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Quantitative Real-Time PCR as a Novel Detection Method for Micro-RNAs Expressed by Cervical Cancer Tissue: A Review

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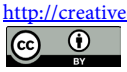

Abstract

The potential of microRNAs (miRNA) as biomarkers of Cervical Cancer (CC) is analyzed extensively by many researchers today. However, some studies have shown that miRNAs are expressed in cancers and may act as a better diagnostic strategy than the Pap smear or even assist it as a screening strategy. MicroRNA expression has been shown to differ in precancerous lesions as well as in cervical cancer tumours from that of normal tissue. With the use of quantitative real-time PCR (qRT-PCR), microRNAs can be detected in many sample types ranging from biopsy samples to blood (serum and plasma). Early detection of the disease is possible due to the aberrant expression of miRNAs in precancerous stages as well as advanced stages of the disease; this proves that they have the potential to be an ideal novel biomarker for CC. This review discusses studies using qRT-PCR to detect the expression of miRNAs within the years 2008-2019 and focuses on giving an insight into the types of samples and kits that have been used. Publications which have used qRT-PCR as a primary or secondary detection method were selected via Google Scholar Search and PubMed. Studies were shown to have used a variety of kits and reagents, but all have applied the main principle of qRT-PCR. Quantitative Real-Time PCR is shown to be a versatile and accurate detection technique for miRNAs of CC.

Keywords

Real-Time PCR, Cervical Cancer, miRNA

1. Introduction

Cervical cancer is the fourth most common type of cancer that affects women globally [1]. It has been reported that an estimated 570,000 new cases were recorded in 2018; this represents 6.6% of all female cancers. Epidemiological statistics show that a high mortality rate of 90% occurred in low- and middle-income countries. However, the global mortality rate can be reduced by implementing comprehensive prevention strategies, early diagnosis, as well as effective screening and treatment programs [1]. It has been shown through many studies that utilizing qRT-PCR to detect miRNA biomarker expression in precancerous lesions and CC tumours can act as a potential screening/diagnostic tool for CC.

The current prevention strategy for CC is the Papanicolaou test (pap-smear) analysis, which is an invasive technique and does not appeal to all female patients [2]. This is primarily a histopathological technique of screening and analysis that can be time-consuming and labor intensive. However, genetic markers have played an important role in tumour dynamics; they are a novel diagnostic technique and help assist the cytological (pap-smear and biopsies) diagnostic results. A possible genetic marker for CC is miRNA and it has the potential to be a novel non-invasive diagnostic criterion in detecting cervical cancer. In order to analyze the expression of these biomarkers only a few techniques could be used, out of which the most effective method would be qRT-PCR.

The study of miRNAs as a potential diagnostic tool for cervical cancer is a novel approach. Circulating miRNAs of different neoplasia reflect the pattern observed in the tumour tissues, giving rise to the possibility of using circulating miRNAs as easily detectable tumour biomarkers, and this property of miRNAs would especially be helpful for early diagnosis [3] [4] [5] [6]. Research shows that due to their small size, miRNAs are more stable compared to messenger RNAs (mRNAs), which allows for expression profiling from fixed tissues or other biological materials. This supports the possible use of miRNAs as the novel, minimally invasive, and robust biomarkers. MicroRNAs can be extracted from frozen and paraffin-embedded tissues, blood, circulating exosomes, and from biological fluids such as urine, saliva and even sputum proving their versatility as biomarkers [7]-[13]. Quantitative real-time PCR is regarded as the best detection method due to miRNAs being highly sensitive to this technique, it is used to amplify DNA/RNA, which depends on fluorescence emission to detect the amplified target gene. This technique is highly sensitive and does not require large amounts of RNA, and it can detect very low levels of miRNA. This technique relies on a variety of steps to detect target sequences, miRNA must be isolated with the appropriate kit and reagents after which it undergoes reverse transcription, and the cDNA of the selected miRNA is synthesized with sequence-specific primers making qRT-PCR very accurate and reliable.

