

Original Article**Increased protein expression of p16 and cyclin D1 in squamous cell carcinoma tissues****Sima Ataollahi Eshkoo¹, Patimah Ismail^{1,*}, Sabariah Abdul Rahman², Mirsaed Mirinargesi¹, Soraya Ataollahi Oshkour³**¹ Department of Biomedical, Faculty of Medicine and Health Sciences. Universiti Putra Malaysia, Serdang, Selangor, Malaysia;² Department of Pathology, Faculty of Medicine and Health Sciences. Universiti Putra Malaysia, Serdang, Selangor, Malaysia;³ Shafa st. Hasan Makiabadi clinic, Sirjan, Iran.**Summary**

Abnormalities of Rb-pathway components are common in the formation of cancer. The immunostaining for cyclin D1 and p16 protein was applied on 1 mm serial tissue microarray (TMA) paraffin sections. Tissue microarray (TMAs) is potentially a good method to find the molecular features of the genes and expressions of them. The aim of this study was to evaluate the protein expressions of cyclin D1 and p16 genes in squamous cell carcinomas (SCCs) of skin and compare with the normal skin tissue. Twenty-five cases of cutaneous SCCs expressed cyclin D1 and p16 proteins. All SCCs samples on the slides showed positive protein expressions of cyclin D1 and p16 genes. Our findings suggested that the increased protein expressions of cyclin D1 and p16 genes might lead to aberrant expressions of these proteins in the affected tumor cells. This study demonstrated that cell cycle controlled deregulation and uncontrolled cell cycle progression might result in the carcinogenesis.

Keywords: p16, cyclin D1, retinoblastoma pathway, tissue microarray, squamous cell carcinoma

1. Introduction

D-type cyclins are the first cyclins expressed in the G1 phase of the cell cycle. Cyclin D1 (CCND1, PRAD1, bcl-1), cyclin D2, and cyclin D3 are three closely related D-type cyclins which are expressed in a cell lineage manner in all proliferating cells. Binding of cyclin D1 with cyclin-dependent kinase (CDK) 4 and CDK6 phosphorylates and inactivates the retinoblastoma protein (pRb). Deregulation of the G1 cyclins and CDKs expression might cause loss of the cell cycle control leading to tumorigenesis (1).

The p16 gene product is encoded by the ARF-INK4 locus. The p16 protein is a specific inhibitor of CDK/cyclin D complexes which prevent the phosphorylation of the pRb protein (2). It can arrest

the cells in G1 phase (2) and thus suppress cell proliferation (1). pRb, p16, and CDK/cyclin D are functionally linked together and the alterations of these cell cycle regulator components might have similar results. Moreover, changes of at least one of these regulators were found in nearly all human cancers (2).

Skin cancer is one of the most common human malignancies. The most common types of skin cancers include basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and malignant melanoma (MM). Chronic exposure to UV irradiation from sunlight is believed to be the cause of the majority of human skin carcinoma types like cutaneous SCCs and BCCs (3).

SCC is currently the second most common type of non-melanoma skin cancer. SCCs of the skin are associated with the risk of metastasis. Typically, SCC is a painless skin lesion that develops on sun-exposed regions of the body (4). Most SCCs occur on skins that have exposed to the sun such as head and neck, forearms, back of the hands, upper part of the torso, and lower legs (5). It may also arise in regions with scars

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from old burns or vaccinations (4).

Many of the SCC lesions develop in precancerous patches called actinic keratoses. SCCs are more common in the old adult population rather than the young. Overall, chance of developing SCCs is about 7-11% in the population (5). Incidence of SCC of the skin is rising worldwide for decades. The most important environmental risk factor for this type of skin cancer is chronic exposure to sunlight which induces the DNA damage (6).

SCC has become an increasing trouble for the general population and it is particularly life threatening in immuno-suppressed patients. The face, head, neck, back, hand, and forearm are preferential sites of chronic UV damage (7). Regarding the alarming rates and disease outcomes, it is more important than ever to understand the regulatory molecular genetic alterations which result in the skin cancer progression and finding correlation of these changes with modified cellular functions (3).

