CD133, MUC1, and KRT19 Chromosomal Rearrangements and Gene Expressions as Potential Biomarkers for Liver Cancer

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ABSTRACT

Liver cancer is one of the most common malignancies characterized by unrestricted proliferation, poor prognosis, aggression, metastasis, and reduced sensitivity to drugs. Despite the advances in diagnosis and treatment, patients with liver cancer are still usually diagnosed at a late progressive stage. Thus, the optimal treatment for liver cancer patients largely depends upon an accurate early diagnosis. Hence, this study evaluated the gene expression profiles and chromosomal rearrangements of CD133, MUC1 and KRT19 genes as potential biomarkers of liver cancer using two cell lines: normal human hepatocytes (hNheps) and human hepatoma (HepG2) cell lines. From the study, it was shown that the gene expression of CD133, MUC1, and KRT19 are indeed up-regulated in liver cancer cells, and that multiple signals of these biomarkers are detected in the liver cancer sample. This suggests that the possible mechanism for elevated expression of the biomarkers is not due to the presence of an enhancer or a highly active gene promoter; rather it is because of the multiple copies, duplication, and possible translocation of these cancer biomarker genes in liver cancer cells. Finally, this study has demonstrated that the detection of chromosomal rearrangements can be used or has a potential as a diagnostic tool for liver cancer.

Keywords: Liver cancer, Biomarkers, Gene expression, Chromosomal rearrangement

INTRODUCTION

Cancer is a genetic disease characterized by numerous mutations in the genome [1]. With a diagnosis of 11 million cases of cancer every year, an estimate of 16 million new cases is predicted for the year 2020 [2]. From these cases, liver cancer is the sixth most common malignancy with 782,000 new cases diagnosed as of 2012 and the second leading cause of death from cancer worldwide with 745,000 counts [3].

Liver cancer is characterized by its unrestricted proliferation, poor prognosis, aggression, metastasis, and reduced sensitivity to drugs [4,5]. Despite the advances in diagnosis and treatment, patients with liver cancer are still usually diagnosed at a late progressive stage [5], which has fewer treatment options and considerably lower survival rates [6]. This implies that the optimal treatment for liver cancer patients largely depends upon an accurate early diagnosis.

This then highlights the importance of providing an early diagnostic technique to circumvent the high mortality and recurrence rate of liver cancer, thus allowing better patient prognosis [7,8].

The most commonly utilized diagnostic tool in cancer involves biomarkers [9] but the successful detection of tumour cells depend heavily on the sensitivity and specificity of these markers. Therefore, in order to comprehensively and accurately analyze the patient's condition, multiple marker genes were utilized [10]. Among these are the *CD133* (liver cancer stem cell), *MUC1* (tumour anti-apoptosis), and *KRT19* (tumour detector) genes.

Assessing the expression level of these genes has enabled the improved survival rates of cancer patients through early diagnosis and development of targeted vaccination [11]. However, the expression of these cancer-related genes changes significantly upon the disease progression, hence limiting the diagnoses of patients and requires constant monitoring of cancer progression. Further, elevated tumour marker levels alone are insufficient to accurately diagnose cancer since there are individuals that are normal but show up-regulation of some biomarkers [6,12]. Hence, the presence of up-regulated gene marker expression does not necessarily indicate that the individual is already positive for cancer. Thus, to improve cancer diagnosis, gene expression analysis must be performed in consideration of other known tests for detecting malignancy.

Currently, there is an existing assay in the Lung Center of the Philippines which makes use of gene expression analysis in combination with Circulating Tumour Cell (CTC) count. The CTC assay is the most commonly used technique in the analysis of tumours. Its premise is based on the fact that malignancy occurs from a primary nodule or tumour site, from which cancer cells are shed off and transported through circulation to distant organs [13]. When CTC is captured, it can then provide a source for the molecular analysis of tumour cells present in the individual. With this, the use of CTC assay has become a well-established routine and a clinical trend which has already gained general acceptance in cancer diagnosis [14,15]. However, this technique, only analyzes surface markers (CD45-, *KRT19*+, DAPI+ and EpCAM+) of the cell. Thus, it provides specific but very limited information, giving rise to false negatives [13,14].

In lieu of this, this study suggests an alternative method by analyzing chromosomal rearrangements, which occur and may already be detected in pre-malignant precursor aberrations using fluorescence *in situ* hybridization or FISH [15]. Moreover, the early stages of chromosomal rearrangement of tumour-associated genes are the primary feature of early tumours. This is validated by the observations that some genetic rearrangements associated with tumour initiation or proliferation can be facilitated by large chromosomal changes [16].

