RESEARCH ARTICLE

Lack of Increased P15INK4B Protein Expression in Basal Cell Carcinomas

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Abstract

Background: The basal cell carcinoma (BCC) is the most common non-melanoma skin cancer (NMSK). BCC might develop because of the faulty cell cycle arrest. P15INK4b is a tumor suppressor gene, involved in cell cycle arrest and inactivated in most human cancers. The role of p15INK4b protein expression in the genesis of BCC is as yet unknown. In a previous study we showed the absence of p15INK4b expression in the majority of tissue microarray cores of cutaneous squamous cell carcinoma (SCCs), another type of non-melanoma skin cancer, indicating that p15INK4b could possibly be involved in the pathogenesis of cutaneous SCC. The aim of this study was to investigate p15INK4b protein expression in BCCs. Materials and Method: Protein expression of p15INK4b in 35 cases of BCC tissue arrays and 19 cases of normal human skin tissue was studied using an immunohistochemical approach. Results: The expression of p15INK4b was not significantly different in the BCC cases as compared with normal human skin (p=0.356; p>0.05). In addition, there were no significant relationship between clinicopathologic variables of patients (age and sex) and p15INK4b protein expression. Conclusions: Our finding may indicate that p15INK4b protein expression does not play a role in the genesis of BCC.

Keywords: Basal cell carcinoma - p15INK4b - immunohistichemistry

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Introduction

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin are the most common malignancies in the white human population, accounting for greater than 95% of nonmelanoma skin cancers (NMSCs) (Miller et al., 1995; Urosevic and Dummer, 2002). BCC is the first most common skin malignancy in fair-skinned persons (Kwa et al., 1992; Miller et al., 1995; Dessinioti et al., 2011), and its incidence is increasing worldwide in recent decades (Asuquo et al., 2008; American Cancer Society, 2009; Skellett et al., 2012). It is estimated that more than 1 million cases of skin cancer will be diagnosed in the United States, 80% of which will be BCC, 16% SCC, and 4% melanoma (American Cancer Society, 2009). Also, in 2009, more than 1 million unreported cases of basal and squamous cell skin cancer are expected to be diagnosed (American Cancer Society, 2009). BCC is a slowly growing cancer and although its mortality is low, this cancer type is associated with substantial morbidity (American Cancer Society 2009; Nakamura et al., 2012). Mortality rates due to BCC are low, but its increasing incidence and prolonged morbidity means the disease is costly to treat (Matanosk et al., 1975).

BCC arises by transformation of basal stem cells located in the hair follicles or basal epidermis and it's only occurring in hair-growing squamous epithelium (Kwa et al., 1992; Dessinioti et al., 2011). It can progress either heredity or a sporadic form (Jemal et al., 2001; Buljan et al., 2008). BCC tends to be locally invasive but rarely metastasizes (Buljan et al., 2008). It develops on chronically sun-exposed skin in elderly people (Kricker et al., 1995; Quinn, 1997). Extensive exposure to UV irradiation from sunlight is the most important causal factor in BCC (Boukam, 2005; Takenouchi, 2006; Simić et al., 2011). Besides chronic UV radiation, other risk factors for the development of BCC include sun bed use, family history of skin cancer, a tendency to freckle in childhood, immunosuppression, previous radiotherapy, and chronic exposure to certain toxic substances such as inorganic arsenic (Friedman et al., 1991; Karagas et al., 1996). In BCC, the possibly modifying effects, such as latency, age when treated, and the type of treatment, are not well understood (Karagas et al., 1996).

BCC has been associated with UV-induced mutations of the patched (PTC) gene and p53 tumor suppressor gene, and to polymorphisms in the melanocortin-1 receptor gene and the xeroderma pigmentosum group D (XPD) gene

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(Hartwell and Kastan, 1994; Naylor et al., 1997). Previous study showed that the development of BCC might be due to defective cell cycle arrest (Hannon and Beach, 1994; Kamb et al., 1994; Naylor et al., 1997). Abnormalities of the cell cycle related to molecular over-expression of cyclins/CDKs and loss of tumor suppressor functions can cause various malignancies (Hanahan and Weinberg, 2000; Serrano et al., 2003).

