ORIGINAL ARTICLE

Detection of miR-210 in formalin fixed human placental tissues using Real Time-PCR

Maimunah Mustakim*, Isatou Jagne

Centre of Medical Laboratory Technology, Faculty of Health Sciences, Universiti Teknologi MARA (UiTM), UiTM Kampus Puncak Alam, 42300 Bandar Puncak Alam, Selangor, Malaysia

***Corresponding Author**

Maimunah Mustakim, PhD
Email: maimunah@uitm.edu.my

Abstract:

MicroRNAs are non-coding regulatory RNAs that are involved in various cell regulation processes. Deregulated expression of miRNAs has been associated with several diseases including preeclampsia. Owing to the huge amount of formalin fixed tissues archived worldwide, this study aims at detecting the presence of miR-210 in formalin fixed placental tissues. Three formalin fixed normal human placentas were used in this study and real time-PCR (RT-PCR) was used as a detection method. Total RNA was extracted from the fixed tissues and miRNA-210 was successfully amplified by RT-PCR. Quantification cycle (Cq) values of three unknown samples were similar; 24.52, 25.06 and 24.32 respectively. Storage of fixed tissues for up to 4 years did not affect the detection of miR-210. In conclusion, RT-PCR is a suitable method in detection of miRNA in formalin fixed tissues including RNAse rich tissues such as placenta. The findings show that archived samples may be used to conduct future studies such as retrospective gene expression studies of miR-210 on both normal and pathological placentas using RT-PCR. This will increase our understanding on the function of miR-210 in the pathogenesis of preeclampsia and pave the way to finding an absolute cure for the disorder.

Keywords: Formalin fixed human placenta tissues, microRNAs, miR-210, RT-PCR

1. INTRODUCTION

MiRNAs are small endogenous non coding RNA about 17-25 nucleotides in length commonly found in plants as well as animals [1]. Since miRNAs are non-coding, they do not make proteins rather they regulate protein synthesis. In other words, they fine-tune the function of other genes. They are involved in various cell regulation processes which include differentiation, proliferation, apoptosis, hematopoiesis, tumour genesis and DNA methylation [2]. About 30% of human genes are thought to be regulated by miRNAs [1]. In mammals, it is anticipated that the activities of over 60% of all protein coding genes are controlled by miRNAs [3]. A number of human diseases including cancers have been associated with miRNA dysregulation [4].

Formalin fixed tissues are increasingly used as a source of RNA for retrospective studies [5]. This is because of their availability since a huge amount of formalin fixed tissues have been archived worldwide for histology purposes [6]. Formalin fixation preserves cellular proteins as well as maintains tissue architecture however it results in enzyme and chemical degradation as well as formation of cross links between RNA and proteins thereby reducing RNA yield, quality and integrity [7]. A study has shown that of all mRNA present in formalin fixed paraffin embedded tissues, 97% is inaccessible to cDNA synthesis and qRT-PCR. Despite the fact that mRNA is not stable in FFPE tissues, various studies have shown that miRNA expression profiles from FFPE samples are comparable to those of fresh samples [8-9]. MicroRNA is preserved in FFPE tissues for extensive periods of time (up to 10 years) since they are not as susceptible to degradation and modification as other types of RNA. This is partly because of their small size and their involvement with large protein aggregates. There may be a reduced detection of miRNAs in FFPE tissues stored for more than 7 years [7].

MicroRNAs have become the focus of researchers due to their roles in various biological processes both in normal physiology and in disease states. The study of miRNA expression paves the way for better understanding of certain diseases and facilitates the diagnosis, prognosis and treatment of those diseases [10]. One of such diseases associated with altered miRNA expression is pre-eclampsia. It is pregnancy specific and characterized by increased blood pressure and urine protein levels. Pre-eclampsia is a major cause of maternal and perinatal morbidity and death and it affects 1 in 20 pregnancies worldwide [11]. Zhu and his colleagues [11] describe preeclampsia as an idiopathic disorder however placenta has been implicated since the only treatment is delivery of the placenta which consequently results in a speedy regression of symptoms. A miRNA, miR-210 has been found to be differentially expressed in pre-eclampsia placentas in comparison to normal placentas [11, 12]. Mir-200b was also found to be differentially expressed in pre-eclampsia + SGA placentas [12].

Studies on miRNA in formalin fixed placenta tissues are limited [2] especially in Malaysia thus this study aims at detecting the presence of mir-210 in formalin fixed normal placenta tissues in order to provide essential information for analysis of the variation in miRNA levels in archived placentas.

2. MATERIALS AND METHODS

2.1 Sample preparation

Forty milligrams of each tissue sample was excised and transferred from the original specimen containers to newly labelled universal containers filled with fresh 10% formalin. The samples were then kept at room temperature prior to extraction of total RNA.