However, this protocol is not yet routinely adapted in histopathological diagnosis, and there is no sufficient data on the chromosomal rearrangements of tumour-associated genes, especially on *CD133*, *MUC1*, and *KRT19* genes. Therefore, this study evaluated the gene expression profiles and chromosomal rearrangements of *CD133*, *MUC1*, and *KRT19* genes as potential biomarkers of liver cancer using two cell lines: normal human hepatocytes (hNheps) and human hepatoma (HepG2) cell lines.

METHODOLOGY

Cell culture

The Human Normal Hepatocytes (hNHeps) and HepG2 cell line was acquired from the Department of Biochemistry and Molecular Biology at the College of Medicine, University of the Philippines Manila.

HepG2 is a purely immortalized cell line derived from the liver tissue of a 15-year-old Caucasian American male, characterized with a high degree of morphological and functional differentiation of hepatic carcinoma cells [4,17,18], while hNHeps is a normal human liver cell line, well-recognized in providing an ideal model for the investigation of normal liver cell function *in vitro* [16].

HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) [Gibco®] and were supplemented with 10% Fetal Bovine Serum (FBS) [Serana] and 1% Penicillin/Streptomycin (Pen/Strep) [Sigma], while hNHeps cells were cultured using the Hepatocyte Basal Medium (HBMTM) [Clonetics®]. Both cells were incubated in 5% CO_2 atmosphere at 37°C, fed every 48 hours, and subcultured once a week.

Cell sample preparation

At 90% cell confluence, the cells were incubated in Phosphate Buffered Saline (PBS) [Gibco®] containing 0.53 mmol/L EDTA and 0.25% trypsin [Millipore] for 5 minutes at room temperature.

The cells were harvested by washing with PBS and 10% FBS. Five mL of feeding media (DMEM) was added and the cells were recovered through centrifugation at 2000 rpm for 2 minutes at 4°C, and immediately stored at -80°C until use.

Gene expression analysis

Total RNA extraction

The total RNA from HepG2 and hNHeps cell lines was extracted using the Total RNA Prep Kit (Biofact) in accordance with the manufacturer's instructions. Concentration of the collected RNA sample was quantified using a spectrofluorometer (Quantus).

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

The gene expression levels were obtained by utilizing qRT-PCR (CFX ManagerTM BIO-RAD). With the gene specific primer sequences for *CD133*, *MUC1* and *KRT19*, 10 ng of total RNA was reverse transcribed into cDNA for 30 mins at 50°C using Oligo(dT)20 Primer and SuperScript™ III Reverse Transcriptase kit (Medical Test Systems Inc.).

Table 1 PCR primer sequences for GAPDH (reference gene), *CD133*, *MUC1*, and *KRT19*

Cancer Biomarkers		Primer Sequences
GAPDH	Forward Primer	ACC CAC TCC TCC ACC TTT G
	Reverse Primer	CTC TTG TGC TCT TGC TGG G
CD133	Forward Primer	GAC CAA AGA GGC GTT GGA GA
	Reverse Primer	TGG ACC AGG CCA TCC AAA TC
MUC1	Forward Primer	CTT ACA GCT ACC ACA GCC CC
	Reverse Primer	AGC TGG GCA CTG AAC TTC TC
KRT19	Forward Primer	ACT ACA GCC ACT ACT ACA CGA C
	Reverse Primer	CAG AGC CTG TTC CGT CTC AAA C

The generated cDNA was used to perform the qRT-PCR reaction using Sso AdvancedTM Universal SYBR® Green Supermix kit [BIO-RAD] at 95°C for 5 mins for initial denaturation, followed by 35 cycles of 94°C for 0.5 min (denaturation), 57°C for 0.5 min (annealing) and 72°C for 0.5 min (elongation), and then a final extension of 72°C for 5 mins. All gene expression analysis for each samples and controls was performed in triplicates with GAPDH as the reference gene. The relative expression of each gene was calculated using the comparative cycle threshold (CT) method. Shown in Table 1 are the primers that were used.

Fluorescence *in situ* hybridization analysis

Preparation of metaphase chromosome spreads

The hNHeps and HepG2 cells were subjected to 1% v/v of 10 µg/mL Colcemid treatment for 6 hours at room temperature. The cells were recovered by centrifugation and then re-suspended in 5 mL of hypotonic KCl. The obtained solution was incubated for 20 minutes at room temperature.

An equal amount of freshly prepared ice-cold Carnoy's Fixative (3:1 methanol: glacial acetic acid) was then added, and the solution was centrifuged at 2000 rpm for 2 minutes. This step was repeated twice.