The $p15^{INK4b}$ tumor suppressor gene is located on chromosome 9, the 9p21 region. $p15^{INK4b}$ is a kinase inhibitor gene that code for protein that functions to inhibit cell cycle transit (Li et al., 1995; Florenes et al., 1996; Sherr, 2001). $P15^{INK4b}$ regulatory pathway is involved in cell cycle arrest and inactivated in most human cancers (Li et al., 1995; Reynisdottir et al., 1995; Florenes et al., 1996). One important essential alteration in cell physiology that manifest growth is insensitivity to antigrowth signals such as $p15^{INK4b}$, retinoblastoma protein (pRB) and transforming growth factor β (TGF-β) receptors (Florenes et al., 1996). For example, in G1 phase, cyclin D bind to CDK4 or 6, and the resulting complexes act as effective growth inhibitory PRB (Reynisdottir et al.,1995).

$P15^{INK4b}$ protein binds to the CDK4- Cyclin D complex, displacing p27kip1, thus freeing p27kip1 to bind to and inhibit the CDK2- Cyclin E complex, which is required for entry into S phase of the cell cycle (Sandhuc et al., 1997). The $p15^{INK4b}$ acts as an effector of TGF-β mediated cell cycle arrest (Hartwell and Kastan., 1994). It is unregulated by TGF-β and inhibits the formation of activated CDK4 (Florenes et al., 1996; Naylor et al., 1997). The TGF-β responsive sequences have been identified in the $p15^{INK4b}$ promoter region (Li et al., 1995). The transcription of $p15^{INK4a}$ is induced in response to the growth inhibitory factor and TGF-β (Li et al., 1995; Reynisdottir et al., 1995; Florenes et al., 1996; Sandhuc et al., 1997).

In addition, interferon-α can induce $p15^{INK4b}$ expression in some hematopoietic cell lines (Sangfelt et al.,1997). A variety of cancers discard $p15^{INK4b}$ gene, which codes for a protein that, in response to signals from TGF-β, then shuts down the machinery that guides the cell through its growth cycle (Sangfelt et al., 1997).

In TGFβ-arrested epithelial cells, $p27^{kip1}$ competes with $p15^{INK4b}$ in binding to cyclin D1/CDK4 (Florenes et al., 1996). Upregulation and binding of $p15^{INK4b}$ to CDK4 serves to destabilize the association of $p27^{kip1}$ with Cyclin D1/CDK4 and promotes $p27^{kip1}$ binding to cyclin E/CDK2 (Robert, 1996). In TGFβ-treated epithelial cells, upregulation of $p15^{INK4b}$ protein and increased binding of $p15^{INK4b}$ to Cyclin D1/CDK4 occurs concomitant with the reduction of CDK4-associated $p27^{kip1}$ and the stabilization of the association of $p27^{kip1}$ with Cyclin E/CDK2 complexes (Moro et al., 2001).

Homozygous deletions of the $p15^{INK4b}$ gene have been detected in many primary malignant cells, including glioblastoma (Jen et al., 1994), leukemia (Hatta et al., 1995), and lung cancer (Okamoto et al., 1995). In addition, detected aberrant methylation of the $p15^{INK4b}$ gene is found in 28.1% of oral squamous cell carcinoma (OSCC) samples (Shintanis et al., 2001). Hypermethylation of the $p15^{INK4b}$ gene has also been found in the majority of non-small cell lung cancers (NSCLC) analyzed, as well as in a subset of pulmonary SCC and OSCC (Viswanathan et al., 2003; Wong et al., 2003; Furonaka et al., 2004).

In previous study, we have shown the absence of $p15^{INK4b}$ expression in the majority of tissue microarray cores of cutaneous SCC and this indicated that $p15^{INK4b}$ could possibly be involved in the pathogenesis of cutaneous SCC (Moad et al., 2009). Alteration of the $p15^{INK4b}$ gene has been reported in several tumour-derived cell lines and primary tumor, but the role played by this gene in the pathogenesis of BCC is not fully elucidated. The aim of this study was to look at $p15^{INK4b}$ protein BCC tissue samples, using immunohistological approach. This research may help towards better understanding of the role played by $p15^{INK4b}$ in the development BCC.