Finding the molecular markers in the tissue provides more information which are not detected by routine histopathology (8). The aim of this study was to evaluate the protein expression status of the cyclin D1 and p16 genes in the skin SCC and compare their expression in the normal skin tissue. It illustrated the molecular alterations which lead to the cell cycle proliferation and uncontrolled cell cycle progression.

2. Materials and Methods

2.1. Samples

Twenty-five spot samples of formalin-fixed, paraffin embedded AccuMax[®] TMA slides were obtained from twenty-five different patients from Korea (CAT#: A216; LOT#: 122120310121, Location: 73 and 74) who were diagnosed as SCC and 4 spot samples of normal skin tissues. Size of each spot is 1.0 mm in diameter. Immunohistochemistry (IHC) was performed on the slides using commercially available monoclonal anti-cyclin D1 antibody obtained from Research Biolabs (Danvers, USA) and anti-p16 polyclonal antibody named anti-p16INK4a from Lab vision (Fremont, CA, USA). Immunohistochemical staining was conducted using DAKO Envision TM system + HRP DAB + Rb /Mo Kit (DAKO Co., Carpinteria, CA, USA) according to the manufacturer's instructions.

Briefly, the slides were dewaxed by heating on hot plate at 60°C and then deparaffinised. The slides were heated in a microwave oven for 20 min in 10 mM citrate-Na (pH 6.0). After incubation with dual endogenous blocking enzyme for 10 min, sections were incubated with primary antibodies with dilution of 1:400 for anti-p16 and 1:300 for anti-cyclin D1 overnight at 4°C. After washing by TBS, the slides were incubated with polymer in the kit for 30 min followed by an

incubation with DAB+ substrate buffer for labelling for 10 min. After counterstaining with hematoxylin and mounting, the slides were viewed by light microscope (BX 51, New York, USA).

2.2. Scoring system

Semi-quantitative assay may improve standardization and reproducibility of the study in the laboratories as well as minimize the impact of many known inconsistencies (9). As the strength of reaction was variable, we graded the intensity of reaction on a numerical scale from + to +++, reflecting weak, moderate, and strong reactions. We also recorded the extent of reactivity in the target cell population as 1 to 4 scales indicating positive cytoplasmic and nucleus reaction in 1-25, 26-50, 51-75, and 76-100% of the cells. Evaluation of IHC staining slides was based on signal intensity and carried out using scoring system (10-12).

The total scores for every spot in slides were recorded as the sum of scores for percentage of positive cells and staining intensity. The intensity of reaction in the procedure varied from gene to gene. All slides were examined at least three times for presence of the genes.

2.3. Statistical analysis

The data of IHC were stored and analyzed by SPSS version 12 software (SPSS Inc, Chicago, IL, USA). The protein expression of p16 and cyclin D1 genes data were evaluated non-parametrically using Mann-Whitney test. Tests were considered significant when p values were less than 0.05 ($p < 0.05$).

3. Results

Samples were obtained from twenty-one SCC patients. In analysis, tissues were compared with control tissues to enable complete analysis. The all SCC tissue samples revealed increasing protein expressions of p16 and cyclin D1 genes. The immunohistochemical study showed that protein expression of cyclin D1 increased in the nuclei of tumoral cells as compared with normal skin tissues. The p16 protein was expressed in the nuclear and cytoplasmic areas of tumor cells.

In IHC method using the SCC tissues of human skin, cyclin D1 and p16 were significantly increased ($p = 0.004$ and $p = 0.019$, respectively). Correlation between protein expression of cyclin D1 and p16 genes in these samples was not shown, which suggested that these genes might work independently ($p = 0.931$). Depending on the genes, the staining was observed in the nucleus, cytoplasm or both. Staining intensity of cyclin D1 varied from +1 to +3, while that of p16 was from +2 to +3. Protein expression of cyclin D1 and p16 in SCC tissues were higher than those in normal



Figure 1. Immunostaining analysis showing nucleus and cytoplasm expression of p16 in TMA sample of normal skin tissue. The nuclei and cytoplasm of epidermal cells show brown staining at moderate intensity (+2). Magnification, 40 \times .