One drop of the obtained sample solution was spread onto a cold slide. The slide was dried immediately over a 50°C warm water bath. The chromosome spreads were then viewed under phase contrast microscope [Evos FL].

Fluorescent probe design

Gene specific fluorescent probes were designed using Primer3Plus software from the GenBank sequences of *CD133* (NC_000004), *MUC1* (NC_000001) and *KRT19* (NC_000017) genes. The BLAST software was then used to verify probe specificity. After designing the probes (Table 2), they were sent to Macrogen, Korea for manufacturing and labelling.

Table 2 FISH probe sequences

Cancer Biomarker	Fluorophores	Probe Sequence
CD133	5' FAM	TCC TGT GCA TGG TTG GGT AT
MUC1	5' TET	CTG AGG CTG GAA AAC CAC TC
KRT19	5' CY3	GGG GTT TAG AAT CTG CCC TC

Chromosome denaturation

Slides initially prepared were pre-treated with 2X sodium chloride-sodium citrate (SSC) buffer for 5 mins at room temperature. The slides were then washed in an ethanol series (70%, 85%, and 100%) at room temperature for 3 minutes and were left to dry at room temperature.

After drying, the slides were immersed in 70% formamide/2X SSC at 73°C for 5 mins and were again passed through a dehydration series of 70%, 85% and 100% cold ethanol for 3 mins each. The slides were left to dry.

Denaturation of probes and chromosome hybridization

Each probe mixture (10 µL) was heated in water at 73°C for 5 mins. This was then immediately applied onto the denatured slides and was sealed onto the slides using a cover slip. The slides were hybridized overnight in a humidified chamber at 37°C.

Detection of hybridization signals

After hybridization, the slides were washed with 0.4X SSC/0.3% Tween 20 for 2 mins at 73°C and 2X SSC/0.1% Tween 20 for 1 min at room temperature. The slides were allowed to dry in a dark room and were then counterstained with 10 µL of 4,6-diamidino-2-phenylindole (DAPI).

Upon incubation of 30 mins in -20°C, the slides were analyzed by fluorescent microscopy (Evos FL) using green (FAM), yellow (TET), and red (CY3) filters for *CD133*, *MUC1* and *KRT19* respectively.

Data analysis

The obtained gene expression profiles generated using normalized expression (ΔCq) analysis were then compared with the detected chromosomal rearrangement patterns of *CD133*, *MUC1* and *KRT19* genes between HepG2 and hNHeps cell lines.

Waste disposal

Cell culture waste (e.g. culture media) was inactivated for at least 2 hours in a solution of hypochlorite (10,000 ppm) or 10% bleach prior to disposal and was drained with ten-fold excess of water. Contaminated pipette tips were placed in hypochlorite solution (2500 ppm) or 10% bleach overnight before disposal by autoclaving and incineration. Solid waste such as flasks, centrifuge/conical tubes, contaminated gloves, tissues, etc., were placed inside heavy-duty sacks for contaminated waste and then incinerated.

RESULTS AND DISCUSSION

Culturing hNHeps and HepG2 cells

The study compared the expression and chromosomal rearrangements of genes *CD133*, *MUC1*, and *KRT19* in two types of cells: normal human hepatocytes (hNHeps) and human hepatoma cells (HepG2). Cell lines, instead of tissue samples from cancer patients were opted as the better source of cells with the aim of providing an almost unlimited supply of liver cancer and normal liver cells characterized with genotypic and phenotypic uniformity to therefore provide an accurate *in vitro* model for studying the biomolecular processes in both malignant and normal hepatocytes [4,19].

HepG2 and hNHeps cells were cultured under the same conditions with regular quality control measures to ensure that the cells are free from contamination. At 90% confluence, cells were then recovered simultaneously for the analysis of gene expression and chromosomal rearrangements.