Materials and Methods

Tissue microarray

BCC tissue samples and normal human skin tissue microarray slides were purchased from Biomax (Rockville, USA) and AccuMax (Seodaemungu, Seoul, Korea). Thirty five BCC tissue cores and 19 normal human skin tissue cores were analyzed for the expression of $p15^{INK4b}$ protein.

Positive/negative control tissue samples

Colon carcinoma tissue was used as a positive control for $p15^{INK4b}$ expression [as recommended by the manufacturer (Abcam plc, Cambridge, UK)]. As for negative control, the primary antibody was omitted.

Immunohistochemical detection of p15 expression in Basal cell carcinoma tissue microarrays and normal human skin tissue

For the immunohistochemical detection of the p15, the antibody used was mouse monoclonal [15p06] to $p15^{INK4b}$ (Abcam). Briefly, before deparaffinization, the tissue microarray slides and control tissue sections were heated at 60ºC for 30 min in horizontal position before proceeding to the staining steps. The deparaffinization steps were followed by rehydration through graded alcohol to water. Endogenous peroxidase activity was blocked by incubating the sections in two changes of 3% H_2O_2 in phosphate buffered saline (PBS) (pH 7.4) at room temperature (RT). For antigen retrieval, 1 mM Tris-ethylenediaminetetraacetic acid buffer, pH 9.0 was first heated (near boiling). The slides were then immersed in the heated buffer and then microwaved for 15 min at 600 W. The slides in buffer were then left to cool using running tap water.

The Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, USA) was used as described by the manufacturer's instruction (Vectastain, Vector Laboratories, Burlingame, CA, USA). Briefly, the slides were rinsed with PBS for 5 min, and non-specific antibody binding was blocked by incubation of the tissue array slides with non-immune horse serum (normal horse serum) at RT for 20 min. A 200 μl of incubation with anti-$p15^{INK4b}$ antibody was at dilution 1/20 for 60 min at RT. Immunostaining was visualized using diaminobenzidine (DAB) (Zymed Laboratories Inc, South San Francisco,

USA).

Positive staining to p15^{INK4b} was recognized under light microscope as a brown color stain in the nucleus.

Assessment of p15 immunostaining

For each array, whole slide cores (1.5 mm diameter each core) were assessed either as positive or negative for p15^{INK4b} immunostain. The positive cores were graded as: weak (+) and strong (++) according to the intensity of staining. Assessment of staining pattern of p15^{INK4b} expression for all the cores was performed by a single independent pathologist, under X40 magnification.

Statistical analysis

After immunohistochemical analysis of all BCC and normal human skin, data were recorded and analyzed statistically. Statistical analysis was performed using the SPSS 10.01 software program. Data was evaluated by Fisher's exact test which was used to determine if there are any differences between the p15^{INK4b} expressions in BCC in comparison to normal skin. The relationship between the p15^{INK4b} expression in BCC and patient's age and sex were also analyzed.

Results

In the present study, the expression of p15^{INK4b} was successfully detected using Immunohistochemistry approach in 54 microarray tissue samples comparing of 35 basal cell carcinoma samples and 19 normal human skin tissue samples. Signals of p15^{INK4b} protein expression were predominantly detected in the nuclei of normal skin and BCC (Figure 1). Infrequent cytoplasmic expression was to a little extent accompanied by additional staining signals in the nuclei. The tissue microarray core details, age and sex subjects and intensity of the p15^{INK4b} protein expression in both BCC and normal skin samples are shown in Table 1. In the normal tissues, eleven samples (57.8%) from normal skin were found to be positive for p15^{INK4b} protein and eight samples (42.2%) of normal skin were negative. Eight cases of normal skin showed strong intensity staining and 3 cases showed weak staining intensity. In regard to BCC, eighteen samples (51.4%) were negative, while seventeen samples (48.6%) were positive (Table 2) (Figure 1A). However, the p15^{INK4b} expression was observed in 17 cases (48.6%); with, 12 cases (34.2%) demonstrated weak expression (weak staining intensity), 5 cases (14.2%) showed strong expression (strong intensity) (Figure 1B-D). There was no significant difference of p15^{INK4b} protein expression between BCC and normal skin ($p=0.356$ and $p>0.05$). A significant greater proportion of the BCC demonstrated weak staining. There was no significant relation to patient age or sex. Fisher's Exact test showed that there was no significant relationship between p15^{INK4b} expression and the type of tissues ($P=0.356$; $P>0.05$) (Table 3).

