

Gene Expression of CDK6 and CCND1 Genes in Basal Cell Carcinoma

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Abstract

Basal cell carcinoma (BCC) is the most common cancer among skin cancers. Cell cycle deregulation in G1-phase is a critical event during the course of carcinogenesis, which is probably much more important than other phases of cell cycle, during the course of skin carcinogenesis. CCND1 and CDK6 are important components of Retinoblastoma regulatory pathway in arrest and uncontrolled proliferation of cell cycle. To determine the expression pattern of CDK6, CCND1 in BCC, this study involved ten samples of paraffin embedded of BCC tissues. Two selected normal skin tissue were investigated using RT *in situ* PCR and Immunohistochemistry (IHC) techniques. Nuclear and cytoplasmic staining intensity of samples within tumor cells and normal tissue illustrated a different mRNA and protein expression. This study represents significant expression of CCND1 and CDK6 genes in BCC (alpha level is 0.05). CDK6 and CCND1 mRNA, and protein of these genes are expressed to induce the cell cycle proliferation and the influence proliferation of cell cycle and BCC.

Introduction

Induction of cancer is a multistage process and its stages have been defined as initiation, promotion, and progression. Carcinogenesis factors, which are inherited and acquired factors, related to exposure for initiation factors like exogenous and endogenous carcinogens, and also promotion and progression factors induce cancer process. The prevention of cancer is concerned with the process to reduce the occurrence of *in situ* or invasive cancers with intervention at earlier stages or cancer morbidity and mortality with intervention or at later stages of cancer (Zbigniew *et al.*, 2005). Genetic damage is a result of endogenous factors or environmental insults. Measurements of an individual's mutation in normal tissues identify individuals with high mutation level and increased disease risk, including cancer (Schlake *et al.*, 2003).

Squamous cell carcinoma (SCC), Basal cell carcinoma (BCC) and Cutaneous malignant melanoma (CMM) are the three most common types of cancer arising on sun exposed skin (Giannetti *et al.*, 2004). Many BCC have high proliferative activity, as well as distinct areas with infiltrative tumor growth, making BCC a suitable model system for studies of invasion and proliferation control (Svensson *et al.*, 2003). BCC, which is one of the most common cancers worldwide, is a slowly growing tumor, only occurring in hair growing squamous epithelium. BCC arise by the transformation of basal stem cells located in hair follicles or basal epidermis. There is no known precursors and rarely metastasizes. BCCs can develop in both a hereditary and a sporadic setting (Jemal *et al.*, 2001; Kricer *et al.*, 1995).

The incidence of BCC is increasing worldwide by up to 10% per year (Wong *et al.*, 2003). Several clinical and histological subtypes of BCC may exhibit different patterns of behavior. Aggressive therapy is often necessary for variants such as micronodular, infiltrating, or morphea form BCC (Ramsey, 2006). The most common BCC is nodular variant (Usatine *et al.*, 2003). Various morphologic subtypes of BCC include solid or nodular, micronodular, cystic, superficial, pigmented, adenoid, infiltrating, sclerosing, keratotic, infundibulocystic, metatypical, basosquamous, and fibroepitheliomatous types (Jiang, 2005).

In cancer, basic alterations are in genetic control of cell division, resulting uncontrolled cell proliferation. Mutations mainly occur in two classes of genes: proto-oncogenes and tumor suppressor genes. Cell cycle deregulation associated with cancer occurs through mutation of genes important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding CDK, Cyclins, CDK-activating enzymes, CKI, CDK substrates, and checkpoint proteins (Vermeulen *et al.*, 2003). p16-CDK/Cyclin D pathway has a main role in cell cycle progression. Protein complexes of D-type cyclins and CDKs induce the phosphorylation of pRb to promote the G1-S phase transition. The phosphorylated pRb releases transcriptional factors such as E2F, which activate the expression of genes which is essential for S-phase entry. CDK inhibitor proteins, including p16, play a critical role in G1-S cell cycle transition by inhibiting cyclin D-CDK4/6-mediated pRb phosphorylation. Alterations in any component of the pathway, such as amplification and over-expression of CDKs or D cyclins or mutations to CDKs result in pRb phosphorylation and subsequent progression of G1 into S-phase and leads to G1-S-phase transition to induce cancer (Omura Minamisawa *et al.*, 2001).

