

A novel tumor suppressor gene in basal cell carcinoma: inhibition of growth factor-2

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Abstract In loss of heterozygosity (LOH) studies at the chromosome 4q22-35 region, it was shown that the amount of deletion was high in basal cell carcinoma (BCC). It has been proposed that genes located in this chromosomal region could be tumor suppressor genes in BCC. It has been thought that deletions in the ING2 gene located in the same region can play a role in the pathophysiology of BCC and that deletions occurring in this region may influence the level of ING2 expression in BCC. Tumoral and non-tumoral tissues from 75 patients with BCC (45 men and 30 women) were included to the

study. Lesions were excised by a surgical margin of 0.5 cm. After excision, RNA was isolated from tumoral and non-tumoral tissue samples. ING2 messenger RNA (mRNA) expression level was determined in tumoral and non-tumoral tissues by the real-time polymerase chain reaction (RT-PCR). It was detected that ING2 mRNA expression level decreased in tumoral tissues when compared to non-tumoral tissues from BCC patients ($p=0.0001$). It was found that expression levels of this gene were comparable among patients with primary, recurrent, or multiple BCC. It is thought that ING2 gene expression level could contribute to the development of BCC but not be associated with the stage and the prognosis of the tumor.

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Introduction

Basal cell carcinoma (BCC) is the most commonly seen cancer arising from the basal layer of the epidermis in humans [1]. It is frequently observed at bodily regions exposing to sunlight. BCC is generally seen at advanced age; however, it has become increasingly frequent in the young population in recent years. The most important factor is exposure to ultraviolet (UV) radiation [2]. Other etiological factors include ionizing radiation, human papilloma virus infections, infrared radiation, inorganic arsenic, trauma, immunosuppression, and genetic disorders [3].

The cell cycle is regulated by proto-oncogenes and tumor suppressor genes. A mutation that may occur in these genes can result in neoplastic transformation. There are several studies on the role of genetic factors in the BCC etiology. It was

shown that there are mutations in the p53 gene in BCC patients exposed to UV radiation [4]. In addition, inactivation of the patched (PTCH) gene located at chromosome 9q22 was reported as the primary mechanism in the development of BCC [5]. Jin et al. reported that genetic mutations occur frequently at chromosome 4q region in patients with BCC [6]. Sironi et al. found a significant amount of loss of heterozygosity (LOH) at the chromosome 9q (9q21–22 and 9q22–qter) and 4q (4q32–35) regions in patients with BCC. The authors showed that these LOHs were associated with BCC development and increased risk for BCC development. The chromosome 4q32–35 region harbors p53, ING1, ING2, and SAP30 tumor suppressor genes [7]. ING2 has a critical role especially in the control of cell cycle and genome stability as a gatekeeper and caretaker gene. Several studies have reported that ING2 protein expression in human tumors and ING2 knockout mice were shown to have spontaneously developed tumors, B cell lymphomas, and soft tissue sarcomas [8]. We think that alterations in this chromosomal region that are thought to be involved in the BCC development could contribute to BCC pathophysiology by influencing the ING2 gene expression level.

In the literature, there are several studies on genetic mutations in patients with BCC. However, to the best of our knowledge, no study exists about the relationship between BCC and ING2 gene expression level in the literature. Thus, we aimed to investigate the relationship between BCC development and ING2 by comparing ING messenger RNA (mRNA) expression levels in tumoral and normal tissues in patients with BCC.

Materials and methods

Patients and tissue samples

The study was approved by the Ethics Committee of the Medicine School of Gaziantep University (voucher number: 5/2010-10). All patients gave written informed consent before participation. The study recruited 96 patients who presented to the Plastic Surgery Department with a non-healing wound. Primary or recurrent cases with a tumor diameter of at least 1 cm were included in the study. Patients who declined to participate, immunocompromised patients, those with genetic susceptibility to BCC development (Xeroderma Pigmentosum, Basal Cell Nevus Syndrome [Gorlin Syndrome], Bazex syndrome), and those with albinism were excluded. In all patients, lesions were excised with a surgical margin of 0.5 cm. Skin samples (0.5 cm³ in volume) were taken from tumoral and non-tumoral tissues without compromising histopathological evaluation and diagnosis. All tissue samples were numbered. Tissue samples taken were stored in liquid nitrogen until RNA isolation. Of these

samples, tissues of 75 patients (45 men and 30 women) diagnosed as having BCC based on histopathological evaluation were used for genetic testing.