The aim of this review is to analyze how different studies have used varying types of samples and qRT-PCR as a primary or secondary detection tool to analyze the expression levels of selected miRNAs specific to cervical cancer.

2. Methodology

Journal articles were filtered according to research methodology and year of publication; studies which have used qRT-PCR as a primary or secondary detection tool for miRNA have been analysed in this review. Online databases such as Google Scholar and PubMed were referred when searching for journal articles. The following search terms: "miRNA + cervical cancer + qRT-PCR", "miRNA detection + cervical cancer + qRT-PCR", and "cervical cancer + specific miRNA + detection", were used to find the appropriate articles on PubMed in all fields. The same terms were searched on Google Scholar. Refer Figure 1 for a detailed view of the literature selection process.

2.1. Inclusion Criteria

- Studies using qRT-PCR as a primary or secondary detection tool;
- Studies which have exclusively evaluated the expression of miRNAs in CC samples as a potential biomarker;
- Studies where full-text was available for free;
- Studies published in English.

2.2. Exclusion Criteria

- Studies which have not used qRT-PCR as a detection tool regardless of evaluating miRNA expression;

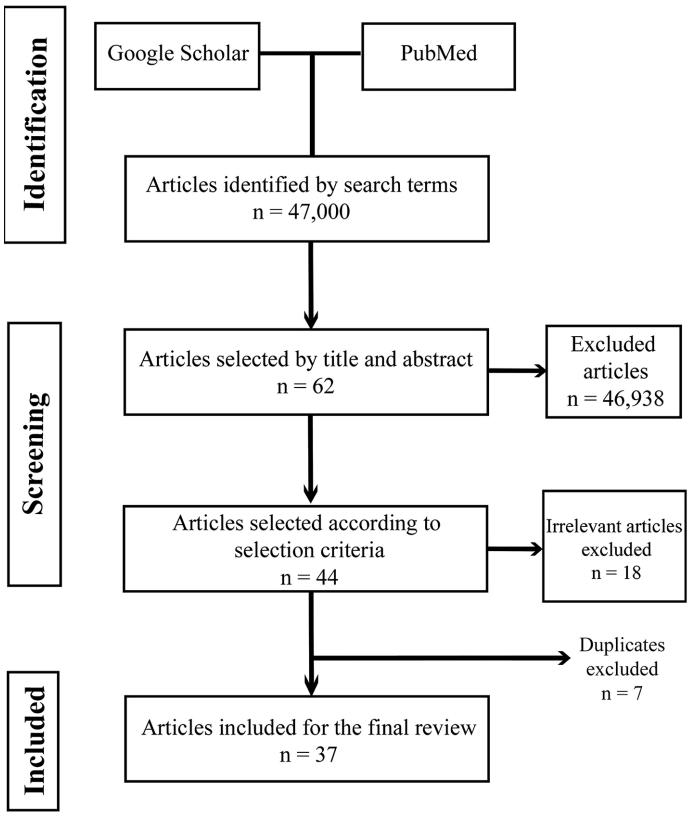

Figure 1. Flow chart demonstrating the literature selection process.

- Studies which have only used cell lines to analyse miRNA expression;
- Studies only analysing the role of miRNA and target gene functions.

There was no discrimination against sample type and size, collection method and storage.

3. Quantitative Real-Time PCR vs the Pap Smear

Using qRT-PCR to detect miRNA biomarkers in CC is a novel technique and there is potential for this to be a good candidate as a new screening/diagnostic procedure. It requires less time and labor compared to the PAP smear technique —which requires clinicians to prepare a slide that will be sent to a lab to be fixed and studied under a microscope before the result is presented. This could take a matter of days or weeks depending on the country, whereas qRT-PCR is an overnight procedure needing only one person to handle and perform making it a more cost effective and versatile procedure overall.