CDK6 and CCND1 are important genes in controlling cell proliferation in normal and cancer tissues. The current study result suggests that changes of CCND1 gene could lead to alteration of the cell cycle and cell proliferation. CCND1 has been demonstrated to be present at high frequency in BCC and leads to cell entering cell cycle proliferation resulting development of the cancerous in BCC (Lu *et al.*, 1999; Kim *et al.*, 2002). The findings of experiments on skin lesions showed that CCND1 over-expression is important in premalignant skin lesions and enhanced skin carcinogenesis (Yamamoto, 2002). CDK6 gene locus at chromosome 7 plays an essential regulatory role in the G1 phase of cell cycle (Tang *et al.*, 1999).

CDK6 and CCND1 cell cycle regulators may play an important role in tumor growth and transformation (Tang *et al.*, 1999). CDK/Cyclin D are functionally interconnected in Rb pathway. Alteration of any of cell cycle regulators in this pathway is likely to have similar consequences. Alterations of at least one of these regulators are found in nearly all human cancers (Vermeulen *et al.*, 2003). CDK6 and CCND1 are not markers of BCC but they can predict the outcome of management of the disease. Gene therapy potentially can control cell proliferation which is the essential process in cancer development.

RT *in situ* PCR and IHC have been used to demonstrate the markers of specific cell and tumor types. There is an abundance of information in the literature on molecular basis of tumors and this is being used with increasing frequency to identify underlying molecular changes or presence of specific molecular markers in tumors, both are contributory to the process of making accurate diagnosis. Acquisition of this knowledge leads to an appropriate choice of therapy which will lead to a favorable outcome for the patient in overcoming the disease (Gown *et al.*, 2002).

The main objective of this study is to determine the levels of expression and activity of CDK6 and CCND1 in samples obtained from normal skin tissue and malignant BCC. The results demonstrated that the differential CDK6 and CCND1 mRNA and protein expression between BCC and normal skin tissue using both RT *in situ* PCR and IHC methods.

Material and Method

Tissue section preparation

Tissues, which were from different parts of the body of ten patients who were diagnosed to have BCC, and were surgically excised adequately and sent for processing to the Shafie Pathology Laboratory, Sirjan, Iran. The tissues were subjected to a standard laboratory processing technique and subsequently stained with Hematoxylin and Eosin. These sections were reviewed and diagnosed by a consultant pathologist in Iran. These sections, which were paraffin embedded, were further processed for RT *in situ* PCR and immunohistochemical studies on CCND1 and CDK6 genes.

RT *in situ* PCR

The sections prepared for this study was 4 µm thick. Firstly, the slides were deparaffinized and then treated with 50 µl of proteinase K digestion of proteinase K (5 µg/ml) for 10 min at 37°C. Slides were treated with RNase free DNase 50 µl (20 U/tissue section) at 37°C overnight in a humid chamber.

Oligonucleotide primer designing

Oligonucleotide primer sequences were designed using primer3 published by Rozen and Skaletsky (2000). Source code is available at: <http://primer3.sourceforge.net>

The primers are following:

CDK6

Sense 5'-AGACCCAAGAAGCAGTGTGG-3'

Anti sense 5'-AAGGAGCAAGAGCATTTCAGC-3'

CCND1

Sense 5'-AACAGAAGTGCGAGGAGGAG-3'

Anti sense 5'-TGAGGCGGTAGTAGGACAGG-3'

One step RT in situ PCR

RT-PCR kit was obtained from promega (Madison, USA). The slides were loaded with 50 µl of reverse transcription reaction mixture solution which contained 1 mM MgSO₄ 2 µl, Nuclease free water 30 µl, 1 X AMV/*Tfl* 5X Reaction buffer 10 µl, 0.2 mM dNTP 2 µl, 2% BSA 1.5 µl, 1 µM forward primer 1 µl, 1 µM Reverse primer 1 µl, 125 nM Digoxigenin-11-dUTP 0.5 µl from Roche (Indianapolis, USA), 0.1 units/ µl AMV Reverse Transcriptase (5 u/µl) 1 µl, 0.1 units/ µl *Tfl* DNA Polymerase (5 u/µl) 1 µl. The slides were covered with autoclave stable plastic cover slips and put into an Eppendorf Thermal Cycler System (Westbury, USA). The cover slips were then removed and the slides washed in DEPC water three times before proceeding to *in situ* PCR. The PCR was performed followed by 35 cycles with an initial denaturation at 94°C for 2 min, Reverse Transcription 45°C for 45 min, denaturation 94°C for 45s, annealing at 53°C for 40s, elongation 68°C for 1 min and final elongation at 68°C for 4 min (Eppendorf Thermal Cycler System, Westbury, USA).

