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ORIGINAL PAPER

# Blocking Wnt as a therapeutic target in mice model of skin cancer

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**Abstract**
Wnt pathway plays an important role in controlling metabolism in cancer cells. It acts as positive modulator for both cell inflammation, through activation of NFκB, and fibrosis, through activation of TGF-β. Therefore, the aim of this study is to investigate the therapeutic effects of blocking Wnt pathway by IWP12 on skin cancer by studying its effects on skin cancer-induced inflammation and fibrosis in a mice model of skin cancer. Skin cancer was induced by application of 7,12-dimethylbenz[a]anthracene (DMBA) and croton oil on the dorsal skin of mice. Dorsal skin was removed for estimation of gene and protein expression of Wnt, β-catenin, SMAD, TGF-β, NFκB, TNF-α, IL-4 and IL-10. Part of the skin is stained with hematoxylin/eosin for assessment of cell structure. Treatment of mice with IWP12 completely blocked Wnt in skin cancer mice without affecting the control mice. Skin of tumorigenic mice showed marked skin hyperkeratosis, parakeratosis, acanthosis and dysplasia. Treatment with IWP12 markedly attenuated epidermal atypia and hyperplasia. In addition, IWP12 reduced expression of β-catenin, SMAD, TGF-β, NFκB and TNF-α associated with increase in the expression of IL-4 and IL-10. In conclusion, blocking Wnt production ameliorated skin cancer via blocking pro-inflammatory cytokines and enhancing the anti-inflammatory cytokines. Moreover, blocking Wnt attenuated skin cancer-induced activation of fibrosis pathway.

**Keywords** β-Catenin · Interleukin (IL)-4/10 · Nuclear factor (NF)κB · Transforming growth factor (TGF)-β · Tumor necrosis factor (TNF)-α · Wnt

## Introduction

Skin cancer is considered as a fatal public health concern all over the world. It can lead to enormous effect on the world economy worldwide. Skin cancer can be initiated by many external and internal factors [1]. However, several strategies are available for treating skin cancer as surgical excision, chemotherapy, curettage and electrodessication, immunotherapy, laser therapy, radiotherapy, cryotherapy and photodynamic therapy. However, many of these strategies are linked with several adverse effects and development of drug resistance. In addition, the cost of skin cancer treatment is very high.

Wnt proteins form a large family of highly conserved glycoproteins, which are involved in embryonic development as well as in cell growth, migration and differentiation. Therefore, any deterioration in the Wnt signaling leads to a wide range of pathological conditions [2]. The signaling pathways of Wnt include canonical, which is β-catenin-dependent and noncanonical, which is β-catenin-independent. The canonical pathway depends on β-catenin stabilization and translocation into the nucleus, leading to stimulation of expression of target genes [3]. On the other hand, instead of β-catenin activation, the signals are transmitted through planar cell polarity and small GTPase proteins [4]. Canonical Wnt pathway was linked with cancer cell metabolism. In skin cancer, Wnt/β-catenin-mediated the metabolic reprogramming inside cancer cells and directly affect vessel density. Skin tumors activates Wnt/β-catenin pathway to induce cell proliferation [5].

NFκB is a positive modulator of both inflammation and immune response. Its signaling pathway plays an important role in early development as well as other physiological functions. It is activated many stimuli such as such as cytokines, growth factors, bacterial lipopolysaccharide and

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ionizing radiation [6]. Next, NFκB enhances transcription of a wide range of target genes, as interleukins, chemokines, apoptosis related genes and proteases. It has high impact on tumor initiation, promotion, metastasis and the resistance to chemotherapy. Therefore, NFκB was highly studied during the tumor progression and genome stability.

Therefore, we conducted this study to investigate the therapeutic effects of blocking Wnt pathway by IWP12 on skin cancer in mice and to illustrate its effect on NFκB inflammatory pathway.

## Materials and methods

### Animals and their treatment outlines

The local ethical committee approved the animal protocol. Swiss albino mice (7–8 weeks old and 26 ± 3 g) were kept in a pathogen-free environment. Mice were subjected to 12 h light–dark cycle and kept at 22 ± 2 °C. They were classified into four groups (with ten mice each):

1. Control group: after shaving the dorsal hair, acetone was applied three times over shaven area for 16 weeks.
2. IWP12-treated control: mice were treated as the control group followed by 25 mg/kg IWP12, intraperitoneal, ip, twice weekly for 16 weeks.
3. Skin cancer group: after shaving the dorsal hair, application of a single dose of 100 mg/100 ml of 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma-Aldrich Chemicals Co., St Louise, MO, USA) in acetone over shaven area. After 2 weeks, croton oil in acetone (1% solution) as a promoter was applied three times weekly for 16 weeks.
4. IWP12-treated cancer group: skin cancer was induced and mice received 25 mg/kg IWP12 (Sigma-Aldrich Co.) ip twice weekly for 16 weeks starting from the first week after skin cancer induction.

### Measurement of scratching behavior frequency and the average number of tumors

Mice were placed into separate cages 2 days before killing. Number of scratching was counted for 10 mins. The measurements were repeated five times. The scratching behavior in mice was defined as movement with hind paws. In addition, on the day of killing, the average number of tumors or papilloma per each mouse was calculated.

### Animal killing and collection of samples

The animals were killed by decapitation. Skin and serum samples were collected and stored at −80 °C.

### Morphologic analysis of skin tissue

The skin was cut into 5-mm-thick sections. Sections were stained with hematoxylin/eosin, Mallory and cytokeratin immunostaining. The slides were examined using phase-contrast microscopy (Leica_DM500 with camera Leica_ICC50HD) with camera software Leica application suite (LAS) EZ, version 3.1.1.

