IDENTIFICATION OF CHROMOSOME BREAKS MEDIATED BY STRESS-INDUCED APOPTOSIS IN NASOPHARYNGEAL CARCINOMA (NPC)

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Master of Science
2012
IDENTIFICATION OF CHROMOSOME BREAKS MEDIATED BY STRESS-INDUCED APOPTOSIS IN NASOPHARYNGEAL CARCINOMA (NPC)

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A thesis submitted in fulfillment of the requirement for the Degree of Master of Science (Cancer Molecular Genetics)

Faculty of Medicine and Health Sciences
UNIVERSITI MALAYSIA SARAWAK
2012
ACKNOWLEDGEMENTS

First and foremost, I owe my deepest gratitude to my lovely supervisor, Assoc. Prof. Dr. Sim Sai Peng who has supported me throughout my study with her supervision, knowledge and steadfast encouragement. I am heartily thankful to her for patiently correcting my writing and for the valuable insights she has shared. I have always benefited by her advice and guidance. This excellent atmosphere for doing research has enabled me to develop a more comprehensive understanding of research work. I am grateful to my former co-supervisor, Ms. Chin Mei Yieng who until her day of emigration had kind concern regarding my progress in the completion of this research study. I greatly appreciate her incessant support, prayers and regards from Vancouver. I gratefully acknowledge the financial support from UNIMAS through the Postgraduate Student Fellowship and the Research Grant under Ministry of Health Malaysia (Grant No. 06-065). I would like to thank Prof. Sam Choon Kook from University of Malaya for giving us the SUNE1 cell line. I offer my gratitude to Prof. Tsao Sai Wah and Prof. Lo Kwok Wai respectively from The University of Hong Kong and The Chinese University of Hong Kong for providing us the HK1 and NP69 cell lines. My special thanks go to my lab mates for science discussion and pleasure working together. It would have been a lonely lab without them. Many thanks go to our scientific officers for their technical assistance. I am indebted to many of my church mates. They were always supporting me with their prayers and encouragement. I would like to show my gratitude to my family who were always there cheering me up and stood by me through the good times and bad. Last but not the least, I would like to give all the glory to God, the one above all of us, for answering my prayers and for giving me the strength to hurdle all the obstacles in the completion of this research work. Thank you so much, Dear Lord.
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Figure 4.1 A potential model for stress-induced chromosomal rearrangement in NPC.
LIST OF ABBREVIATIONS