RNA isolation from tissues

Tumoral and non-tumoral tissue samples of 25 mg were homogenized on ice by using a homogenizer (Kinematica, Gmdh, Switzerland). Total RNA was obtained in accordance with the manufacturer's instruction by using a High Pure RNA Tissue Kit (Roche, Cat. No. 12 033 674 001, Mannheim, Germany).

Quantitative real-time polymerase chain reaction

Firstly, complementary DNA (cDNA) of mRNAs of all samples was obtained by using miScript II RT Kit (Qiagen, Hilden, Germany, Cat. No. 218161) in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Primer sets for specific reverse transcription including ING2 and endogenous control ActB were obtained from Qiagen (Hilden, Germany). The qRT-PCR was carried out using LightCycler 480 II (Roche, Mannheim, Germany). The PCR master mix (Qiagen, Hilden, Germany, Cat. No. 218073) containing 2× QuantiTect SYBR Green PCR Master Mix, 10× miScript Universal Primer, 10× miScript Primer Assay (related mRNA primer), RNase-free water, and template cDNA in 25 µL volume were processed as follows: 95 °C for 15 min and then 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. The signal was collected at the endpoint of every cycle.

Statistical analysis

The collected data were analyzed by using the SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Tumor and non-tumor tissues of BCC patients were compared with the Wilcoxon signed rank test. *P* values of <0.05 were interpreted as statistically significant.

Results

Mean age was 68 (44–86) years and mean follow-up was 1.5±0.5 years. No recurrence or wound healing problem was detected in any patient. It was seen that BCC lesions were at the nose (*n*=23), the eyelid (*n*=16), the malar region (*n*=18), the forehead (*n*=9), the ear (*n*=8), and the leg (*n*=1) of the patients. To close the defects, primary closure was employed in 16 patients, while grafting was performed in 26 patients, and defects were closed by appropriate local flaps in the remaining 33 patients. Of the patients, there were synchronous multiple lesions in 11 patients while the remaining 64 patients had a

single lesion. Of the patients with a single lesion, there was recurrent tumor in 12 patients while there was newly diagnosed tumor in 52 patients.

The mRNA expression of ING2 and β -actin genes was assessed in tumoral and non-tumoral tissue samples from 75 patients by using qRT-PCR. It was found that the ING2 mRNA level was lower in tumoral tissue than non-tumoral tissue samples in all BCC patients included ($n=75$) ($p=0.0001$; Table 1 and Fig. 1). With the ING2 gene expression levels in tumoral and non-tumoral tissues from 52 patients with newly diagnosed tumor, 12 patients with recurrence and 11 patients with synchronous multiple lesion were compared, and it was found that the ING2 gene expression level was decreased in all three groups (Table 1; Fig. 2a–c). However, it was found that there was no significant difference when ING2 mRNA expression levels were compared within groups (Fig. 2d).

Discussion

BCC displaying slow progression and local invasion is the most common tumor of the skin, consisting of 50–75 % of all skin tumors [1, 9]. It develops in elderly patients, particularly in the head and neck regions. Although BCC has low mortality and little potential for metastasis, it progresses with local destruction if not treated appropriately. Exposure to ultraviolet radiation is the most important factor in BCC etiology [2]. Intermittent and/or brief periods of UV radiation exposure at early ages increase BCC risk more than cumulative lifetime exposure [10]. In skin cancers, genetic mutations are seen by 30–60 % in patients with intact DNA repair mechanisms, whereas it is 50–80 % in patients with disease such as xeroderma pigmentosum (XP) where the DNA repair mechanism is defective. UV radiation leads to DNA mutations; however, this damage is corrected by cellular repair mechanisms. Hereditary diseases such as nevoid basal cell carcinoma syndrome (NBCCS; Gorlin syndrome), Bazex syndrome, Rombo syndrome, and unilateral basal cell nevus syndrome predispose BCC [11]. Mutations at PTCH genes are defined by sporadic BCCs [12–15]. Thus, patients with genetic

Table 1 ING2/ β -actin values in tumoral and non-tumoral tissues

	Tumoral ^a	Non-tumoral ^a	<i>p</i> value ^b
Total ($n=75$)	1.32 (1.24–1.49)	1.59 (1.49–2.31)	0.0001
Primary ($n=52$)	1.32 (1.16–1.44)	1.55 (1.47–2.31)	0.003
Recurrent ($n=12$)	1.44 (1.26–1.62)	2.54 (1.68–2.87)	0.001
Multiple ($n=11$)	1.36 (1.29–1.54)	1.76 (1.59–2.32)	0.03

