# OPTIMIZATION OF FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) ANALYSIS TO DETECT TERT GENE AMPLIFICATION IN A CANCER CELL LINE MODEL, K562

by

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# CERTIFICATE

This is to certify that the dissertation entitled "Optimization of Fluorescence *in situ* hybridization (FISH) analysis to detect TERT gene amplification in a cancer cell line model K562" is the bonafide record of research work done by Ms Kalaiselvi Manveeran during the period from July 2008 to October 2008 under my supervision.

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| | |
|---|---|
| BAC | Bacterial artificial chromosome |
| CCND1 | Cyclin D1 |
| CDK4 | Cyclin-dependent kinase 4 |
| cDNA | Complementary DNA |
| CGH | Comparative genomic hybridization |
| CML | Chronic myeloid leukemia |
| CNS | Central Nervous system |
| $CO_2$ | Carbon dioxide |
| dmins | Double minutes |
| $dH_2O$ | Distilled water |
| DHFR | Dihydrofolate reductase |
| DKC1 | Dyskerin |
| DNA | Deoxyribonucleic acid |
| EGFR | Epidermal growth factor receptor |
| ERBB2 | Human Epidermal growth factor Receptor 2 |
| ESP | End sequence profling |
| FBS | Foetal bovine serum |
| FISH | Fluorescent in situ hybridization |
| g | Gram |
| HER2 | Human Epidermal growth factor Receptor 2 |
| HSR | Homogeneously staining region |
| hTERT | Human telomerase reverse transcriptase |
| hTR | Human telomerase RNA |
| KCl | Potassium chloride |
| M | Molar |
| MB | Medulloblastomas |
| Mg | Milligram |
| min | Minute |

| | |
|---|---|
| ml | Milliliter |
| p | Short arm chromosome |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| q | Long arm chromosome |
| QuMA | Quantitative microsatellite analysis |
| RNA | Ribonucleic acid |
| RF10 | RPMI medium supplemented with 10% FBS |
| rpm | Revolution per minute |
| TERC | Telomerase RNA component |
| TERT | Telomerase Reverse Transcriptase |
| TR | Telomerase RNA |
| TYMS | Thymidylate synthetase |
| V | Volume |

# ABSTRACT

TERT gene located on chromosome 5p15.33 is one of the core components of telomerase enzyme which encodes human telomerase reverse transcriptase (hTERT) and commonly amplified in human cancers. With this regards, the detection of TERT gene amplification in cancer cell may have useful application in cancer diagnosis and prognosis. Hence, in this study, K562 cell line, a chronic leukemic cell line was used to demonstrate the TERT gene amplification by using Fluorescence *in situ* hybridization (FISH) method in interphase stage.

Maintenance and cultivation of the K562 cell line was performed where the cells were grown in tissue culture flask as suspension culture in RF10 medium. Normal peripheral blood also was used in this study as the normal control. In this study, TERT gene amplification was examined in K562 cell line by using a dual-color probe (Poseidon$^{TM}$) that covered the genomic region of TERT gene at region 5p15 together with the control DNA probe, EGRI (5q31) gene to facilitate identification of chromosome 5. FISH analysis was successfully performed based on the recommendation protocol provided by the manufacturer with a minor modification. The FISH signals were visualized using fluorescence microscope with Leica system and CytoVision system. Amplification involving the TERT gene region showed several red signals, while the control at the chromosome 5q31 region (EGR1) will provide 2 green signals. In normal cells, the FISH signal pattern indicated the presence of 2 red (R) 2 green (G) signals. Our findings showed that the ratio of TERT/5q31 signal varied between 1 and 3 per cell. The cells which have 3-4 red (TERT) signals/cell and two green (5q31) signals/cell were considered to be a low

grade of amplification. There was also an occurrence of chromosome 5 aneusomy, where there were 3 green signals indicating the presence of 3 copies of chromosome 5 or trisomy 5. Some cells also showed 3 green signals with more than 2 red signals.

Our study suggests that TERT gene amplification was detected in K562 cells but further investigation by observing the frequency of signal pattern in 200 cells or using metaphase FISH or cytospin samples should be done to reveal the pattern of TERT gene amplification in K562 cells or other types of cancer cells. Additional optimization of FISH technique should be done to ensure cost-effectiveness before utilizing it for routine diagnosis of TERT gene amplification in cancer patients.