### ELISA determination

Commercially available ELISA kits were used for evaluation of serum levels of biochemical parameters. ELISA kits for β-catenin, SMAD3 (Abcam, Cambridge, MA, US), TGF-β, TNF-α (MyBiosource, San Diego, CA, USA), IL-4 and IL-10 (R&D Systems, Minneapolis, MN, United States) were used following the manufacture protocol using microplate reader (BioTek, Winooski, VT, USA).

### Quantitative real-time polymerase chain reaction (RT-PCR)

Gene expression of Wnt1, β-catenin, TGF-β, NFκB, TNF-α, IL-4 and IL-10 was evaluated as described previously [7, 8]. In addition, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed to serve as an internal reference. Specific primers are shown in Table 1.

### Statistical analysis

Mean ± standard error was used. Normality of sample distribution was tested with the Kolmogorov–Smirnov (K–S) test. ANOVA was used to compare means between groups. Once differences were found, post hoc Bonferroni correction tests were used. Statistical computations were performed using SPSS version 20. Statistical significance was predefined as $p \leq 0.05$.

## Results

### IWP12 blocked skin cancer-induced activation of Wnt pathway

Skin cancer resulted in 2.43- and 2.96-fold increase in gene expression of Wnt and β-catenin, respectively, associated with 2.47-fold increase in the protein expression of

**Table 1** The primer sets used

| Primer | Accession number | Sequence (sense, antisense) |
|---|---|---|
| Wnt1 | NM_021279 | 5′-CGAGAGTGCAAATGGCAATTCCG-3′<br>5′-GATGAACGCTGTTTCTCGGCAG-3′ |
| β-Catenin | NM_001165902 | 5′-GTTCGCCTTCATTATGGACTGCC-3′<br>5′-ATAGCACCCTGTTCCCGCAAAG-3′ |
| NFκB | NM_008689 | 5′- GAAATTCCTGATCCAGACAAAAAC -3′<br>5′- ATCACTTCAATGGCCTCTGTGTAG -3′ |
| TNF-α | X02611 | 5′- TACTGAACTTCGGGGTGATTGGTCC -3′<br>5′ CAGCCTTGTCCCTTGAAGAGAACC -3′ |
| IL-4 | NM_021283 | 5′-ATCATCGGCATTTTGAACGAGGTC-3′<br>5′-ACCTTGGAAGCCCTACAGACGA-3′ |
| IL-10 | NM_010548 | 5′CGGGAAGACAATAACTGCACCC-3′<br>5′-CGGTTAGCAGTATGTTGTCCAGC-3′ |
| TGF-β | NM_011577 | 5′-CGGGGCGACCTGGGCACCATCCATGAC-3′<br>5′-CTGCTCCACCTTGGGCTTGCGACCCAC-3′ |
| GAPDH | M32599 | 5′ ACCACAGTCCATGCCATCAC -3′<br>5′- CACCACCCTGTTGCTGTAGCC -3′ |

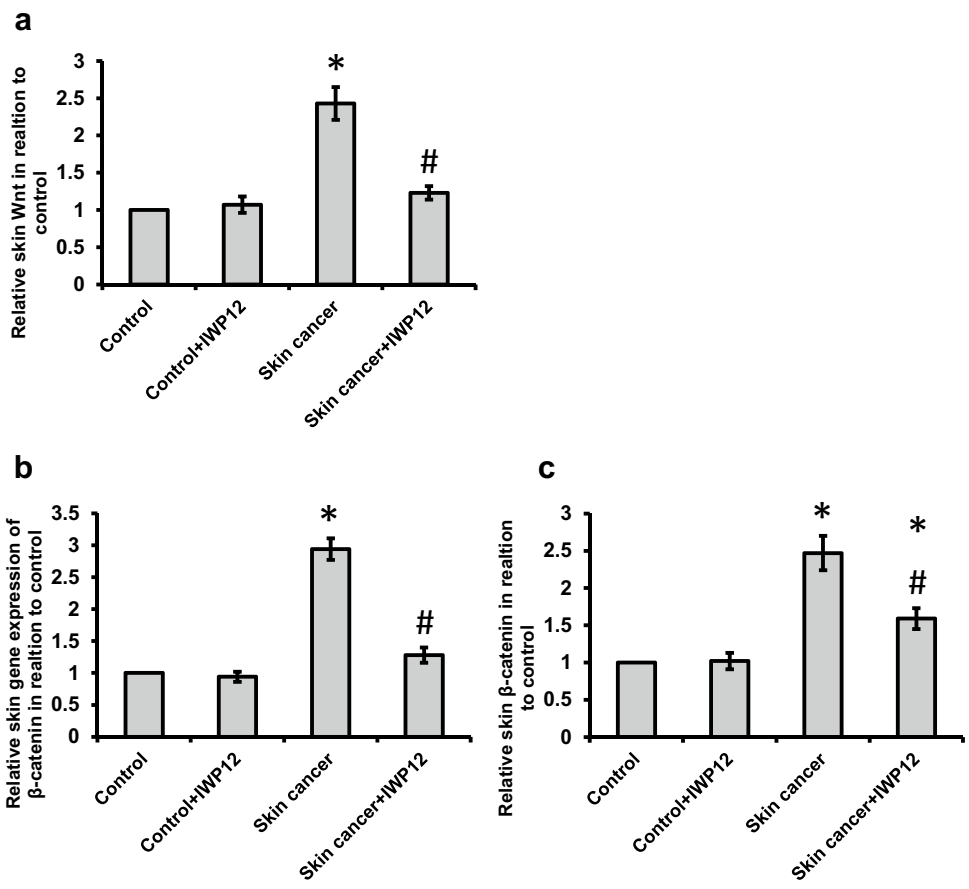


**Fig. 1** Effect of IWP12 on skin cancer-induced gene expression of Wnt (**a**), β-catenin (**b**) and protein level of β-catenin (**c**) in the skin of mice. *Significant difference as compared with the control groups at $p < 0.05$. #Significant difference as compared with skin cancer group at $p < 0.05$

β-catenin when compared with control mice. Treatment of skin cancer mice with IWP12 significantly attenuated all these effects in the skin cancer group without affecting the control group (Fig. 1).