AIF  apoptosis inducing factor
ALL  acute lymphocytic leukaemia
AML  acute myelogenous leukaemia
ANLL  acute non lymphoblastic leukaemia
Apaf-1  apoptotic protease activating factor 1
APL  acute promyelocytic leukaemia
ASK1  apoptosis signal-regulating kinase 1
ASR  age-adjusted rates
ATP  adenosine triphosphate
BCR  breakpoint cluster region
BL  Burkitt’s lymphoma
BLAST  Basic Local Alignment Research Tool
bp  base pair
BPE  Bovine Pituitary Extract
BSA  bovine serum albumin
CAD  caspase-activated deoxyribonuclease
°C  degree Celsius
C-FLIP  cellular FLICE (FADD-like IL-1p-converting enzyme)-inhibitory protein
CGH  comparative genomic hybridisation
(CH3)2CHCH2CH2OH  Isoamyl alcohol
CH3COONH4  Ammonium acetate
CHO  Chinese Hamster Ovary
CI  caspase inhibitor
CLL  chronic lymphocytic leukaemia
CML  chronic myelogeneous leukaemia
CMML  chronic myelomonocytic leukaemia
CPT  camptothecin
CO2  Carbon dioxide
cyt c  cytochrome c
DD  death domain
DDR  DNA damage response
Δψ  membrane potential
DAB  Disabled Homolog 2
DIABLO  direct IAP (inhibitor of apoptosis)-binding protein with low pl
DISC  Death Inducing Signalling Complex
DMSO  Dimethyl sulfoxide
DNA  deoxyribonucleic acid
dNTP  Deoxynucleotide triphosphate
DLCL  diffuse large-cell lymphoma
DSB  double-strand break
(EBNA)-1  EBV nuclear antigen
EBV  Epstein-Barr Virus
EDTA  Ethylenediaminetetracetic acid
Endo G  endonuclease G
FADD  Fas-associated death domain protein
Fas-L  Fas ligand
FITC  fluorescein isothiocyanate
FL  follicular lymphoma
G6PDH  glucose-6-phosphate dehydrogenase
gDNA  genomic DNA
GPX  glutathione peroxidase
GR  glutathione reductase
GST  glutathione-S-transferase
H$_3$BO$_3$  boric acid
HBV  hepatitis B virus
HCV  hepatitis C virus
HMGIC  High mobility group protein gene
HMW  high-molecular-weight
HR  homologous recombination
H$_2$O$_2$  hydrogen peroxide
IAP  inhibitor of apoptosis
ICAD  inhibitor of CAD
IPCR  Inverse Polymerase Chain Reaction
JC-1  1st J-aggregate-forming cationic dye (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide)
JMML  juvenile myelomonocytic leukaemia
KCl  potassium chloride
KH$_2$PO$_4$  potassium dihydrogen phosphate
LHFPL3  Human Lipoma HMGIC Fusion Partner-Like 3
LOH  loss of heterozygosity
LTR  long terminal repeats
MAR  matrix attachment region
MDA  malondialdehyde
MDRs  minimal deletion regions
MDS  myelodysplastic syndrome
MgCl$_2$  magnesium chloride
ml  millilitre
MLL  Mixed Lineage Leukaemia
mM  milimolar
mm  millimeter
MRS  MAR/SAR recognition signature
NaAc  sodium acetate
NaCl  sodium chloride
Na$_2$HPO$_4$.7H$_2$O  disodium hydrogen phosphate
ng  nanogram
NHL  non-Hodgkin’s lymphoma
nm  nanometer
NHEJ  non-homologous end joining
NPC  nasopharyngeal carcinoma
O$_2$-  superoxide
O.D. optical density
OH· hydroxyl radical
PBS Phosphate-Buffered Saline
PCR Polymerase Chain Reaction
PKC protein kinase C
PI Propidium Iodide
PS phosphatidylserine
PTP permeability transition pore
RE restriction enzyme
rEGF Recombinant Epidermal Growth Factor
RNA ribonucleic acid
RNS reactive nitrogen species
RO- peroxyl
ROS reactive oxygen species
SAR scaffold attachment region
SD standard deviation
SDS Sodium dodecyl sulfate
Smac second mitochondrial derived activator of caspases
SOD superoxide dimutase
t-ALL therapy related-acute lymphocytic leukaemia
t-AML therapy related-acute myelogenous leukaemia
t-MDS therapy-related myelodysplastic syndrome
TBE Tris/borate/EDTA
TE Tris/EDTA
TMHS Tetraspan Membrane Protein of Hair Cell Sterocilia
TNF tumour necrosis factor
TNFR-1 tumour necrosis factor receptor
TOP2 topoisomerase II
TRADD TNFR-associated death domain protein
TRAIL TNF-related apoptosis-inducing ligand
xg multiples of earth's gravitational force
µg microgram
UL uterine leiomyoma
µl microlitre
µM micromolar
UV ultraviolet
v volume
VDAC voltage-dependent anion channel
VP16 etoposide
WHO World Health Organization