4. Research Evidence of Using qRT-PCR for miRNA Detection in Cervical Cancer

There are many studies which utilized qRT-PCR as a tool to evaluate miRNA expression in CC patients. However, there are many differences between each study when it comes to the type of sample, storage, the brand of the kits and reagents, the PCR machine, etc. A thorough methodology is important to prove the validity of a study and the potential of the detection technique as a diagnostic/screening tool.

4.1. Sample Types Used to Detect the Expression of miRNAs in CC Patients and Healthy Controls

Most studies used tumour tissue samples along with their adjacent normal tissues as well as known cell lines. The most common sample types were Formalin Fixed Paraffin Embedded (FFPE) tissue samples and fresh cervical tissue biopsy samples. Exfoliated cells, and blood samples (serum/plasma) were amongst the more rare sample types. Homogenized tissue samples are said to be more reliable as the concentration of miRNAs are high, but it is an invasive diagnostic procedure. Studies that have used peripheral blood samples have an upper-hand as it is a minimally invasive procedure. When it comes to blood circulating miRNAs, they are found in cell-free lipid carriers (exosomes, microvesicles, and apoptotic bodies), that are released from apoptotic/necrotic cells [14] [15]. Blood circulating miRNAs are also secreted from cells bound to protein complexes like Argonautes or high-density lipoproteins which prevent the miRNAs from degradation and from reacting with RNases [14] [15]. Since miRNAs are aberrantly expressed in cancer, the levels of blood circulating miRNA may differ from that of normal levels (cut-off value) found in a healthy person [16].

A majority of the studies have obtained control samples from the healthy tissues of the patient, whilst only one study specified the size of the biopsy sample [17]. Most studies collected samples from CC patients before they underwent

any treatment except for two studies; one study that took samples from patients previously diagnosed with Low Grade Squamous Intraepithelial Lesion (LGSIL)/ High Grade Squamous Intraepithelial Lesion (HGSIL) and had undergone treatment [17] and the other from patients that have undergone radiotherapy [18]. One other study recruited women who had a history of cancer other than CC [19].

The most common sample types were snap-frozen tissue samples (5) [20] [21] [22] [23] [24], FFPE samples (8) [25]-[33], and tissue lesion/biopsy samples (13) [17] [25] [26] [34]-[44]. Other studies used serum or plasma (7) [18] [39] [45] [46] [47], exfoliated cells (4) [48] [49] [50] [51], and tissue samples frozen in liquid nitrogen (5) [33] [50] [52] [53] [54]. Refer **Table 1** for a detailed summary of all sample types and storage conditions used in the studies discussed. Some studies followed a pre-storage sample processing protocol: One study homogenised their tissue samples in TRIzol reagent and the isolated RNA was dissolved in RNase free water and stored at −70˚C, whilst another study rinsed the tissue samples in sterile saline before storing at −70˚C [17] [35]. A 2012 study embedded their samples in OCT compound before storage and, a 2014 study, divided their sample set into two and used two different storage methods; one set of tissue samples were stored in RNA Later, and the other in 4% formaldehyde for 2 days before embedding in Paraffin wax blocks [25] [36].

Table 1. Summary of sample types used by the studies in this review.

Authors	Sample type	Sample size	Normal tissue samples	Storage temperature
Yao and Lin, 2012 [34]	Tumour/lesion tissue	126	Adjacent samples collected 1 cm away from tumour/lesion tissue	-
Xie *et al.*, 2012 [20]	Snap-frozen tissue	30	-	−80˚C
Wang *et al.*, 2014 [35]	Cervical biopsy	-	-	−70˚C
Zhang et.al., 2013 [21]	Snap-frozen tissue	92	-	-
Wen *et al.*, 2017 [22]	Snap-Frozen tissue	40	Healthy controls	−80˚C
Han *et al.*, 2015 [17]	Tumour/lesion tissue	30	Adjacent samples collected 5 cm away from tumour/lesion tissue	−80˚C
Cheung *et al.*, 2012 [36]	Tumour/lesion tissue embedded in OCT compound	48	Healthy controls	−80˚C
Jiminez-Wences *et al.*, 2016 [37]	Biopsy samples	49	-	-
Villegas-Ruiz *et al.*, 2014 [25]	Biopsy samples (one part in RNA later the other embedded in paraffin wax—FFPE)	45	-	−70˚C (RNA later samples)
Gozce *et al.*, 2015 [26]	Tumour/lesion tissue—FFPE	98	-	n.a.
Deftereos *et al.*, 2011 [27]	Tumour/lesion tissue—FFPE	104	23 healthy controls	n.a.
Bumrungthai *et al.*, 2016 [28]	Fresh tumour/lesion tissue and FFPE samples	79	no	-
Shishodia et.al., 2015 [38]	Tumour/lesion tissue samples in 1X PBS	102	23 healthy controls	-

