UNIVERSITI PUTRA MALAYSIA

APPLICATION OF COMPARATIVE GENOMIC HYBRIDIZATION AND FLUORESCENT IN SITU HYBRIDIZATION TECHNIQUES ON HUMAN GLIOMA CELL LINES TREATED WITH BIS[S-METHYL-a-N-(2 - FURYLMETHYLKETONE) DITHIOCARBAZATO] CADMIUM(II)

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APPLICATION OF COMPARATIVE GENOMIC HYBRIDIZATION AND FLUORESCENT *IN SITU* HYBRIDIZATION TECHNIQUES ON HUMAN GLIOMA CELL LINES TREATED WITH BIS[*S*-METHYL-β-*N*-(2-FURYLMETHYLKETONE) DITHIOCARBAZATO] CADMIUM(II)

By

SUHAILI ABU BAKAR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Master of Science

September 2004

DEDICATION

This thesis is dedicated to my beloved husband, Mohd Taufik Mahpop, my son, Muhammad Haiman Fakhrudin and my parents, Siti Besah and Abu Bakar, without whom none of this would have been even possible.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

APPLICATION OF COMPARATIVE GENOMIC HYBRIDIZATION AND FLUORESCENT *IN SITU* HYBRIDIZATION TECHNIQUES ON HUMAN GLIOMA CELL LINES TREATED WITH BIS[*S*-METHYL-β-*N*-(2-FURYLMETHYLKETONE) DITHIOCARBAZATO] CADMIUM(II)

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Comparative genomic hybridization (CGH) and fluorescent *in situ* hybridization (FISH) have become invaluable tools for the diagnosis and identification of numerous chromosomal aberrations either in haematological malignancies or solid tumors. CGH is a modified *in situ* hybridization technique that allows detection and mapping of DNA sequence copy differences between two genomes in a single experiment whereas FISH is a quantitative analysis of specific chromosomes and genes. In this study, both techniques were used in three gliomas cell lines; A172 (glioblastoma), U87 MG (astrocytoma grade III) and T98G (glioblastoma multiforme) to investigate the genomic imbalance and to detect cancer-related genes before and after treatment with a new synthetic cadmium compound. Bis[*S*-methyl-β-*N*-(2-furylmethylketone) dithiocarbazato] cadmium(II) (SMDB-Cd) was synthesized at the Chemistry Department, Faculty of Science, Universiti Putra Malaysia and has shown to have potential as an anticancer agent. The EC_{50} values for SMDB-Cd on A172, U87MG, T98G and HCN-2 were at 0.7, 0.3, 0.4 and 1.5 µg/ml respectively, while tamoxifen

which is commonly used to treat brain cancer were at 7.0, 5.0, 4.0 and 6.0 μg/ml. CGH data indicated that these three cell lines have various DNA copy number changes; the most frequent DNA gains found were at 7p and 13q, and losses of chromosome 9p, 17p and 19q indicate that these regions contain candidate tumor suppressor genes involved in gliomas. Upon treatment with SMDB-Cd at those EC_{50} concentrations, U87 MG was shown to be more sensitive to SMDB-Cd compared to A172 and T98G. Chromosome 7p did not show any changes in DNA amplification or deletetion. Involvement of one of the important tumor suppressor genes in many human cancers, p53, which is mapped to the short arm of chromosome 17, was then examined. The amplification status of this region was evaluated by using FISH through the locus specific p53 (17p13.1) probe. About 60% of cells were detected to have deletion on one or both copies of the p53 gene in A172 and U87MG. However, two copies of the p53 gene were detected in T98G, which means there is no deletion of p53. These results agree with the previous study on the association of p53 mutation with different subtypes of gliomas. After treatment with SMDB-Cd, p53 level was observed to be amplified in T98G. Increasing of p53 level may have been induced by the action of SMDB-Cd on the cells that inhibit cell growth and lead to cell damage. Thus, the combined use of CGH and FISH provided an efficient method for resolving the origin of aberrant chromosomal material unidentified by conventional cytogenetic analysis. CGH was observed to be a powerful tool in assisting the screening of amplification and deletion regions in glioma cell lines upon treatment with SMDB-Cd. Use of FISH to confirm the involvement of the p53 gene further enhances the validity of the technique.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGGUNAAN TEKNIK PERBANDINGAN PENGHIBRIDAN GENOM DAN PENGHIBRIDAN *IN SITU* FLUORESEN KE ATAS SEL - SEL GLIOMA MANUSIA SELEPAS DIRAWAT DENGAN BIS[*S*-METHYL-β-*N*-(2-FURYLMETHYLKETONE) DITHIOCARBAZATO] CADMIUM(II)