The mean age for male was 63.1 years and the mean age of female was 71.5 years. 13 cases (37.1%) of BCC are seen in patients of 60 years or below whereas 22 cases (62.9%) were in patients over 60 years of age. Fisher's Exact test showed that there was no significant relationship

Table 1. Tissue Microarray Core Details, Their Clinicopathological Features and the Expression of p15^{INK4b} Expression in Basal Cell Carcinoma Cases and Normal Skin Cases

No.	Age	Sex	Organ	Pathology Diagnosis	P15^{INK4b} expression
1	50	F	Skin	Basal cell carcinoma of ear	-
2	73	F	Skin	Basal cell carcinoma of left lower eyelid	+
3	67	M	Skin	Basal cell carcinoma of left orbit	-
4	76	M	Skin	Basal cell carcinoma	-
5	82	F	Skin	Basal cell carcinoma of left thigh	++
6	60	M	Skin	Basal cell carcinoma of left malar	++
7	62	F	Skin	Basal cell carcinoma of left lower eyelid	++
8	70	F	Skin	Basal cell carcinoma of right upper eyelid	++
9	44	M	Skin	Basal cell carcinoma of face	-
10	50	M	Skin	Basal cell carcinoma of nasal dorsum	+
11	55	M	Skin	Basal cell carcinoma of head	-
12	85	M	Skin	Basal cell carcinoma of face	-
13	87	M	Skin	Basal cell carcinoma	-
14	43	M	Skin	Basal cell carcinoma	+
15	83	M	Skin	Basal cell carcinoma	-
16	45	M	Skin	Basal cell carcinoma	+
17	59	M	Skin	Basal cell carcinoma	+
18	76	F	Skin	Basal cell carcinoma	-
19	81	M	Skin	Basal cell carcinoma	++
20	70	M	Skin	Basal cell carcinoma	+
21	76	M	Skin	Basal cell carcinoma	+
22	69	M	Skin	Basal cell carcinoma seborrheic keratosis	-
23	66	M	Skin	Basal cell carcinoma	-
24	62	M	Skin	Basal cell carcinoma	-
25	74	F	Skin	Basal cell carcinoma	+
26	82	F	Skin	Basal cell carcinoma	+
27	49	M	Skin	Basal cell carcinoma	-
28	41	M	Skin	Basal cell carcinoma	-
29	45	M	Skin	Basal cell carcinoma	-
30	49	M	Skin	Basal cell carcinoma	-
31	75	F	Skin	Basal cell carcinoma	-
32	73	M	Skin	Basal cell carcinoma	+
33	46	M	Skin	Basal cell carcinoma	-
34	72	M	Skin	Basal cell carcinoma	+
35	88	M	Skin	Basal cell carcinoma	+
36	50	F	Skin	Skin tissue	++
37	69	M	Skin	Skin tissue	-
38	1	M	Skin	Skin tissue	-
39	75	F	Skin	Skin tissue	++
40	75	F	Skin	Skin tissue of right hand	+
41	63	M	Skin	Skin tissue of chest	++
42	49	F	Skin	Skin tissue	++
43	49	F	Skin	Non-malignant normal skin tissue	-
44	75	M	Skin	Non-malignant normal skin tissue	-
45	42	F	Skin	Non-malignant normal skin tissue	++
46	50	F	Skin	Non-malignant normal skin tissue	-
47	58	F	Breast	Non-malignant normal skin tissue	-
48	58	F	Breast	Non-malignant normal skin tissue	-
49	35	M	Skin	Non-malignant normal skin tissue	+
50	22	M	Skin	Non-malignant normal skin tissue	-
51	45	M	Skin	Non-neoplastic normal skin	++
52	70	M	Skin	Non- neoplastic normal skin	++
53	45	M	Skin	Non- neoplastic normal skin	++
54	75	F	Skin	Non- neoplastic normal skin	+