Continued

Zeng *et al.*, 2015 [29]	Lesion tissues—FFPE	103	13 normal tissues	-
Zheng *et al.*, 2015 [30]	FFPE cervical tissue samples	140	15 normal tissue samples	-
Li *et al.*, 2011 [22]	Snap-frozen tissue samples	125	20 normal	−70˚C
Bierkens *et al.*, 2012 [31]	FFPE samples	75	6 frozen HPV+ normal samples	-
Chen *et al.*, 2013 [39]	Tumour/lesion tissue and matched serum samples	-	-	−80˚C
Ma *et al.*, 2014 [40]	Tumour/lesion tissue and matched serum samples	60	6 healthy controls	−80˚C
Zhang *et al.*, 2016 [45]	Whole blood samples—serum	89	20 healthy controls	-
Ye *et al.*, 2019 [48]	Exfoliated cells	177	-	-
Ribeiro *et al.*, 2015 [49]	Exfoliated cells	65	49 healthy controls	Room temperature
Malta *et al.*, 2019 [50]	Exfoliated cells	73	38 normal controls	-
Hou *et al.*, 2014 [44]	Fresh tissue samples	10	10 normal controls	-
Huang *et al.*, 2016 [24]	Snap-frozen tissue samples	126	64 normal controls	−80˚C
Ma *et al.*, 2015 [32]	FFPE samples	412	208 cervicitis cases used as controls	-
Zhang *et al.*, 2017 [41]	Fresh tissue samples	105	-	-
Kawai *et al.*, 2017 [51]	Exfoliated cells from cervical mucus	230 (total number including normal samples)	N/A	−80˚C
Lukic *et al.*, 2018 [33]	FFPE samples	40	10	-
Shen *et al.*, 2013 [52]	Fresh tissue samples frozen in liquid nitrogen	126	126 (adjacent healthy samples)	-
Luo *et al.*, 2015 [53]	Fresh tissue samples frozen in liquid nitrogen	88	88 (adjacent healthy tissues)	−70˚C
Jiang *et al.*, 2017 [46]	Serum samples	182	12	−80˚C
Leitao *et al.*, 2014 [42]	Biopsy samples stored in RNAlater	46	19	−80˚C
Babion *et al.*, 2018 [43]	Frozen fresh cervical tissue samples cervical scrape samples in ThinPrep Medium	51 187	8 -	−80˚C
Wei *et al.*, 2017 [18]	Plasma samples	120	120	−80˚C
Zhang *et al.*, 2019 [54]	Fresh tissue samples frozen in liquid nitrogen	67	20	-
Xin *et al.*, 2016 [47]	Serum samples	126	60	−80˚C

According to the literature discussed in this review samples have been collected from women of varying stages of CC. Even pre-cancerous stages were included as some of the miRNAs were shown to be expressed early on in disease progression. Most studies have opted for normal samples taken from healthy tissues of the cancer patient itself as it is an unnecessarily invasive procedure for a healthy individual. Taking adjacent healthy tissue biopsies is more reliable as it eliminates any inter-individual discrepancies [55]. Many studies have used tissue

samples, only a minority of the studies discussed here has used blood samples to detect miRNA expression levels. Other studies that have analyzed the levels of miRNA expressed in both tissue and corresponding plasma/serum samples have shown a correlation in expression levels. Micro RNA does not easily degrade, therefore the sample type may not be an issue, however, this depends on the type of miRNA and cancer to be detected.