# ABSTRAK

Gen TERT yang terletak pada kromosom 5p15.33 merupakan salah satu komponen teras telomerase dimana ia mengkodkan transkriptase membalik telomeras manusia (hTERT) dan biasanya berganda dalam kanser manusia. Oleh itu, pengesanan amplifikasi gen TERT dalam sel kanser boleh menjadi aplikasi berguna dalam diagnostik dan prognostik kanser. Maka, dalam kajian ini, titisan sel K562, sel kronik leukemia digunakan untuk menunjukkan amplifikasi gen TERT dengan mengunakan teknik Fluorescence *in situ* hybridization (FISH).

Pengkulturan dan penuaian sel K562 telah dilakukan di mana sel dibiakkan dalam kelalang kultur tisu sebagai kultur cairan dalam medium RF10. Darah periferi normal digunakan dalam kajian ini sebagai kawalan normal. Dalam kajian ini, amplifikasi gen TERT telah diperiksa dalam titisan sel K562 dengan mengunakan prob dwi warna (Poseidon$^{TM}$) yang meliputi gen TERT dikawasan 5p15 bersama dengan probe control DNA, gen EGRI (5q31) bagi memudahkan pengenalpastian kromosom 5. Analisis FISH berjaya dilakukan berpandukan protokol yang disediakan oleh syarikat pengeluar dengan sedikit modifikasi. Isyarat FISH telah diperhati dengan mengunakan mikroskop fluoresen dengan system Leica dan system Cytovision. Amplifikasi yang meliputi kawasan gen TERT akan menunjukan beberapa isyarat merah, manakala kontrol pada kromosom kawasan 5q31 (EGR1) akan memberi 2 isyarat hijau. Didalam sel normal, isyarat FISH menunjukkan kehadiran 2 isyarat merah (R) dan 2 isyarat hijau (G). Kajian ini, menunjukan nisbah isyarat TERT/5q31 berlainan diantara 1 and 3 dalam satu sel. Sel yang mempunyai 3-4

isyarat/sel merah (TERT) and dua isyarat hijau (5p31) dianggap mempunyai amplifikasi gred rendah. Terdapat juga kewujudan aneusomy kromosom 5, dimana terdapat 3 isyarat hijau menunjukkan kehadiran 3 salinan kromosom 5 atau trisomi 5. Sesetengah sel juga menunjukkan 3 isyarat hijau dengan lebih dari 2 isyarat merah.

Kajian kami mancadangkan bahawa amplifikasi gen TERT dikenalpasti dalam sel K562 tetepi penyiasatan lanjutan dengan memerhati kekerapan bentuk isyarat dalam 200 sel atau mengunakan sample metafasa FISH atau cytospin harus dilakukan untuk menunjukkan corak amplifikasi gen TERT dalam sel K562 atau jenis sel kanser yang lain. Optimesasi tambahan bagi teknik FISH sepatutnya dilakukan bagi memastikan keberkesanan kos sebelum ia digunapakai dalam untuk diagnosis rutin bagi mengesan amplifikasi gen TERT dalam pesakit-pesakit kanser.

# CHAPTER 1

# INTRODUCTION

## 1.1 General introduction

Gene amplification is a copy number increase of a restricted region of a chromosome arm. It is prevalent in some tumors and is associated with overexpression of the amplified gene(s). Gene amplification is a relatively rare event in haematological malignancies but it is more common in the development of many solid tumors (Schwab, 1998). The development of tumors is associated with the acquisition of genetic and epigenetic alterations and the corresponding changes in gene expression that modify normal growth control and survival pathways. These changes can be brought about at the genomic level in a variety of ways, including altered karyotypes, point mutations and epigenetic mechanisms. Genomic DNA copy number aberrations are frequent in solid tumors and are expected to contribute to tumor evolution by copy number-induced alterations in gene expression (Donna, 2006). The analysis of amplified DNA in mammalian cell lines and tumors has revealed that it can be organized as extrachromosomal copies, called double minutes; in tandem arrays as head-to-tail or inverted repeats within a chromosome, often forming a cytologically visible homogeneously staining region (HSR); or distributed at various locations in the genome (Donna *et al.*, 2003). The unit of amplified DNA in some cases can involve sequences from two or more regions of the genome, indicating a complex

process of formation involving multiple chromosomes. The unit of amplified DNA in tumors can range in size from kilobases to tens of megabases (Donna *et al.*, 2003, Donna, 2006).