## IWP12 reversed skin cancer-induced activation of SMAD3 and TGF-β

Skin cancer produced 2.34-fold elevation in skin levels of SMAD3 as well as 3.76- and 2.94-fold increase in the gene and protein expression of TGF-β, respectively, as compared with the control group. However, treatment of

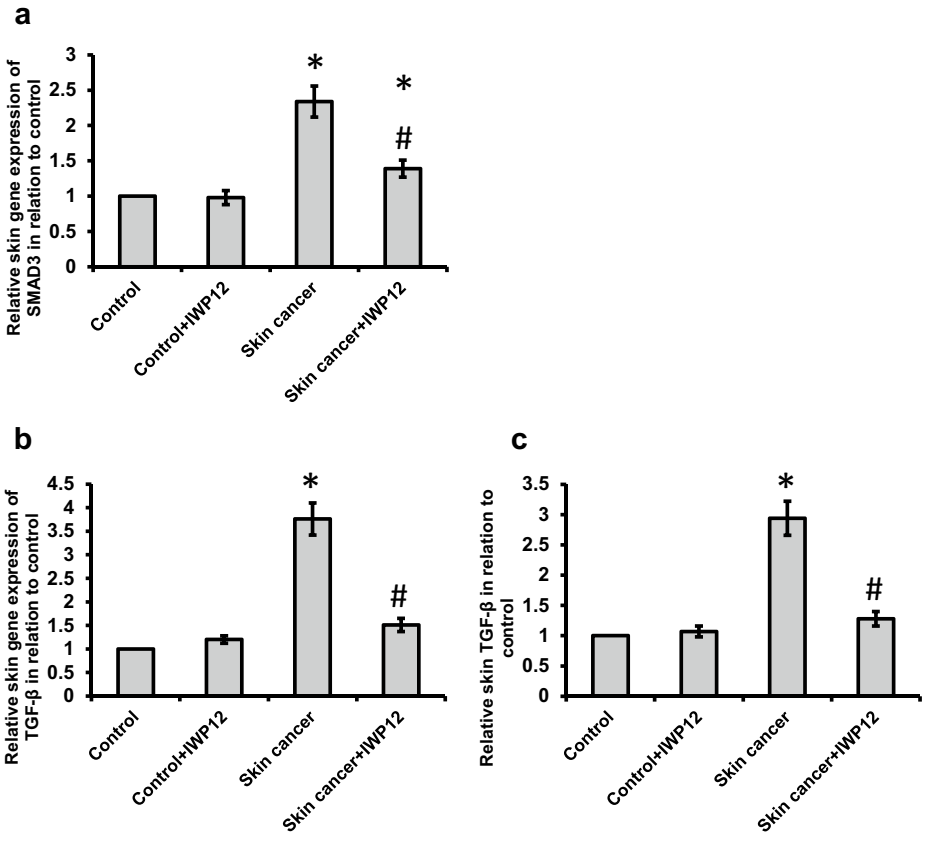


**Fig. 2** Effect of IWP12 on skin cancer-induced expression of SMAD3 (**a**), gene expression of TGF-β (**b**) and protein expression of TGF-β (**c**) in the skin of mice. *Significant difference as compared with the control groups at $p < 0.05$. #Significant difference as compared with skin cancer group at $p < 0.05$

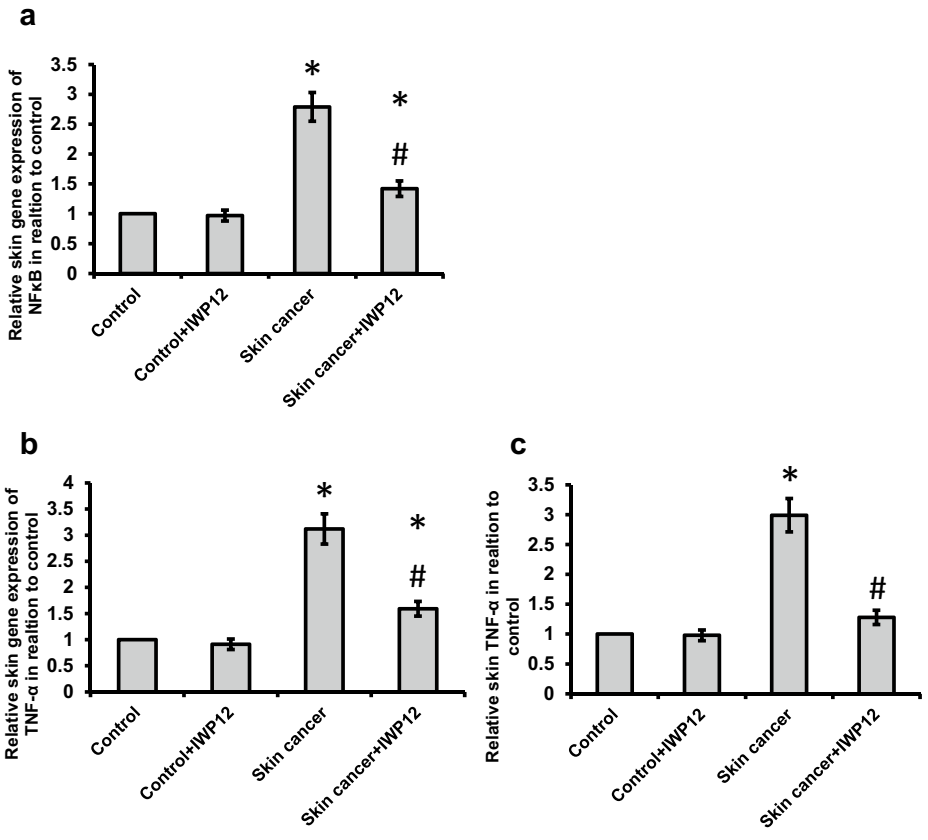


**Fig. 3** Effect of IWP12 on skin cancer-induced gene expression of NFκB (**a**), TNF-α (**b**) and protein level of TNF-α (**c**) in the skin of mice. *Significant difference as compared with the control groups at $p < 0.05$. #Significant difference as compared with skin cancer group at $p < 0.05$