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Perbandingan penghibridan genom (CGH) dan penghibridan *in situ* fluoresen (FISH) telah menjadi teknik yang sangat bernilai dalam diagnosis dan pengenalpastian pelbagai kesilapan kromosom sama ada di dalam pertumbuhan kanser darah atau kanser solid. CGH adalah teknik penghibridan *in situ* yang telah diubah suai untuk mengesan dan merajahkan perbezaan rangkaian DNA di antara dua gen dalam satu kajian manakala FISH adalah teknik yang berasaskan analisa kuatitatif terhadap kromosom dan gen yang khusus. Di dalam kajian ini, kedua-dua teknik di gunakan keatas tiga jenis sel glioma; A172 (glioblastoma), U87MG (astrocytoma gred III) dan T98G (glioblastoma multiforme) untuk menyelidik ketidakseimbangan genom dan mengesan gen-gen yang berkait rapat dengan pembentukan kanser glioma sebelum dan selepas dirawat dengan sintetik kompoun kadmium. Bis[*S*-methyl-β-*N*-(2-furylmethylketone) dithiocarbazato] cadmium(II) (SMDB-Cd) yang disintesiskan di jabatan kimia, fakuli sains dan alam sekitar, Universiti Putra Malaysia telah menunjukkan potensi sebagai anti kanser. Nilai EC_{50} untuk SMDB-Cd pada A172, U87MG, T98G dan HCN-2 adalah 0.7, 0.3, 0.4 dan

1.5 µg/ml dibandingkan dengan tamoxifen yang selalu digunakan dalam merawat kanser otak iaitu 7.0, 5.0, 4.0 dan 6.0 µg/ml. CGH telah mengenal pasti pelbagai perubahan salinan DNA didalam ketiga – tiga sel tersebut; yang paling kerap dilihat adalah pertambahan DNA pada 7p dan 13q manakala kehilangan DNA adalah pada kromosom 9p, 17p dan 19q di mana bahagian – bahagian tersebut mengandungi gen supresor tumor yang terlibat dalam gliomas. Selepas dirawat dengan SMDB-Cd pada kepekatan EC_{50}, U87MG telah menunjukkan tindak balas yang lebih sensitive terhadap kompoun tersebut berbanding dengan A172 dan T98G. Kromosom 7p tidak menunjukkan sebarang penambahan atau kehilangan DNA. Penglibatan satu gen supresor kanser, p53 yang terletak pada bahagian pendek kromosom 17 telah.diuji. Status peningkatan bahagian ini telah dinilai dengan menggunakan FISH melalui probe khusus untuk loci p53 (17p13.1). Hampir 60% dari sel – sel A172 dan U87MG menunjukkan tiada salinan atau satu salinan gen p53 sahaja yang dilihat. Walau bagaimanapun, terdapat dua salinan gen p53 yang dikenal pasti didalam T98G bermakna tidak ada kehilangan gen p53. Keputusan ini bersetuju dengan kajian terdahulu mengenai hubungan gen p53 dengan jenis – jenis gliomas. Selepas diberi SMDB-Cd, terdapat peningkatan paras p53 di dalam T98G. Peningkatan paras p53 ini di dalam sel – sel yang dirawat mencadangkan kemungkinan tindakan kompoun sintetik tersebut keatas sel yang menghalang pertumbuhan sel dan menyebabkan kemusnahan sel. Maka, kombinasi CGH dan FISH memberikan kaedah yang berkesan dalam meyelesaikan permulaan kepelbagaian kromosom yang tidak dapat dikenal pasti melalui analisis sitogenetik konventional. Keputusan ini menunjukkan CGH adalah satu teknik yang boleh digunakan dalam penyaringan peningkatan dan kehilangan bahagian-

bahagian yang terlibat dalam sel-sel glioma sama ada sebelum atau selepas dirawat dengan SMDB-Cd. FISH sebagai pengesahan teknik kepada gen p53 tersebut yang meningkatkan lagi kesahihan kaedah tersebut.

