstico	qRT-PCR	Metástase	KIM et al., 2013
miR-25	↑	40 AGNC/40 C Plasma	Diagnóstico	Taqman low-density array/ qRT-PCR	-	ZHU et al., 2014
	↑	40 Pré CG/20 PósCG	Diagnóstico Prognóstico	qRT-PCR	TNM Invasão	ZHANG et al., 2014
miR-26a	↓	285 CG/ 285 C Plasma	Diagnóstico	microarray qRT-PCR	-	QIU et al., 2016
miR- 27a	↑	164 CG/127 C Soro	Diagnóstico	Solexa sequencing	-	LIU et al., 2011

miR-34	↑	164 CG/127 C Soro	Diagnóstico	Solexa sequencing	-	LIU et al., 2011
miR-92a	↑	40 AGNC/40 C Plasma	Diagnóstico	Taqman low-density array/ qRT-PCR	-	ZHU et al., 2014
miR-93	↑	40 PréCG/20 PósCG	Diagnóstico Prognóstico	qRT-PCR	TNM Invasão	ZHANG et al., 2014
miR-100	↑	50 CG/47 C Plasma	Diagnóstico	qRT-PCR	TNM Metástase	WANG et al., 2014
miR-106a	↑	90 CG/27 C Soro	Diagnóstico Prognóstico	qRT-PCR	Detecção CTCs	ZHOU et al., 2010
	↑	69 CG/30 C Plasma	Diagnóstico	qRT-PCR	-	TSUJIURA et al., 2010
miR-106b	↑	87 AGNC/114 C Soro	Diagnóstico	qRT-PCR	*H.pylori* Pior prognóstico	SHIOTANI et al., 2013
	↑	69 CG/30 C Plasma	Diagnóstico	qRT-PCR	-	TSUJIURA et al., 2010
	↑	90 CG/90 C Plasma	Diagnóstico	qRT-PCR	TNM	CAI et al., 2013

	↑	40 PréCG/20 PósCG	Diagnóstico Prognóstico	qRT-PCR	TNM Invasão	ZHANG et al., 2014
miR-122	↓	36 CGDM/36 CGNDM/36 C PlasmaPlasma	Prognóstico	qRT-PCR	Metástase	CHEN et al., 2014
	↑	441 CG/233C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-132-3p	↑	441 CG/233C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-142-3p	↓	285 CG/285 C Plasma	Diagnóstico	microarray qRT-PCR	-	QIU et al., 2016
miR-146a	↑	16 MLN+/15 MLN-/ 15 C Soro	Prognóstico	qRT-PCR	Metástase	KIM et al., 2013
miR-148a	↑	16 MLN+/15 MLN-/15 C Soro	Prognóstico	qRT-PCR	Metástase	KIM et al., 2013

	↓	285 CG/285 C Plasma	Diagnóstico	Microarray qRT-PCR	-	QIU et al., 2016
miR-185-5p	↑	441 CG/233C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-191	↑	57 CG/58 C Soro	Diagnóstico	qRT-PCR	TNM Tamanho do tumor	PENG et al., 2014
miR-192	↑	36 CGMD/36 CGSMD/ 36 C Plasma	Prognóstico	qRT-PCR	Metástase	CHEN et al., 2014
miR-195	↓	285 CG/285 C Plasma	Diagnóstico	microarray qRT-PCR	-	QIU et al., 2016
miR-195-5p	↑	441CG/233 C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
	↓	20 CG/190C Plasma	Diagnóstico	qRT-PCR	-	GORUR et al., 2013
miR-196a/b	↑	98 CG/126C Plasma	Diagnóstico Prognóstico	qRT-PCR	TNM Metástase Sobrevida	TSAI et al., 2016
miR-199a-3p	↑	30 CG/70 C Plasma	Diagnóstico	qRT-PCR	-	LI et al., 2013
miR-200c	↑	52 CG/15 C Sangue	Diagnóstico Prognóstico	qRT-PCR	Pior prognóstico	VALLADARES-AYERBES et al., 2012

miR-203	↓	130 CG/22 C Soro	Prognostico	qRT-PCR	TNM Metástase Pior prognóstico	IMAOKA et al., 2016
miR-218	↓	60 CG/60 C Plasma	Diagnóstico	qRT-PCR	-	LI et al., 2012
	↓	68 CG/56 C Soro	Prognóstico	qRT-PCR	TNM Grau de diferenciação Metástase Pior Prognóstico	XIN et al., 2014
miR-221	↑	68 CG/68 CG Soro	Diagnóstico	qRT-PCR	-	SONG et al., 2012
miR-223	↑	50 CG/47 C Plasma	Diagnóstico Prognóstico	qRT-PCR	TNM Metástase	WANG et al., 2014
	↑	60 CG/60 C Plasma	Diagnóstico	qRT-PCR	*H. pylori*	LI et al., 2012
miR-296-5p	↑	441 CG /233 C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	HUANG et al., 2017
miR-375	↓	20 ACGD/20 C Soro	Diagnóstico	qRT-PCR	Detecção de ACGD	ZHANG et al., 2012

miR-376c	↑	68 CG/68 CG Soro	Diagnóstico	qRT-PCR	-	SONG et al., 2012
miR-378	↑	61 CG/61C Soro	Diagnóstico	miRNA microarray qRT-PCR	-	LIU et al., 2012
miR-421	↑	90 CG/90 C Soro CMSPs	Diagnóstico	qRT-PCR	-	WU et al., 2015
miR-423-5p	↑	164 CG/127 C Soro	Diagnóstico	Solexa sequencing qRT-PCR	-	LIU et al., 2011
miR-451	↑	40 AGNC/40 C Plasma	Diagnóstico	Taqman low-density array qRT-PCR	-	ZHU et al., 2014
	↑	56 CG/30 C Plasma	Diagnóstico Prognóstico	miRNAmicroarray qRT-PCR	-	KONISHI et al., 2012
miR-486	↑	56 CG/30 C Plasma	Diagnóstico Prognóstico	miRNAmicroarray qRT-PCR	-	KONISHI et al., 2012
miR-486-5p	↑	40 AGNC/40 C Plasma	Diagnóstico	Taqman low-density array/ qRT-PCR	-	ZHU et al., 2014

miR-627	↑	58 CG/46 C Plasma	Diagnóstico Prognóstico	microarray qRT-PCR	TNM Progressão	SHIN et al., 2015
miR-629	↑	58 CG/46 C Plasma	Diagnóstico Prognóstico	microarray qRT-PCR	TNM Progressão	SHIN et al., 2015
miR-652	↑	58 CG/46 C Plasma	Diagnóstico Prognóstico	microarray qRT-PCR	TNM Progressão	SHIN et al., 2015
miR-744	↑	68 CG/68 CG Soro	Diagnóstico	qRT-PCR	-	SONG et al., 2012
miR-940	↓	115 CG/105 C Plasma	Diagnóstico Prognóstico	microarray qRT-PCR	Metástase	LIU et al., 2016

ACGD: Adenocarcinoma gástrico difuso; AGNC: adenocarcinoma gástrico não cárdia; C: controle; CG: câncer gástrico; CGMD: câncer gástrico com metástase distante; CGSMD: câncer gástrico sem metástase distante; CMSP: células mononucleares sanguíneas periféricas; CTC: célula tumoral circulante; GAS: gastrectomia.

1.4 Papel do miR-218 no câncer

O RNA não-codificante miR-218 (hsa-miR-218-5p) é específico de vertebrados, codificado por dois genes: MIR-218-1, localizado no braço curto do cromossomo 4 (4p15.31) (TATARANO *et al.* 2011); e o MIR-218-2, localizado no braço longo do cromossomo 5 (5q34) (OMIM: 616771), como representado na Figura 7. As localizações citadas correspondem aos introns dos genes supressores tumorais *SLIT2* e *SLIT3* e, quando há supressão ou redução da expressão destes genes, evidências relacionam à angiogênese e metástase (LI *et al.* 2018; TIE *et al.* 2010).

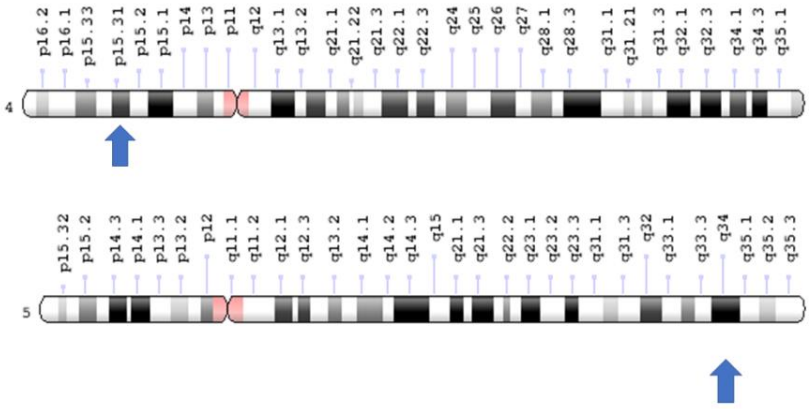

Figura 7. Ilustração da localização dos genes MIR-218-1 no cromossomo 4 (4p15.31); e, do gene MIR-218-2 no cromossomo 5 (5q34).

As alterações que levam a diminuição da expressão do miR-218 podem ser a nível genético, como demonstradas no primeiro estudo à abordar a atividade supressora tumoral do mir-218 realizado em células de câncer de bexiga, que correlacionou a perda de regiões do cromossomo 4 com o desenvolvimento e progressão da carcinogênese (TATARANO *et al*, 2011). Além disso, alterações como metilação do DNA nos genes *SLIT2* e *SLIT3* e modificações de histonas podem interferir na expressão do miR-218, interferindo no cenário do CG (KIM *et al.* 2016).

Recentemente, um estudo caso-controle composto por 897 pacientes com CG e 992 controles na subpopulação chinesa Han, conduzido por Wu et al. (2017), reportou a associação do polimorfismo de nucleotídeo único (SNP) rs11134527 do miR-218 com a menor sobrevida global dos pacientes com CG. Além disso, 48,5% dos pacientes que

carregavam o genótipo rs11134527 A/A morreram de CG em comparação com apenas 39,5% de morte dos pacientes que apresentavam o genótipos A/G-G/G. Demonstrando que os genótipos A/G-G/G de rs11134527 possuíam melhor sobrevida em relação ao genótipo A/A. Uma possível explicação seria que a transição do genótipo A para G do rs11134527 é capaz de alterar a estrutura secundária do pri-miR-218 (SHI *et al.* 2013). Os SNPs são variações genética de apenas uma base nitrogenada comuns em até 1% da população que podem alterar a susceptibilidade de desenvolvimento de doenças (MOCELLIN *et al.* 2015). Segundo RYAN; ROBLES; HARRIS (2010), um polimorfismo no gene do miRNA pode afetar a função por diferenças no transcrito primário, pelo processamento do pri-miRNA e pre-miRNA e por efeitos na interação do miRNA maduro com o RNAm.