^a Median \pm interquartile ranges (Q1 and Q3)

^b Wilcoxon signed rank test

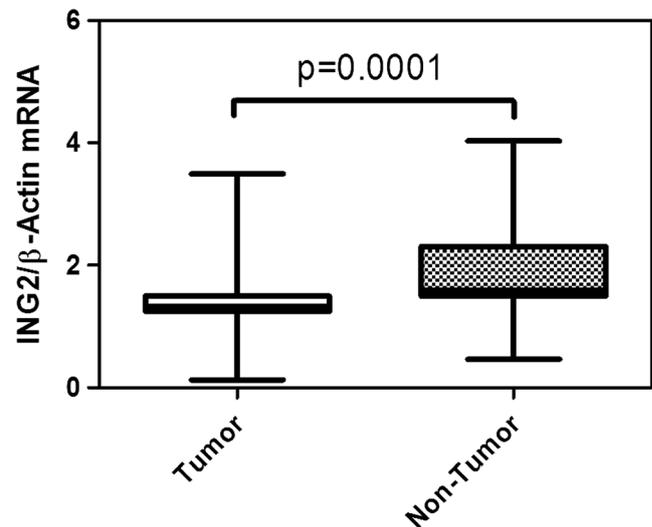


Fig. 1 ING2 gene expression levels in tumoral and non-tumoral tissues from patients diagnosed with BCC. ING2 and β -actin gene expression levels were determined by using the qRT-PCR method following total mRNA isolation in tumor and non-tumoral parts of BCC lesions excised surgically. Expression levels were normalized by β -actin. Results are expressed as median \pm interquartile ranges (IQR1 and IQR3)

predisposition to BCC development were excluded as they can affect results.

Members of the ING family (ING1, ING2, ING3, ING4, and ING5) play a role in the activation of the pathway that stimulates apoptosis when cell division must be controlled or needed [16]. It has been thought that members of the ING gene family are involved in the regulation of gene transcription as they contain methylated histone groups and phosphoinositol with histone acetyltransferase and histone deacetylase [8]. This feature functions as a transcription factor of some genes accounting from cell growth and transformation [8]. One of these pathways involves the p53 gene [17]. A relationship between inactivation of ING genes and tumor development and progression was established in studies on human tumor cells [18]. Additionally, the results of forced overexpression studies performed in tissue culture have indicated that several of the ING proteins can interact with the p53 tumor suppressor protein and/or the nuclear factor-kappa B (NF-kappaB) protein complex. As a result of these interactions, several vital events such as DNA repair, cell growth and survival, inflammation, and tumor suppression are affected either through p53 and NF- κ B or independently [18]. It is found that alterations in ING gene expression are effective in the development and progression of different cancer types such as head and neck cancers [19], melanoma [20, 21], lung cancers [22], lymphomas [23, 24], breast cancers [26, 27], and colon cancer [28]. However, there are a limited number of studies of the relationship between ING2, the ING gene family, and BCC development.

The p53 is a tumor suppressor protein with potent proapoptotic activity [25]. In normal conditions, the