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Last but not least, I would like to express my heartfelt thanks to my beloved husband Mohd Taufik, mother Siti Besah and father Abu Bakar for their endless encouragement, patient, care, financial and sacrifies that had helped me in my undertakings and to complete this research study successfully. To my son, Haiman Fakhrudin, thank you for allowing me to take part of your growing time.

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ATCC	American Typed Culture Collection
BACs	Bacterial artificial chromosomes
CCD	Charged couple device
Cd	Cadmium
CDK	Cyclin-dependent kinase
CGH	Comparative genomic hybridization
CIN	Chromosomal instability
CNS	Central Nervous System
DAPI	(4',6 – diamidino – 2 - - phenylindole)-
dATP	Deoxyadenosine triphosphate
del	Deletion
der	Derivative chromosome
DMEM	Dulbecco's Modified Eagle's media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EC_{50}	Fifty-percent effective concentration
EGFR	Epidermal Growth Factor receptor
EMEM	Eagle's Minimal Essential media
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
FR	Fluorescent ratio
G banding	Giemsa banding
GBM	Glioblastoma multiforme
ISCN	The International System for Human Cytogenetic Nomenclature
ISH	*in situ* hybridization
LOH	Loss of heterozygosity

LOH	Loss of heterozygosity
LSI	Locus specific interphase
MDM2	Mouse double minute 2
MIN	Microsatellite instability
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
PACs	P1 filamentous phage artificial chromosomes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PTEN	Phosphatase and tensin homolog
RB1	Retinoblastoma 1
RNA	Ribonucleic acid
SKY	Spectral karyotyping
SMDB-Cd	Cd(II) complex, bis [*S*-methyl-β-*N*-(2-furylmethyl-ketone)dithiocarbazato]Cd(II)
SSC	Standard sodium citrate
t	Translocation
TRITC	Texas red isothiocyanate
VEGF	Vascular endothelial growth factor
UV	Ultra violet
WCP	Whole chromosome probe
WHO	World Health Organization
YACs	Yeast artificial chromosomes

CHAPTER 1

INTRODUCTION

Currently, molecular cytogenetic techniques that are based on fluorescence *in situ* hybridization (FISH), have become invaluable tools for the diagnosis and identification of the numerous chromosomal aberrations which are associated with neoplastic disease, including both haematological malignancies and solid tumors. This is because the etiologies of these genetic diseases involve a complex interplay of numerous acquired genetic abnormalities, including amplification of oncogenes, deletion of tumor suppressor genes, gene rearrangements and loss or gain of function mutations (McNeil *et al.*, 2000).

Molecular cytogenetic techniques focus on specific chromosomes, chromosome regions and unique DNA sequences or genes as compared to standard cytogenetic techniques. With these techniques, some chromosomal abnormalities in nondividing cells can be detected with interphase nuclei instead of using standard cytogenetics which require actively dividing cells with metaphase nuclei. In other words, the molecular cytogenetic techniques can be used as a complementary technique which will expand the capabilities for making more accurate and refined cytogenetic diagnosis either for constitutional abnormalities or acquired chromosomal changes in cancer cells (Table 1) (Rautenstrauss and Liehr, 2002).

Table 1: Milestones in karyotyping and molecular cytogenetics (adapted from Rautenstrauss and Liehr, 2002)

Early findings	Several authors in the 19th century	- Cell theory - Mendel's law of inheritance - First identification of miotic figures
	1882 Flemming 1883 Roux	Behavior of chromosomes in a dividing cell
	1944 Avery *et al.*	DNA – transforming factor
	1952 Hsu	Hypotonic treatment of cells
	1953 Watson	DNA double helix and genetic implications
Pre – banding era	1956 Tjio & Levan	46 chromosomes
	1958 Ford, Jacobs & Lajtha	First chromosome analysis in leukemia
	1959 Lejeune *et al.*	First chromosomal diseases: - (Down's syndrome) trisomy of one of the smallest chromosomes - Turner's syndrome (monosomy X)
	1960 Ford *et al.*	Denver Conference 23 human chromosome pairs divided by morphology into 7 groups (A - G)
	1960 Edwards *et al*; Patau *et al*	- Trisomy for a D – group chromosome - Trisomy for an E – group chromosme
	1960 Nowell & Hungerford	First identification of chromosomal disorder related to leukaemia
Banding era	1968 Carpesson *et al.*	Quinacrine mustard banding
	1970 Chaudhuri *et al.*, Caspersson *et al.*	G and Q banding refined 450 – 550 bands/metaphase
	1970 Smith & Wilcox Kelly & Smith	Birth of molecular genetics specific restriction endonucleases
	1971	Paris Conference – first cytogenetic nomenclature of all 23 chromosome pairs