Immunodetection of PCR products

The digoxigenin-labeled PCR products were detected after incubation with 50 µl of anti-digoxigenin-11-dUTP-gold diluted (1:30) in PBS and BSA(1 mg BSA/1 ml PBS) for 30 min at Room temperature (Rt) in dark humid chamber and the slides were treated with 50 µl DAB chromogen solution at Rt in dark humid chamber. Sections were kept in darkness and monitored at intervals until color development (brown) was visible. After that the slides counter stained was done with hematoxylin and the slides were mounted and viewed under light microscope (BX 51, New York, USA) and analysis of image was carried out.

Both positive and negative controls were prepared to assess the result of this study. Four negative controls include (1) omission of the AMV/*Tfl* DNA polymerase; (2) omission of the primer respectively CDK6 and CCND1 (3) omission of digoxigenin-11-dUTP 4) omission of anti-digoxigenin-11-dUTP-gold and positive control is omission of DNase treatment step.

Immunohistochemistry (IHC)

IHC studies were performed on 4 µm thick of tissue sections mounted on silanized slides using commercially available monoclonal antibodies: Anti-CCND1 and anti-CDK6 obtained from Research Biolabs (Danvers, 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 for over night at 4°C with an antibody dilution of 1:10 for anti-CDK6 antibody and 1: 300 for anti-CCND1 antibody. After washing by Tris buffered saline (TBS) the slides were incubated with polymer in the kit for 30 min and for labeling the slides were incubated with DAB + substrate buffer in the kit for 10 min. After counter staining with hematoxylin and mounting, the slides were viewed under light microscope (BX 51, New York, USA).

Scoring system

IHC and RT *in situ* PCR interpretations are qualitative but it is semi-quantitative, it minimized the impact of many known inconsistencies among laboratories with regard to reagents and methods. It was recognized as the semi-quantitative assay, it may improve intra and inter-laboratories standardization and reproducibility of the study (Sompuram *et al.*, 2002).

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 within target cell population on 1 to 4 scales indicating convincingly positive cytoplasmic and nucleus reaction in 1_25, 26_50, 51_75, and 76_100% of cells. The evaluation of RT *in situ* PCR (Sanno *et al.*, 1997; Guo *et al.*, 2003) and IHC (Argani *et al.*, 2001, Franklin *et al.*, 2005 and Sanno *et al.*, 1997) staining slides were based on signal intensity and it was carried out by using scoring system. The total scoring for every single spot in slides counted as the sum of score for percentage of positive cells and score for staining intensity. The intensity of reaction in every procedure is varied from gene to gene (Table 1).

Statistical Analysis

The data of IHC and RT *in situ* PCR were stored and analyzed by means of SPSS version 12 software (SPSS Inc, Chicago, IL, USA). Relationship between expression of CDK6 and CCND1 genes was evaluated non-parametrically using Mann-Whitney test. Tests were considered significant when the alpha level was 0.05.

Results

Site of reaction product

Paraffin embedded tissue of BCC samples obtained from ten patients revealed the increasing of CDK6 and CCND1 mRNA expression in RT *in situ* PCR method. IHC analysis showed good intensity of an increased expression of CCND1 protein while the staining intensity for CDK6 appeared weakly. There were nuclei showing visible expression of CDK6 and CCND1 protein. The immunohistochemical study represented an increasing protein expression of CDK6 and CCND1 in nuclei of tumoral cells as compared with normal skin tissue. All slides were tested at least three times for presence of both CDK6 and CCND1 genes on the different slides.

Depending on the gene, the staining reaction in IHC was seen in the nucleus. CDK6 was mildly expressed in IHC and the staining intensity is +1. CCND1 shows moderate to strong staining intensity (Table 1). Staining intensity of CCND1 gene varied between +2 to +3 and also the expression of CCND1 gene was significant ($p=0.028$). Protein expression of CCND1 in BCC is more than normal skin tissue. In normal tissue these genes are expressed in normal basal cell layer of the epidermis and follicular epithelium.