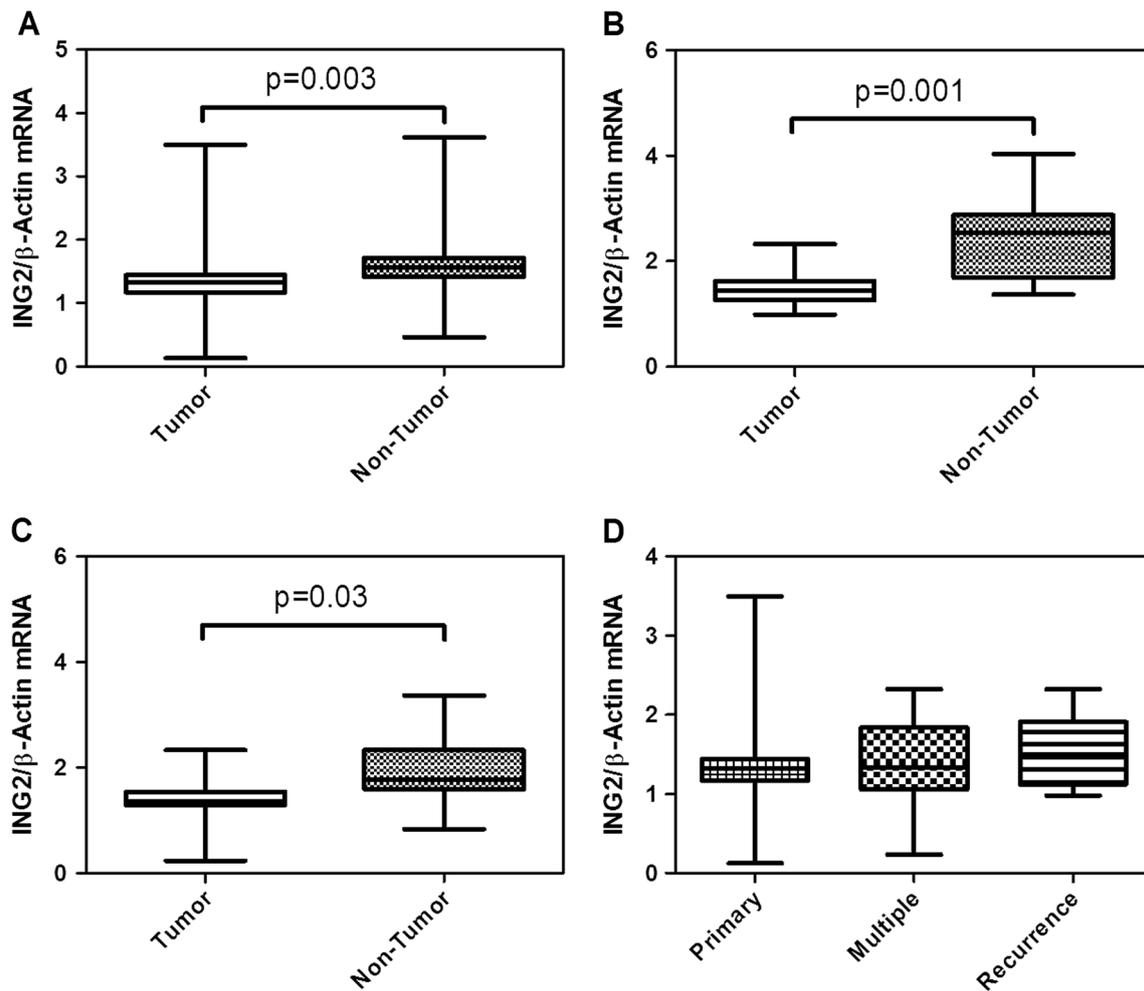


Fig. 2 ING2 mRNA expression levels in patients diagnosed as BCC. ING2 mRNA expression levels in tumoral and non-tumoral tissues from **a** patients with newly diagnosed BCC, **b** patients with recurrent tumor, **c** patients with synchronous multiple lesions, and **d** comparisons of ING2

mRNA expression levels in tumor tissues among groups (patients with primary, recurrent, and multiple BCC). Results are expressed as median \pm interquartile ranges (IQR1 and IQR3)

expression level of this protein is maintained at extremely low levels [26]. The p53 is convened at the nucleus after undergoing chemical modifications such as phosphorylation, acetylation in several cellular stress conditions such as DNA damage, oncogenic activity, hypoxia, nucleotide imbalance, and oxidative damage. This causes triggering of apoptosis through the mitochondrial pathway by stimulating BAX, PUMA, NOXA, and p53 AIP1 activity [27]. The ING protein activates p53 through an acetylation mechanism [28]. ING2, acting together with p53, functions as a tumor suppressor protein by influencing cell growth arrest and apoptosis [29–31]. In our study, it was found that the ING2 expression level was lower in patients with BCC when compared to normal tissue. We think that a decreased ING2 expression level in tumor tissues can contribute to tumor development through p53. In the support of this idea, it was shown that BCC patients harbored p53 gene mutations by 33–56 % [32].

In the studies attempting to elucidate pathogenesis of BCC, it was proposed that alterations in the hedge-hog signaling pathway are the most important factors in the development of the disease [33]. This pathway is regulated by many genes, mainly by PTCH, while its primary function is to control cell division [13]. Sironi et al. suggested that PTCH gene is inactivated as a result of allelic loss in chromosome 9q22 due to mutations induced by UV radiation and in the p53 gene and that PTCH gene inactivation is the primary genetic mechanism accounting for BCC development [7]. PTCH protein is located in the phospholipid layer of the plasma membrane, serving as a transmembrane receptor for the sonic hedgehog (SHH) protein [14]. The normal function of the PTCH protein is to inhibit a second transmembrane protein, the so-called smoothed, frizzled class receptor (SMO) [34]. The binding of SHH signal protein to PTCH protein abolishes inhibition of SMO by PTCH, releasing SMO protein [34]. SMO protein