Os estudos em CG demonstram a associação da expressão diminuída de miR-218 a sua função de supressor tumoral (TIE *et al.* 2010; LI *et al.* 2012; DENG *et al.* 2017; ZHANG *et al.* 2017).

TIE *et al.* (2010), demonstraram em linhagens celulares de CG (MKN28-Me SGC7901-M) e em tumores primários de CG vs. linfonodos metastáticos de 10 pacientes com estágio III/IV de GC por meio de qRT–PCR, a relação da expressão de miR-218, com o eixo SLIT- Robo1- mir-218 e metástase e progressão tumoral. O receptor Robo1 é um dos alvos do miR-218 e com a expressão diminuída de mir-218, maior a expressão do receptor e maior capacidade invasiva das células, estimulando a metástase e progressão tumoral. Além disso, ZHANG *et al.* (2017) relacionaram a diminuição da expressão de miR-218 e aumento da expressão de Robo1, com a neovascularização tumoral e angiogênese em CG. O grupo ainda sugeriu a utilização de miR-218 como uma potencial terapia anti-angiogênica, já que a inserção de miR-218 no meio extracelular alterou a estrutura dos vasos em modelos *in vitro* com células endoteliais e, em modelos xenográficos de tumor CG tratados com miR-218 os vasos sanguíneos tumorais apresentaram densidade reduzidas, o que diminui o índice de crescimento tumoral *in vivo*.

A propriedade anti-angiogênica de miR-218 também foi demonstrada por TANG *et al.* (2016). Ao comparar três linhagens celulares de CG (NCI-87, MGC80-3 e HGC-27), esses autores observaram que a expressão de miR-218 estava significativamente mais reduzida na linhagem NCI-87, que ainda apresentava elevada expressão de Angeopoietina (Ang) – 2, o que conferia à linhagem uma característica mais invasiva e agressiva comparada a MGC80-3 E HGC-27. Adicionalmente, os autores reportaram

que a via miR-218/Ang-2 está diretamente relacionada a formação de novos vasos por se ligar ao receptor Tie2 e inibir a sinalização Ang2/Tie2, o que interferiria na progressão tumoral e desenvolvimento do tumor.

Recentemente, DENG *et al.* (2017) demonstraram que a expressão reduzida de miR-218 está inversamente associada aos níveis de CDK6 e Cyclin D1 nos tecidos, o que poderia indicar um estágio clínico avançado e pior prognóstico para os pacientes.

Análise de miR-218 circulante, foi demonstrada primeiramente por LI *et al.*(2012) no plasma, ao comparar a expressão de três miRNAs (miR-223, miR-21 e miR-218) no plasma, os quais foram diferencialmente expressos, e associar a redução da expressão de miR-218 como possível biomarcador de diagnóstico de CG, além de ressaltar seu comportamento supressor tumoral.

Ao avaliar a expressão de miR-218 no soro de 68 pacientes de CG e 56 controles em população chinesa, XIN *et al.* (2014), demonstraram associação positiva entre menor expressão de miR-218 com maior invasão tumoral, presença de metástases de linfonodo e maior avanço da doença, sugerindo assim, pior prognóstico em CG. Entretanto, por ser o único estudo a associar a diminuição de expressão de mir-218 no soro de pacientes com CG à um pior prognóstico é necessário mais estudos para confirmar o potencial prognóstico do miR-218 em um número amostral maior e em outras populações diferentes da população chinesa.

Por esse motivo, a importância desse trabalho que tem como objetivo avaliar a expressão de miR-218 no soro de pacientes com CG e a sua relação com as características clínico-patológicas, confirmando assim, a funcionalidade do miR-218 como biomarcador de prognóstico em CG.

2. APLICABILIDADE CLÍNICA

A biópsia líquida surge como uma importante ferramenta para auxiliar no diagnóstico, prognóstico e monitoramento do câncer. A utilização precoce de métodos minimamente invasivos na prática clínica melhora as chances de restabelecimento e tratamento adequando, enquanto que como ferramentas de prognóstico, podem auxiliar na melhor escolha de conduta frente à evolução clínica, aumentando as chances de um tratamento adequado.

Devido a sua grande estabilidade e abundância na corrente circulatória, os miRNAs circulantes são potenciais candidatos à biomarcadores, sendo vários os estudos que avaliam sua utilização para diagnóstico e prognostico em pacientes com câncer. Em específico o miR-218, já demonstrou por meio de estudos na população asiática, um biomarcador de prognóstico bastante promissor, fazendo-se necessária a demonstração de sua aplicabilidade e possível utilização em estudos ocidentais.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar a expressão de miR-218 no soro de pacientes com CG e sua relação com as características clínico-patológicas.

3.2 Objetivos Específicos

1. Avaliar a expressão do miR-218 em pacientes de CG e controle saudáveis;
2. Comparar a expressão relativa entre pacientes de CG e controle saudáveis;
3. Relacionar a quantificação relativa dos pacientes de CG com as suas características clínico-patológicas;

4 METODOLOGIA

4.1 Pacientes e Processamento do Sangue

Foram coletados 4ml de sangue periférico de 302 pacientes com diagnóstico confirmado de CG por endoscopia e biópsia. Dos 302 pacientes, 195 pacientes eram homens e 107 eram mulheres, com idade variável entre 28 e 92 anos.

Os pacientes foram selecionados no Hospital Oncológico Ophir Loyola (HOL) e no Hospital Universitário João de Barros Barreto (HUJBB) entre os anos de 2015 e 2018, e assinaram o termo de consentimento livre e esclarecido (TCLE) para a participação voluntária do projeto (protocolo 3316737, comitê de ética HUJBB).

Ademais, foram utilizadas amostras controle de 120 indivíduos sadios, sendo60 homens e 60 mulheres que foram pareados em gênero e idade, sem histórico de câncer ou doenças inflamatórias associadas.

O sangue foi coletado em tubo com gel separador BD Vacutainer® SST® II Advance® (REF 367955, 5ml), sendo centrifuga do a 3000rpm por 15 minutos, em seguida, o soro foi dividido em alíquotas e transferido para criotubos e armazenado no freezer -80°C até sua utilização.

4.2 Extração do RNA Total

Para extração do RNA total das amostras, foi utilizado TRIzol reagente (Invitrogen, ThermoFisherScentific®), segundo recomendação do fabricante, na proporção 10:1 de reagente/ amostra. Com adição do miRNA sintético cel-miR-39 para a normalização.

4.3 RT-PCR qPCR

Foram utilizados os *"primers"* TaqMan® MicroRNA Assays em conjunto com TaqMan® MicroRNA Reverse Transcription Kit para 200 reações para converter miRNA em cDNA, de acordo com as instruções do fabricante. A sequência dos "primers" está descrita na tabela abaixo:

Tabela 2: Identificação dos primes utilização no método RTqPCR para analíse de expressão no soro de pacientes com CG.

miRNA	Identificação miRBase	Sequência
hsa-miR-218-5p	MIMAT0000275	UUGUCGUUGAUCUAACCAUGU
cel-miR-39-5p	MIMAT0020306	ACGUGAUUUCGUCUUGGUAAUA

Após a obtenção do cDNA, foi realizado a análise por *Real Time Polymerase Chain Reaction* (qPCR) em triplicata. A quantificação relativa do miR-218 em CG e controles foi calculada pelo método 2-ΔΔCT.

4.4 Testes Estatísticos

Foi realizado o teste Shapiro-Wilk para a verificação da normalidade e após teste Mann-Whitney para dados não paramétricos, sendo a associação entre a expressão de miR-218 com as características clínico-patológicas considerada estatisticamente significante quando $p < 0,05$. Foi utilizada a versão mais recente do software SPSS para análise estatística (https://www.ibm.com/br-pt/marketplace/spss-statistics).

4. RESULTADOS

Dentre os 302 pacientes, 195 pacientes eram homens e 107 eram mulheres e foram classificados em grupos etários com ≤ 45 anos e > 45 anos, gênero e dados clínicos-patológicos. Os resultados das análises de expressão do miR-218 e sua comparação com as características clínico-patológicas estão destacadas na Tabela 3.

Tabela 3: Expressão do miR-218 correlacionado aos dados clínico-patológicos de pacientes com CG (mediana ± IQR).

	N= 302	miR-218	P* (≤0,05)
Gênero			
Masculino	**195**	0,36±0,12	0,174
Feminino	**107**	0,34±0,10	
Idade			
≤45	**54**	0,37±0,13	0,614
>45	**248**	0,35±0,11	
Local			
Cárdia	**104**	0,37±0,15	0,157
Não- Cárdia	**198**	0,35±0,10	
Histopalotogico			
Difuso	**136**	0,30±0,13	0,000*
Intestinal	**166**	0,38±0,09	
Estadiamento			
I – II	**43**	0,45±0,12	0,000*
III – IV	**259**	0,34±0,10	
T			
T1	**12**	0,45±0,17	0,037*
T2 - T4	**290**	0,35±0,11	
N			
N0	**16**	0,43±0,18	0,004*
N1 – N3	**286**	0,35±0,11	

Ao fazer a comparação os resultados da expressão de miR-218 entre pacientes e controle pelo método 2-ΔΔCtfoi possível observar a quantificação relativa diminuída (Rq<1) em pacientes em relação ao grupo saudável.

Ao comparar a expressão de miR-218 no soro das amostras e correlacionando-as com as características clínico-patológicas, foi considerada a expressão significativamente (p<0,05) menor no CG do tipo difuso (p=0,00) em relação ao tipo intestinal. Adicionalmente, segundo a classificação para estadiamento do CG, a

expressão do miR-218 foi considerada significante para os estágios III-IV, em relação aos estágios I-II.

Quanto a classificação TNM, os resultados demonstram significância para o tamanho do tumor (T) nos estágios T2-T4, indicando menor expressão do miR-218 para CG avançado quando comparado ao estágio precoce (T-1).