In RT *in situ* PCR all slides were tested by standard one step RT *in situ* PCR. In RT *in situ* PCR CCND1 ($p=0.021$) and CDK6 ($p=0.015$) genes illustrated a significant expression. In IHC the expression of CCND1 protein ($p= 0.028$) was significant while there is not significant expression of CDK6 in this study ($p= 0.815$). There was no correlation between CCND1 and CDK6 genes in both methods IHC ($p= 0.706$ and $r= 0.122$) and RT *in situ* PCR ($p= 0.107$ and $r=0.489$). It suggests that these genes may work independently. The CDK6 gene expression in RT *in situ* PCR procedure varied between +2 and +3. A positive reaction was identified by the presence of visible specific PCR products observed during microscopic examination of the tissue sections.

The intensity of differential mRNA expression varied from gene to gene and predominantly present in the cytoplasm. All slides of BCC tissue sections showed brown cytoplasmic staining for CDK6 and CCND1 mRNA expression and varied at moderate to strong intensity (Table 1).

Table 1: staining intensity in different groups of genes according to protein expression level using IHC in skin BCC samples.

Genes		Intensity level				Total
		0	+1	+2	+3	
CCND1	IHC	0	0	5	5	10
	RT <i>in situ</i> PCR	0	0	3	7	10
CDK6	IHC	0	10	0	0	10
	RT <i>in situ</i> PCR	0	0	4	6	10

Much lower expression of CDK6 and CCND1 mRNA was observed in normal tissue as compared with BCC tissue. In RT *in situ* PCR, the negative control preparations of gene expression were not observed while in positive control high nuclear intensity was observed in more than 50% of cells based on average evaluation of five fields at 10X magnification from tested slide.

Hematoxylin and Eusin (H&E) section staining of BCC illustrates the cells which have scant cytoplasm, vesicular nuclei and small nucleoli. Some cells show hyperchromatism with increased staining nuclear intensity. There is an increased nuclear-cytoplasmic ratio and nuclei are markedly enlarged. The maturation of cytoplasm is decreased and mitotic activity increase (Fig 1).

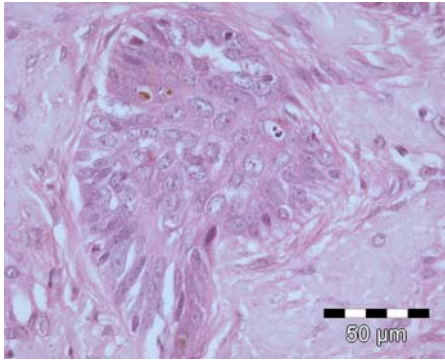


Fig 1: H&E staining of this section at magnification 40X shows morphological and cellular changes of tissue due to the effect of basal cell carcinoma (BCC).

CDK6 (A) and CCND1 (C) proteins are visible in normal tissue but CDK6 (B) and CCND1 (D) proteins in BCC tissue which are seen as brown staining of the involved cells demonstrating the protein expression. CDK6 in the tissue in IHC method showed a lack of good intensity of the brown color but number of stained cells in malignant (B) tissue is more than that noted in normal (A). CCND1 protein staining in malignant tissue is more than normal tissue (Fig 2).