In one study the miRNA expression in breast cancer was detected using FFPE samples that were put through severe thawing processes, showing that this did not degrade miRNA [56]. They concluded that this makes miRNAs a reliable biomarker for cancer detection [43]. It has also been proven that the miRNA levels of serum and tissues correlated [39] [40]. When collecting samples it is important that participants have not undergone any treatments (*i.e.*; chemotherapy, curative surgery) as this can affect miRNA expression, however this depends on the objectives of the study.

4.2. The Isolation of Micro RNAs and the qRT-PCR Procedure

Running a qRT-PCR reaction requires certain reagents and kits, however, the quality may vary between manufacturers. The studies discussed here have purchased kits from a variety of manufacturers.

Most studies were shown to have used real-time PCR machines from Applied Biosystems, the machines used are as follows: 7300 Real-Time PCR System, 7500 Fast Real-Time PCR system, 7500 Real-Time PCR system, 7900 Real-Time PCR system [17] [18] [20] [21] [24] [29] [31] [41] [44] [46] [51] [52] [54]. However, two studies opted for Stratagene machines; the MX-3000p and MX-3500P, whilst the iCycler by BioRad was used by another [33] [34] [38]. A Rotor-Gene 6000 thermocycler was used by Leitao *et al.* [42] and the iQ5 multicolor PCR detector system by Bio-Rad was used by Ma and colleagues [32]. A 2018 study used the ViiA™ 7 real-time PCR system (ThermoFisher Scientific) for qRT-PCR [43]. The study by Cheung and colleagues used two different machines, the ABI Gene Amp 9700 PCR system for reverse transcription, and the ABI 7900 PCR system for qRT-PCR. The Gene Amp 9700 may have helped increase the yield of miRNA produced during the reverse transcription process as miRNAs are found in very low concentrations in samples [36]. Another study also used two machines: reverse transcription was performed using the QuantScript RT kit by TianGen and real-time PCR was performed using the SuperReal PreMix plus also by TianGen [30].

A commonly used miRNA isolation kit was the Ambion mirVana miRNA Isolation Kit by Life Technologies (ThermoFisher Scientific). The study conducted in 2012 used the mirVana miRNA isolation kit to isolate RNA, and applied small RNA enrichment for tissue sample [20]. Deftereos *et al.* [27] used the Recover All Total Nucleic Acid Isolation Kit by Ambion (ThermoFisher Scientific). One study [30] removed paraffin from tissue samples using xylene and ethanol, the RNA was extracted using the miRNeasy FFPE Kit by Qiagen, and

the RNA samples were stored at −80˚C till reverse transcription and qRT-PCR. A majority of the studies were shown to have used Trizol from Invitrogen (Thermofisher Scientific) as the preferred RNA isolation reagent. Refer Table 2 for a list of all the studies that have used Trizol.

Table 2. Summary of Kits and reagents used for qRT-PCR by studies discussed.