Em relação a metástases em linfonodo, é possível observar significância para N1-N3, ou seja, um valor menor da expressão de miR-218 em metástases de linfonodos locais e mais de distantes, quando comparo a ausência de metástases de linfonodo (N0).

5. DISCUSSÃO

Os miRNAs apresentam um papel importante na carcinogênese gástrica e são potenciais biomarcadores de diagnóstico e prognóstico devido sua maior estabilidade em fluídos corporais, principalmente na circulação sanguínea (OLIVEIRA *et al.* 2016).

Os resultados encontrados nesse estudo demonstraram a expressão diminuída do miR-218 no soro de pacientes com CG em relação aos indivíduos saudáveis. Além disso, a associação da expressão do miR-218 com as características clinicopatológicas encontrou expressão significantemente diminuída em tumores com estágio mais avançados (p=0,000), maior invasão tumoral (p=0,037), presença de metástase de linfonodos (p=0,004), menor diferenciação celular (p=0,000).

Alguns estudos demonstraram uma associação da diminuição da expressão do mir-218 aos tipos tumorais mais agressivos e pior prognóstico dos pacientes em diferentes tipos de câncer, incluindo bexiga (CHENG *et al*. 2015), esôfago (YANG *et al.* 2015), nasofaríngeo (ALAJEZ *et al.* 2011), hepatocelular (YANG *et al.* 2016) e mama (AHMADINEJAD *et al.* 2017).

Evidências acumuladas têm demonstrado a relação da expressão de miR-218 com proteínas como Robo 1 (ZHANG *et al.* 2017), Ang2 e Tie2 (TANG *et al.* 2016), CDK 6 e Cyclin D1 (DENG *et al.* 2017), responsáveis por funções como regulação do ciclo celular e angiogênese. Geralmente, esse miRNA é caracterizado como tsmiR de acordo com os seus alvos.

Na literatura, há dados insuficientes quanto a utilização do miR-218 circulante em pacientes com CG, sendo este estudo, o segundo a analisar e comparar a expressão de miR-218 no soro de pacientes com CG com as características clinicopatológicas.

Os nossos resultados corroboram os resultados obtidos por XIN *et al.* (2014), além de resultado semelhante ao correlacionar a diminuição da expressão do miR-218 com as características clínico-patológicas já citadas, confirmando mais uma vez o potencial prognóstico do miR-218 em CG, agora na população ocidental e com um número amostral maior.

Foram encontrados resultados significantes para menor expressão de miR-218 em pacientes que apresentavam CG em estágio mais avançado, confirmando que a diminuição do miR-218 é um fator de risco para progressão tumoral.

Além disso foi observada significância para baixa expressão de miR-218 no CG do tipo difuso de Lauren (p=0,000), com pouca diferenciação celular, considerado mais agressivo e com pior evolução (JIMÉNEZ FONSECA *et al.* 2017; PETRELLI *et al.* 2017).

Resultados semelhantes foram encontrados em tecido por WANG *et al*.(2015) que avaliaram a expressão de miR-218 e sua associação com pior prognóstico em 112 amostras de pacientes de CG, que não haviam sido submetidos a nenhuma espécie de tratamento prévio. Por meio do método *qPCR*, foi demonstrada expressão diminuída de miR-218 em comparação com o tecido adjacente, além da baixa expressão significantemente associadas à baixa diferenciação celular, metástase de linfonodo, invasão tumoral e classificação TNM, dados que sugerem que a baixa expressão de miR-218 está correlacionada progressão agressiva de CG.

A correlação entre pior prognóstico e menor diferenciação tumoral também foi descrita em outros tipos de câncer. YU *et al.* (2013) avaliaram a expressão de miR-218 em tecido de 186 pacientes com câncer colorretal e compararam com os resultados obtidos em 30 controles, confirmando o papel supressor tumoral do miRNA e sua correlação com maior invasão tumoral, metástase de linfonodos, estágio mais avançado e menor diferenciação tumoral. Contudo, diferentemente de outros resultados, o grupo também avaliou os níveis séricos do miR-218 após a cirurgia de ressecção, afirmando uma fraca elevação na expressão, mas suficientemente significativa. Sugerindo que a secreção de miR-218 para o sistema circulatório varie de acordo coma a exposição das células normais às células cancerígenas.

LI; LIU; YANG (2015), avaliaram por meio do método de qPCR, a expressão de miR-218 e sua associação com pior prognóstico e sobrevida em 107 amostras pareadas com tecido adjacente de pacientes de câncer pancreático. A expressão do miR-218 foi significativamente menor nas amostras de tecido tumoral do que as amostras adjacentes. Além do mais, expressão diminuída do miR-218 nos tecidos foi fortemente associada a maior estágio tumoral segundo classificação TNM, metástase à distância, menor diferenciação tumoral e com o menor tempo de sobrevida global dos pacientes com câncer pancreático.

O grupo espanhol de MEDINA-VILLAAMIL *et al.* (2014) demonstrou a relação de um painel de miRNAs (hsa-miR-337-3p, hsa-miR-330-3p, hsa-miR-339-3p, hsa-miR-124, hsa-miR-218, hsa-miR-128, hsa-miR-10a, hsa-miR-199b-5p, hsa-miR-200b e hsa-miR-15b) como biomarcadores de diagnóstico em amostras de sangue total em

câncer de próstata, confirmando a expressão diminuída de miR-218 em pacientes quando comparados a saudáveis.

No soro, a correlação significativa para baixa expressão do miR-218 também foi relatada em outros tipos de câncer (JIANG *et al.* 2015; YANG *et al.* 2016; YU *et al*. 2013). Yang *et al*. (2016) observaram que níveis séricos de miR-218 estavam notavelmente diminuídos em paciente com carcinoma hepatocelular *versus* controle, demonstrando resultados significantes para a correlação da expressão de miR-218 com o tamanho do tumor, invasão, e maior estágio TNM.

Em câncer de esôfago, JIANG *et al.* (2015) relataram menor expressão sérica de miR-218 nos pacientes que apresentavam menor diferenciação celular, estágio avançado e metástase de linfonodo, reforçando que a baixa expressão no soro pode estar relacionada ao desenvolvimento do tumor e progressão deste tipo de câncer.

Portanto, embora o miR-218 não seja um biomarcador específico para câncer gástrico, podendo ser analisado em outros tipos de câncer, como de mama (AHMADINEJAD *et al.* 2017), esôfago (JIANG *et al.* 2015), próstata (GUAN *et al.* 2017) e pulmões (YANG *et al.* 2017b) e em diversas amostragens (cultura de células, tecidos, sangue), os resultados das pesquisas são unanimes em correlacionar a baixa expressão de miR-218 com estágios avançados, metástases de linfonodos e pior prognóstico.

7. CONCLUSÃO

No nosso estudo foi possível verificar o potencial uso do miR-218 no soro como biomarcador de prognóstico em pacientes com CG, confirmando sua expressão reduzida no estadiamento, tamanho do tumor (T), presença de metástase em linfonodos e classificação de Lauren. Apesar dos resultados concordarem com os achados da literatura, faz-se necessária a análise da sobrevida global dos pacientes de CG para um melhor valor prognóstico.

No cenário futuro, pode avaliar se as vias de sinalização miR-218 têm validades na prática clínica, aprofundando os estudos de linhagens celulares para modelos *in vivo* que contribuam para uma melhor compressão sobre os mecanismos carcinogênicos do miR-218.

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ANEXO I

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Review Article

The Emerging Role of miRNAs and Their Clinical Implication in Biliary Tract Cancer

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Biliary tract cancers are aggressive malignancies that include gallbladder cancer and tumors of intra- and extrahepatic ducts and have a poor prognosis. Surgical resection remains the main curative therapy. Nevertheless, numerous patients experience recurrence even after radical surgery. This scenario drives the research to identify biliary tract cancer biomarkers despite the limited progress that has been made. Recently, a large number of studies have demonstrated that deregulated expression of microRNAs is closely associated with cancer development and progression. In this review, we highlight the role and importance of microRNAs in biliary tract cancers with an emphasis on utilizing circulating microRNAs as potential biomarkers. Additionally, we report several single nucleotide polymorphisms in *microRNA* genes that are associated with the susceptibility of biliary tract tumors.

1. Background

Despite their relatively rare incidence, biliary tract cancers (BTCs) are an aggressive tumor group with poor prognosis and are characterized by early lymph node and systemic metastases [1]. These tumors include gallbladder cancer (GBC) and cholangiocarcinoma (CCA), which is divided into intrahepatic cholangiocarcinoma (iCCA) and extrahepatic cholangiocarcinoma (eCCA). Currently, surgical resection remains the only curative treatment for BTCs, and neoadjuvant chemoradiotherapy is not a standard option for patients with these malignancies. Moreover, many cases present with recurrence even after radical surgery, and patients with recurrent or metastatic BTCs usually have a poor outcome [2]. Therefore, there is a need for additional investigations to determine potential biomarkers of BTCs for early diagnosis, determining patient prognosis and the development of targeted therapy.

Recent studies have described microRNAs (miRNAs) as potential biomarkers in different cancer types [3–6]. However, miRNA expression and their implications in the diagnosis of, prognosis of, and therapeutic applications towards BTCs remain elusive.

miRNAs are small noncoding RNAs (18–25 nucleotides) that play important roles in the regulation of a large number of essential biological functions that are critical to the development of different cancer types, including cell proliferation, differentiation, apoptosis, migration, and invasion [7]. miRNA biogenesis initiates in the nucleus, where miRNA genes are usually transcribed by RNA polymerase II, resulting in a primary transcript of miRNA (pri-miRNAs) [8]. During the initial processing of pri-miRNAs, the DroshaDGCR8 complex cleaves the pri-miRNA, releasing a hairpin structure named pre-miRNA (~70 nucleotides). Pre-miRNAs are transferred to the cytoplasm and converted into an miRNA duplex by Exportin-5 and the Dicer-TRBP complex, respectively. Then, a helicase separates the double-stranded miRNA to produce one stable single-stranded miRNA, while the other strand is processed for autolytic degradation. The stable mature miRNA strand is loaded into the RNA induced silencing complex (RISC) to mechanistically target the 3′ untranslated regions (3′UTRs) of protein coding mRNAs, thereby acting as post transcriptional regulators by two mechanisms: mRNA degradation (when the sequences are perfect complements) and inhibition of translational initiation (when there is partial complementarily) [9]. Thus, miRNAs act as negative regulators of post transcriptional gene expression of target mRNAs.