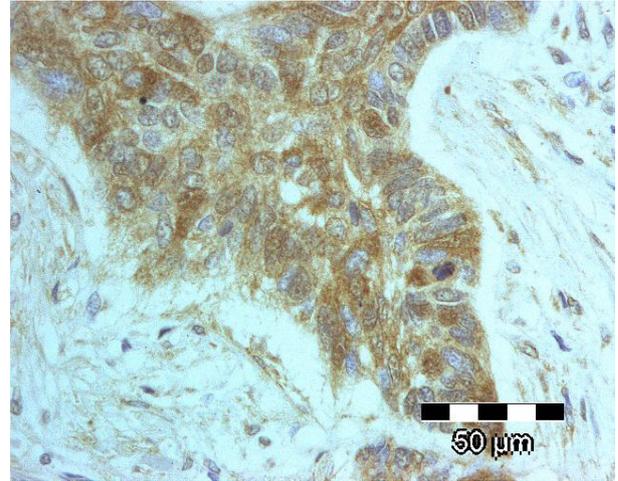


Figure 2. Immunohistochemical study for p16 on SCC of TMA sample. Strong brown staining intensity (+3) is shown in the nuclei and cytoplasm of the cells in SCC of skin. Magnification, 40 \times .

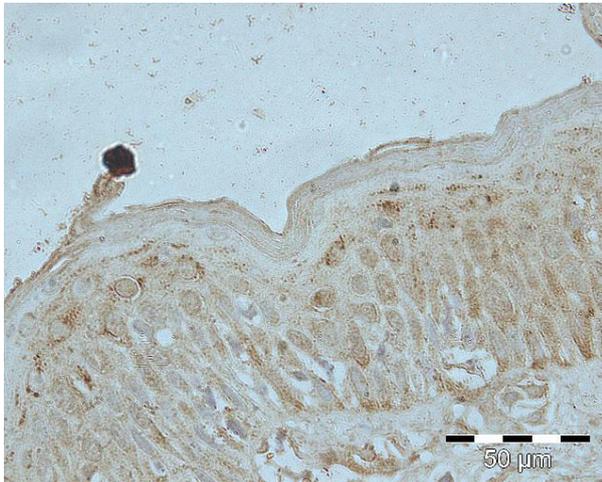


Figure 3. Immunostaining analysis of CCND₁ on normal skin tissue. Mild brown staining (+1) is illustrated in nuclei of normal epidermal cells. Magnification, 40 \times .

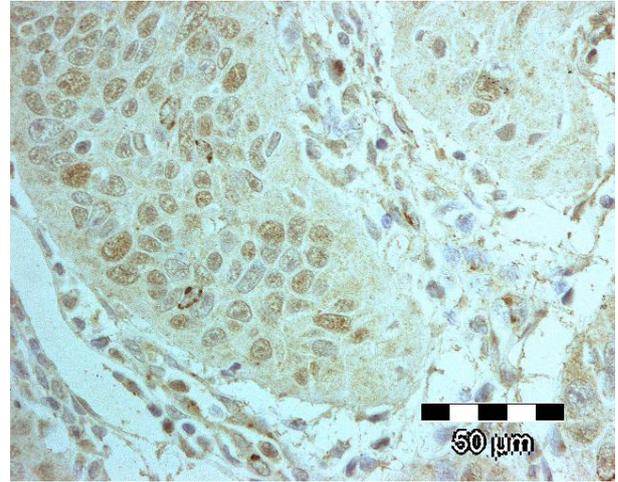


Figure 4. Immunohistochemical study on TMA sample of SCC tested for CCND₁. Moderate brown staining is shown in nuclei of the cells (+2). Magnification, 40 \times .

skin tissues.