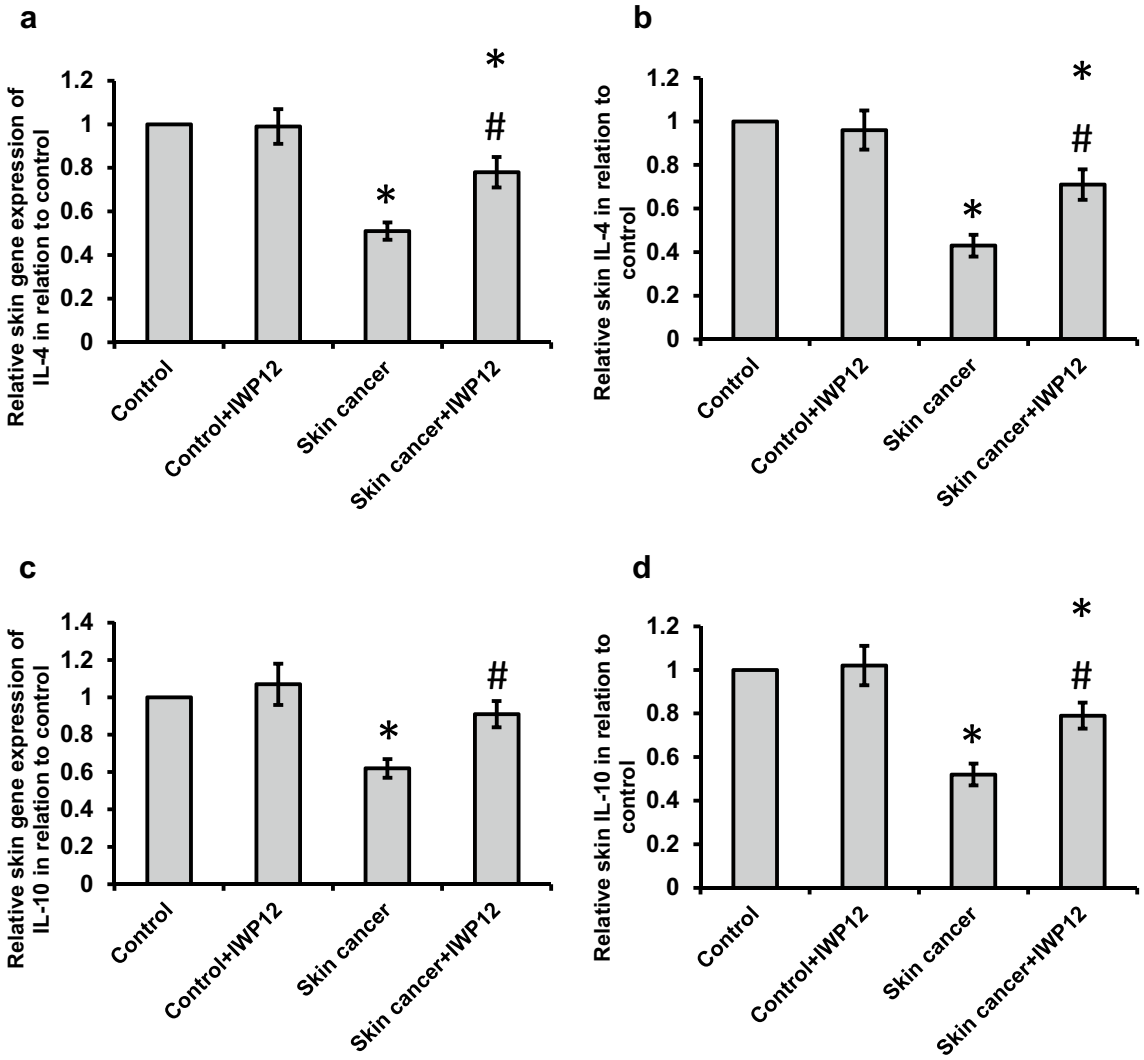


**Fig. 4** Effect of IWP12 on skin cancer-induced gene expression of IL-4 (**a**) and IL-10 (**c**) as well as protein levels of IL-4 (**b**) and IL-10 (**d**) in the skin of mice. *Significant difference as compared with the control groups at $p < 0.05$. #Significant difference as compared with skin cancer group at $p < 0.05$

mice with skin cancer with IWP12 significantly attenuated these without producing any effect in control mice (Fig. 2).

## IWP12 attenuated skin cancer-induced activation of inflammatory pathway

Analysis of skin samples from mice with skin cancer showed significant elevation in the gene expression of NFκB and TNF-α as well as increased TNF-α release in the skin of skin cancer mice (Fig. 3). In addition, skin cancer resulted in reduced gene and protein expression of the anti-inflammatory cytokines, IL-4 and IL-10, as compared with the control group (Fig. 4). Treatment of skin cancer mice with IWP12 reversed all these effects.

