



UNIVERSITI PUTRA MALAYSIA

**EXPRESSION OF CCND₁, p16 AND CDK₆ IN HUMAN BASAL CELL
CARCINOMA**

SIMA ATAOLLAHI ESHKOOR

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By

SIMA ATAOLLAHI ESHKOOR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
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EXPRESSION OF CCND₁, p16 AND CDK₆ IN HUMAN BASAL CELL CARCINOMA

By

SIMA ATAOLAH ESHKOOR

December 2007

Chairman: Associate Professor Patimah bt Ismail, PhD

Faculty: Medicine and Health Sciences

Basal cell carcinoma (BCC) is the most common cancer among skin cancers. The incidence of cutaneous malignant melanoma (CMM) and non-melanoma skin cancer (NMSC) has increased more than 600% worldwide since the 1940s. Carcinogenesis is a multi-step process involving multiple genetic alterations. The connection between cell cycle proliferation and cancer resulting in deregulated cellular proliferation leads to cancer. Cancer has been associated with disturbances in cell cycle regulation. Recent studies have shown that p16, CDK₆ and CCND₁ mRNA genes and protein expression are involved in the tumorigenesis of skin cancer. These genes play a role in cell cycle proliferation. In this study, we assessed the expression of a cyclin, a cyclin dependent kinase, and a cyclin kinase inhibitor in skin BCC tissue. Reverse Transcription *in situ* polymerase chain reaction (RT *in situ* PCR) and immunohistochemistry (IHC) were used to detect the expression of p16, CDK₆ and CCND₁ mRNA genes through them of protein expression in human skin BCC tissue.



The results show that p16, CDK₆ and CCND₁ mRNA genes and protein are expressed in both normal and human skin BCC tissues. CCND₁, CDK₆ and p16 mRNA can be found to be expressed mostly in cytoplasm. The mRNA expression in BCC is higher than normal skin tissue. Protein expression of CCND₁ and p16 in different BCC tissue are greater than normal skin tissue. p16 mRNA and protein expression is stronger than other genes. RT *in situ* PCR and IHC analysis data showed significant expression of CCND₁, p16 mRNA and protein in BCC compared to normal skin tissue ($p < 0.05\%$). Investigation on Iranian samples showed the protein expression of CDK₆ is not significant ($p > 0.05\%$) but the expression of mRNA for CDK₆ gene is significant ($p < 0.05\%$). The findings of IHC study on tissue microarray (TMA) demonstrated significant protein expression of p16 and CCND₁ genes ($p < 0.05\%$) which support findings on Iranian samples. Taken together, these data provide evidence that cell cycle deregulation in G₁-phase is a critical event during the course of carcinogenesis of BCC.

In conclusion, this study showed that p16, CDK₆ and CCND₁ are involved in the process of tumorigenesis in human BCC. p16, CDK₆ and CCND₁ mRNA genes are expressed to induce cell cycle proliferation and also the protein expression of these genes can influence proliferation of the cell cycle. RT *in situ* PCR study on ten Iranian samples illustrated significant expression of p16 ($p = 0.026$), CDK₆ ($p = 0.015$) and CCND₁ ($p = 0.021$) mRNA genes ($p < 0.05\%$). There is a direct correlation between p16 and CCND₁ and also between p16 and CDK₆. There is no correlation between CCND₁ and CDK₆. IHC analysis on the Iranian samples demonstrated significant protein expression of p16 ($p = 0.019$) and CCND₁ ($p = 0.021$) ($p < 0.05\%$) but CDK₆ protein expression is not significant ($p = 0.082$). Direct correlation between p16 and CCND₁ was obtained. TMA



samples were used for the IHC study only for p16 ($p=0.008$) and CCND₁ ($p=0.024$) due to insufficient tissue to perform complete study with IHC. Even CDK₆ could not be done because of insufficient samples then the result of TMA samples using IHC supports the findings on the Iranian samples about protein expression of CCND₁ and p16 genes. RT *in situ* PCR is a sensitive method to study specific mRNA genes. However there are problems in getting good results as well as their interpretation. IHC on the other hand shows more reliable results. These methods may be used in the clinical setting and as it can be used to predict tumor behavior including cellular proliferation which can affect the mode of therapy.