IHC analysis demonstrated the protein expressions of p16 and cyclin D1 in both SCC and normal skin tissues. Figures 1 and 3 showed the protein expressions of p16 and cyclin D1 genes, respectively, in the normal skin tissues. Figures 2 and 4 showed the protein expressions of p16 and cyclin D1 genes, respectively, in the SCC tissues. Compared with the normal skin tissue, the protein expressions of p16 and cyclin D1 in the cells of malignant tissues were significantly higher.

4. Discussion

TMA is used like whole tissue sections for immunohistochemical studies of the human SCC tissues. Two SCC cores are representative of whole tissue of one case in assessing expression patterns of proteins for cyclin

D1 and p16 in this study. P16 and cyclin D1 proteins were expressed in all SCC samples which demonstrated the high sensitivity and specificity of this approach.

The present study assessed protein expression of these genes in SCCs and explained their different expressions in the malignant and normal human skin tissues. P16 and cyclin D1 genes are important in controlling cell proliferations in the normal and malignant tissues. The current study suggested that the changes of cyclin D1 gene altered the cell cycle proliferation. The high level of cyclin D1 protein in SCCs might result in the development of cancerous in skin tissue (13,14).

Over-expression of cyclin D1 in tumoral skin lesions in the other studies revealed the relevance and pathogenesis of this gene in skin carcinogenesis. Recent studies have provided evidence suggesting that

disruption of cyclin function might play a critical role in the tumorigenesis (7,14-19).

Meanwhile, the present study demonstrated the expression of p16 gene in the SCCs and normal skin tissues. P16 is an important gene in the cells of normal and tumoral tissues in the proliferation process. P16 protein expression in the SCCs was higher level of staining intensity presentation rather than the normal skin tissues (17,18,20) unlike the expected low intensity of staining which has been noted in the other cancers (21-23).

P16 gene might function as general regulator which mediates the switch between high proliferation and low proliferation in the cells (23). The acquired results from the current study like the other studies supported the role of p16 gene as a factor causing tumors (2,23-25).

The results showed the protein expression of these genes was detectable in all SCC tissue samples but the intensity is different from tissue to tissue. The detection of p16 and cyclin D1 genes has thus considerable potential as an assay to detect occult metastatic tumor cells from SCC and other neoplasms. p16 and cyclin D1 are not markers of SCCs but they can predict the outcome of management of the disease. Gene therapy can potentially control cell proliferation which is the essential process in cancer development.

Our results and those from other authors support the argument of demonstrating enhanced signals for multiple genes in malignant tissue and identifying the presence of small numbers of infiltrative deposits of SCC cells in the tissues. Taken together, the p16INK4 plays an important role in the skin carcinogenesis. The highly up-regulation of p16INK4 mediates growth control which likely contributes to the benign behavior of the tumor type. In neoplasm, the high level of p16 gene seems to activate the spontaneous regression process though the exact mechanism of this regulatory function needs to be determined. On the other hand, the loss of p16 gene represents a crucial step in the progression of SCC (7).

5. Conclusion

Our findings demonstrated the over-expression of cyclin D1 and p16 proteins in SCC tissues using IHC method. The over-expression of these genes probably induces the cell cycle proliferation which may result in the SCC in skin tissues. In conclusion, the present study provided a clear evident that the protein expression of cyclin D1 and p16 genes was highly detected in the SCCs as compared with normal skin tissues. Our results indicated functional genes in Rb pathway such as p16 and cyclin D1 which probably mediates the phenotype changes from normal proliferation to abnormal proliferation activity in the SCC cells.

The data implicated that cyclin D1 and p16 genes might be candidates for further evaluation as therapeutic

targets in this cancer. This project showed that IHC is a sensitive method to assess protein expression of the genes. IHC presents the structure of tissue well to detect the small number of cancerous cells. The current study demonstrated the value of IHC using TMA as pre-screening tool in selecting patients suitable for analysing the alteration of cyclin D1 and p16 genes in diagnostic and research settings, respectively.

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