The TERT gene is one of the main components in telomerase enzyme which encodes human telomerase reverse transcriptase (hTERT) catalytic subunit which is located at human chromosome band 5p15.33. Telomerase is a ribonucleoprotein enzyme complex that adds telomeric repeats to the ends of chromosomes. This enzyme also consists of RNA component known as telomerase RNA (TR) template subunit which is encoded by TERC gene located at chromosome 3q26.3. Chromosomal gains and gene amplifications involving chromosome arms 5p and 3q are among the most frequent in human tumors (Ying *et al.*, 2008). Telomerase activity has been detected in germ cell line and most cancer cells, but in normal human somatic cell it is either undetectable or present at low level. A chronic myeloid leukemic cell line, K562 has been found to express TERT gene amplification (Ying *et al.*, 2008).

The development of fluorescence *in situ* hybridization (FISH) techniques has allowed the rapid and sensitive detection of single-copy DNA sequences in metaphase and interphase nuclei, thus greatly facilitating gene mapping by allowing the unambiguous assignment of genomic probes to specific chromosomal segments with great precision (Shapiro *et al.*, 1993). Fluorescence *in situ* hybridization (FISH) provides a simple, fast, and reliable means to assess genetic instability in cancer. Perhaps the simplest method for detecting the genetic instability in cancerous tissue is to examine for abnormal numbers of chromosomes

(aneusomies) in the affected tissue. Myeloid leukemias, including acute and chronic myeloid leukemia and myelodysplastic syndrome, have long been known to demonstrate the presence of a third copy (trisomy) of chromosome 8 (Fox *et al.*, 1995). Increased knowledge of cancer genetics has led to the development of new assays for the detection of malignancy. One such assay, fluorescence *in situ* hybridization (FISH), has become a valuable tool for detecting and monitoring cancer FISH utilizes fluorescently labeled DNA probes to assess interphase or metaphase cells for chromosomal alterations. Studies have shown that FISH is able to identify malignant cells in a variety of cytologic specimens such as fine needle aspirates, effusions, and urine (Benjamin *et al.*, 2004).

## 1.2 Aim of study

K562 has been found to demonstrate TERT gene amplification. Therefore, this cell line was used as a model to validate TERT gene amplification status using Fluorescent in situ hybridization (FISH) technique in interphase stage. This study may facilitate the optimized FISH method to detect gene amplification in K562 cell line using a commercial dual color probe. The most reliable method for detection of TERT gene may have useful application in cancer patients.

The specific aims of the study were as follows:

1) To optimize detection method for TERT gene amplification in K562 cancer cell line using Fluorescence *in situ* Hybridization (FISH) in interphase cells.

2) To determine the TERT gene amplification signal pattern using FISH technique in K562 cell line.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Telomerase Reverse Transcriptase (TERT) gene

The core telomerase components are the telomerase reverse transcriptase (TERT) catalytic subunit, and the telomerase RNA (TR) template subunit. In most cancers, telomerase activity is expressed at levels that are substantially higher than in normal cells. A known consequence of telomerase upregulation which is considered to play a critical role in oncogenesis is maintenance of telomere length, and thus evasion by cancer cells of the normal limits on proliferation that are associated with the steady decrease in telomere length that accompanies proliferation of normal cells. It has also been suggested that telomerase upregulation confers other advantages on cancer cells independent of its enzymatic activity (Ying *et al.*, 2008). The mechanisms responsible for up-regulation of telomerase in cancer are incompletely understood. Here we review evidence suggesting that this frequently results from increased copy number of the genes encoding telomerase components. The TERT gene is located at human chromosome band 5p15.33, and the telomerase RNA component (TERC) gene that encodes TR is at 3q26.3 (Ying *et al.*, 2008). Increased TERT and TERC gene dosage has been detected frequently in a variety of human cancers and clonal evolution of cells with increased TERT or TERC copy number has been

observed, suggesting a growth advantage in cells with increased TERT or TERC gene dosage. (Ying *et al.*, 2008).

### 2.1.1 Telomerase and its associated protein

Telomerase enzyme maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis. Studies in mouse suggest that telomerase also participates in chromosomal repair, since de novo synthesis of telomere repeats may occur at double-stranded breaks. Alternatively spliced variants encoding different isoforms of telomerase reverse transcriptase have been identified; the full-length sequence of some variants has not been determined. Alternative splicing at this locus is thought to be one mechanism of regulation of telomerase activity.(Ying *et al.*, 2008)

The active human telomerase enzyme is composed of human telomerase reverse transcriptase (hTERT), human telomerase RNA (hTR) and dyskerin (Cohen *et al.*, 2007). hTERT (encoded by the TERT gene) is the catalytic reverse transcriptase component (Nakamura *et al.*, 1997), hTR (encoded by the TERC gene) serves as the RNA template for

the addition of telomeric repeats (Feng *et al.*, 1995) and dyskerin (encoded by the DKC1 gene) is an RNA binding protein (Mitchell *et al.*, 1999). Mutations in any of these components may result in dyskeratosis congenita, a human disease syndrome associated with short telomeres (Kirwan and Dokal, 2008).