+: weak stained Nucleus, ++: strong stained Nucleus, -: negative stain

Table 2. P15^{INK4b} Expression among 54 Tissue Microarray; 35 BCCs and 19 Normal Human Skin Tissue Microarray

Tissue array	Total Analyzable (n=54)	p15^{INK4b} Immunoreactivity* 0	1	2	P value
BCC†	35	18	12	5	0.356
Normal skin	19	8	3	8	

*Immunoreactivity of 0 indicates negative; 1, weak positive; 2, strong positive, †There was no significant difference in staining between BCC and normal skin. P=0.356; P<0.05 (Fisher's Exact test)

Table 3. Clinicopathologic Variables and p15^{INK4b} Expression among 35 BCC

Variable	Total Analyzable (n=35)	p15^{INK4b} Immunoreactivity* 0	1	2	P value
Age: ≤60	13	8	4	1	0.285
>60	22	10	8	4	
Sex: Male	26	15	9	2	0.315
Female	9	3	3	3	

*Immunoreactivity of 0 indicates negative; 1, weak positive; 2, strong

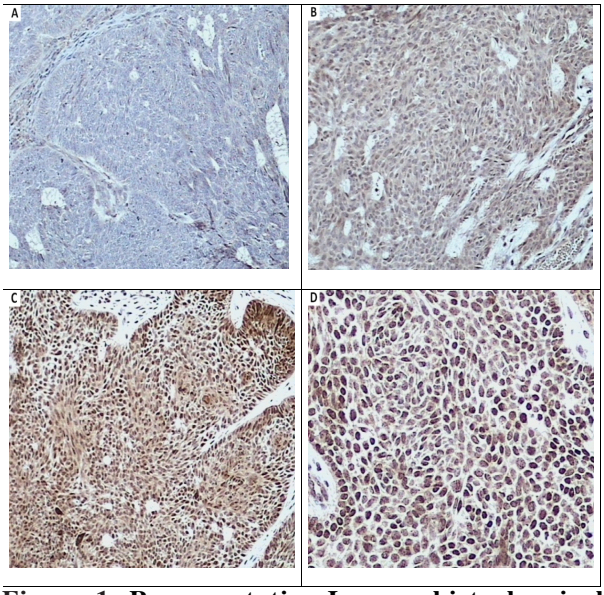

Figure 1. Representative Immunohistochemical Staining of p15^{INK4b} Protein Expression in BCC. A) Immunohistochemical staining of p15^{INK4b} shows an absence of p15^{INK4b} protein expression in the BCC tumour cells (Original magnification x 100). B) Higher magnification shows weak nuclear expression of p15^{INK4b} protein expression in the BCC tumour cells (Original magnification x 100). C) Immunohistochemical staining of p15^{INK4b} shows strong nuclear expression of p15^{INK4b} protein expression (Original magnification x 100). D) Higher magnification of C shows strong nuclear expression of p15^{INK4b} protein expression in the BCC tumour cells (Original magnification x 200)

between age of patients and p15^{INK4b} protein expression in BCC tissues (P=0.285; P>0.05). There were 26 male subjects (74.2%) and 9 female subjects (25.8%) with BCC. The expression of p15^{INK4b} was not significantly different between male and female in the BCC cases. Fisher's Exact test showed that there was no significant relationship between sex of patients and the expression of p15^{INK4b} protein in the BCC tissues (P=0.315; P>0.05).