2.2 RNA extraction

The concentrated wash solution provided was diluted by adding 50 ml of 95% ethanol resulting in a final volume of 72 ml. All the blades, forceps, microcentrifuge tubes, elution tubes, pipette tips and pipettes used were autoclaved at 121°C for 15 minutes.

Total RNA was isolated using the Phenol free Total RNA Purification Kit (N788-KIT, AMRESCO) according to the manufacturer's protocol with some alterations.

2.3 RNA quantification

The total RNA obtained was quantified using a Nanodrop 1000 spectrophotometer (ThermoScientific).

2.4 cDNA synthesis

Total RNA was reverse transcribed into cDNA using a cDNA synthesis kit (Hp0042,Origene, USA) and performed as described in the manusfacturer's instruction. The cDNA samples were kept at -20°C for further analysis.

2.5 Real Time-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the Thermo scientific DyNAmo SYBR Green PCR kit (F-400L). MiR-210 (Human) qSTAR miRNA pimer pairs and template standard kit by Origene was used (Accession no: MI0000286; Catalog no: HK300247). Amplifications was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). The reaction solution was assembled in a volume of 20 µl, which comprised of 1X master mix, 0.2 µM of the primer pair mix, and cDNA template. Real-time PCR cycling condition was as follows: 95°C for 10 minutes for initial denaturation, followed by a denaturation step at 94°C for 10 seconds. The annealing temperature was set at 55°C for 20 seconds and the extension step was at 72°C for 20 seconds. The tubes were subjected to 40 cycles after which a melting curve step was performed at a temperature of 60°C to 95°C for 5 seconds.

3. RESULTS AND DISCUSSION

3.1 Quantification of total RNA

In this study, the three placenta samples used were collected in 2009 and had been fixed in 10 % formalin since then. There was no information with regards to demographic characteristics such as the type of delivery or gestation age at the time of delivery however they are all considered to be normal/non diseased placentas. A previous study mentioned that factors such as tissue hypoxia, the manner in which the tissue is handled and the process of protection from RNAse degradation may affect the quality of total RNA extracted from placentas [13]. The results in Table 1 showed very low concentrations (8.1 ng/µl, 7.2 ng/µl and 7.6 ng/µl) and yields (810 ng, 720 n, 760 ng per 100 µl) of total RNA extracted from samples 001, 002 and 003, respectively. The low concentrations and yields may be due to the small quantity (9mg) of tissue used for extraction of RNA, the tissue type (placenta) or the extraction method used. Previous studies have used larger amounts of tissue, about 20 to 50 mg in one study by Jung et al. [8] and Zhu et al. [11] mentioned that RNA was isolated from pooled tissue fragments suggesting that it wasn't a small quantity. The kit used for total RNA extraction in this study is optimized for use of up to 10 mg of tissue and it is recommended that the starting material should not be more than 10 mg therefore much less tissue was used in comparison to other studies. The purity obtained in this study is way below the optimal purity of RNA which is >1.8 or 2. Low purity may be attributed to presence of RNAses and or DNA contamination. Placentas are known to be RNAse rich [13] thus extraction of RNA requires aggressive methods to minimize the effect of RNAses. One of such ways is to use buffers containing guanidium thiocyanate and 2-mercaptoethanol to inactivate RNases [14]. The latter cleaves disulphite bonds which make the enzymes stable, thus denaturing them [15]. The phenol free total RNA purification kit (Amresco) used in this study has a lysis buffer containing guanidium thiocyanate but lacks 2-mercaptoethanol.

Table 1: Concentration, purity and yield of total RNA extracted from each placenta sample.

Sample ID #	Concentration (ng/µl)	A_{260}/A_{280}	Yield (ng)/100µl
001/09	8.1	1.53	810
002/09	7.2	1.49	720
003/09	7.6	1.49	760

The quality of starting material (total RNA) is very important to subsequent assays such as RT-PCR thus low quality RNA such as the one used in this assay may not produce optimum results however a previous study obtained similar low quality total RNA from different extraction methods which was used in both microarray and RT-PCR assays [16].