## IWP12 ameliorated skin cancer-induced elevation in the number of tumors and scratches

Skin cancer results in about 14- and 39-fold increase in number of scratches/10 mins and number of tumors in each mouse, respectively. However, treating skin cancer mice with IWP12 caused nearly 67% and 56% reduction in number of scratches and number of tumors, respectively (Fig. 5).

## Effect of IWP12 on skin cancer-induced changes in skin cell structure

Skin images of control mice stained with hematoxylin and eosin revealed normal epidermis and dermis layers. Images from skin cancer mice revealed epidermal hyperplasia, acanthosis, dysplasia, hyperkeratosis and dermal leukocytic

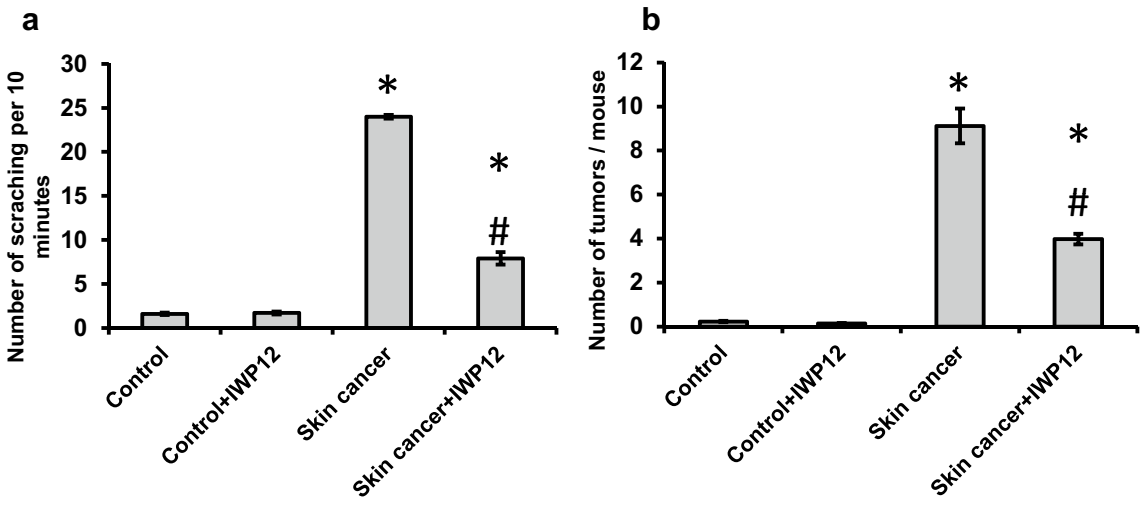


**Fig. 5** Effect of IWP12 on skin cancer-induced elevation in number of scratches per 10 min (**a**) and number of tumors per mouse (**b**). *Significant difference as compared with the control groups at $p < 0.05$. #Significant difference as compared with skin cancer group at $p < 0.05$

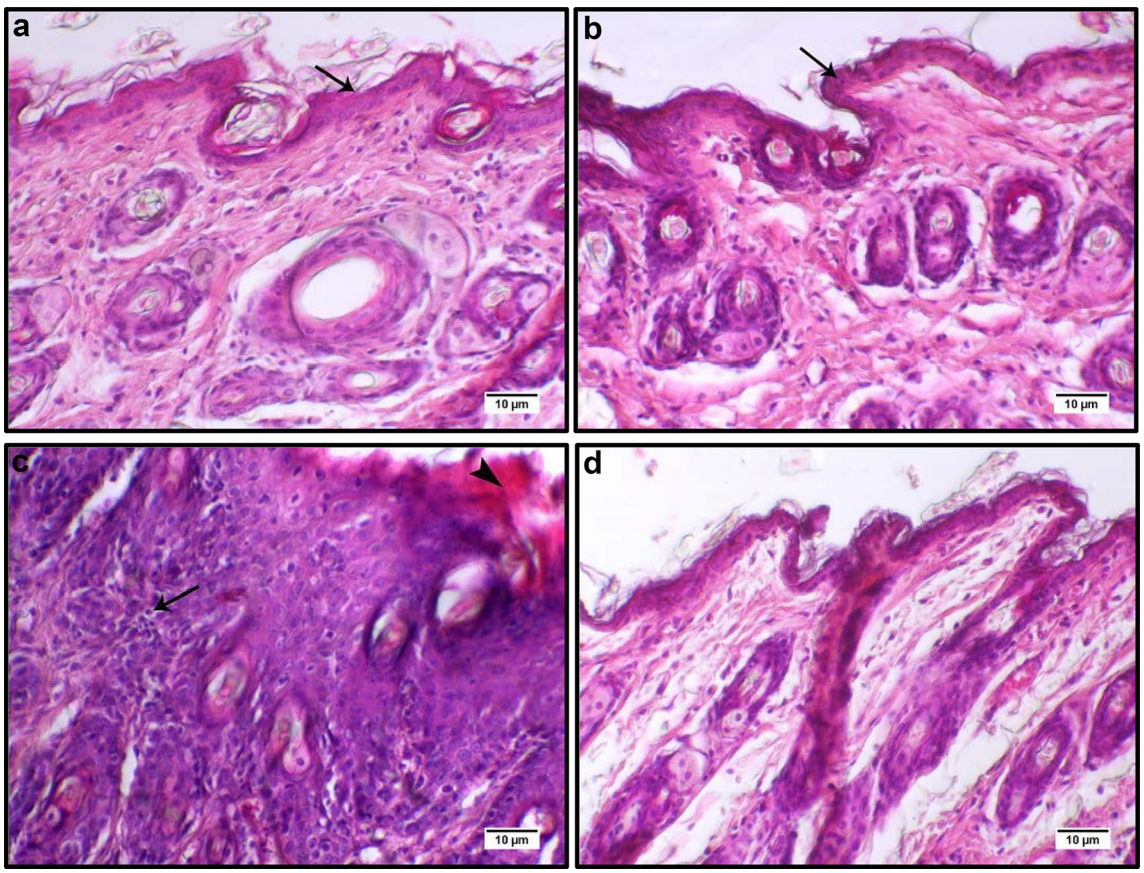


**Fig. 6** Skin sections stained with hematoxylin/eosin from control group with normal epidermis (arrow) and dermis layer (**a**); control group treated with IWP12 with normal epidermal (arrow) and dermal structure (**b**); skin cancer group with epidermal hyperplasia, acanthosis, dysplasia, hyperkeratosis (arrowhead) and dermal leukocytic infiltration (arrow) (**c**) and skin cancer treated with IWP12 (**d**) with x 200 magnification

