CHAPTER IV

RESEARCH METHODOLOGY

4.1 RESEARCH FIELD

This research is in the field of molecular genetics

4.2 LOCATION AND RESEARCH PERIOD

Research will be performed in Department of Human Genetics, Radboud University of Medical Center, the Netherlands for the period of one year.

4.3 RESEARCH DESIGN

This is an explorative study.

4.4 POPULATIONS AND SAMPLES

Populations in this research are colorectal cancer patients that were admitted to RUNMC, the Netherlands.

Samples selected for the study cohort are those with the inclusion criteria of:

- No *MLH* and *MSH* deletion (stable microsatellites)
- Diagnosed with CRC at the age of less than 40 years old or
- Diagnosed with CRC at the age of less than 50 years old with at least one 1st degree relative and/or recessive inheritance pattern (affected by CRC).

4.5 OPERATIONAL DEFINITION

Deletions, insertions, duplications, and complex multi-site variants of DNA segments, collectively termed copy number variants (CNVs) or copy number polymorphisms (CNPs), are found in all humans. MicroRNAs (miRNAs) are functional RNA molecules that are transcribed from the DNA sequence of RNA genes, but not translated into protein and to profile the CNVs affecting miRNA genes a high resolution microarray (385K) is used. Validation of CNVs found will be performed using PCR and q-PCR.

4.6 RESEARCH PROTOCOLS

4.6.1 Microarray design

CGH measures DNA copy number differences between a test and reference genome. Human microarrays used in the current study comprised approximately 380×10^3 oligonucleotides probes (385K) tiling the positive strand.Each perturbed oligonucleotide originated from a perfect match oligonucleotide in which the GC content of the perturbed oligonucleotide was identical to the GC content of the original oligonucleotide in an attempt to maintain the same melting temperature. Each perturbed oligonucleotide can therefore be associated with a specific perfect match oligonucleotide present on the array and the difference in fluorescence intensity between the pair should be a reflection of the perturbation applied.

During the time of the design, the array design was based on the miRNA database version of 2006 (miRBASE v1.1) containing 695 annotate miRNAs.

Next the probes in the array were combined with miRNAs latest database (miRBASE v1.5) that currently covers 939 annotated miRNA. In total 495 of annotated miRNAs in the latest database are covered also covered in our array design.

The design of the array is that the each region oh human DNA covered in the array has a 20kb probes span. The 20kb probes are divided into two major areas. The first area has a length of 5kb in total. Each probe in this location has a length of 10bp thus making it a high density area. The high density area is located at the miR genes and predicted miR genes. The second area are flanking on each side of the first area, has a length of 7.5kb each. The probes positioned in this area, each has a length of 50bp making it a low density area.

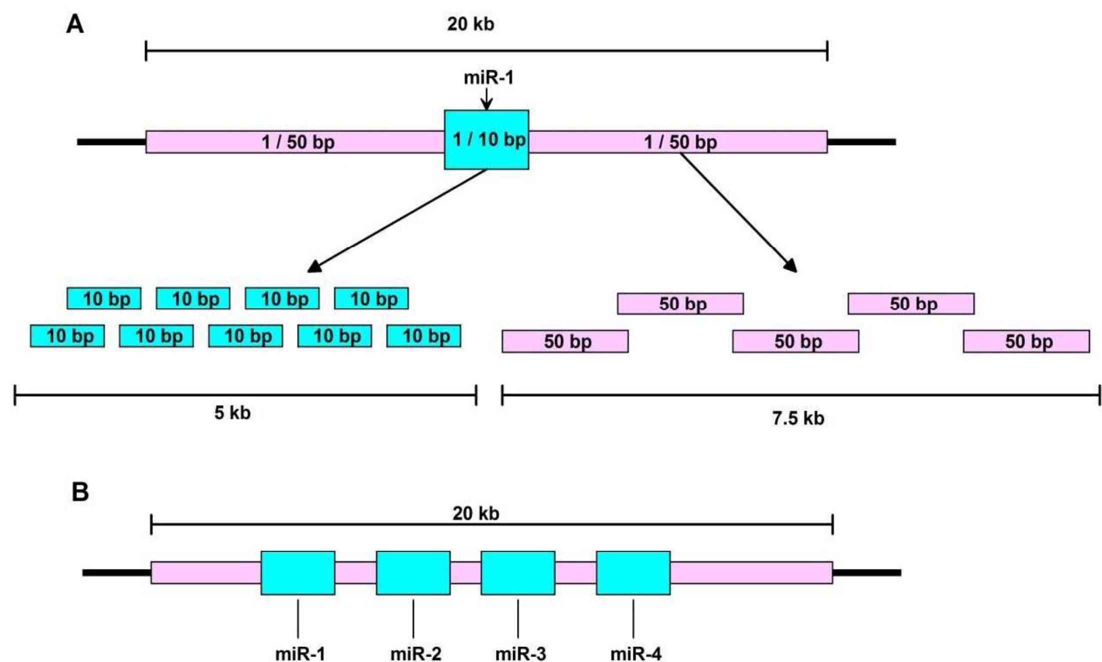

Figure 3.Representative of Array Comparative Genomic Hybridization (aCGH) design.**A**. The blue region representing the high density area with 1 probe in every 10 bp that stretches for 5 kb. The red region represents low density with 1 probe placed in every 50 bp that stretches for 7.5 kb in each side of the high density area. **B**. The array design for miRNAs cluster, in total the region covered is 20 kb.