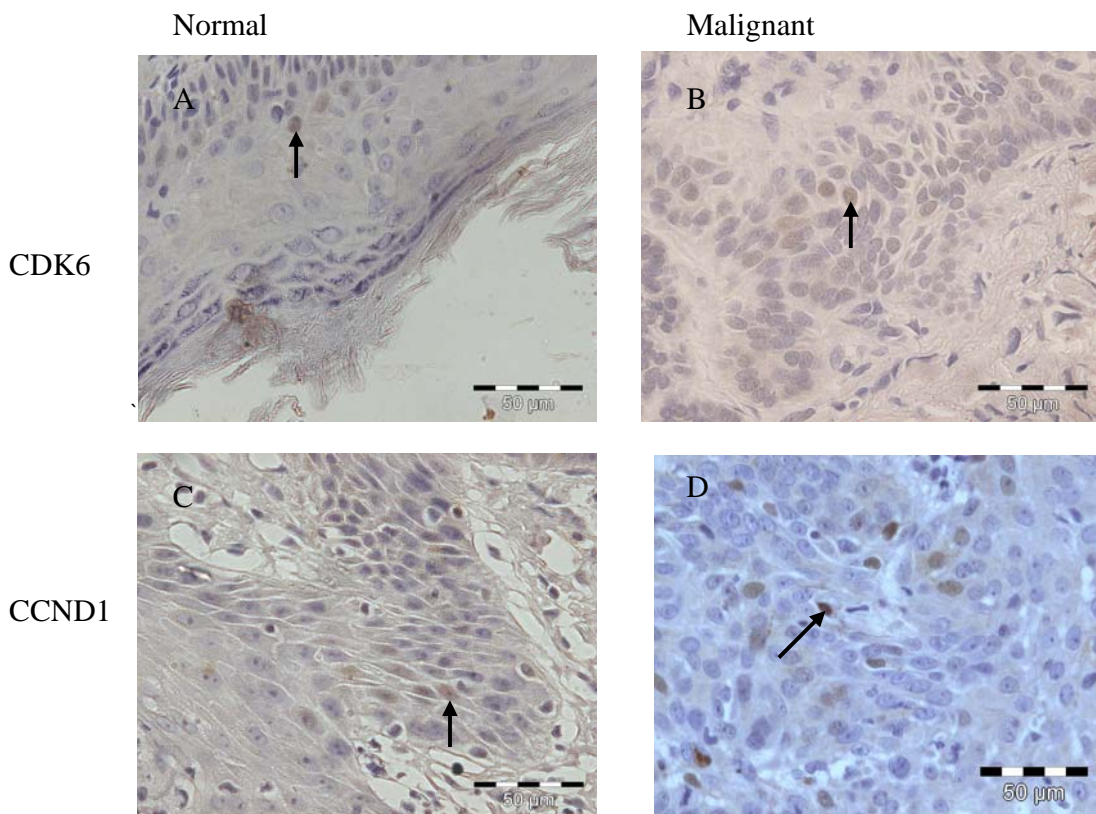


Fig 2: Immunostaining analysis shows the expression of CDK6 and CCND1 in normal tissue and Basal cell carcinoma (BCC) at magnification of 40X. There is more intense staining of the nuclei which represents the more expression of CDK6 (B) and CCND1 (D) protein in BCC compared to normal skin tissue (arrow).

RT *in situ* PCR sections show stronger cytoplasmic staining in malignant tissue compared to normal tissue. CDK6 (B) and CCND1 (D) demonstrate more intense staining in malignant sections compared to normal skin tissue section of CDK6 (A) and CCND1 (C) mRNA expression (Fig 3).