3.2 Amplification of miR-210 by RT-PCR

The standard curve on Figure 1 shows a linear curve with an efficiency of 108.7 % which is within the acceptable range of 100 ± 10 %. Its coefficient of correlation is also acceptable (0.995) as it is > 0.99 [17]. The standards were run in duplicates as recommended in the manufacturer's protocol; the replicate Cq values were similar with standard deviations (SD) ranging from 0.018 to 0.581. The biorad CFX 96 real time PCR system recommends a standard deviation of ≤0.2 between replicates. Two pairs of replicates (Standards 1 and 7) and (standards 6 and 12) had SD > 0.2. The increase in SD is probably due to pipetting errors which is caused by the use of uncalibrated pipettes to a large extent. The errors were minimized to a great extent by the use of a mastermix however the use of calibrated pipettes and better pipetting skills could have produced more desirable results. The curve was fit after automatic baseline subtraction. The threshold was automatically calculated by the software. The Cq values of all 3 samples were within range of the standard curve as they all fell on the least square fit.

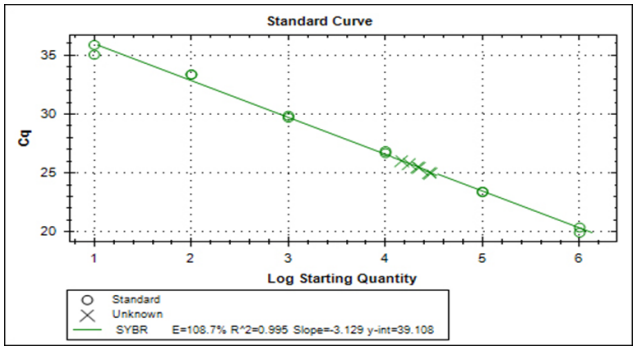

Figure 1: Standard curve showing the quantification cycles (Cq) against the log starting quantity/copy number of the template standards and unknown samples.

Real-Time PCR amplification of miR-210 is presented in Figures 2, 3 and Table 2. Figure 2 shows amplification of miR-210 in the template standards, controls and unknown samples. Figure 3 shows amplification of miR-210 in controls and unknown samples as shown in Figure 2. All samples were all run in triplicates however replicates whose Cq values were too high or too low as compared to the other two were omitted from the data. Replicates showed similar Cq values with SD ranging from 0.027 to 0.403. As mentioned above, the increased SD values are probably due to pipetting errors. The results on Table 2 showed that the NTC and NRT samples were also amplified. NTC and NRT were run as controls to rule out contamination. The NTC sample was template deficient thus under normal circumstances it should not showed any amplification. However, the NRT sample was also amplified contrary to ideal results. Since it does not contain reverse transcriptase, the RNA would not have been transcribed to cDNA therefore there shouldn't have been any amplification. The amplification seen in the NTC was probably due contamination of reagents. The amplified NRT control indicated that the RNA sample was contaminated most probably with genomic DNA since the purity of the samples were low. Comparing the Cq values of the NTC and NRT to that of the 3 unknowns, there was a huge difference as the Cq values of the unknowns were within 20-30 cycles whilst that of NTC and NRT were above 30 cycles. Table 2 also showed the starting quantities for the controls and unknowns. It was clear that the average starting quantity of the NTC (127.48) and NRT (27.08) was significantly lower than those of the unknown samples (001,002 and 003) whose average starting quantities were 45856.37, 30878.80 and 53132.68 respectively indicating that the contamination was minimal.

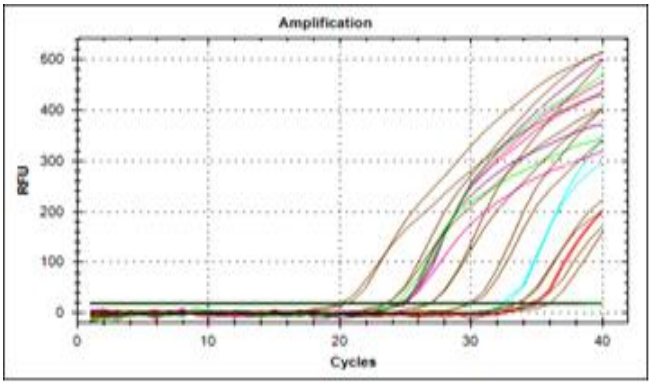

Figure 2: Amplification with SYBR Green and miR-210 specific primers of Template standard, NTC, NRT and Unknowns. The standard is represented in brown, NTC in turquoise, NRT in Red. For the unknowns, Sample 1 is coloured pink, Sample 2 Purple and sample 3 green. NTC: No Template Control, NRT: No Reverse Transcriptase

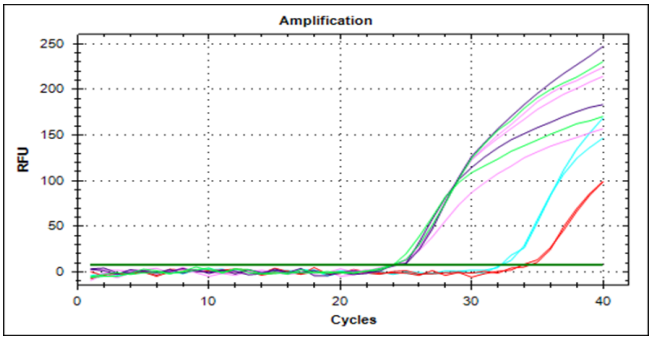

Figure 3: Amplification plot of NTC, NRT and unknown samples 001, 002 and 003 as shown in figure 2 above. NRT is in red and NTC is in turquoise. Sample 1 is coloured pink, Sample 2 Purple and sample 3 green. NTC: No Template Control, NRT: No Reverse Transcriptase

Table 2: Cq values and starting quantities of the controls and unknown samples.