Technique	Kit	Manufacturer	Estimated cost	Authors
miRNA isolation	Ambion mirVana miRNA isolation kit	Life technologies (ThermoFisher Scientific)	USD 384.00	Xie *et al.*, 2012 [20]; Hou *et al.*, 2014 [44]; Villegas-Ruiz *et al.*, 2014 [25]; Ribeiro *et al.*, 2015 [49]; Zhang *et al.*, 2016 [45]; Shishodia *et al.*, 2015 [38]; Luo *et al.*, 2015 [53]; Wei *et al.*, 2017 [18]
	Recover all total nucleic acid isolation kit from applied biosystems	Ambion (ThermoFisher Scientific)	USD 374.00	Deftereos *et al.*, 2011 [27]
	miRNeasy FFPE kit	Qiagen	USD 474.00	Zheng *et al.*, 2015 [30]
	RNeasy FFPE kit	Qiagen	USD 484.00	Ma *et al.*, 2015 [32]
	Invitrogen™ TRIzol™ Reagent	Invitrogen (ThermoFisher Scientific)	USD 209-374.00	Gozce *et al.*(2015) [26]; Wen *et al.*, 2017 [22]; Jiminez-Wences *et al.*, (2016) [37]; Wang *et al.*, 2014 [35]; Burmrungthai *et al.*, 2016 [28]; Cheung *et al.*, 2012 [36]; Ye *et al.*, 2019 [48]; Han *et al.*, 2015 [17]; Huang *et al.*, 2016 [24]; Zhang *et al.*, 2017 [41]; Shen *et al.* 2013 [52]; Leitao *et al.*, 2014 [42]; Babion *et al.* 2018 [43]
	Mirneasy mini kit	Qiagen	USD 402.00	Kawai *et al.*, 2017 [51]; Xin *et al.*, 2016 [47]
	High pure miRNA isolation kit	Roche	USD 504.50	Lukic *et al.*, 2018 [33]
	Invitrogen mirVANA miRNA isolation kit	ThermoFisher Scientific	USD 326.00	Luo *et al.*, 2015 [53]
	Total RNA isolation kit	Beijing Tiandz gene technology	N\A	Zhang *et al.*, 2019 [54]
Reverse Transcription	TaqMan MicroRNA reverse transcription kit	Applied biosystems (ThermoFisher Scientific)	USD 397-1014.00	Xie *et al.*, 2012 [20]; Han *et al.* 2015 [17]; Ribeiro *et al.*, 2015 [49]; Malta *et al.*, 2015 [50]; Jiminez-Wences *et al.*, 2016 [37]; Deftereos *et al.*, 2011 [27]; Cheung *et al.*, 2012 [36]; Wang *et al.*, 2014 [35], Kawai *et al.* [51], Jiang *et al.*, 2017 [46]; Babion *et al.*, 2018 [43]; Wei *et al.*, 2017 [18]
	High capacity cDNA reverse transcription kit	Applied biosystems (ThermoFisher Scientific)	USD 353-1360.00	Zhang *et al.*, 2016 [45]
	MiraMas™ kit	Bioo scientific	N\A	Chen *et al.*, 2013 [39]
	M-MLV kit	Promega	USD 25-364.00	Zhang *et al.*, 2013 [21]; Luo *et al.*, 2015 [53]
	PrimeScript RT reagent kit	Takara Bio	N/A	Wen *et al.*, 2017 [22]