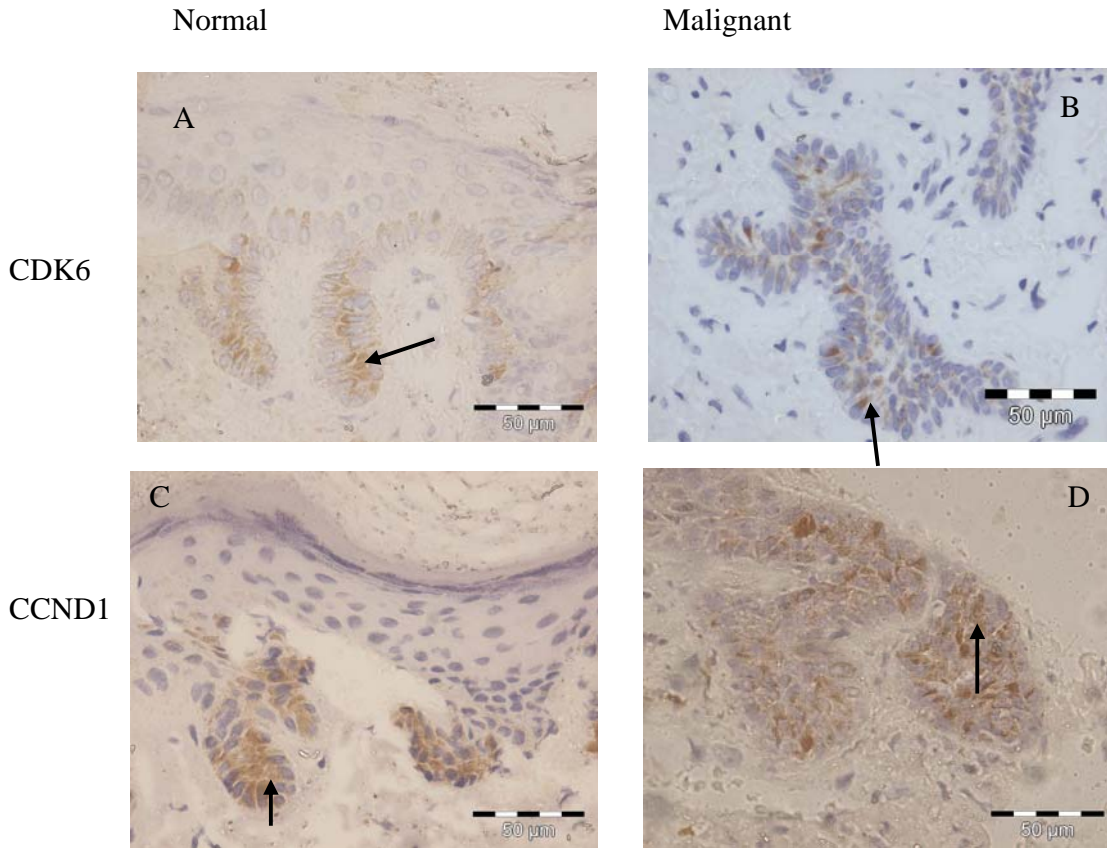


Fig 3: RT *in situ* PCR study on normal and basal cell carcinoma (BCC) tissue at magnification of 40X represents different cytoplasmic mRNA expression of CDK6 and CCND1 (arrow in B and D). The intensity of staining in malignant tissue is more than normal skin tissue (A and C).

RT *in situ* PCR in positive control (F) with omission of DNase treatment shows mostly nuclear staining in tested slide. Negative control (E) with omission of primer shows no stains in the tested tissue (Fig 4).

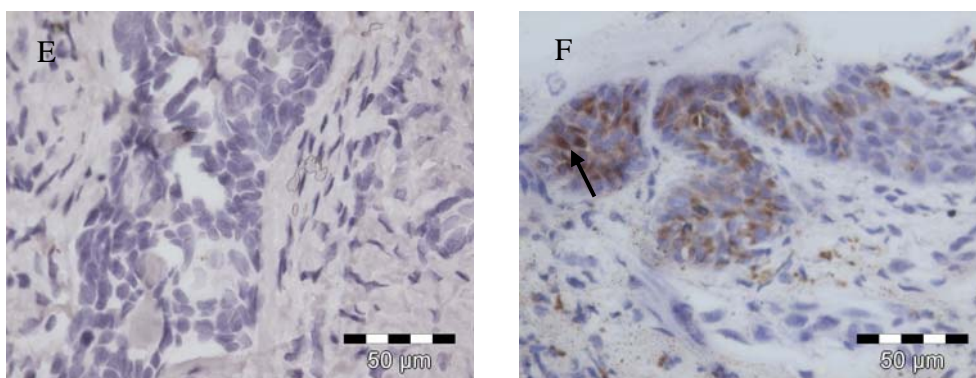


Fig 4: Photomicrograph at magnification 40X showing negative control (E) and positive control (F). The positive control illustrates much more nuclear staining.

Discussion

IHC and RT *in situ* PCR were used to identify minute number of tumor cells in BCC tissue sections. It is essential to know that retrieval of antigen and the pH required for IHC are essential preliminary steps in this process in order to get good results. In RT *in situ* PCR susceptibility to the digesting enzymes vary from tissue to tissue and hence their morphological appearances may also be different. It is essential to know the type and concentration of fixative employed, duration of tissue exposure to it and also size and thickness of tissue section. However determination of optimum incubation time in every step of IHC is an important step which will determine the outcome of the reaction. In RT *in situ* PCR protease digestion time and DNase treatment are crucial steps in producing reliable results.