4.6.2 DNA fragmentation and labeling

Cy3 and Cy5 dye-labeled random 9-mers were diluted into 1 O.D./42 μL of buffer containing 0.125 M Tris-HCl (pH 8.0), 0.125 M $MgCl_2$, 1.75 μL/mL β-mercaptoethanol. Patient's DNA samples were labeled with Cy3 and the control/reference's DNA samples were labeled with Cy5. One microgram of genomic DNA was added to each random 9-mer buffer solution, denatured at 98°C for 10 minutes, and then chilled on ice for 2 minutes in 0.2 mL PCR tubes. A total of 10 μL of 50× dNTP mixture (1× TE buffer, 10 mM each of dATP, dCTP, dGTP, and dTTP), 8 μL of DI water, and 2 μL of Klenow fragment (exo-) was added to each tube and mixed well with a pipet. Samples were centrifuged and incubated at 37°C for 12-16 h and 10 μL of 0.5 M EDTA was added and mixed well to stop the labeling reaction. DNA was precipitated by adding 11.5 μL of 5 M NaCl and 110 μL of isopropanol, vortexing, incubating in the dark for 10 min at room temperature, and centrifuging at 12,000*g* for 10 min. The supernatant was removed and the DNA pellet was washed with 500 μL of 80% ethanol. After centrifugation at 12,000*g* for 2 min, the supernatant was removed, and the pellet was dried in a SpeedVac on low heat for 5 min before being rehydrated in 15 μL of DI water. DNA concentration was measured using a spectrophotometer.

4.6.3 Sample hybridization, imaging and data analysis

The applied microarray platform, Roche NimbleGen, Human Whole-Genome Array CGH Analysis v1 (Roche Diagnostics, Mannheim, Germany), provides measurements from 385 000 unique genomic loci.Samples were hybridized in the NimbleGen Service Facility using standard operating

procedures, as previously described [74]. Briefly, 15 μg of each labeled test and reference DNA sample were added to a single 1.5 mL tube and dried down in the dark in a SpeedVac on low heat. The DNA was resuspended in 3.5 μL of DI water and vortexed; 41.5 μL of NimbleGen hybridization buffer was added to the tube, mixed well, and heated at 95°C for 5 min in the dark. Samples were hybridized at the NimbleGen Service Facility for 16–20 h at 42°C and then washed with NimbleGen wash buffers and scanned on an Axon scanner (Model GenePix 4000B). For mapping of genomic breakpoints the segMNT v1.1 CGH segmentation analysis algorithm were run in NimbleScan™ software v2.4.

To determine the threshold for scoring of gain and loss, normalized, log2-transformed ratios were used. Based on the variation in autosomal genomic regions, which should not vary between the two reference samples, thresholds for averaged log2 ratio data were set to 0.25 and -0.25 for gains and losses, respectively.

4.6.4 Data Analysis

This is a descriptive study and the data analysis being performed was done majorly to illustrate the size distribution of the CNVs found affecting miRs in CRC patients.

4.6.5 CNVs Validation

CNVs detected through the array are deletion and duplication DNA copy number. To validate the results of CNVs generated through microarray, PCR and q-PCR were used. PCR was used because the profile CNVs are in the DNA within

the nucleus and is used for the longer length of CNVs. Q-PCR was used for the shorter length of CNVs found. However there were no specific criteria to be used for the selection of validation method technique.

4.6.5.1 Polymerase Chain Reaction (PCR)

Approximately 100 nanogram DNA solution was amplified in a final volume of 50 µl mix solution containing 5 µl of 10X PCR Buffer, 2 µl $MgCl_2$, 2 µl 10mM dNTP, 2 µl 10mM forward primer, 2 µl 10mM reverse primer, 0,4 AmpliTaq Enzyme, 35 µl milli Q water and 2 µl DNA. Samples were denatured initially for 5 minutes at 95°C followed by 30 cycles of 95°C for 15 seconds, 64°C for 2 minutes, 72°C for 2 minutes; with a final extension of 5 minutes at 72°C. Five microliters of each sample was then runned on an 1.5% agarose gel with 130V for 30 minutes and stained with ethidium bromide, to confirm PCR amplification product.

4.6.5.2 Quantitative Polymerase Chain Reaction (qPCR)

The qPCR reactions were carried out in final volumes of 25 µL using the Applied Biosystems 7900HT Fast Real-Time PCR System. Reaction mix consisted of 12.5 µL SYBR Green Supermix, 0.75 µL 10 µM forward primer, 0.75 µL 10 µM reverse primer, 6 µL milli Q water and 5 µL 2ng/µL of DNA. The PCR reactions were initiated with 10 minutes incubation at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 2 minutes; with a final extension of 5 minutes at 72°C.

4.7 ETHICAL CLEARANCE

The study was part of an ongoing main research. The main research was approved by Institutional Review Board of Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands.

4.8 RESEARCH FLOW