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ABSTRACT

Chromosomal rearrangements such as additions and deletions are genomic instability frequently observed in nasopharyngeal carcinoma (NPC). However, the molecular mechanism underlying the NPC chromosomal rearrangements remains elusive. Recently, there is increasing evidence that the apoptotic nuclease caspase-activated deoxyribonuclease (CAD) is one of the players leading to translocation process in leukaemia. In normal cells, CAD exists as a complex with inhibitor of CAD (ICAD). ICAD possesses two caspase-3 cleavage sites which are cleaved by caspase-3 when the apoptotic inducer is present. This releases the CAD from ICAD and allows it to degrade the chromosomal DNA. Although apoptosis is a cell death process, cells have the potential to survive apoptosis upon DNA repair. In human cells, chromosomal double-strand breaks (DSB) are primarily repaired by multiple repair pathways including non-homologous end joining (NHEJ) system. Non-homologous end joining pathway joins two cleaved DNA ends with microhomology, thus potentially resulting in erroneous DNA repair. Eventually, the surviving cells may carry chromosomal rearrangements. Apoptosis can be triggered by oxidative stress which occurs when the reactive oxygen species (ROS) generation exceeds the antioxidant defence capability. Oxidative DNA damage is strongly associated with carcinogenesis. It has recently become apparent that DNA breaks do not randomly distribute throughout a gene but usually cluster in certain regions containing specific chromatin structures. Matrix attachment region/scaffold attachment region (MAR/SAR) is a binding site of DNA loop structure to nuclear scaffold proteins. It has DNA unwinding property which makes it to be a region of DNA fragility. This study hypothesised that stress-induced apoptosis may cause DNA breaks at MAR/SAR and subsequently contribute to NPC chromosomal rearrangements in cells that survive apoptosis.
upon DNA repair. This study focused on the \textit{AF9} gene at 9p22 and the \textit{ABL} gene at 9q34 because they are involved in translocations in leukaemia and are located at NPC common deletion sites. We aimed to identify DNA breaks mediated by stress-induced apoptosis, to relate the breakpoints to MAR/SAR sites and to investigate the role of CAD in DNA cleavage mediated by stress-induced apoptosis. Upon hydrogen peroxide (H$_2$O$_2$) treatment, apoptotic evidence was observed in NPC and normal nasopharyngeal epithelial cells by flow cytometry. Numerous DNA breaks were detected in H$_2$O$_2$-treated NPC and normal nasopharyngeal epithelial cells by nested Inverse Polymerase Chain Reaction (IPCR). All breakpoints were mapped within close proximity to the MAR/SAR sites. Besides, translocations were identified in H$_2$O$_2$-treated normal nasopharyngeal epithelial cells. Regions of microhomology were found at the translocation junctions. Furthermore, pre-treatment of caspase inhibitor which indirectly inhibits CAD significantly reduced the DNA breaks in H$_2$O$_2$-cotreated NPC and normal nasopharyngeal epithelial cells. In addition, a comparison of SAR and non-SAR regions showed that there were more chromosomal breaks detected within the non-SAR region which is occupied by nearly 60% of repeat elements. However, these breaks are not H$_2$O$_2$ dependent. This result shows that repeat elements might play an important role in inducing spontaneous chromosomal breaks, but not in chromosomal cleavage mediated by stress-induced apoptosis. Taken together, these findings suggested that under oxidative stress, surviving apoptosis involving compromised DNA repair could be one of the mechanisms contributing to NPC carcinogenesis. Matrix attachment region/scaffold attachment region located at the base of chromosomal loop structure could be the preferential sites of chromosomal cleavage. The apoptotic nuclease CAD may play an important role in DNA cleavage mediated by oxidative stress-induced apoptosis. Therefore, a potential model for oxidative stress-induced chromosomal rearrangements in NPC is proposed.
ABSTRAK

Penyusunan semula kromosom seperti penambahan dan delesi adalah ketidakstabilan genomik yang sering didapati dalam kanser nasofaring (NPC). Namun, mekanisme molekul yang mendasari penyusunan semula kromosom NPC tetap sukar difahami. Baru-baru ini, terdapat semakin banyak bukti yang menunjukkan bahawa nuklease apoptosis (kematian terancang sel), iaitu deoksiribonuklease (caspase-activated deoxyribonuclease, CAD) merupakan salah satu pemain utama untuk proses translokasi dalam leukemia. Dalam sel yang normal, CAD wujud sebagai satu kompleks dengan perencatnya, ICAD. Terdapat dua lokasi pembelahan dalam ICAD yang akan dibelah oleh caspase-3 semasa inducer apoptosis wujud. Pembelahan ICAD oleh caspase-3 akan melepaskan CAD daripada inhibitornya, iaitu ICAD, seterusnya membentukkan CAD untuk memdegradasi DNA kromosom. Walaupun apoptosis adalah sejenis proses kematian sel, sel mempunyai potensi untuk mempertahankan diri daripada apoptosis dengan pembaikan DNA. Dalam sel manusia, lesi DNA dibaiki dengan pelbagai jenis sistem pembaikan DNA, termasuk sistem ‘non-homologous end joining (NHEJ)’. Sistem NHEJ menggabungkan dua unitai terbelah DNA yang berakhir dengan microhomolog. Oleh demikian, sistem ini berpotensi menyebabkan kesilapan dalam pembaikan DNA. Akibatnya, sel-sel yang berjaya mempertahankan diri daripada apoptosis bermungkinan besar akan membawa kromosom yang telah mengalami penyusunan semula. Apoptosis dapat dirangsangkan oleh tekanan oksidatif yang berlaku ketika penghasilan spesies oksigen reaktif (ROS) melebihi kemampuan pertahanan antioksidan. Kerosakan DNA yang disebabkan tekanan oksidatif amat berkaitan dengan karsinogenesis. Baru-baru ini, terdapat suatu hakikat yang menjadi semakin jelas, iaitu pembelahan DNA tidak menyebar secara rawak dalam seluruh gen malah biasanya terkumpul pada lokasi tertentu yang
mempunyai struktur kromatin khusus. Matrix attachment region/scaffold attachment region (MAR/SAR) merupakan suatu lokasi pengikatan struktur pusingan DNA pada protein perancah nukleus. Salah satu ciri-ciri yang didapati dalam MAR/SAR adalah pelonggaran struktur DNA yang menjadikannya sebagai suatu lokasi kerapuhan DNA. Hipotesis penyelidikan ini adalah apoptosis yang dirangsang oleh tekanan oksidatif boleh menyebabkan pembelahan DNA dalam MAR/SAR, seterusnya mengakibatkan penyusunan semula kromosom NPC dalam sel-sel yang berjaya mempertahankan diri dariapada apoptosis dengan pembaikan DNA. Penyelidikan ini focus pada gen AF9 yang berada di kromosom 9p22 dan gen ABL yang berada di kromosom 9q34. Hal ini kerana kedua-dua gen tersebut terlibat dalam translokasi leukemia dan berada dalam lokasi delesi yang umum dalam NPC.