infiltration. Treating skin cancer mice with IWP12 showed marked decrease of epidermal hyperplasia (Fig. 6).

Staining of skin sections from control mice with Mallory's stain showed thin keratin layer with minimal amount of keratin staining in epidermal layer. Sections from skin cancer mice showed hyperkeratosis, while treating skin cancer mice with IWP12 markedly decreased epithelial hyperkeratosis (Fig. 7).

Investigation of skin sections from control mice immunestained with cytokeratin revealed thin epidermal layer. On the other hand, skin sections from skin cancer mice showed marked elevation in the thickness of epidermal layer. Sections from mice treated with IWP12 revealed marked decrease of epidermal layer thickness (Fig. 8).

## Discussion

Skin cancer is an aggressive high-mortality cancer with increasing incidence in the last decades. There are many risk factors; however, ultraviolet radiation, age and family history are the major factors. It is the most prevalent type of malignancy in USA, Australia and New Zealand. It is about tenfold more common in whites as compared with African-Americans [9]. The available therapeutic options are not sufficient with minor improvement in overall survivor rate [10].

The progression of skin cancer is mediated by many molecular changes that assure cells proliferation, invasion and metastasis. Many previous studies illustrated the role of Wnt/β-catenin pathway in embryonic development as well as adult tissue homeostasis such as migration, proliferation, hematopoiesis and repair. Any mutation developed in Wnt/β-catenin pathway is involved in tumor formation and progression [11]. Many previous studies illustrated the role of Wnt signaling pathway in carcinogenesis and neoplastic transformation in many types of cancer, such as breast cancer, prostate cancer and skin cancer [12, 13]. Therefore, Wnt signaling is a potential therapeutic target for treating skin cancer. In addition, activation of the Wnt/β-catenin is a predisposing factor in skin cancer [14, 15]. In canonical Wnt pathway, β-catenin tends to accumulate inside the nucleus

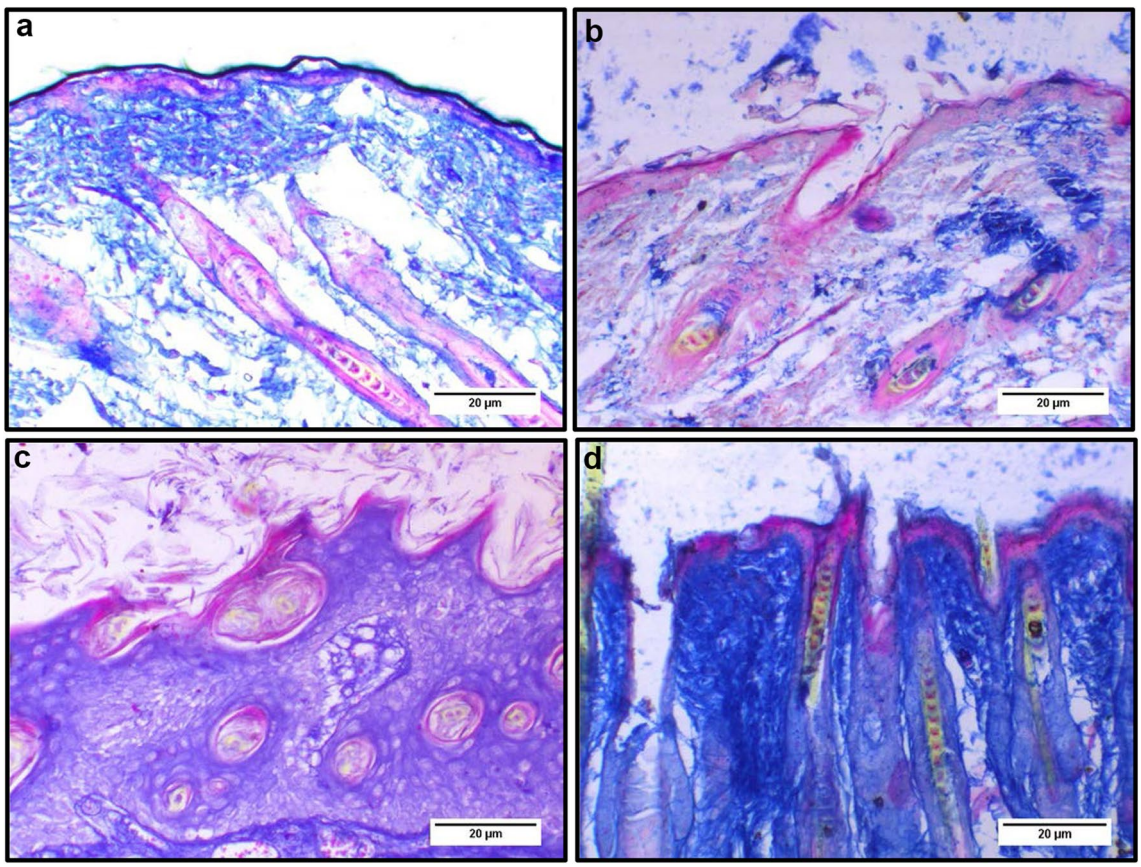


**Fig. 7** Skin sections stained with Mallory from control group showing normal epidermis (arrows) (**a**); control group treated with IWP12 showing normal epidermis (arrow) (**b**); skin cancer group showing epidermal hyperplasia, acanthosis, dysplasia, hyperkeratosis (arrow head) and dermal leukocytic infiltration (arrow) (**c**), and skin cancer treated with IWP12 (**d**) with x 200 magnification