It has been well established that miRNAs could regulate approximately 60% of human genes, including many oncogenes and tumor suppressor genes; this phenomenon strengthens the importance of these noncoding RNAs as relevant regulators in cancer [10].

In this review, we focus on the roles and importance of miRNAs in BTCs and highlight the potential of circulating miRNAs as diagnostic and prognostic biomarkers. Therefore, we reported several single-nucleotide polymorphisms (SNPs) in *miRNA* genes associated with BTC susceptibility.

2. Roles and Clinical Significance of miRNAs in BTCs

A large number of deregulated miRNAs have been categorized as oncomiRs (oncogene miRNAs) and/or tsmiRs (tumor suppressor miRNAs) in cancer depending on the effect of the target mRNA.

In BTCs, several studies on miRNA expression have identified many upregulated oncomiRs and downregulated tsmiRs as well as their potential targets (Table 1).

One of the best-described miRNAs in BTCs is hsa-miR21, which is usually identified as an oncomiR since its overexpression has been associated with invasion and metastasis [11–19, 21–25, 27, 30, 41, 47–50]. Liu et al. [13] observed that overexpression of hsa-miR-21 significantly promotes cell migration, invasion, and xenograft growth after transfection of hsa-miR-21 into CCA cell lines (QBC939 and RBE). Moreover, these authors showed decreased E-cadherin expression and increased N-cadherin and vimentin expression after hsa-miR-21 overexpression. Thus, hsa-miR-21 could induce the epithelial-mesenchymal transition (EMT) in CCA. In this process, epithelial cells lose their cell polarity and cell adhesion—probably due to the decrease of E-cadherin expression—which

allows cells to migrate and invade surrounding tissues; this the loss of E-cadherin expression plays a key role in tumor invasion and metastasis.

Similarly, aberrant expression of miRNAs also induces EMT and enhances the metastatic potential of GBC cells [24, 51]. Bao et al. [51] reported that hsa-miR-101 overexpression inhibits the proliferation, migration, and invasion of GBC cells, induces the increased expression of E-cadherin and β-catenin, and causes decreased expression of vimentin. Furthermore, these authors observed that hsa-miR-101 downregulation was correlated with tumor size, invasion, lymph node metastasis, TNM stage, and poor survival in GBC patients. These results indicate that hsa-miR-101 plays a tsmiR role and attenuates EMT and metastasis in GBC.

Accumulating evidence has indicated that hsa-miR-146b5p presents critical tumor suppressor properties [52, 53]. Its expression was significantly downregulated in GBC tissue compared with adjacent nonneoplastic tissues. In addition, the overexpression of hsa-miR-146b-5p in the SGC-996 GBC cell line inhibited cell growth by enhancing apoptosis and arresting the cells at G1 phase. However, the enforced expression of *EGFR*, a cell surface protein that binds to epidermal growth factor (which inducing cell proliferation), reversed the ability of hsa-miR-146b-5p to inhibit proliferation. Moreover, hsa-miR-146b-5p expression levels were significantly correlated with tumor size and cancer progression [46].

Recent studies have described hsa-miR-135a-5p as having a tsmiR role [54–56]. In GBC, Zhou et al. [44] found that hsa-miR-135a-5p levels were significantly downregulated in tumors compared to adjacent nontumor gallbladder tissues and were correlated with neoplasms of histological grades III and IV. Additionally, this study identified *VLDLR* as a direct and functional target gene of hsa-miR-135a-5p in GBC tissues. Furthermore, the transfection with a hsa-miR-135a5p mimetic inhibited the proliferative and colony-forming abilities of GBC cells by arresting the cells in G1/S phase. These data suggest that hsa-miR-135a-5p may inhibit the proliferation of GBC cells.

3. Circulating miRNAs as PotentialBTCs Biomarkers

Several studies have reported that detectable miRNAs in bodily fluids (e.g., plasma, serum, urine, and saliva) are more stable in comparison with other circulating nucleic acids [57]. Therefore, circulating miRNAs may be noninvasive and specific diagnostic and/or prognostic molecular biomarkers for human diseases, including cancer [4, 7, 58, 59]. In BTCs, many circulating miRNAs seem to be reproducible and reliable potential biomarkers as well as possible therapeutic targets [60]. Table 2 summarizes the circulating miRNAs with potential diagnostic, prognostic, and predictive biomarker applications in BTCs.

In CCA patients, Cheng et al. [64] observed different expression levels of circulating hsa-miR-106a not only between CCAs and healthy controls but also among CCAs and benign bile duct diseases (e.g., primary bile duct stone and congenital biliary duct cysts). Furthermore, they identified decreased hsa-miR-106a levels in patients with lymph node metastasis compared with those without metastasis, indicatingthepossibleroleofhsa-miR-106aintheoccurrence of lymph node metastasis.

Interestingly, Voigtlander et al. [65] found a distinct¨ circulating miRNA profile in the bile and serum samples from CCA patients and patients with primary sclerosing cholangitis (PSC), a noncancerous disease. Furthermore, bile samples from patients with concomitant PSC and CCA (PSC/CCA) were also included in this study. Their results showed higher expression levels of hsa-miR-126, hsa-miR-26a, hsa-miR-30b, hsa-miR-122, and hsa-miR-1281 in PSC patients than those

Table 1: Deregulated miRNAs in BTCs.

miRNA	Tumor	Expression	Target	Roles in BTCs	Reference
hsa-miR-21	CCA	↑	*PTEN*	Invasion Migration Chemoresistance	[11–14]
		↓	*TPM1*	DNA methylation	[15]
		↓	*15-PGDH* *HPGD*	Histone deacetylation Inflammation	[16]
		↑	*PDCD4*	Lymph node metastasis Migration	[17–20]
		↑	*RECK*	Migration Metastasis	[21, 22]
		↑	*TIMP3*	Apoptosis	[18]
		↑	—	pTNM Prognosis	[23]
hsa-miR-20[a]	GBC	↑	*SMAD7*	Invasion Metastasis Migration Prognosis	[24]
hsa-miR-34a	CCA	↓	*C-MYC*	Progression	[25]
	GBC	↓	*PNUTS*	Proliferation Prognosis	[26]
hsa-miR-335	GBC	↓	*BMI1*	Invasion Lymph node metastasis pTNM Prognosis	[27, 28]
hsa-miR-148a	CCA	↓	*DNMT1*	Prognosis	[29]
hsa-miR-31	CCA	↑	*RASA1*	Apoptosis Proliferation	[30]
hsa-miR-200b/c	CCA	↓	*SUZ12*	Chemoresistance Invasion Migration	[28]
hsa-miR-210	CCA	↑	*MNT*	Progression	[25]
Let-7a	CCA	↓	*RAS*	Progression	[21]
hsa-miR-370	CCA	↓	*MAP3K8*	Inflammation pTNM	[31]
hsa-miR-29b	CCA	↓	*C-MYC*	Apoptosis	[32]
hsa-miR-101	CCA	↓	*VEGF* *COX-2*	Angiogenesis	[33]
hsa-miR-200b/c	CCA	↓	*ROCK2*	Migration	[28]
hsa-miR-138	CCA	↓	*RHOC*	Migration	[34]
hsa-miR-376c	CCA	↓	*GRB2*	Migration	[35]
hsa-miR-124	CCA	↓	*SMYD3*	Migration	[36]
hsa-miR-204	CCA	↓	*SLUG*	Migration	[37]
hsa-miR-214	CCA	↓	*TWIST*	Migration	[38]
hsa-miR-200c	CCA	↓	*NCAM1*	Migration	[39]
hsa-miR-200b	CCA	↑	*PTPN12*	Chemoresistance	[14]
hsa-miR-29b	CCA	↓	*PIK3R1*	Chemoresistance	[40]
hsa-miR-205	CCA	↓	*MMP2*	Chemoresistance	

hsa-miR-221	CCA	↓	*PIK3R1*	Chemoresistance	
hsa-miR-182	GBC	↑	*CADM1*	Invasion Migration Metastasis	[27]

Table 1: Continued.

miRNA	Tumor	Expression	Target	Roles in BTCs	Reference
hsa-miR-155	GBC	↑	*SMAD7*	Invasion Lymph node metastasis Proliferation Prognosis	[41]
hsa-miR-130a	GBC	↓	*HOTAIR*	Invasion Proliferation	[42]
hsa-miR-26a	GBC	↓	*HMGA2*	pTNM Proliferation	[43]
hsa-miR-135a-5p	GBC	↓	*VLDLR*	pTNM Proliferation	[44]
hsa-miR-218-5p	GBC	↓	*BMI1*	Invasion Migration Proliferation	[45]
hsa-miR-146-5p	GBC	↓	*EGFR*	Apoptosis pTNM Proliferation	[46]
hsa-miR-1 hsa-miR-145	GBC GBC	↓	*VEGF-A* *AXL*	Apoptosis Proliferation	[47]
hsa-miR-143 hsa-miR-122 hsa-miR-187	GBC GBC	↓ ↑	*AXL*	Lymph node metastasis pTNM stage	

in CCA patients. However, bile samples showed hsa-miR-640,hsa-miR-1537,andhsa-miR-3189 downregulation, as well as hsa-miR-412 upregulation in PSC and PSC/CCA patients. These results demonstrated that PSC and CCA patients have distinct miRNA profiles in their bile and serum, which could be used to discriminate these diseases.

A small number of studies have described circulating miRNAs in patients with GBC. Kishimoto et al. [62] demonstrated an increase in the hsa-miR-21 expression levels in plasma from GBC patients before curative resection when compared with postsurgical patients and healthy volunteers. These findings suggest that hsa-miR-21 plasma levels were significantly affected by cancer occurrence and might have the potential to be a diagnostic biomarker for GBC patients.

Recently, Li and Pu [47] described significantly deregulated miRNAs in the peripheral blood samples of GBC patients compared with healthy volunteers. The expression levels of hsa-miR-187, hsa-miR-192, and hsa-miR-202 were upregulated while hsa-miR-143 was downregulated. These results were associated with lymph node metastasis, inflammation, immune reaction, and poor prognosis and could be translated to clinical practice as biomarkers for the early diagnosis, prognosis, and predictive response in patients with GBC.