Objektif-objektif penyelidikan ini termasuk mengenalpasti pembelahan DNA yang dimediasi oleh apoptosis yang dirangsang oleh tekanan oksidatif, mengaitkan lokasi pembelahan DNA dengan MAR/SAR serta menyelidik peranan CAD dalam pembelahan DNA yang dimediasi oleh apoptosis yang dirangsang oleh tekanan oksidatif. Setelah rawatan hidrogen peroksida (H₂O₂), bukti-bukti yang menunjukkan apoptosis telah diperhatikan dalam sel NPC dan sel epitelium nasofaring normal dengan menggunakan aliran sitometri (flow cytometry). Dengan menggunakan Reaksi Polimerisasi Rantai Terbalik Bersarang (nested Inverse Polymerase Chain Reaction, nested IPCR), terdapat banyak pembelahan DNA telah dikekan dalam sel NPC dan sel epitelium nasofaring normal yang telah dirawati dengan H₂O₂. Semua pembelahan DNA dipetakan dalam jarak dekat dengan MAR/SAR. Selain itu, translokasi dikenalpasti dalam sel epitelium nasofaring normal yang telah dirawati dengan H₂O₂. Kawasan mikrohomologi ditemui di persimpanan translokasi. Selain itu, prarawatan inhibitor caspase yang menghambat CAD secara tidak langsung telah mengurangkan pembelahan DNA dalam sel NPC dan sel epitelium nasofaring normal yang dirawati dengan
H₂O₂. Di samping itu, perbandingan kawasan yang mempunyai SAR dengan kawasan yang tidak mempunyai SAR menunjukkan bahawa terdapat lebih banyak pembelahan DNA dalam kawasan yang tidak mempunyai SAR. Hampir 60% kawasan yang tidak mempunyai SAR tersebut terdiri daripada elemen berulang (repeat element). Namun, pembelahan DNA dalam kawasan yang tidak mempunyai SAR tidak bergantung kepada H₂O₂. Keputusan ini menunjukkan bahawa elemen berulang mungkin memainkan peranan penting dalam perangsangan pembelahan kromosom yang spontan, tetapi tidak memainkan peranan dalam pembelahan kromosom yang dimediasi oleh tekanan oxidatif. Kesimpulannya, penemuan penyelidikan ini menyarankan bahawa di bawah tekanan oksidatif, apoptosis yang melibatkan pembaikan DNA berkompromi mungkin merupakan salah satu mekanisme yang mengakibatkan karsinogenesis NPC. Matrix attachment region/scaffold attachment region yang berada di dasar struktur pusingan kromosom mungkin merupakan lokasi preferensial bagi pembelahan kromosom. CAD yang menjadi sebagai nuklease apoptosis mungkin memainkan peranan penting dalam pembelahan DNA yang berlaku ketika apoptosis yang dirangsang oleh tekanan oksidatif. Dengan ini, suatu model berpotensi bagi penyusunan semula kromosom yang dimediasi oleh tekanan oksidatif dalam NPC telah dicadangkan.
CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Cancer and Chromosomal Rearrangements