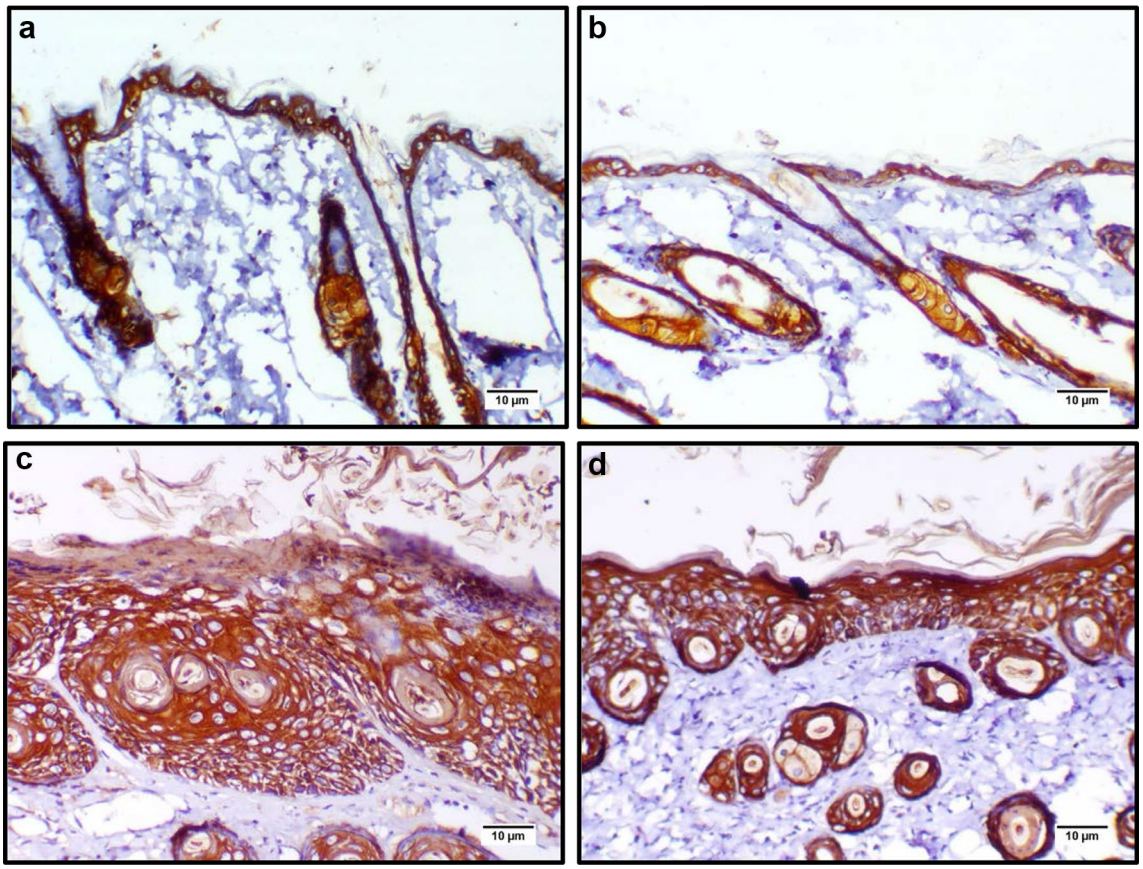


**Fig. 8** Skin sections stained with cytokeratin immunostaining from control group (**a**); control group treated with IWP12 (**b**); skin cancer group (**c**) and skin cancer treated with IWP12 (**d**) with x 200 magnification

and bind to T cell factor/lymphoid enhancer binding factor (TCF/LEF). This binding initiates the activity of many target genes, which plays a major role in monitoring proteasomal degradation [16]. It has been reported as predisposing factor in progression and deterioration of skin cancer [11, 17]. Moreover, β-catenin enhances cell–cell adhesion at the tumor site [18]. However, we found that treatment of mice with IWP12 blocked skin cancer-induced elevation in the expression of both Wnt and β-catenin associated with reduced course of skin cancer as indicated by reduction in the tumor number as well as reduced skin hyperkeratosis, parakeratosis, acanthosis and dysplasia.

SMADs are intracellular proteins that are engaged in TGF-β signaling and helps in the transducing of receptor signals to nucleus, where, they recognize the SMAD-binding elements [19]. SMADs are divided into three functional classes. SMAD1, 2, 3, 5 and 8 are formed by phosphorylation of TGF-β receptors substrate and called receptor-regulated SMADS or R-SMADs. SMAD4 is associated with R-SMADs for transfer of the signal to nucleus and called Co-SMAD. SMAD6 and SMAD7 are called inhibitory SMADs (I-SMADs) and antagonize the other SMADs [20]. TGF-β activates SMAD2 and 3 [2]. A previous study illustrated the relation between Wnt/β-catenin signaling and TGF-β-mediated fibrosis [21]. TGF-β is widely involved in skin cancer progression, invasiveness and metastasis [22]. It has two different roles in carcinogenesis. At early stages, it suppresses tumor progression by activating pro-apoptotic genes, while in the advanced stages, it activates tumor growth [23] as well as metastasis [24]. TGF-β deactivates antitumor response by many mechanisms, such as suppressing T-cell differentiation, promoting regulatory T cells and attenuation of tumor-specific cytotoxic T lymphocytes [25]. However, agents that block TGF-β are considered adjuvants for chemotherapy and radiotherapy in a wide range of cancers. The TGF-β blockage can be achieved by inhibiting receptor, reducing its binding to its receptor or reducing its expression [26]. However, we found that reduced SMAD3 and TGF-β by treatment of mice with IWP12 is associated with reduced skin cells abnormality and tumors numbers in mice.