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

EKSPRESI CCND₁, p16 DAN CDK₆ PADA BARAH TISU BASAL MANUSIA

Oleh

SIMA ATAOLAH ESHKOOR

Disember 2007

Pengerusi: Profesor Madya Patimah bt Ismail, PhD

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Kanser sel asas (*basal cell carcinoma*, BCC) adalah kanser kulit yang paling biasa berbanding kanser-kanser kulit yang lain. Kes-kes penyakit cutaneous malignant melanoma (CMM) dan non-melanoma kanser kulit (MSC) di serantau dunia telah meningkat sebanyak 600% dari tahun 1940. Karsinogenesis adalah satu proses pelbagai langkah yang meliputi pelbagai perubahan kandungan genetik. Hubungan di antara kitaran sel dan penyakit kanser menjadi begitu rapat jika mengambil pertimbangan bahawa perkembangan dan pergandaan sel-sel yang tidak normal merupakan tanda yang mustamat bagi penyakit kanser. Penyakit kanser telah dihubungkan dengan gangguan dalam proses regulasi kitaran sel. Kajian yang terbaru menunjukkan bahawa gen-gen mRNA p16, CDK₆ dan CCND₁ dan pernyataan protein gen-gen tersebut terlibat dalam tumorigenesis penyakit kanser. Gen-gen tersebut memainkan peranan dalam perkembangan kitaran sel. Hasil kajian kami telah menunjukkan pernyataan cyclin, satu cyclin dependent kinase, dan satu cyclin kinase inhibitor yang terdapat pada basal sel



kulit kanser dengan menggunakan blok tisu. Transkripsi Terbalik Tindakbalas Rantaian Polimerase (RT *in situ* PCR) dan immunohistochemistri (IHC) telah kami gunakan untuk mengesan ekspresi gen-gen mRNA p16, CDK₆ dan CCND₁ serta pernyataan protein dalam tisu kulit kanser sel basal manusia.

Hasil kajian kami juga telah menunjukkan bahawa gen-gen mRNA p16, CDK₆ dan CCND₁ serta protein di ekspres dalam kedua-dua tisu yang sihat serta tisu kanser kulit sel basal manusia. Keputusan kami juga menunjukkan ekspresi gen-gen mRNA CCND₁, CDK₆ dan p16 banyak didapati pada sitoplasma sel. Walau bagaimanapun, mRNA tersebut di ekspres secara berlebihan dengan intensi yang lebih tinggi pada tisu kanser berbanding dengan tisu sihat. Pengekspresan protein jenis CCND₁ dan p16 dalam pelbagai tisu di Iran adalah lebih tinggi berbanding tisu normal kulit di mana gen p16 mRNA dan pengekspresan protein lebih kuat berbanding gen-gen lain. Data daripada RT *in situ* PCR dan analisis IHC menunjukkan pertambahan regulasi yang jelas bagi CCND₁ dan p16 mRNA serta protein-proteinnnya pada tisu basal barah berbanding dengan tisu kulit yang sihat ($p < 0.05\%$). Satu kajian yang dijalankan ke atas orang Iran telah menunjukkan pengekspresan protein CDK₆ tidak signifikan ($p > 0.05\%$) manakala pengekspresan mRNA adalah signifikan ($p < 0.05\%$). Tambahan pula, keputusan kajian yang dijalankan oleh IHC ke atas TMA (tissue microarray) menunjukkan keputusan yang signifikan bagi pengekspresan protein p16 dan CCND₁ genes ($p < 0.05\%$). Keputusan ini telah membuktikan bahawa ketidakpengawalaturan kitaran sel dalam fasa G₁ adalah kejadian yang kritikan semasa pembentukan BCC.