Cancer is a developmental disorder of cells in which a group of abnormal cells proliferate infinitely without control from the normal growth regulation mechanism. Cancer is a genetic disease. It is commonly initialised by chromosomal rearrangements such as deletion, addition, inversion and translocation. The rearrangements occurring at the deoxyribonucleic acid (DNA) level will subsequently cause the transcription of mutated ribonucleic acid (RNA) and results in synthesis of dysfunctional protein. Chromosomal rearrangement is especially critical when it occurs in proto-oncogene or tumour suppressor gene. Proto-oncogene encodes for protein that stimulates growth while tumour suppressor gene encodes for protein that controls growth. Either activation of proto-oncogene or inactivation of tumour suppressor gene may contribute to cancer development (Holland, 2003).

Carcinogenesis is a multi-stage process which usually involves complex chromosomal rearrangements. Some chromosomal rearrangements are primary events occurring early in the development of cancer and are likely to be a crucial event in its development. Others are secondary events and may play a role in the subsequent biological behaviours of the cancer, such as metastasis and invasiveness (Macdonald et al., 2004).
The first consistent chromosomal rearrangement to be recognised was the Philadelphia chromosome observed in chronic myelogeneous leukaemia (CML) which was discovered by Peter Nowell in 1960 (Nowell and Hungerford, 1960). In the 1970s, with improved cytogenetic techniques, Philadelphia chromosome was found to be resulted from a translocation between chromosomes 9 and 22 (Rowley, 1973). In the 1980s, molecular techniques demonstrated that the critical genes involved in this reciprocal translocation as the tyrosine kinase $ABL$ gene on chromosome 9 and the breakpoint cluster region ($BCR$) gene on chromosome 22 (Groffen et al., 1984; Shtivelman et al., 1985). The product of $ABL$-$BCR$ fusion was later found to be an abnormal kinase acting as stimulant for proliferation of myeloid cells leading to CML (Lugo et al., 1990).

The discovery of the Philadelphia chromosome has provided the first direct link between chromosomal rearrangements to malignancy, albeit an association that has been debated for more than one hundred years (Koretzky, 2007). Since then, many other chromosomal rearrangements have been reported in various cancers.

More recently, a therapeutic agent, imatinib mesylate (Gleevec) which has been proved to have major positive therapeutic effects in CML patients by targeting the $ABL$-$BCR$ kinase has been developed (Hernandez-Boluda and Cervantes, 2002; Druker et al., 2006). Imatinib mesylate represents the paradigm of how a more comprehensive understanding of the chromosomal abnormalities as well as the underlying pathogenetic mechanisms in a malignancy plays an essential role in the development of a genetically targeted molecular therapy.
Basically, there are two categories of cancers, namely solid and haematological cancers. Nasopharyngeal carcinoma is one of the examples of solid cancers while leukaemia is one of the examples of haematological cancers.

1.2 Nasopharyngeal Carcinoma (NPC)

1.2.1 Subtypes of NPC

Nasopharyngeal carcinoma (NPC) is a malignant neoplasm derived from mucosal epithelium of the nasopharynx. According to the World Health Organization (WHO), NPC can be classified into three subtypes according to the degree of epithelial differentiation, namely keratinizing squamous cell carcinoma (Type I), non-keratinizing squamous cell carcinoma (Type II) and undifferentiated or poorly differentiated carcinoma (Type III) (Shanmugaratnam, 1978).

1.2.2 Geographic Variation in NPC Incidence Rates

Nasopharyngeal carcinoma is a rare malignancy in most parts of the world, the incidence rates are below one per 100,000 persons per year (Hirayama, 1978; Chang and Adami, 2006). However, there are a few well-known notable exceptions, including South-Eastern Asia, Northern Africa, Middle-East and Arctic Region (Chang and Adami, 2006).

The intermediate rates are reported in several countries such as Thailand, Vietnam, Philippines, Malaysia and Singapore (South-Eastern Asia), Algeria (Northern Africa), Israel