Analysis of gene expression profiles

Reverse transcription of RNA to cDNA

For every reaction mixture, 30 ng of the extracted total RNA of HepG2 and hNHeps cells were reverse transcribed to cDNA. The reverse transcribed cDNA was then analyzed through AGE.
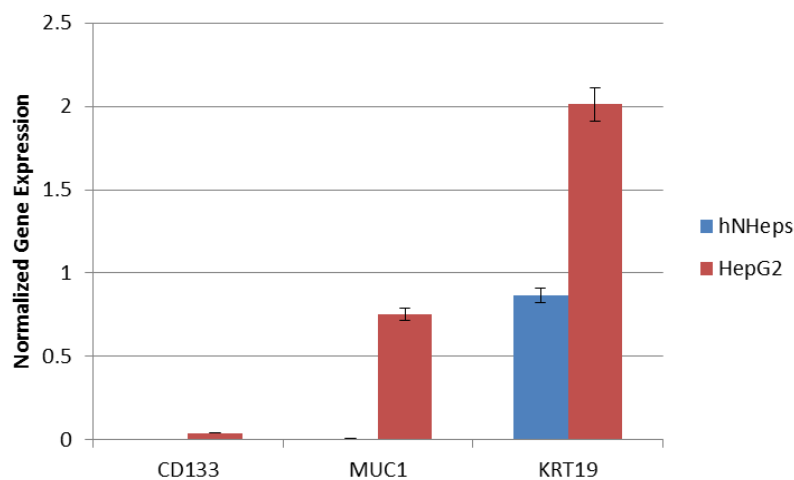
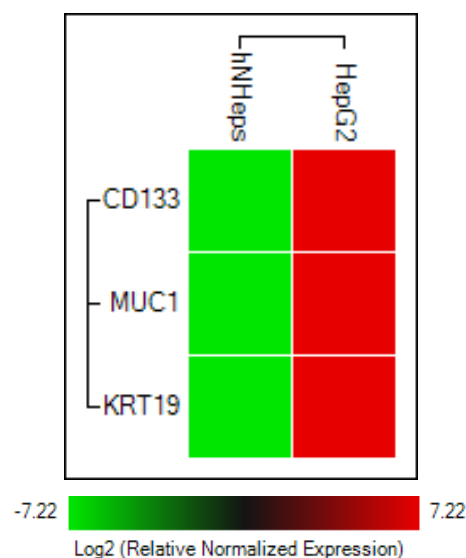**Figure 1** Bar chart of CD133, MUC1 and KRT19 normalized gene expression in HepG2 and hNHeps cells

Figure 2 Clustergram of CD133, MUC1 and KRT19 normalized gene expression in HepG2 and hNHeps cells

Analysis of gene expression profiles

Upon synthesis of cDNA, qRT-PCR was then used to analyze the gene expression profiles of HepG2 and hNHeps cells. Figure 1 shows the normalized expression of *CD133*, *MUC1*, and *KRT19* using GAPDH as reference. Gene expression was also compared using a clustergram (Figure 2).

From Figures 1 and 2, it can be observed that the gene expression of *CD133, MUC1,* and *KRT19* is higher in HepG2 as compared to that of hNHeps. This suggests that there is indeed an up-regulation of the cancer biomarkers *CD133*, *MUC1* and *KRT19* genes in liver cancer cells as evidenced by other studies [20-22].

The *KRT19* tumour detector gene first distinguishes the cancer from the non-cancer liver cells. *CD133*, on the other hand, enables the maintenance [5] and unlimited self-renewal [23] of liver cancer cells. Lastly, *MUC1* gene expression facilitates the development of drug resistance in liver cancer cells [24]. Although the up-regulation of these cancer biomarker genes is clearly seen, other methods are still required for the accurate liver cancer diagnosis. One of these methods is the detection of chromosomal rearrangements.

Detection of chromosomal rearrangements

Well spread chromosomes are fundamental for the fluorescence *in situ* hybridization (FISH) procedure. Thus, prior to subjecting the HepG2 and hNHeps cell samples to FISH treatment, the conditions for the preparation of ideal chromosome spreads were initially optimized on A549 lung cancer cell line, which have comparable growth and doubling time to the liver cell lines.

Optimization of colcemid treatment

In the recent studies, a range of incubation time from 4 to 6 hours is usually employed in the preparation of chromosome spreads. However, inconsistencies in the optimum incubation time for colcemid treatment still remain a major problem in cytogenetic studies [25]. Therefore, in this study, the metaphase spreading process was optimized in order to obtain properly differentiated chromosome spreads. A549 cells were first subjected to 4, 5, and 6 hours of colcemid treatment to arrest the cells at metaphase.