Although most studies involving circulating miRNAs utilize real-time PCR for detection, Kojima et al. [68] used a highly sensitive microarray denoted as “3D Gene” that was capable of simultaneously analyzing more than 2,500 miRNAs in serum samples from patients with pancreato biliary cancers. These authors found several significantly dysregulated miRNAs, including 30 upregulated miRNAs and 36 downregulated miRNAs in BTCs. However, none of these miRNAs could be used as single biomarker for this type of cancer. The best results were achieved with a panel of eight miRNAs (hsa-miR-6075, hsa-miR-4294, hsa-miR6880-5p, hsa-miR-6799-5p, hsa-miR-125a-3p, hsa-miR-4530, hsa-miR-6836-3p, and hsa-miR-4476).

4. *miRNA* Single-Nucleotide Polymorphisms in BTCs

In general, aberrations in miRNA expression result from either epigenetic modifications or genomic changes, which include chromosomal rearrangements, mutations, or SNPs [69].

Several SNPs in miRNAs can lead to distinctions in the miRNA expression levels, which can modulate miRNA-target gene expression and, subsequently, affect cancer susceptibility [4, 70]. However, few studies have been performed to identify SNPs in miRNAs in BTCs patients until now.

The SNPs hsa-miR-27a rs895819, hsa-miR-570 rs4143815, and hsa-miR-181a rs12537 have been found to play important roles in many cancer types [71–78], and their contribution in BTCs has been explored. Gupta et al. [70] observed that the combination of hsa-miR-27a rs895819, hsa-miR570 rs4143815, and hsa-miR-181a rs12537 was the best gene-gene interaction model for predicting the susceptibility and treatment response in GBC patients. Moreover, the SNPs hsamiR-27a rs895819 and hsa-miR-181a rs12537 were associated with treatment toxicity but had no influence on the survival outcomes of GBC patients with locally advanced and/or metastatic tumors.

Table 2: Circulating miRNAs in patients with BTC as potential diagnostic, prognostic, and predictive biomarkers.

miRNA	Expression	Samples	N samples	Potential biomarker	Method	Clinical implication	Reference
hsa-miR-9	↑	Bile	BTCs (9) HV (9)	Diagnostic Prognostic	RT-PCR	Metastasis	[61]
hsa-miR-145	↑	Bile	BTCs (9) HV (9)	Diagnostic	RT-PCR	—	[61]
hsa-miR-21	↑	Plasma	BTCs (94) HV (50) BBD (2)	Diagnostic	qRT-PCR	Inflammatory reaction	[62]
		Peripheral blood	GBC (40) HV (40)	Diagnostic	qRT-PCR	—	[47]
hsa-miR-150	↑	Plasma	iCCA (15)	Diagnostic	qRT-PCR	Tumor progression	[63]
hsa-miR-106a	↓	Serum	CCA (103) HV (20)	Prognostic	qRT-PCR	Lymph node metastasis	[64]
hsa-miR-126	↑	Serum	PSC (40) CCA (31) HV (12)	Diagnostic	RT-PCR	—	[65]
hsa-miR-26a	↑	Serum	PSC (40) CCA (31) HV (12)	Diagnostic	RT-PCR	—	[65]
hsa-miR-30b	↑	Serum	PSC (40) CC (31) HV (12)	Diagnostic	RT-PCR	—	[65]
hsa- miR-122	↑	Serum	PSC (40) CC (31) HV (12)	Diagnostic	RT-PCR	—	[65]
hsa-miR-1281	↑	Serum	PSC (40) CC (31) HV (12)	Diagnostic	RT-PCR	—	[65]
hsa-miR -187	↑	Peripheral blood	GBC (40) HV (40)	Diagnostic Prognostic Predictive	qRT-PCR	Lymph node metastasis Poor prognosis	[47]
hsa-miR-192	↑	Peripheral blood	GBC (40) HV (40)	Diagnostic Prognostic Predictive	qRT-PCR	Inflammatory reaction Immune reaction Lymph node metastasis	[47]
		Serum	iCCA (11) HV (09)	Diagnostic Prognostic	miRNA RT-PCR array	Lymph node metastasis Poor prognosis	[66]
hsa-miR-194	↑	Serum	CCA (70) HV (70)	Diagnostic	qRT-PCR	Tumor progression	[58]

hsa-miR -202	↑	Peripheral blood	GBC (40) HV (40)	Diagnostic Prognostic Predictive	qRT-PCR	Lymph node metastasis	[47]
hsa-let- 7a	↓	Peripheral blood	GBC (40) HV (40)	Diagnostic	qRT-PCR	—	[47]
hsa-miR -143	↓	Peripheral blood	GBC (40) HV (40)	Diagnostic Prognostic Predictive	qRT-PCR	Inflammatory and immune reaction Lymph node metastasis	[47]
hsa-miR-335	↓	Peripheral blood	GBC (40) HV (40)	Diagnostic	qRT-PCR	—	[47]
hsa-miR-1307-3p	↓	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-1275	↑	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]

Table 2: Continued.

miRNA	Expression	Samples	N samples	Potential biomarker	Method	Clinical implication	Reference
hsa- miR-320b	↑	Plasma	iCCA (13) HVs (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-874	↑	Plasma	iCCA (13) HVs (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-483-5p	↑	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]
		Serum	CCA (70) HV (70)	Diagnostic	qRT-PCR	Tumor progression	[58]
hsa-miR-885-5p	↑	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-92b-3p	↑	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-505-3p	↑	Plasma	iCCA (13) HV (5)	Diagnostic	qRT-PCR	—	[67]
hsa-miR-6836-3p	↑	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-6075	↑	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa- miR-4634	↑	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-4294	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-6880-5p	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-6799-5p	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]

hsa-miR-125a-3p,	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	Tumor progression	[68]
hsa-miR-4530	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-7114-5p	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]
hsa-miR-4476	↓	Serum	BTCs (98) HV (150)	Diagnostic	3D-Gene	—	[68]

BBD: benign biliary disorders; BTCs: biliary tract cancers; CCA: cholangiocarcinoma; iCCA: intrahepatic cholangiocarcinoma; GBC: gallbladder cancer; HV:heath volunteers; PSC: primary sclerosing cholangitis.

SNPs in pri-miRNAs and pre-miRNAs could also affect miRNA processing, miRNA expression, and cancer susceptibility. Srivastava et al. [79] reported genetic polymorphisms in pre-mir-196a2 rs11614913 (C>T), pre-hsa-mir-196a rs11614913, and pre-hsa-mir-499 rs3746444 (T>C) that were associated with an increased overall risk of developing GBC development. In CCA, Mihalache et al. [80] investigated the G/Cvariantin pre-hsa-miR-146ars2910164andfoundnosignificant relationship between genetic susceptibility and CCA.

Additional studies addressing the identification of miRNA SNPs could be useful to assess the individual susceptibility of BTCs and improve our understanding of their potential contribution to the disease as well as aid in the development of potential clinical applications.

5. Conclusion

miRNAs are profoundly involved in tumor onset and progression [81–84]. However, the implications of miRNA for the diagnosis, prognosis, and therapeutic options for patients with BTCs remain unsatisfactory. This review highlighted some miRNAs that are dysregulated in BTCs, their targets, and the possible clinical implications. A better understanding of the therapeutic applications of miRNAs could lead to future clinical trials involving the inhibition of oncomiRs or the promotion of expression of tsmiRs as new approaches against diverse cancer types, including aggressive BTCs.

Here, we also reported several circulating miRNAs as possible diagnostic, prognostic, and/or predictive biomarkers in BTCs. Circulating miRNAs could be promising potential biomarkers for cancers because detectable miRNAs in the bodily fluids are stable and can be measured using noninvasive methods [57]. BTCs are usually

asymptomatic; therefore, the use of miRNAs as early diagnostic biomarkers could be a useful tool to improve the long-term survival of BTC patients. However, more studies with clinical outcomes are needed to identify which miRNAs could serve as either a potential therapeutic target or diagnostic and prognostic biomarkers of BTCs.

Moreover, several SNPs in miRNAs can affect the expression of target genes, leading to a cellular disorder and, consequently, tumorigenesis [4, 70]. However, few studies have been performed to identify SNPs in the miRNAs expressed by BTC patients until now; this review emphasizes the need to expand the knowledge in this field of study.

Competing Interests

The authors declare no conflict of interests for this article.

Authors' Contributions

Danielle Queiroz Calcagno conceived the review design; Nina Nayara Ferreira Martins, Kelly Cristina da Silva Oliveira, and Danielle Queiroz Calcagno collected the data; Nina Nayara Ferreira Martins, Kelly Cristina da Silva Oliveira, Amanda Braga Bona, and Danielle Queiroz Calcagno wrote the paper; Mar´ılia de Arruda Cardoso Smith and Geraldo Ishak performed corrections and made suggestions; Paulo Pimentel Assumpc¸ao and Rommel Rodr˜ ´ıguez Burbano critically revised the paper.

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ANEXO II

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Review

Liquid biopsy provides new insights into gastric cancer

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ABSTRACT

Liquid biopsies have great promise for precision medicine as they provide information about primary and metastatic tumors via a minimally invasive method. In gastric cancer patients, a large number of blood-based biomarkers have been reported for their potential role in clinical practice for screening, early diagnosis, prognostic evaluation, recurrence monitoring and therapeutic efficiency follow-up. This current review focuses on blood liquid biopsies' role and their clinical implications in gastric cancer patients, with an emphasis on circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and circulating non-coding RNAs (ncRNAs). We also provide a brief discussion of the potential and limitations of liquid biopsies use and their future use in the routine clinical care of gastric cancer

INTRODUCTION

Among the various types of cancer that affect humans, gastric cancer (GC) is the fifth most frequent tumor type and the third leading cause of cancer death worldwide [1]. The high mortality rate presents a major clinical challenge because most GC cases are diagnosed at an advanced stage with poor prognosis, limited treatment options, and frequent metastasis and recurrence [2].

The sensitivity and specificity of current blood biomarkers for GC are insufficient to define the diagnosis and prognosis. In general, a GC diagnosis relies on an upper digestive endoscopy, an invasive procedure with a relatively high cost and unusual but serious adverse events [3, 4]. In addition, a single tumor-biopsy may not represent the intratumoral heterogeneity and can contribute to treatment failure and drug resistance [5]. Thus, new promising analyses of liquid biopsies should be further explored and validated for use in a clinical setting in GC patients.

Liquid biopsies have emerged as a new strategy for use in the clinical treatment of different cancer types to provide early disease detection, determine the tumor genomic profile, monitor treatment responses, assess the emergence of therapy resistance, quantify minimal residual disease, and perform real-time cancer management [6].