Continued

	MirCute miRNAs kit	Tiangen	USD 652.00	Han *et al.*, 2015 [17]
	Quant reverse transcriptase		N\A	Zeng *et al.*, 2015 [29]
	Takara reverse transcription kit	Takara Bio	N/A	Huang *et al.*, 2016 [24]
	SuperScript™ III reverse transcriptase kit	Invitrogen	USD 111-1368.00	Zhang *et al.*, 2017 [41]
	Brilliant II SYBR Green QRT-PCR, AffinityScript two-step	Agilent technologies	USD 849.00	Lukic *et al.*, 2018 [33]
	Reverse transcriptase kit	Hangzhou Bioer technology	N\A	Zhang *et al.*, 2019 [54]
Real-Time PCR	TaqMan MicroRNA assays	Applied biosystems (ThermoFisher Scientific)	USD 313-2616.00	Xie *et al.*, 2012 [20]; Han *et al.*, 2015 [17]; Ribeiro *et al.* 2015 [49]; Malta *et al.*, 2015 [50]; Jiminez-Wences *et al.*, 2016 [37]; Deftereos *et al.*, 2011 [27]; Cheung *et al.*, 2012 [36]; Wang *et al.*, 2014 [35], Bierkens *et al.*, 2012 [31]; Zhang *et al.*, 2017 [41]; Kawai *et al.* [51]; Lukic *et al.*, 2018 [33]; Shen *et al.*, 2013 [52]; Jiang *et al.*, 2017 [46]; Babion *et al.*, 2018 [43]; Wei *et al.*, 2017 [18]
	SYBR Green MIX kit	Takara Bio	N\A	Wen *et al.*, 2017 [22]
	SYBR Premix Ex Taq II	Takara Bio	N\A	Huang *et al.*, 2016 [24]
	SYBR Green specific primers	Takara Bio	N/A	Li *et al.*, 2011 [23]
	SYBR Green real-time PCR universal reagent	GenePharma Co. Ltd.	N/A	Yao and Lin, 2012 [34]
	Fluorescent quantitative detection kit specific primers	Shanghai biological engineering and Guangzhou ruibo	N/A	Han *et al.*, 2015 [17]
	miScript miRNA detection system	Qiagen	N\A (discontinued)	Zhang *et al.*, 2016 [45]; Xin *et al.*, 2016 [47]
	mirVANA qRT-PCR kit	Applied biosystems (ThermoFisher Scientific)	N\A (discontinued)	Shisodia *et al.*, 2015 [38]
	SuperReal PreMix plus (SYBR green)	Tiangen	USD 2830.90	Zeng *et al.*, 2015 [29]
	SYBR™ Green PCR master mix	Applied biosystems	USD 127-3880.00	Ma *et al.*, 2015 [32]
	Hairpin-itTM miRNA qPCR quantitation kit	Genepharma	N\A	Luo *et al.*, 2015 [53]
	QuantiTect SYBR Green PCR kit	Qiagen	USD 473-1946.00	Leitao *et al.*, 2014 [47]
	RT-PCR mixture	Bio-Rad	N\A	Zhang *et al.*, 2019 [54]

Some studies were shown to have checked RNA purity and quantity, however, they were shown to have used microarrays for miRNA detection other than qRT-PCR. Microarrays require a specific RNA concentration for detection, whereas RNA quantity may not be much of an issue for qRT-PCR as RNA is expected to be in low volumes especially miRNA as they are small in size. Han *et al.* [17] performed gel electrophoresis on RNA samples to check quality and only

selected the 28S bands as eligible for qRT-PCR.

Ma *et al.*, used reverse transcription reagents from ThermoFisher, however they have not mentioned the name of the kit used, they combined these reagents with stem-loop primers from Sangon Biotech similar to Huang and colleagues who used stem-loop primers from Invitrogen [24] [32]. Studies that did not use the applied biosystems miRNA PCR assay which includes predesigned primers, opted for specific primers from Invitrogen, miScript Primer Assays (Qiagen) [24] [43]. Some studies were consistent in using the applied biosystems miRNA assay for their qRT-PCR procedure instead of opting for different kits for reverse transcription and quantitative real-time PCR [41] [43] [51] [52].

The most common RT-PCR and qRT-PCR kits used were the TaqMan Reverse Transcription kit and TaqMan MicroRNA Assays by applied biosystems. Refer **Table 2** for a detailed summary of the kits and assays used by the studies discussed.

4.3 The Effect of Different Kits on miRNA Expression

Almost all the studies showed similar results for miRNA expression despite using different samples and processing techniques, however, most studies did use similar kits. The expression of specific miRNAs differed from one another but their level of expression remained consistent in all studies, with the exception of a few. Some studies have evaluated a panel of miRNAs instead of studying the expression levels and properties of one miRNA, this can have a better diagnostic value than detecting only one miRNA biomarker [26] [27] [29] [35] [36] [37] [38] [42] [43] [47] [49] [51].

Studies which evaluated the expression level of miRNA-196a all showed significantly high levels in diseased samples as well as cell lines compared to normal cells [21] [26] [44], except for one study which stated that there was no significant difference between miRNA-196a expression in cancer cells and healthy cells [25]. They assumed that this could be due to the heterogeneity of CC tumour cells compared to cancer cell lines [25].