Discussion

In the present study, the application of TMA technology allowed us to determine the protein expression pattern and clinical relevance of p15^{INK4b} in BCC. The present study showed almost similar results for the expression of p15^{INK4b} protein in microarrays of basal cell carcinoma samples and normal human skin samples. In addition, there was no significant relationship between patient's age and sex although more intense expression in nuclei was evident in some of tumors.

p15^{INK4b} gene is frequently inactivated in various human cancers (Jen et al., 1994; Hatta et al., 1995; Okamoto et al., 1995). However, previous study on breast cancer, showed that loss of p15^{INK4b} gene to be a rare event in primary breast cancer (Musgrove et al., 1995). Also, previous study on p15^{INK4b} gene in tongue sequamous cell carcinoma (SCC) showed that p15^{INK4b} protein was expressed in the majority of tongue SCC samples (Liu et al., 1998).

We have shown previously the absence of the expression of p15^{INK4b} protein in the majority of tissue microarray cores of cutaneous squamous cell carcinoma (Moad et al., 2009). The reasons for low p15^{INK4b} protein levels in cutaneous SCC are not known. To date, two mechanisms have been implicated as primary causes of inactivation of p15^{INK4b} gene; homozygous deletion and hypermethylation of the p15^{INK4b} promoter (Kamb, 1998). Loss of p15^{INK4b} protein function is known to initiate continual activation of the CDK2-cyclin E complex, which is necessary for entry into the S phase of the cell cycle, resulting in an avoidance of cell cycle arrest and promotion of the neoplastic process (Hartwell and Kastan, 1994). In another way, binding of p15^{INK4b} cell cycle regulatory protein to the cyclin dependent kinase CDK4 inhibits CDK4-cyclin D dependent phosphorylation of retinoblastoma protein and thus prevents Rb from being hyperphosphorylated, resulting in Rb-dependent cell cycle arrest in the G1 phase of the cell cycle (Kamb,1998). Previous study has used multiplex polymerase chain reaction (PCR) to determined homozygous deletions at the p15^{INK4b} locus gliom. The above mentioned study on Renal cancer cells detected homozygous deletion of p15^{INK4b} gene in 43% of these cells (Kawakami et al., 2003).

Inactivation of p15^{INK4b} gene is common genetic events in acute leukemia and plays an important role for the retinoblastoma (RB) protein/ p16^{INK4a} pathway in the pathogenesis of acute leukemia. The p15^{INK4b} gene was found to be methylated in around 34% of acute lymphoblastic leukemia (ALL), 52% of acute myeloid leukemia (AML), and 18% of chronic myelogenous leukemia (CML) (Guo et al., 2000; Uehara et al., 2012). Alteration of the p15^{INK4b} gene was also linked to the genetic events in bladder cancer which occurs more frequently in schistosomiasis-associated bladder cancer (SABC) and SCC, and may play an important role in the pathogenesis of SABC. Deletion of p15^{INK4b} gene was found in 21.4% of cases (Eissa et al., 2000). Promoter methylation of the p15^{INK4b} gene, is involved in the pathogenesis of many different types of cancer (Guo et

al., 2000; Jha et al., 2012)

To our knowledge, this is the first study to investigate the association between the expression of $p15^{INK4b}$ protein and basal cell carcinoma. The results of this study showed that 51.4% of BCC demonstrated negative staining for $p15^{INK4b}$ expression. Fisher's exact test showed that there was no significant relationship between $p15^{INK4b}$ expression and the type of tissues. No significant difference in positively was found for BCC and normal tissues (P=0.356; P>0.05). Approximately half of the BCC showed that $p15^{INK4b}$ protein was not expressed in these tissues, which indicated that $p15^{INK4b}$ does not contribute to the genesis of BCC.

In addition, the relationship between clinicopathologic variables of the patients (based on tissue cores) and $p15^{INK4b}$ protein were analyzed. However, there were no significant relationship between the clinicopathologic variables and $p15^{INK4b}$ expression. Age and sex of the subjects were not significantly associated with $p15^{INK4b}$ expression in this study.

In conclusion, this study may suggest that $p15^{INK4b}$ gene does not play a role in the development of BCC. Further experimental studies are needed to further elucidate the role played by $p15^{INK4b}$ in the genesis of BCC.

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