Sebagai kesimpulan, kajian ini menjelaskan bahawa p16, CDK₆ dan CCND₁ telah terlibat dalam proses permulaan tumor dalam BCC manusia. p16, CDK₆ dan gen CCND₁ mRNA diekspreskan untuk merangsangkan kitaran pembiakan sel dan ekspresan protein gen ini akan mempengaruhi pembiakan kitaran sel. Kajian RT *in situ* PCR yang dijalankan ke atas sepuluh orang Iran telah menunjukkan keputusan signifikan ekspresan p16 ($p=0.026$), CDK₆ ($p=0.015$) dan CCND₁ ($p=0.021$) mRNA genes ($p<0.05\%$). Terdapat satu korelasi terus di antara p16 dan CCND₁ dan di antara p16 dan CDK₆. di samping itu, tiada korelasi didapati di antara CCND₁ dan CDK₆. Analisis IHC yang dijalankan ke atas sample orang Iran menunjukkan keputusan yang signifikan ke atas ekspresi protein p16 ($p=0.019$) dan CCND₁ ($p=0.021$) ($p<0.05\%$) tetapi tidak signifikan bagi ekspresi protein CDK₆ ($p=0.082$). tambahan pula, terdapat korelasi terus antara p16 dan CCND₁. TMA hanya telah digunakan untuk menjalankan IHC bagi p16 ($p=0.008$) dan CCND₁ ($p=0.024$). Keputusan IHC dan TMA telah menyokong menemukan terhadap sample orang Iran tentang ekspresi protein gen-gen tersebut. RT *in situ* PCR adalah kaedah yang sensitive untuk mengkaji gen mRNA spesifik dan IHC menunjukkan ekspresi protein semasa pengawalaturan kitaran sel dan peningkatan kanser. Kaedah ini boleh digunakan untuk mengenalpasti sel metastatic tumoral dan meramal tanda-tanda awal dan memilih terapi yang terbaik untuk kanser.



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I certify that an Examination Committee has met on **date of viva** to conduct the final examination of **Sima Ataollahi Eshkoo** on her Master of Science entitled "Expression of CCND₁, p16 and CDK₆ in Human Basal Cell Carcinoma" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

SIMA ATAOLLAHI ESHKOOR

Date:



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LIST OF ABBREVIATIONS

AMV	alfalfa mosaic virus
bp	base pair
BSA	bovine serum albumin
BCC	basal cell carcinoma
BER	base excision repair
C	cytosine
cDNA	complementary deoxyribonucleic acid
CCND1	Official full name is Cyclin D ₁
CPD	cyclobutane pyrimidine dimer
CIS	carcinoma in situ
CKI	Cyclin kinase inhibitor
CMM	cutaneous malignant melanoma
DAB	3, 3'-diaminobenzidine
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DPX	DePex
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleic triphosphate
dTTP	deoxythymidine triphosphate



dUTP	deoxy-uridine-5' triphosphate
EDTA	ethylene diamminetetraacetate
<i>et al.</i>	<i>et alii</i>
ETOH	etanol
FISH	fluorescence in situ hybridization
G	guanine
HPV	Human papilloma virus
IHC	immunohistochemistry
ISH	<i>In situ</i> hybridization
H&E	Hematoxylin and Eosin
min	minute
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NER	nucleotide excision repair
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
pRb	protein retinoblastoma
RNA	ribonucleic acid
rt	room temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT <i>in situ</i> PCR	Reverse Transcriptase <i>in situ</i> Polymerase Chain Reaction
SCC	squamous cell carcinoma
SK	seborrhoeic keratosis



T	thymine
TBE	Tris-boric acid-disodium EDTA
TE	Tris EDTA
<i>Tfl</i>	<i>Thermus flavus</i>
Tris	Tris (hydromethyl) aminomethane
TSG	Tumor suppressor gene
UV	Ultraviolet



CHAPTER 1

INTRODUCTION

Human cytogenetics was born in 1956 with the discovery of 46 chromosomes in normal human cells. Three years after the structure of the DNA helix was elucidated cytogeneticist could do karyotype analysis since 1968. The first observation of human chromosomes was in the 1880s after which the full complement of the 46 human chromosomes were counted. However, soon after the number 46 was firmly established scientists readily applied the new cytogenetic technique to the investigation of phenotype-genotype correlations in humans and began to utilize useful information from naturally occurring chromosomal rearrangements (Barbara, 2002).