Samples	Cq Value	Starting Quantity (SQ)
NTC-1	32.41	138.24
NTC-2	32.64	116.71
Mean ±SD	**32.525 ± 0.163**	**127.48± 15.22**
NRT-1	34.94	21.48
NRT-2	34.37	32.68
Mean ±SD	**34.655 ± 0.403**	**27.08 ± 7.92**
001/09-1	24.55	44668.36
001/09-2	24.51	46279.46
001/09-3	24.50	46621.28
Mean ±SD	**24.52 ± 0.027**	**45,856.37 ± 1042.94**
002/09-1	25.04	31,333.23
002/09-2	25.08	30,424.36
Mean ±SD	**25.06 ± 0.032**	**30,878.80 ± 642.67**
003/09-1	24.41	49,813.54
003/09-2	24.24	56,451.81
Mean ±SD	**24.32 ± 0.11**	**53,132.68 ± 4693.97**

Figure 4 represents the melt curve of the controls and unknown samples. The melt curve shows a single peak indicative of a single PCR product however the melting temperature of the individual samples ranged from 71.5°C to 73.5°C. Average melting temperatures were 72.25°C, 71.5°C, 73°C, 73°C and 73.50°C for NTC, NRT, and unknown samples 001, 002 and 003, respectively. The three unknown samples had almost identical melting temperature which is suggestive of the same PCR product. The temperatures for NTC and NRT vary slightly probably due to the presence of DNA contaminants as discussed earlier on. In spite of the slight difference in melting temperatures of the PCR products, the primers can be considered specific since there is an absence of primer dimers which would have shown on the melt curve as additional peaks at lower temperatures. However the specificity is probably not optimal because the melting temperatures are not within 80°C and 90°C. The primer pair used in this study was obtained commercially and is supposedly specific to miR-210. The specificity could have been improved by running a gradient to determine the optimum annealing temperature of the primer pair. Optimizing the primer concentration would probably have also improved the specificity. However, neither the annealing temperature nor the primer concentration was optimized due to the limited volume of SYBR Green available for use.

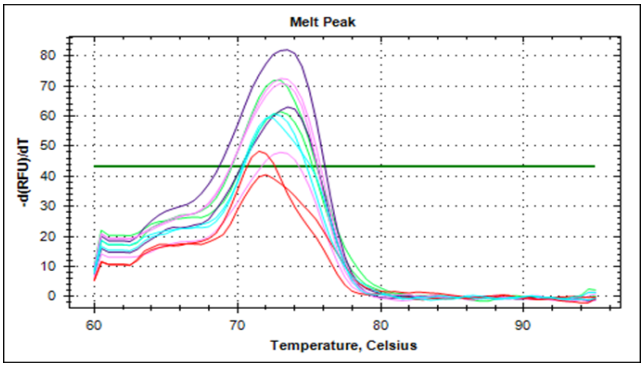

Figure 4: Melt curve plot showing the melt peaks for each of the controls and unknown samples. NRT is in red and NTC is in turquoise. Sample 1 is coloured pink, Sample 2 Purple and sample 3 green. NTC: No Template Control, NRT: No Reverse Transcriptase

4. CONCLUSION

In conclusion, miR-210 was successfully detected in the unknown samples used in this study. Despite the fact that the total RNA used to synthesize cDNA for subsequent amplification had low concentration as well as low purity, the RT-PCR assay was suitable for use to amplify the desired amplicon. In spite of formalin fixation for more than 3 years and that the target amplicon miR-210 is not placenta specific, the RT-PCR protocol used was able to detect miR-210 in the placenta samples. Therefore it is proven that RT-PCR is feasible to use for the detection of miRNAs in formalin fixed tissues of up to 4 years. The findings of the study may however be used as a basis to conduct gene expression studies using RT-PCR on normal placenta tissues in comparison to preeclamptic placentas to improve our understanding of the relationship between levels of miR-210 and preeclampsia. These studies may pave the way to finding an absolute cure for preeclampsia.

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