Originally, the term liquid biopsy had been assigned only to the investigation of circulating tumor cells (CTCs) in the blood of patients with cancer, but has now been extended primarily to include the analysis of circulating tumor DNA (ctDNA) and circulating non-coding RNAs (ncRNAs) [7].

In the current review, we highlight the recent advances in liquid biopsies and examine how different forms of liquid biopsies can be exploited to improve GC patient care. We argue that they should ultimately be integrated into clinical practice for GC management, with a focus on CTCs, ctDNA and circulating ncRNAs.

CIRCULATING TUMOR CELLS (CTCs)

Circulating tumor cells (CTCs) have been identified as predictive and prognostic biomarkers that are useful for clinical approaches. They can determine risk for metastasis and can provide real-time monitoring of the therapeutic response in cancer patients [7]. Table 1 summarizes known CTCs as potential prognostic biomarkers and their utility for the evaluation of therapeutic efficacy in GC [8–29].

In general, CTCs are rare in peripheral blood circulation and are found at a concentration of less than 5 CTCs per 7.5 ml of blood [30]. Furthermore, these CTCs originate from either primary or metastatic tumors [31], present a heterogeneous population and express the antigenic or genetic characteristics of a specific tumor type [32].

Early studies of CTCs characterized them as nucleated cells that express markers of epithelial cells EpCAM and cytokeratin 8, 18, and 19 (CK8, CK18, CK19), but are negative for CD45 (CD45-) [33]. Recent studies have described subpopulations of CTCs undergoing the epithelial mesenchymal transition (EMT) that may show decreased expression of EpCAM and cytokeratin with potential overexpression of mesenchymal markers, including Vimentin and Twist [34, 16]. In addition, it is also possible for these CTCs to undergo the reverse process, termed the mesenchymal epithelial transition (MET), resulting in subpopulations of CTCs that present mesenchymal and epithelial markers [35].

CTCs with mesenchymal phenotypes could have a greater propensity for tumor escape due to larger plasticity, thus facilitating the invasion and migration process [36, 37]. Moreover, CTCs with mesenchymal markers seem to be more resistant to chemotherapeutic drugs [38].

Concerning GC, Li *et al.* [16] indicated five types of cells including exclusively epithelial (E^+) CTCs, exclusively mesenchymal (M^+) CTCs and intermediate CTCs ($E^+ > M^+$; $E^+ = M^+$; $M^+ > E^+$), using a filtrationbased method and EpCAM, CK8, CK18, CK19, Vimentin and Twist as markers. These authors observed that approximately 11% (4/35) of patients formed a subgroup exclusively with M^+ CTCs and 29% (10/35) of patients had subgroups M^+ and $M^+ > E^+$, just one patient had the CTCs subgroups $E^+ > M^+$ and no patient with the CTCs subgroup that was exclusively E+. Taken together, these findings demonstrated the heterogeneity of CTCs and their predominantly mesenchymal phenotype, suggesting a limitation of the methodologies that only just epithelial markers to enumerate CTCs in GC.

Although the CellSearch™ platform (Veridex LLC, Huntingdon Valley, PA, USA) uses antibodies against the adhesion molecule (EpCAM)-coated with magnetic beads, cytokeratin (CK8, CK18 and CK19) antibodies and negative staining for the CD45 (CD45-) antibody to isolate and exclusively quantify the E+ CTCs, it remains the main method used in GC studies and is the only technique approved for the

enumeration and isolation of CTCs by the Food and Drug Administration (FDA) for clinical use in the prognosis of breast, colorectal and prostate cancer [39].

Additional characterization of CTCs can identify specific morphological, phenotypic and molecular features for each cancer type over time, disease stage and therapeutic definition [40]. For instance, Iwatsuki *et al.* [14] evaluated CTCs and their HER2 status in gastrointestinal cancer patients; overexpression of HER2 is a selective biomarker for treatment with the monoclonal antibody Trastuzumab in metastatic GC. In GC patients, these authors detected at least one CTC (CTC ≥ 1) in 73.5% (25/34) of samples that were 28% (7/25) HER2 positive. However, a discordant HER2 status was found between CTC-positive cases and corresponding primary tumors (HER2-positive CTCs/ HER2-negative primary tumor tissue), suggesting that primary HER2-negative tumors acquired *HER2* gene amplification in their CTCs during cancer progression. Therefore, the HER2 status of CTCs might be required as a liquid biopsy to provide personalized treatment strategies in GC.

Several studies have observed the aneuploidy of chromosome 8 in CTCs from GC patients [41, 17, 25], a frequent genetic abnormality reported in GC tumors and cell lines [42–47]. Interestingly, Li *et al*. [41] established an integrated subtraction enrichment (SET) and immunostaining-fluorescence *in situ* hybridization (iFISH) platform to detect and characterize CTCs that correlated with different ploidies of chromosome 8 in advanced GC (AGC) patients. These authors suggested that SET iFISH is significantly more sensitive than the CellSearch™ method to enumerate CTCs.

Recently, two studies performed SET-iFISH to enumerate CTCs with chromosome 8 aneuploidy before and after treatment in advanced gastric cancer (AGC) patients. Ma *et al*. [17] observed a marked expressive reduction in CTCs number with chromosome 8 amplification in patients after neoadjuvant therapy with Docetaxel/Oxaliplatin/5-FU (DOF) plus Bevacizumab compared to patients treated with DOF alone, suggesting that the addition of bevacizumab, a VEGF inhibitor, could decrease CTC counts. In addition, Li *et al*. [23] quantified CTCs and analyzed their chromosome 8 multiploidy in patients before and after therapy with the first-line (paclitaxel or cisplatin) or targeted therapy (anti-HER2 and cisplatin) and correlated these findings with the patient's clinical prognosis. AGC patients who have an unfavorable CTC value (≥ 4 CTCs) and an unfavorable CTC multiploidy value (≥ 2 per 7.5 mL) following therapy showed a significant association with poor progressionfree survival (PFS) and overall survival (OS). Moreover, patients with ≥ 10% increase in multiploid CTCs after the first 2 cycles of therapy had a greater risk of progression and mortality than patients with a decrease number of

Table 1: CTCs and their clinical implication in GC patients

Markers	Methodology	Samples	Country	Clinical Implications	References
CEA, CK19, hTERT and MUC1 mRNA	qRT-PCR	64 GC	China	The membrane array-based method is a potential tool for detecting CTCs for early diagnosis and postoperative surveillance.	[8]
EpCAM, CK8, CK18, CK19 and CD45-	CellSearch	41 GC	Japan	CTCs number associated with advanced stage, peritoneal dissemination, metastasis and poor survival.	[9]
BIRC5, CEA, CK19 and VEGF	qRT- PCR	70 GC	Italy	BIRC5 has a significant prognostic value to the current TNM staging system.	[10]
EpCAM, CK8, CK18, CK19 and CD45-	CellSearch	52 AGC	Japan	CTCs enumeration may be useful as a marker for determining response to S1-based or paclitaxel regimens in AGC.	[11]
piR-651 and piR-823	qRT-PCR	93 GC	China	Levels of piR-651 and piR-823 could be useful to diagnosis GC with high sensitivity and specificity.	[12]
EpCAM, CK8, CK18, CK19 and CD45-	CellSearch	265 GC	Japan	CTCs associated with significantly worse OS.	[13]
EpCAM, CK8, CK18, CK19, CD45- and HER2	CellSearch	34 GCM	Japan	HER2 status of CTCs might be helpful for stratification of HER2-directed therapy.	[14]
CD44+ and CD45-	FACS	31 GC	China	CD44+/CD45- CTCs were associated with stronger malignant behavior and relatively sensitive to fluorouracil, cisplatin and paclitaxel, but relatively resistant to irradiation, oxaliplatin, cetuximab and trastuzumab.	[15]
EpCAM, CK8, CK18, CK19, Vimentin, Twist and CD45-	CanPatrol (RNA-ISH)	44 GC	China	Mesenchymal CTCs have a potential relevance to therapy response and can be useful on a therapeutic resistance.	[16]
Chromosomes 7 and 8	FISH	8 AGC	China	Reduction in CTCs count showed beneficial results to the patients treated with docetaxel/oxaliplatin/5-FU (DOF) regimen plus bevacizumab.	[17]
EpCAM, MUC1, *KRT19*, *MUC1*, *CEACAM5*, *EPCAM* and *BIRC5*	Immunomagnetic and RT-PCR	62 AGC	Germany	A combination of immunomagnetic separation of CTC followed by a real-time RT-PCR analysis of *KRT19*, *MUC1*, *EPCAM*, *CEACAM5* and *BIRC5* can serve as a prognostic tool for PFS and OS in patients with AGC.	[18]
CD133 and ABCG2	Flow cytometry and Immunomagnetic	36 GC	China	Presence of CD133 in bloodstream is potentially correlated with potentially be used as a marker of CTCs.	[19]
EpCAM, CK8, CK18, CK19, CD45-, CD19, CD20, CD40, CD44, CD133, CEA and HLA	CellSearch and Flow cytometry	42 AGC	Japan	CD44 is an appropriate biomarker of tumorigenic cells on peripheral blood.	[20]
EpCAM, CK8, CK18, CK19 and CD45-	CellSearch	136 AGC	Japan	Detection of CTCs was an independent predictor of a shorter PFS and could be a useful biomarker in the selection of patients who require intensive treatment in AGC. In addition, combined status of CTC and CY would be useful in selecting patients for radical gastrectomy.	[21]
OBP-401	FP-CTC Assay	37 GC	Japan	The number of CTCs (S-GFP+ cells) was relatively high in samples from GC patients who had received postoperative chemotherapy. However, no significant association between the change in the number of CTCs, treatment or prognosis in gastric cancer patients who underwent curative surgery.	[22]
CK4, CK5, CK6, CK8, CK10, CK13, CK18, CD45- and Chromosome 8	SE-iFISH	31 AGC	China	Aneuploidy of chromosome 8 in CTCs is associated with a poor prognosis.	[23]
EpCAM, CK7, CK18, CK19,CK20, CD45-, CD68, MUC1, HER2 and EGFR	MetaCell	22 GC	Poland	Higher sensitivity of CTC detection could be using a cytomorphological and molecular analysis.	[24]
EpCAM, CK8, CK18, CK19 and CD45-	CellSearch	136 GC	China	Post-treatment CTCs levels can help to evaluate therapeutic response and predict their prognosis in patients with AGC.	[25]