There are over 3,000 diseases known to be caused by a single gene defect and chromosomal anomalies are found in over 1 in 700 live births (Nora and Fraser,1989). In addition, research has demonstrated that genetic viability affects many aspects of health, ranging from heart disease to cancer (Evans, 1988; Ashby and Richardson, 1985). It has long been speculated that genetically determined variation in susceptibility may predispose some workers to occupational disease while others in the same environment seem to be unaffected (Hart and Brusick,1987; Puck and Waldren, 1987; Sorsa and Yager, 1987).

In addition, certain environmental agents are known to cause mutation in normal somatic cells which lead to cause diseases. Recognition of genetic factors in disease presents



new opportunities for detection, prevention, and treatment. Because of uncertainties about the exact nature of the relationship between genes and environment, genetic monitoring and screening of otherwise healthy populations remain problematic (Schulte and Halperin, 1987). The relationships between genes, mutations, and disease are becoming clearer with the development of molecular techniques that enhance both the quantitative and qualitative evaluation of mutation (Honard, 1988).

In many cases, mutagens are also carcinogens, so at high exposure levels, the most common manifestation of genetic damage is in the form of cancer (Ward, 1985). The damage will be resolved in one of three ways: cell death, successful DNA repair or viable mutation. It is difficult to establish the causal relationships between the mutation and cancer because of the long latency of human cancer (Nora and Fraser, 1989). The type of alteration produced by physical and chemical agents depends on the lesions induced in the DNA and, therefore, on the chemical structure of the genotoxic substance (Carrano and Natarajan, 1988).

Epidemiological, clinical, and biological studies have indicated that solar ultraviolet radiation is the major etiological agent in skin cancer development (Miller and Weinstock, 1994; Brash *et al.*, 1991). The common, wavelengths in the UVB (280–320 nm) region of the solar spectrum are absorbed by the skin, producing erythema, burns, and eventually skin cancer (Young, 1990; Parrish *et al.*, 1982). Studies using laboratory animals have shown that UVB radiation is very efficient in inducing skin cancer (De Gruijl, 1995; Urbach, 1978). In fact, some early investigators began to analyze the process, and they defined the concepts of tumor initiation and promotion as well as co-



carcinogenesis in operational terms (Friedelwald and Rous, 1944; BoutweHll, 1964). These sequential events were also found to occur in liver, urinary bladder, breast, cheek pouch, esophagus, colon, stomach, lung, and prostate (Walaszek *et al.*, 2004; Slaga *et al.*, 1995; Slaga, 1983). DNA damage together with the cellular response to that damage can establish genomic instability through multiple pathways (Limoli *et al.*, 1997).

In the present study RT *in situ* PCR and immunohistochemistry (IHC) was performed in order to determine CDK₆, p16, CCND₁ mRNA and protein expression in the human skin BCC. Positive and negative regulatory proteins control exit from or entry into the G₀ quiescent state. G₁ cyclin-dependent kinases (CDKs) serve as positive regulators. D-type cyclin (D₁, D₂, D₃) complex with CDK₄ and CDK₆ to stimulate their kinase activities, which in turn cause the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein. By binding to E₂F, Rb recruits histone deacetylases to the promoters of E₂F-responsive genes and represses their transcription (Harbour and Dean, 2000).

In this pathway p53 and pRb genes are involved in the control of G₁/S phase cell cycle progression and proliferation. Inactivation of these tumor suppressor genes (TSG) results in dysfunction of proteins that normally inhibit cell cycle progression. pRb is the most important CDK substrate during G₁ and it is frequently mutated in human cancers. Absence or loss of function of pRb is associated with unrestrained cell cycle progression (Vermeulen 2003).