As shown in Figure 3, it can be observed that the chromosome spreads in the 4th hour of colcemid treatment are still not at metaphase. In the 5th hour of colcemid treatment, the chromosomes cannot be differentiated since there is overlapping of chromosomes observed in the slide. However, in the 6th hour of incubation with the colcemid treatment, it can be observed that the cells are well spread with a larger metaphase area and chromosomes can easily be differentiated, thereby ensuring that the signals in chromosomes for the FISH analysis are easily distinguishable.
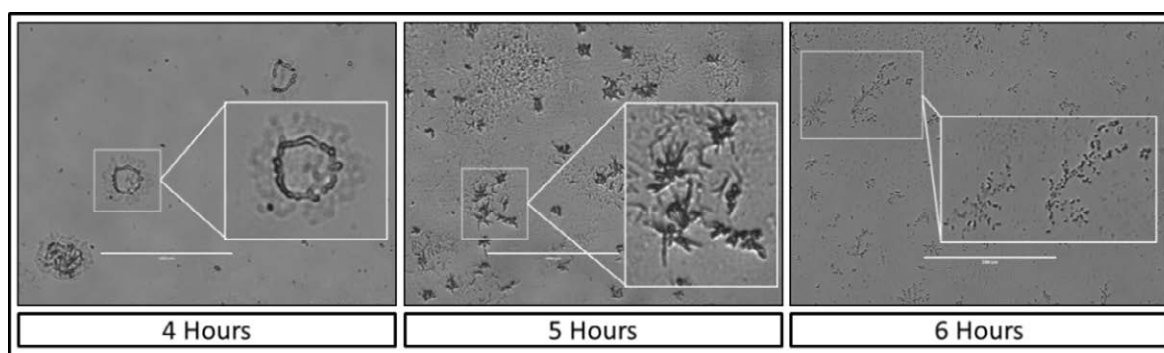

Figure 3 Optimization of colcemid treatment in A549 cells for 4, 5 and 6 hours

Dropping cells from a height and inclining a slide at an angle are known to relatively enhance metaphase chromosome spreading [25]. In the study, the angle and height of drop was also optimized and it was determined that the cell suspension dropped onto a cold slide from a height of 30 cm and the slide that was inclined at an angle of 45 degrees resulted to the best metaphase chromosome spreads (Figure 4).
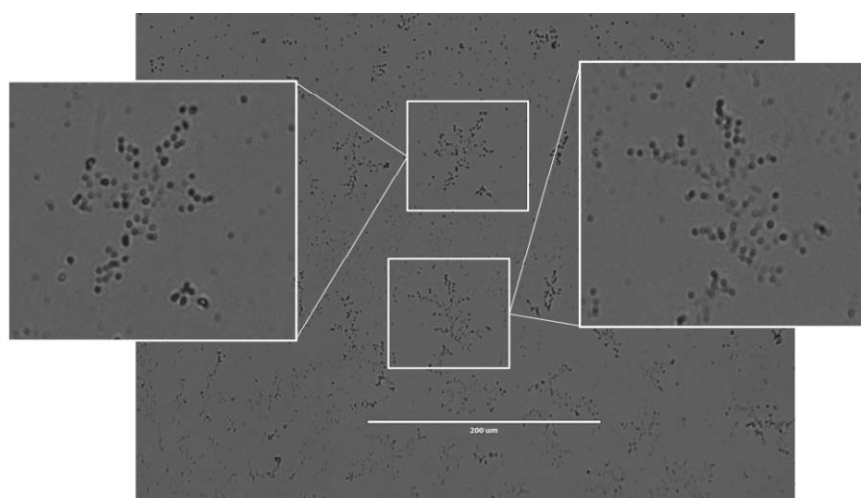

Figure 4 Metaphase chromosome spreads dropped from a height of 30 cm onto a 45° inclined cold slide

Slides were also dried over a 50°C water bath and a hot plate. It can be seen that immediate drying through the hot plate led to clumping of chromosomes belonging to more than one cell, evident from the multiple chromosomes observed in Figure 5. This unfavourable metaphase chromosome spreading may be due to the insufficient time for
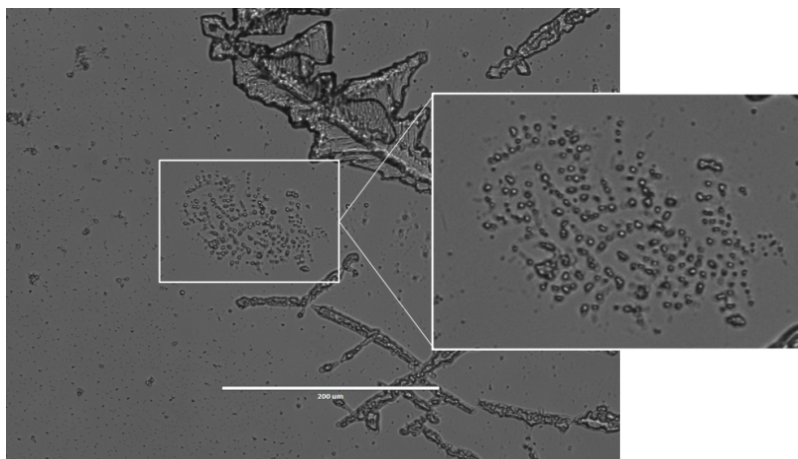