EpCAM, CK8, CK18, CK19, CD45- and c-MET	CellSearch and Immunomagnetic	7 GEA	USA	c-MET CTCs might be useful as a predictive biomarker for c-MET directed therapies.	[26]
EpCAM, CK and CD45-	FAST-disc	116 GC	Korea	Potential role of FAST-based CTC detection as an early diagnostic biomarker of GC.	[27]
Vimentin, CK8, CK18, CK19, CD45- and CA125	ISET	86 GC	China	CTCs could be divided into epithelial CTCs, epithelial/ mesenchymal CTCs, and mesenchymal CTCs, whereas CTM could be divided into two subpopulations, including mesenchymal CTM and partially mesenchymal (epithelial/ mesenchymal) CTM. Moreover, CTM were a independent predictor of worse PFS and OS in stage IV patients.	[28]
EpCAM, CK8, CK18, CK19, CD45- and HER2	CellSearch and IF-FISH	118 GC	Japan	IF-FISH method is applicable for select patients for trastuzumab therapies.	[29]

AGC: advanced gastric cancer; CTM: circulating tumor microemboli; CY: peritoneal lavage cytology; EMT: epithelial-mesenchymal transition; FACS: fluorescence activated cell sorter; FAST: fluid-assisted separation technique; GC: gastric cancer; GCM: gastric cancer with metastasis; GEA: gastroesophageal adenocarcinoma; IF-FISH: immunofluorescence integrated with immunostaining-fluorescence in situ hybridization; ISET: isolation by size of epithelial tumor cells; PFS: progression-free survival; OS: overall survival; SE-iFISH: enrichment (SE) integrated with immunostaining-fluorescence *in situ*

hybridization; RNA-ISH: RNA *in situ* hybridization.

multiploidy CTCs. These studies suggested that the use of SET-iFISH to enumerate CTCs with chromosome 8 aneuploidy is an efficient method to monitor the treatment response of GC patients.

In addition, many studies have reported that the presence of CTCs in circulating tumor microemboli (CTM) confer a survival advantage in the circulatory system compared to single CTCs, which indicate poor prognosis and influence disease progression [48, 49]. In GC, Zheng *et al*. [28] observed CTCs in 59% (51/86) of GC patients in clinical stage I to IV, but CTMs were only found in 24% (10/41) of GC patients in stage IV. They concluded that the group that was CTM-positive had worse PFS and OS than the CTM-negative group ($p < 0.001$). Thus, CTM could be useful to predict prognosis in GC.

CIRCULATING TUMOR DNA (ctDNA)

DNA fragments available in the blood stream, known as ctDNA, derived from primary tumor cells, CTCs and/or distant metastasis can reflect specific genetic cancer alterations, including mutations, amplifications, copy number variation (CNV), rearrangement and methylation [50, 51].

Accumulating evidence has demonstrated that ctDNA detection is a minimally invasive method with potential clinical applications in cancer, including i) early detection of cancer; ii) monitoring of intratumoral heterogeneity and metastasis; iii) therapeutic target identification; iv) real-time evaluation of treatment response and tumor relapse; and v) real-time evaluation of drugs resistance [52].

So far, limited studies on the identification and monitoring of ctDNA levels in GC patients have been performed. Hamakawa *et al.* [53] reported that 30% (3/10) of AGC patients had *TP53* mutations in their primary tumors and preoperative ctDNA, suggesting that identification of the *TP53* mutation (c.103delT; c.747G>C; c.166G>T) is a useful tool to monitor progression and residual disease during the clinical follow-up.

In 2016, Fang *et al*. [54] analyzed the mutational profile of eight genes (*ARID1A*, *TP53*, *PIK3CA*, *PTEN*, *AKT3*, *BRAF*, *AKT2* and *AKT1*) and the altered levels of ctDNA in 277 patients with primary gastric tumors. The authors found that *TP53*, *ARID1A* and *PI3KCA* were the most frequently mutated genes in AGC patients. Furthermore, they also found that patients with greater ctDNA levels were more likely to exhibit vascular invasion and a poor 5-year global survival rate than patients without detected ctDNA. Therefore, the highest ctDNA detectable levels were associated with peritoneal recurrence and a poor outcome in patients with AGC.

In addition, Shoda *et al*. [55] reported on potential of ctDNA for the detection of *HER2* amplification determined by real-time quantitative PCR (qRT-PCR) in AGC patients before surgery and during postoperative treatment, highlighting spatial and/or temporal tumor heterogeneities. Unfortunately, quantitative information using qRT-PCR is obtained from the cycle threshold (Ct) and these values can be affected by amplification imperfections that reduce efficiencies and limit the accuracy of this method for absolute quantification. On the other hand, the digital droplet PCR (ddPCR) method improves upon these limitations of nucleic acid quantification.

In 2017, Shoda *et al*. [56] showed the clinical utility of *HER2* ratios in GC patients during treatment progression and demonstrated the *HER2* status during real time evaluations using ddPCR method. Postoperative follow-ups revealed high plasma *HER2* ratios at the time of recurrence in 53.84% (7/13) cases, even in cases that were diagnosed as being *HER2* negative at the time of surgery. Overall, detection of the *HER2* ratio by digital droplet PCR (ddPCR) could provide a window of opportunity for novel decision-making treatment strategies based on *HER2* status at different periods in a clinical setting.

In a GC ctDNA meta-analysis, Gao *et al*. [57] demonstrated a significantly association between the ctDNA level based on gene methylation with the TNM stage, tumor depth, lymph node metastasis and distant metastasis in GC patients with high specificity (0.95, 95% CI 0.93–0.96) and relatively moderate sensitivity (0.62, 95% CI 0.59−0.65).

Recently, periodic mutation profiling of ctDNA from stage IV GC patients by Next Generation Sequencing (NGS) revealed the complex and heterogeneous molecular mechanisms for crizotinib resistance after two months of treatment, including reoccurrence of *MET* amplification, multiple secondary *MET* mutations (D1228, Y1230, V1092, G1163 and L1195), a remarkable increase in the relative copy number of the *FGFR2* gene as well as mutations in other downstream and related elements [58]. Crizotinib, a potent *MET* inhibitor, has demonstrated promising effects for the treatment of *MET*-amplified esophagogastric cancer [59, 60]. Moreover, *MET* amplification has been reported to occur in approximately 5% of GC patients and is

targeted by crizotinib, which is currently undergoing a clinical trial in advanced *MET*positive GC. However, tumors experienced progression shortly after crizotinib treatment [59]. Therefore, ctDNA profiling for treatment decision-making and prognosis in clinical practice have demonstrated great potential to elucidate mechanisms of resistance.

Overall, regular analysis by NGS is more expensive than ddPCR for ctDNA quantification method. Also, NGS practical use reveals an information reservoir unnecessarily for objective decision-making in clinical setting. Consequently, ddPCR could be well applied in clinical practice to identify relevant genetic aberrations in the ctDNA that facilitate GC management.

CIRCULATING NON-CODING RNAS (ncRNAs)

Deregulated ncRNAs expression has largely been reported in the literature acting as oncogene or with tumor suppressor role in several cancers types, including GC [61–63]. Since ncRNAs are important mediators of intracellular activities with tissue specific characteristics, quantification of deregulated ncRNAs expression in blood can indicate disease state, disease progression and/ or response to a particular treatment, therefore directing initial clinical practice. Secretion to the bloodstream is usually the results of cell death (necrosis or apoptosis) or due to active secretion from the cell, therefore, the expression profile reflects the primary tumor in the corresponding tissue [64].

A large number of studies have highlighted the potential importance of circulating ncRNAs as diagnostic, prognostic, and/or predictive biomarkers in cancer, mainly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). These molecules are remarkably stable as they are often incorporated into exosomes and microvesicles, thus providing resistance to RNase activity, extreme pH and multiple freeze-thaw cycles [62–63, 65–67].

microRNAs

miRNAs are a class of single-stranded small ncRNAs of 19–25 nucleotides (nt) in length that play an essential role in the negative post-transcriptional gene regulation of at least 50% of all protein-coding gene [64]. Supplementary Table 1 summarizes a large number of circulating miRNAs as potential diagnosis and prognosis biomarkers in GC and their clinical implications [68–88]. Recently, Tsai *et al.* [80] demonstrated that miR196a/b expression in the serum of GC patients could be more sensitive and specific for GC diagnosis than CA 19-9 (carcinoembryonic antigen 19-9) or CEA (carbohydrate antigen). Moreover, circulating miR-196a/b was also associated with TNM stage, a poor survival rate and cancer outcome, suggesting that miR-196a/b is a potential diagnostic and prognostic biomarker in GC.

Several studies also described associations between miRNA expression, *H. pylori* and EBV infection, which are well-known causes of GC [61, 62]. For instance, Shiotani *et al.* [73] observed an association between upregulated circulating miR-21 and miR-106b and *H. pylori* infection. The authors also suggested that the upregulation of both miRNAs in the serum of patients after *H. pylori* eradication could be used for the detection of high risk GC in individuals with extensive atrophy.

A single candidate approach can reveal deregulated miRNA, however, the search for a miRNA signature profile that can predict prognosis and monitor cancer progression has been a common focus of many studies. Among these studies, it was reported that the miRNA profile in the serum/plasma of patients with GC displayed unique miRNA changes or a miRNAs signature.

Using MiSeq sequencing, Jiang *et al.* [89] performed an initial screening of serum miRNAs in ten GC patients with lymph node metastasis (LNM+), ten patients without lymph node metastasis (LNM-) and ten healthy controls. Then, the candidate miRNAs (miR-501-3p, miR-143-3p, miR451, and miR-146a) were validated in serum samples from
73 controls, 103 LNM+ and 103 LNM- patients by qRTPCR. Prediction of LNM+ in GC restricted to the mucosa prior to surgery with a circulating miRNA panel could help determine the need for surgical lymph node resection. This would allow endoscopic mucosal resection, a less invasive treatment, to be immediately conducted without delay to provide more effective treatment for early gastric tumors. On the other hand, endoscopic resection of tumors should be avoided when the miRNA panel to indicates LNM+. In the case, surgical resection with an extensive lymphadenectomy would be recommended for a better outcome in GC patients.

Despite numerous efforts, no consensus has been found for miRNA biomarkers that can be incorporated into GC clinical practice. For this occur, a number of obstacles must be overcome, for example, the quantification of miRNAs can suffer from variations due to inadequate processing, storage, RNA extraction, and reference genes choice for qRT-PCR quantification.