Human TERT subunit (hTERT) is a protein of 1132 amino acids with a highly conserved domain homologous to reverse transcriptase (RT) (Nakamura *et al.*, 1997). In contrast to hTR, hTERT is only expressed in a limited number of normal tissues, such as germ cells, stem cells, and activated lymphocytes, but is highly expressed in immortalized cells and most tumor tissues (Yinhua *et al.*, 2002). Telomerase activation requires the interaction between TERT and TR. In vitro studies showed that telomerase activity could be reconstituted using only hTR and hTERT although a variety of additional factors may participate in regulating or promoting in vivo activity (Yinhua *et al.*, 2002). Mouse fibroblast cells lacking the RNA component of telomerase exhibited progressive telomere shortening and chromosomal instability (Hande *et al.*, 1999). Late generations of TR knockout mice had shorter telomeres and an increased incidence of spontaneous tumors (Rudolph *et al.*, 1999). All these data show that the combination of both subunits is necessary for telomerase function. However, where and exactly how these two parts are assembled to form the active RNP complex (Telomerase RNP) in mammalian cells remains unknown (Yinhua *et al.*, 2002).

### 2.1.2 Involvement of TERT gene amplification in cancer

Telomerase activity has been detected in more than 85% of human tumors (Kim *et al.*, 1994) whereas in normal human somatic cells it is either undetectable or present at low levels. In normal cells, telomeres shorten with every cell division, and this eventually results in senescence, a state characterized by permanent withdrawal from the cell division cycle. The increased telomerase activity found in cancers prevents telomere shortening, and allows cancer cells to escape the normal limits on cellular proliferation. (Ying *et al.*, 2008). Several studies demonstrated copy number increases of the TERT gene in multiple tumors or immortalized cell lines (Ying *et al.*, 2008). Previous study (Anju *et al.*, 2000), shows that FISH analysis using a probe that covered the genomic region encoding TERT together with a specific sequence at 5q31 as a marker probe detected two TERT gene copies located on band 5p15.33 and a 1:1 ratio of TERT/5q31 signal in normal cells . However, only 5 of 26 human tumor cell lines from different origins and 28 of 58 human primary tumors carried two TERT and two 5q31 marker copies. The remainder of the cell lines and primary tumors had more than two copies of TERT and a TERT/ 5q31 ratio ≥1. Some of these (50% of cell lines and 22% of primary tumors) displayed 3–4 TERT copies/cell while 31% of cell lines and 30% of human primary tumors had ≥5 copies of TERT per cell (Anju *et al.*, 2000). These included cell lines derived from neuroblastomas (Lan2, Lan5 and SHEP1) and carcinomas of breast (578T), cervix (HeLa and CaSki) and lung (H125, U1285, U1752, SHP77/97, H1688, Colo677/97, H446/97, BEN and H209). (Anju *et al.*, 2000) (Zhang *et al.*, 2002) (Saretzki *et al.*, 2002). Cell lines derived from bladder and epidermal carcinomas (5637 and A431) were reported to have ≥3 copies of h*TERT* per cell (Bryce *et*

*al.*, 2000). In primary tumors, increased TERT copy number has been detected in neuroblastomas (12%), CNS embryonal tumors (42%), hepatocellular carcinomas (22%) and cancers of the lung (30–63%), cervix (24–30%), breast (26%) and colon (48%). In addition, FISH analysis revealed 2–60 copies of TERT in leukemic cells (Nowak *et al.*, 2006).

#### 2.1.2.1 Role of TERT gene and in regulation of Telomerase activity

Most human somatic cells do not produce active telomerase and do not maintain stable telomere length with proliferation. Most or all do have telomerase RNP, which raises the possibility of a second telomerase function independent of DNA synthesis. The significance of telomerase function in any given cell is dependent on both telomere length and the number of future cycles of proliferation. It becomes more difficult to interpret the importance of telomerase regulation in settings where this is means something other than just the constitutive absence or overabundance of activity (Kathleen Collins and Mitchell, 2002).

In humans, telomerase activity detectable at the blastocyst stage and in most embryonic tissues before 20 weeks of gestation is subsequently lost (Wright *et al.*, 1996). Some correlation can be made between telomerase inactivation, differentiation and increased rate of telomere loss with time (Ulaner *et al.*, 2001). Several levels of regulation combine to determine the amount of active telomerase in any given cell.