5. Discussion

There are many things to consider in methodology design. When it comes to evaluating miRNA expression in tissue/blood samples using the qRT-PCR method many things come into play. Steps like sample processing, storage and, RNA extraction can have a significant impact on results but are easily overlooked. In miRNA profiling regardless of the type of technique, choosing the right miRNA isolation kit is of great importance and this solely depends on the sample type. The quality of the kits matter, this is why it is important to procure kits from renowned manufacturers like life technologies and applied biosystems which are part of the ThermoFisher Scientific group. Most of the studies in this review used the Ambion mirVANA miRNA isolation kit by Life Technologies, the second most common RNA isolation technique was the use of Trizol by In-

vitrogen (ThermoFisher Scientific) which is a Chaotropic salt. Overall, products from ThermoFisher Scientific were commonly used amongst most studies discussed in this review. Sample type may also affect RNA quality, high quality miRNA can be extracted from tissues and cell lines, however, extraction from blood samples can be challenging due to endogenous RNase activity. However, it is important to note that endogenous plasma miRNA is stable in its native form therefore, extraction methods which can inactivate RNase activity rapidly are required [57]. Vigneron and colleagues [58], validated that the use of the NucleoSpin miRNA plasma/serum kit as being able to isolate more miRNA from blood samples than the widely used kits like mirNEasy kit (Qiagen) and Ambion mirVANA miRNA isolation kit (Life Technologies) agreeing with two other studies [59] [60]. The amount of miRNA extracted has a major influence on qRT-PCR results, therefore it is imperative to choose the best kit and optimize the methodology for the most reliable results. Other factors that may affect miRNA nature and quantity in blood samples are centrifugation, white blood cell count and hemolysis. It is important to note that miRNAs in plasma exist within vesicles/exosomes, or are associated with argonaute-2 (AGO2) containing protein complexes. Sample processing conditions which alter vesicle content can influence miRNA expression during evaluation [57].

The most commonly used RT-PCR and qRT-PCR kits were both from the same manufacturer, the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan MicroRNA Assays by applied biosystems (ThermoFisher Scientific). The TaqMan MicroRNA Assays are shown to be the best to evaluate miRNA expression; it accommodates high-throughput platforms and is highly reproducible. The applied biosystems assay is pre-designed, making it a versatile and efficient product for miRNA detection, whereas, the mirScript kit was shown to have lower detection sensitivity [60]. Two kits have been discontinued, the mirVana qRT-PCR kit by applied biosystems is now replaced by the Applied Biosystems™ TaqMan™ Advanced miRNA cDNA Kit, and the miScript PCR system (Qiagen) is replaced by miRCURY LNA miRNA PCR System by Qiagen [61] [62]. The cost of the experiments depends solely on the products used, most reagents are expensive due to the advanced technologies used to make them, and another factor that may affect is shipping costs depending on the region the study is conducted in. The performance of miRNA extraction kits can be indicated via the purity and yield of extracted RNA measured using a spectrophotometer [60]. However, phenol contamination and RNA degradation can cause inconsistencies in the results, hence why direct qRT-PCR expression results are a better indicator of miRNA recovery. Sampling bias is another factor that affects RNA levels, especially when collecting blood samples. Phlebotomy process and participant's physiological state can cause changes in miRNA levels in plasma. Within healthy individuals circulating miRNAs can remain stable over time. The serum has lower levels of miRNAs leading to problems in accuracy and detection rate, hence why plasma is a better option to detect miRNA expression.

Sample processing time and sample type can also influence miRNA levels [60].

Almost all studies agree that miRNA biomarkers detected via qRT-PCR show potential as a screening/diagnostic tool for CC or at most may be able to aid clinical diagnosis alongside pre-existing tests. However, more research and optimization of existing methodologies are needed to make this a possibility.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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