Figure 5 Metaphase chromosome spreads dried over a hot plate

In comparison, when dried over a warm water bath, well defined metaphase chromosome spreads were produced (Figure 6). This may be attributed to the slow drying of the Carnoy's fixative enabling the spreading of the chromosomes. This is because it takes time for the covered warm water bath to build enough moisture to coincide with the chromosome spreading as the fixative evaporates [25].
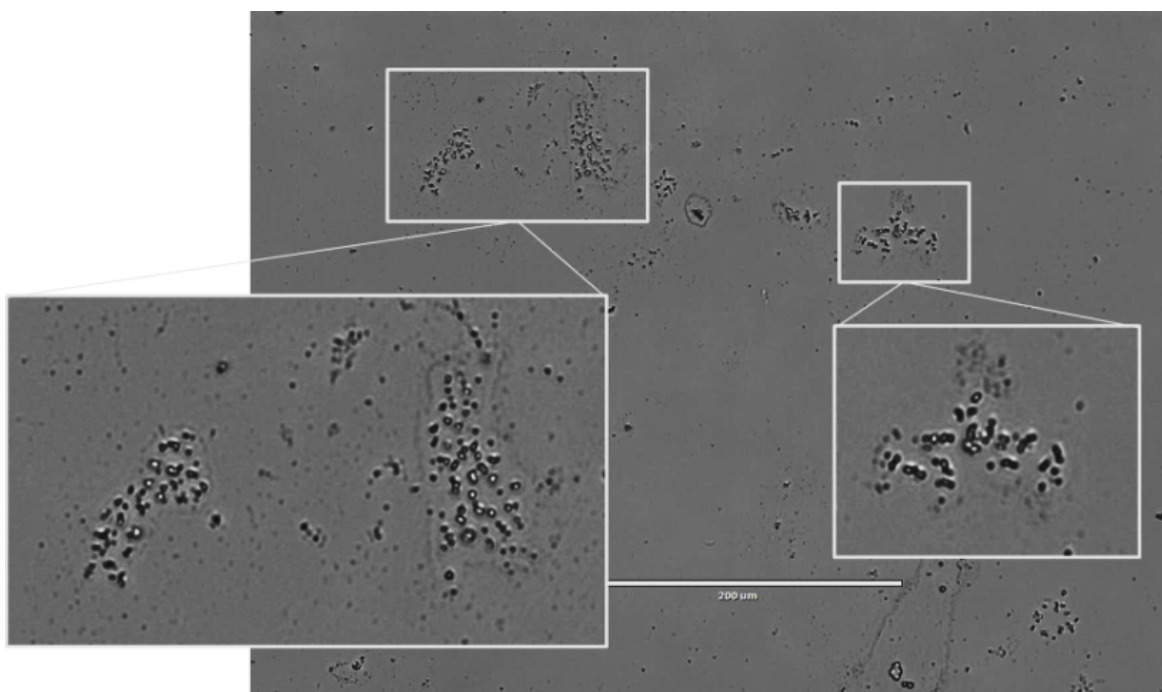

Figure 6 Metaphase chromosome spreads dried over 50°C water bath

Preparation of optimized metaphase chromosome spreads

Upon the recovery of HepG2 and hNHeps cells, the optimized conditions were employed on the preparation of chromosome spreads. The cell samples were subjected to six-hour incubation with colcemid which was then dropped from a height of 30 cm onto a cold slide inclined at a 45° angle. The slides were dried over a warm 50°C water bath,

and shown in Figure 7 are the optimized metaphase chromosomes spreads obtained for HepG2 and hNHeps cells, respectively.
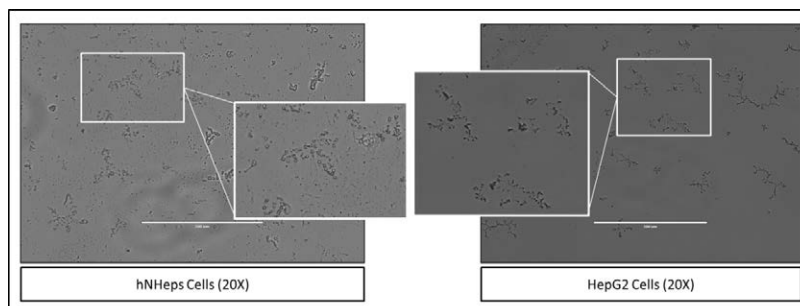

Figure 7 Metaphase chromosome spreads of HepG2 and hNHeps cells prepared under optimized conditions