triggers a transforming growth factor- β (TGF- β) by increasing glioblastoma Gli-1 and Gli-2 expression [35]. This causes dissolution of basal membrane material through fibroblast proliferation and increased metalloproteinases. Moreover, Gli-2 also binds to Bcl (B cell lymphoma)-2 protein to induce its expression. Bcl-2 is a protein that inhibits apoptosis. It is proposed that an increase in Gli-2 protein may have a more important role in the initiation of BCC via Bcl-2 activation. It was shown that Bcl-2 expression is increased in sporadic BCCs while Bcl-2 overexpression was demonstrated in slowly progressing BCC subtypes [36]. To the best of our knowledge, there is no study establishing a relationship between the ING2 and the PTCH gene. However, it has been proposed that SMO is within the same pathway with PTCH and ING2 and serves as a transcription factor of SMO [13]. Based on these findings, we think that decreased levels of ING2 gene expression alter PTCH activity by interaction through SMO, suggesting a mechanism for BCC development.

Jin et al. reported that there might be a correlation between losses in chromosome 4q and BCC [6]. Sironi et al. reported there was >20 % of LOH at two chromosome regions (9q21-qter and 4q32-35), which could be accounted for from BCC development [7]. In addition, the authors reported that there were allelic losses in other chromosome by <15 %. To the best of our knowledge, the first study on LOH at 4q32-35 genes in sporadic BCC was performed by Sironi et al. In that study, it was suggested that p53 and PTCH genes were inactivated due to allelic losses at chromosome 9q22 caused by mutations induced by UV radiation in BCC, and this is the primary genetic mechanism accounted for from the development of BCC. It was suggested that further studies are needed to identify clearly gene alterations at the chromosome 4q32-35 region, where deletions were detected by Sironi et al., which harbor genes to regulate the control point of the cell cycle such as p53, ING2, ING1, and Sap30. ING2 acting by p53 leads to reduction of cell proliferation due to inhibition of cell growth caused by upregulation of p21^{waf1/cip1}. This finding was demonstrated previously in studies on BCC [37]. It is well known that p21^{waf1/cip1} protein inhibits cell cycle-dependent kinase and contributes to apoptosis induced by genomic stimulation through growth arrest mediated by Tp53 [38]. The absence of ING2 causes increased Bax gene expression by reduction of p21^{waf1/cip1} protein expression. As a result, apoptosis fails as there are dysfunctional cells and potentially transformed cells in the G1 phase of the cell cycle. In light of these data, it plays a key role in skin cancers, particularly in BCC.

In previous studies, it was shown that ING2 expression was decreased in many cancers such as lung cancer, melanoma, and colon cancers without mutation [22, 39]. In addition, it was seen that ING2 expression was decreased in melanoma when compared to dysplastic nevus. However, the decrease in ING2 expression was found to have no influence on tumor

stage, subtype, and 5-year survival. On the contrary, ING2 expression was found to be effective in tumor progression and survival in hepatocellular carcinoma. In light of these data, it is thought that decreased or no ING2 expression has influence on tumor onset or progression. In our patients, it was seen that ING2 mRNA expressions did not change among patients with newly diagnosed, recurrent, or multiple BCC when groups were compared (Fig. 2d). This finding suggests that ING2 may have a role in the development, but not in the clinical course, of BCC.

Conclusion

In conclusion, we revealed that ING2 expression level was increased in tumoral tissues compared to non-tumoral tissues from BCC patients. This was true for all cases including those with new diagnosis, recurrent tumors, and multiple lesions, and that there was no significant difference. Based on these findings, we think that ING2 gene expression level could contribute to the development of BCC but not be associated with the stage and the prognosis of the tumor. We think that ING may be involved in the development of BCC through two mechanisms: 1) through interacting with transcription and/or activity of proteins such as SMO and p 53 with a critical role in cell signaling pathways and 2) through the effects of deletions in the chromosome 4q region harboring the ING2 gene on the expression level of the gene (based on the assumption that the abovementioned chromosomal region is involved in the development of BCC). ING2 expression level is decreased in tumoral tissue in both conditions, suggesting that this gene may be a candidate tumor suppressor gene in BCCs. However, further studies are needed to elucidate this condition.

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Conflicts of interest None

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