Deregulation of cell cycle G1-restriction point control via abnormalities of Rb-pathway components is a frequent event in formation of cancer (Utikal *et al.*, 2005). CDK6 and CCND1 in Rb-pathway play as important genes in controlling cell proliferation in normal and cancer tissues. The present study was undertaken to assess expression of some genes in BCC and explain location of CDK6 and CCND1 genes of Rb-pathway in BCC and normal skin tissue. The current study suggests that changes of CCND1 gene could lead to alteration of the cell cycle and cell proliferation activity.

CCND1 has been demonstrated to be present at high frequency in BCC and leads to cell entering cell cycle proliferation resulting epidermal hyperplasia (Sauter *et al.*, 1999; Xu *et al.*, 2003) or development of cancerous in BCC (Lu *et al.*, 1999; Kim *et al.*, 2002). CCND1 over-expression in tumoral lesions shows the relevance and pathogenesis of CCND1 gene in the skin malignant lesions which is important to take a growth advantage (Kim *et al.*, 2002; Bianchi *et al.*, 1993).

Recent studies have provided evidence suggesting that disruption of cyclin function may play a critical role in tumorigenesis (Bianchi *et al.*, 1993; Yamamoto, 2002). It suggests that decreasing of expression may lead to cell death (Sauter *et al.*, 1999). Over-expression of CCND1 has been reported in various human malignant tumors, including esophageal cancers, breast cancers, hepatocellular carcinomas, mantle cell lymphoma (Sauter *et al.*, 1999; Xu *et al.*, 2003) and melanoma (Sauter *et al.*, 2002). CDK6 mRNA in RT *in situ* PCR procedure has been shown to be more in BCC compared to normal skin tissue and this study supports the finding of other authors (Tang *et al.*, 1999). The expression of CDK6 in IHC was so mild although the number of staining cells is high. Abnormality of these cell cycle regulators may play an important role in cancer growth and transformation (Tang *et al.*, 1999).

The amount of RT-PCR products by RT *in situ* PCR depends on kind of primers used in the reaction. Sometimes RT-PCR solution and IHC preparation process have resulted in suboptimal reaction tissue. The change in actual cellular composition is a major factor which affects signaling enhancement process; hence the mRNA and protein expressions can never be determined with certainty. The RT *in situ* PCR and IHC studies on tissue sections provide the opportunity to identify the individual cells for detection of any signaling present in the affected area. The techniques have great potentials in cancer diagnostic work as they have capacity for detecting cancer cells even though in small numbers. Hence these methods will be good for early cancer detection which is the trend in cancer management for optimal outcome.

The knowledge of presence or absence of genes in invasive groups of cancer cells will be one of the most powerful prognostic factors in cutaneous BCC. The observation of that some patients who have a positive RT *in situ* PCR or IHC in face of negative histology and immunohistology, allows prediction of increased risk for further disease progression. Such finding can certainly be interpreted as supporting the view that new approach is able to identify minute numbers of tumor cells which have the capacity to proliferate and even disseminate.

The results of this present study indicate that multiple genes approach is feasible with RT *in situ* PCR and IHC techniques. CDK6 and CCND1 genes were expressed by all different BCC samples which demonstrate the high sensitivity and specificity of these approaches. The *in situ* detection of multiple genes has a considerable potential as an assay to detect occult metastatic tumor cells from cancers of skin and other neoplasms. The results showed that the expression of genes was detectable in all BCC tissue but the intensity is different for different genes. Our result supports the argument of demonstrating enhanced signals for multiple genes in intact tissue and identifying the presence of small numbers of BCC cells in tumoral tissues.

Conclusion

The results of molecular tumor analysis will make it possible to accurately predict which strategies are applicable to a given tumor, and empirical choices of treatment are likely to be replaced by rational ones. This line of research, or a derivative one, holds promise for the development of new, clinically effective agents. Our findings demonstrated the overexpression of protein and mRNA of CDK6 and CCND1 in BCC tissue using IHC and RT *in situ* PCR methods.

In addition, there was an increased expression of CDK6 and CCND1 mRNA gene and protein as compared with normal human skin tissue which probably induce abnormal proliferation activity and may relate to BCC. In conclusion, the present study offers clear evidence that the expression of CDK6 and CCND1 mRNA and protein were more detected in BCC as compared with human normal skin tissue. These results suggest that the alteration of both CDK6 and CCND1 genes could lead to the abnormal proliferation activity and resulting in BCC.

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