	1973 Cohen *et al.*	First report on cloning DNA using plasmids as vectors
	1976 Yunis	High resolution chromosome banding
	1981	(up to 2000 bands/prophase)
FISH era	1981 Harper & Saunders; Malcolm et al.; Gerhard et al.	Localization of single copy genes by insitu hybridization
	1981 Langer et al.	Biotin – labeled nucleotides
	1985	International System for Human Cytogenetic Nomenclature (ISCN 1985)
	1986 Saiki et al	PCR technology
	1986 Pinkel et al.	Fluorescence hybridization
	1986 Deaven et al.	Chromosome specific DNA libraries
	1988 Pinkel et al. Cremer et al.	Fluorescence in situ hybridization with human chromosome specific libraries
	1988 Lichter et al.	CISS hybridization
	1990 Nederlof et al.	Multi – color FISH
	1991	Supplement to ISCN now including cancer cytogenetics
	1992 Telenius et al.	DOP – PCR
	1992 Kallioniemi et al.	CGH – analysis
	1995 Mitelman	Supplement to ISCN now including FISH nomenclature
	1996 Schrock et al. Speicher et al.	24 – color karyotyping spectral karyotyping multicolor karyotyping
	1998 Choudoba et al.	High resolution bar coding

Improvement of the cytogenetic techniques from a conventional method such as G-banding to modern cytogenetic techniques such as fluorescence *in situ* hybridization (FISH) has enabled the finding of abnormalities in chromosome number and structure more quickly. FISH probes have been designed to detect specific regions of DNA and thus to elucidate abnormalities even at the level of the gene which cannot be detected by conventional techniques. Some of the advantages of FISH include the confirmation of chromosome breakpoints, an assessment of specific nucleic acid sequences and the ability to detect such sequences in non-dividing cells (*i.e.* interphase nuclei). Although FISH is an extremely useful technique, until recently only a few target sequences could be visualized simultaneously (McNeil *et al.*, 2000).

By contrast, comparative genomic hybridization (CGH) is widely used as a powerful method for detection and identification of chromosomal imbalances in a wide range of tumor samples (Kallioniemi *et al.*, 1992). The technique gives an overview of gains and losses of whole chromosomes or regions as well as amplifications and deletions of smaller segments. Identification of the chromosomal gains and losses is carried out by using differentially labeled tumor DNA and normal DNA. On visualizing the two different fluorochromes, differences in the intensity of fluorescence along the chromosome correspond to the loss or gain of genetic material in the tumor sample (McNeil *et al.*, 2000). Currently, the amplification of single genes have been documented, while deletions are detectable only on a larger scale (band size) (Barch *et al.*, 1997). The technique of CGH, which has also been referred to as copy number of

karyotyping may target a locus for molecular analysis or indicate pathways leading to tumor progression, recurrence and metastasis (Barch *et al.*, 1997).

In solid tumors, classical cytogenetic preparation has been less successful because of the difficulty of obtaining high quality representative metaphase chromosome preparations and to analyze the chromosomes that may not be representative of the tumor. Sometimes, the preparation of chromosome may have contamination of the specimen with normal tissue. The potential for multiple abnormal clones which contain high level of chromosomal rearrangements makes karyotyping analysis complicated. The complex nature of those rearrangements makes them indecipherable by banding techniques (Barch *et al.*, 1997). Therefore, the advent of molecular cytogenetic techniques in the late 1960s, which is the visualization of loci using the dynamic biochemical technique of *in situ* hybridization, has helped to detect chromosomal aberrations. In the case of solid tumors and leukaemia, obtaining of the metaphase chromosome spreads in conventional technique is difficult therefore in molecular cytogenetic, preparation of metaphase chromosome spreads from the sample is unnecessary that will help to analyze the chromosome in those diseases.

Research in cancer biology unequivocally shows that cancer tumors have a wide variety of aberrations in the organization and content of their genomes as compared with the genomes of normal cells. This genomic instability which is defined as the dynamic process of genomic changes in a tumor cell over time is a general feature of cancer cells. It displays an elevation of mutation rates during tumor progression that results in