Moreover, in order to verify the presence of chromosomes on the metaphase spread, a sample of hNHeps chromosome spread was stained with DAPI, which is a nucleic acid stain. As shown in Figure 8, the prepared spreads were successfully stained with DAPI confirming the presence of an optimized metaphase chromosome spread in the slide which is suitable for fluorescence *in situ* hybridization (FISH).
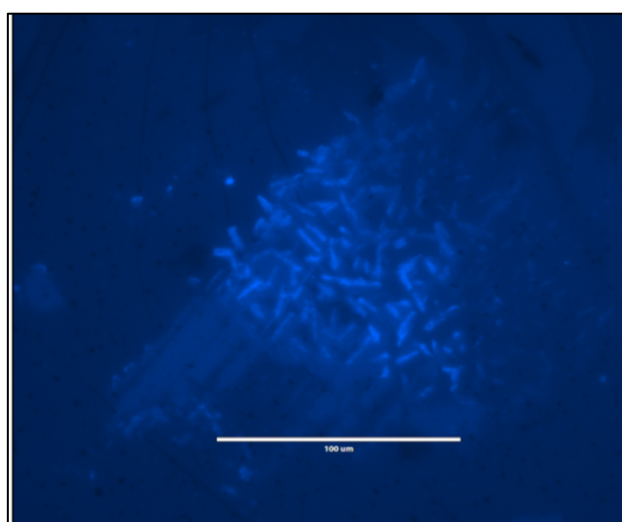

Figure 8 Metaphase chromosome spreads of hNHeps stained with DAPI

Fluorescent probe design

For the success of FISH, designing probes that are specific and sensitive is required. Thus, in the study, three oligo-probes specifics to *CD133*, *MUC1* and *KRT19* genes were designed using Prime3Plus. Specific probes sequences were designed with low self-complementarity, no dimer formation capability, and optimum melting temperature (T_m) at 59°C to 60°C and GC% of 50% to 55% (Appendix E).

This ensures that the designed probes will bind specifically to the target sequence and will be stable upon the hybridization process. The designed probes were then labelled with fluorescent dyes FAM, TET and CY3. Thus, in the FISH analysis, *CD133* appears as green signals, *MUC1* appears yellow, and the *KRT19* gene appears as red signals.

Fluorescence *in situ* Hybridization (FISH) analysis

Slides prepared were then subjected to FISH using fluorescent probes against *CD133*, *MUC1*, and *KRT19* genes, respectively. As seen in Figure 9, single bands of *CD133* (green), *MUC1* (yellow) and *KRT19* (red) were identified upon hybridization with the metaphase chromosome spreads of hNHeps viewed using the blue (DAPI), green, yellow, and red filters.
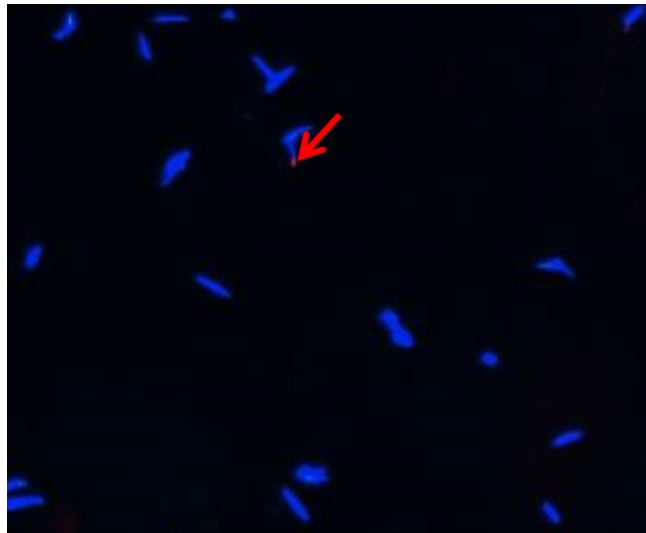
Figure 9 Metaphase chromosome spreads of hNHeps after fluorescence in situ hybridization with gene-specific probes: *CD133* [green], *MUC1* [yellow], and *KRT19* [red] under the DAPI filter, green (FAM) filter, red (CY3) filter and yellow (TET) filter