Variation is such a problem that and even differences between serum and plasma miRNA quantification have been observed [86, 69, 90–93]. Moreover, there is no unique protocol to control for these parameters. Divergences in the analysis of the circulating miRNA make it difficult for perform a comparison among them.

Long non-coding RNAs

lncRNAs comprise a diverse class of RNA transcripts >200 nt in length. They regulate gene expression through a variety of transcriptional and posttranscriptional mechanisms, including i) chromatin modification and remodeling; ii) direct transcriptional regulation; iii) regulation of RNA processing events such as splicing, editing, localization, translation and turnover/ degradation;, iv) induction of DNA methyltransferases; v) protein scaffolding; vi) modulation of miRNA regulation; vii) miRNA precursor processing; viii) regulation of translation; and ix) protein binding [94].

Similar to miRNAs, a number of circulating lncRNAs also have emerged as diagnostic and/or prognostic biomarkers in GC (Supplementary Table 2) [95–103]. For instance, HULC (highly up-regulated in liver cancer) was significantly higher in the serum of GC patients than healthy controls. Interestingly, the serum HULC level was significantly decreased in post-treatment patients to a level similar to that of healthy individuals. In addition, serum HULC levels expression was associated with tumor size, lymph node metastasis, distant metastasis and *H. pylori*, a strong risk factor for both GC development and progression. Furthermore, a ROC curve to evaluate the diagnostic utility of HULC revealed that serum HULC levels provides a more powerful differential ability than CEA and CA72-4, followup detection and Kaplan-Meier curve analysis showed that HULC is a good predictor of GC prognosis. Taken together, these findings indicate that HULC may be a potential tumor biomarker for early diagnosis, progression monitoring and GC prognosis of GC [100].

According to Chao *et al*. [104], elevated circulating levels of AA174084 were associated with invasion and lymph node metastasis in GC patients. Their levels dropped markedly on day 15 after surgery compared to preoperative levels. However, the measurement of plasma-based AA174084 has obvious limitations, because AA174084 levels in plasma do not differ between healthy individuals and GC patients. Thus, the authors suggested that AA174084 may have potential as a prognostic biomarker for GC.

Several studies investigated the levels of circulating H19 (H19, imprinted maternally expressed transcript) from GC patients as potential diagnostic biomarker [96–98]. Arita *et al*. [96] found that H19 levels were significantly higher in the plasma of GC patients than in healthy controls. However, there was no correlation between plasma H19 levels and the clinicopathological factors of these GC patients. In comparison with the levels in pre- and postoperative paired plasma samples, H19 levels were significantly lower in postoperative plasma.

In 2015, Zhou *et al*. [97] validated the expression of eight lncRNAs (HOTAIR, CCAT1, PVT1, H19, MALAT1, MRUL, GHET1 and HULC) by test-scale analyses in tissue and plasma using qRT-PCR. Among them, H19 and another five lncRNAs (HOTAIR, PVT1, MALAT1, GHET1 and HULC) were significantly higher in tumor tissues compared to matched normal samples. Of these lncRNAs, only H19, MALAT1 and HOTAIR were significantly higher in the plasma of ten GC patients compared to ten healthy controls. Among these three lncRNAs, only H19 expression was significantly higher in GC patient plasma compared to heathy controls, when plasma lncRNAs levels were examined on a large scale using plasma from 70 GC patients and 70 healthy controls. That analysis involved the comparison of plasma H19 concentrations in paired plasma obtained from pre- and postoperative samples; H19 levels were significantly reduced postoperatively in patients with high preoperative plasma H19. Clearly, these findings demonstrated that plasma levels of H19 are useful as a potential biomarker for the diagnosis of GC, particularly for early tumor screening.

Many of the obstacles that exist for the effective application of circulating lncRNAs in GC clinical practice are similar to those described for circulating miRNAs.

FUTURE DIRECTIONS

Liquid biopsy approaches have enormous implications for cancer, ranging from early diagnosis to the monitoring of treatment response, and have transformed clinical care. Currently, the liquid biopsy does not replace the conventional biopsy, however, it has been applied to tumor growth control and in deciding on therapeutic choice to improve the overall survival rate of patient with different cancer types. Nevertheless, liquid biopsies remain removed from GC clinical management. Figure 1 summarizes the putative outlook of liquid biopsy use and their potential

application in clinical care in GC management (classified as strong, moderate and weak evidence).

In GC, we believe that the earliest use of liquid biopsy in clinical practice should focus on therapies that target detection and monitoring. Indeed, *HER2* status in CTCs or ctDNA has emerged as a therapeutic marker of effective molecular targeted therapy and therapeutic response monitoring in GC patients [14, 56, 57]. Figure 2 shows timeline of CTC and ctDNA analysis in pre-treatment and post-treatment (immediately and monitoring) period of GC patients.

Before liquid biopsies are incorporated into clinical practice as a precision medicine tool to drive GC management, pre-analytical steps must be standardized in order to ensure reproducible processing techniques. Moreover, analytical steps must be validated, such as the enumeration of CTCs and ctDNA, the quantification of circulating ncRNAs, subsequent CTCs characterization and genetic or epigenetic alterations in ctDNA analysis. Finally, CTCs markers or assays applied to circulating ncRNAs or ctDNA measurements must have strong and reproducible sensitivity and specificity, beyond having the suitable internal and external quality controls.

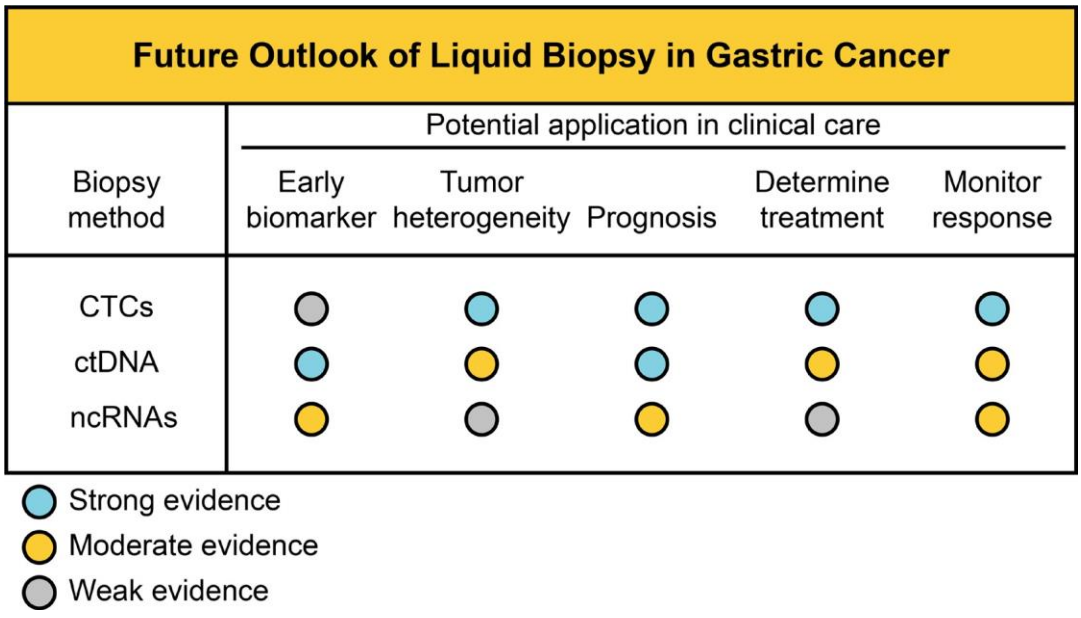

Figure 1: Potential application of liquid biopsies in GC management. Establishment of standard analysis of CTCs, ctDNA and circulating ncRNAs in the future.

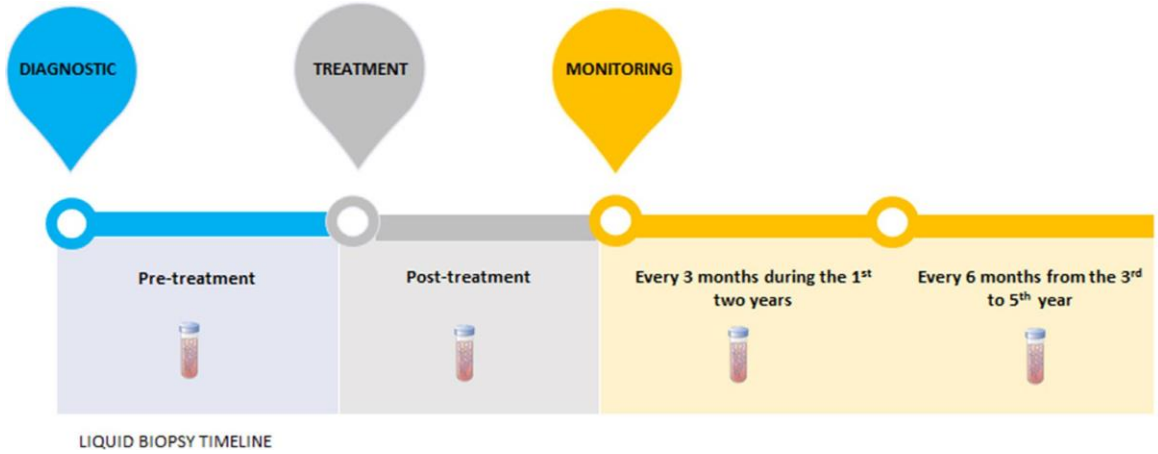

Figure 2: Liquid biopsies timeline. At the diagnostic, the pretreatment ctDNA demonstrates the molecular characteristics of the tumor, as well as the patient's prognosis. After treatment, ctDNA quantification could measure the treatment's efficacy, since ctDNA bloodstream half-time is about 1-2.4hours. In case of advanced gastric tumors, CTCs analysis could also be used in the same manner. Moreover, monitoring of ctDNA or CTCs, every three months during the first two years and every six months from the third to fifth year, could evaluate therapeutic response and recurrence disease before the patients shows clinical symptoms or metastasis is observed by computed tomography.

Consequently, many questions remain about liquid biopsies from blood that require resolution: i) how should the blood samples be collected to ensure quality biomarker detection; ii) which is the ideal method and marker for CTC enumeration and characterization; iii) what gene alterations are key for ctDNA measurement; iv) what reference genes are stable and suitable for circulating ncRNA measurement in GC patients; v) what criteria should be adopted for the CTC, ctDNA and circulating ncRNA validation analysis.

CONFLICTS OF INTEREST

None.

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