However, when compared with the metaphase chromosome spreads of HepG2 cells (Figure 10), intense red signals were observed implying that there may be multiple copies of *KRT19* present in the chromosomes. Moreover, it can also be observed that there is multiple faint green (*CD133*) and yellow (*MUC1*) signals. This suggests that there may be duplication or possible translocation of *CD133*, *MUC1*, and *KRT19* gene in liver cancer cells.
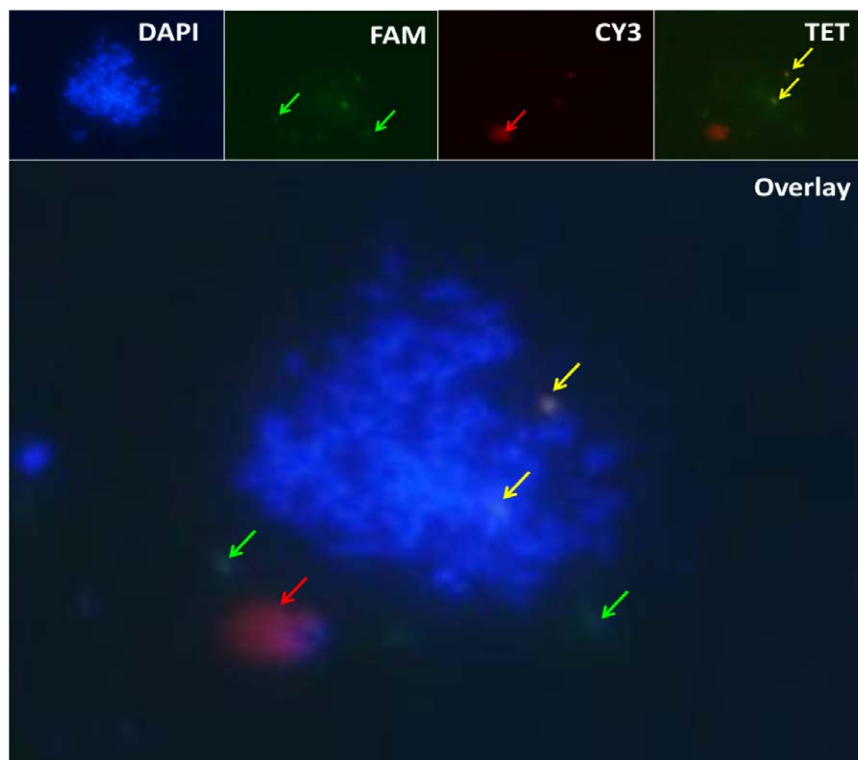

Figure 10 Metaphase chromosome spreads of HepG2 after fluorescence in situ hybridization with gene-specific probes: *CD133* [green], *MUC1* [yellow], and *KRT19* [red] under the DAPI filter, green (FAM) filter, red (CY3) filter and yellow (TET) filter

Chromosomal rearrangements, as demonstrated by the multiple signals (Figure 10) rose from the imprecise repairs in the course of DNA replication. These faulty repair processes induce damage and mutations through several mechanisms such as duplication and translocation of genes usually caused by unequal crossing over and nondisjunction. When these accumulate, they may lead to the development of cancer [26,27]. As such, chromosomal rearrangements may be a precursor to the onset of cancer, once again highlighting its potential as a cancer hallmark.

Correlating the gene expression with the chromosomal rearrangements of *CD133*, *MUC1*, and *KRT19*, the study suggests that the possible mechanism for the up-regulation of gene expression is not due to the presence of an enhancer or a highly active gene promoter; rather it is because of the duplication and possible translocation of these cancer biomarker genes in liver cancer cells. Moreover, since the duplication and translocation of tumour-associated genes are common causes of many types of cancer [28], the detection of these chromosomal rearrangements in the cancer biomarkers, *CD133*, *MUC1,* and *KRT19* genes can then be utilized as a prospective biomarker for cancer.

CONCLUSION AND RECOMMENDATIONS

From the data, this study has demonstrated that gene expression of *CD133*, *MUC1*, and *KRT19* genes are indeed up-regulated in liver cancer cells in corroboration with previous studies and that the up-regulation of these cancer biomarkers may be due to chromosomal rearrangements in liver cancer cells, evident from the multiple green and yellow signals specific to *CD133* and *MUC1* genes and the intense red signals observed for the *KRT19* gene. This implies multiple copies, duplication or possible translocation of genes not observed in the normal liver cell samples. This study has also shown that chromosomal rearrangements are indeed found in cancer cells, which implicates its potential as a diagnostic marker for liver cancer.

Thus, in order to provide more information for future treatment and prognosis of liver cancer, it is recommended that the specific location of chromosomal rearrangements i.e., where the duplication or translocation was observed be identified by karyotyping. This is essential to help target the specific cause and direct the type of treatment that can be adapted to liver cancer. Moreover, it is also recommended that the FISH procedure be applied to cancer and non-cancer patient samples, in order to evaluate chromosomal rearrangement as a new tool for the early diagnosis and prognosis of liver cancer in patients..

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