Tumor microenvironment consists of a large set of inflammatory cells as neutrophils, macrophages, mast cells; killer cells and T- and B-lymphocytes that affect tumor initiation and progression. All these cells activate the production of

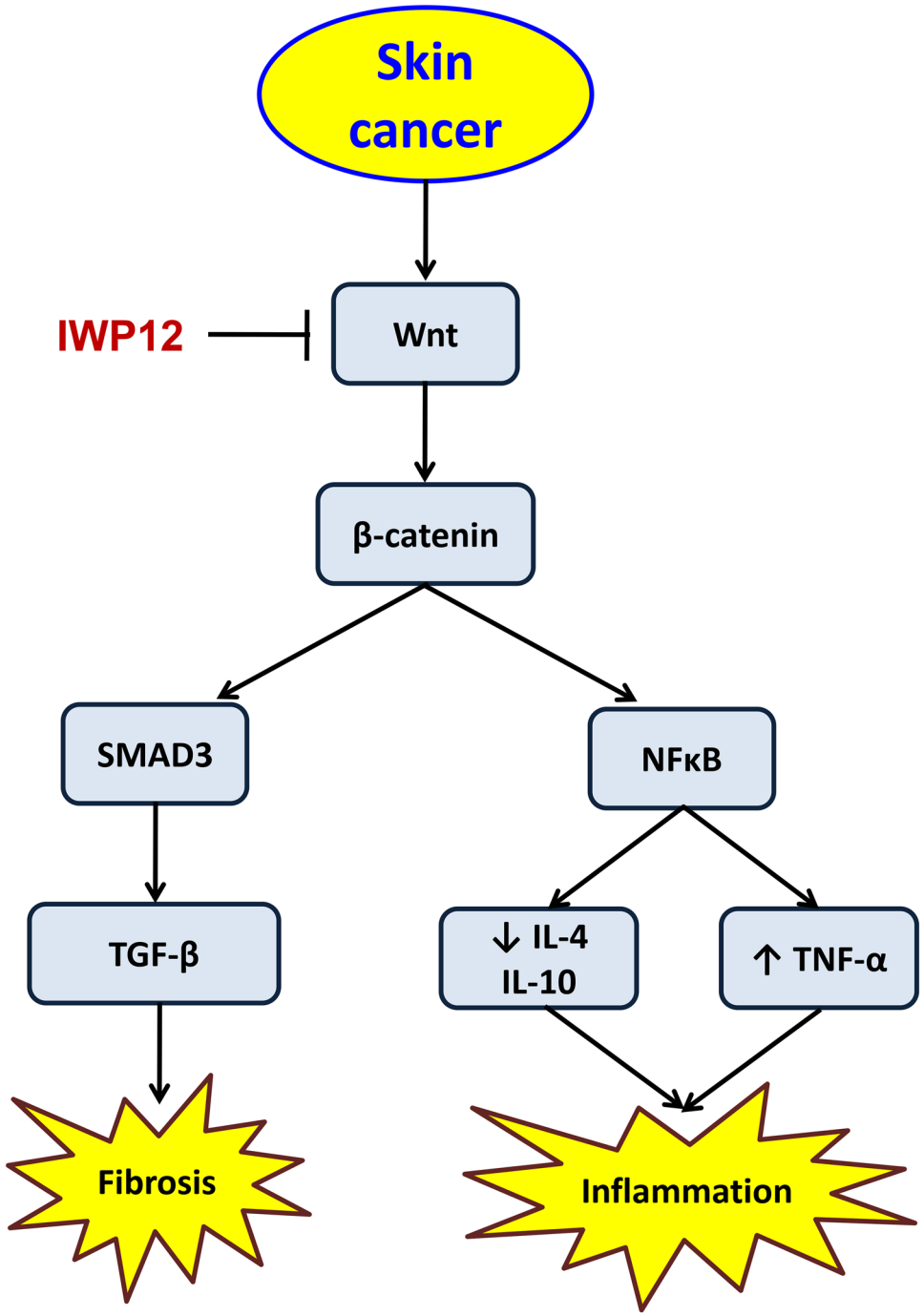


**Fig. 9** Mechanism of action of IWP12 in attenuating skin cancer induced in mice

interferons, TNF-α and interleukins [27]. In addition, different cytokines helps in promoting tumor development [28]. Skin cancer activates NFκB pathway leading to enhancement of developing drug resistance [29]. NF-κB is the major transcription factor in inflammation, which contributes to tumorigenesis. In skin, there are many NFκB-dependent genes, which are essential for initiation of cutaneous inflammation such as cytokines, chemokines, adhesion molecules and selectin [30]. TNF-α is correlated with many steps of carcinogenesis such as transformation, proliferation, angiogenesis, invasion, and metastasis [31]. In addition, genetic deletion of TNF-α in transgenic mouse is correlated with protection against ultraviolet radiation-induced skin cancer highlighting the direct relation between TNF-α and tumor progression [32]. Finally, we found that treatment of skin cancer mice with IWP12 blocked skin cancer-induced

elevation in the expression of inflammatory cytokines as well as enhanced the expression of anti-inflammatory cytokines. All these effects are associated with amelioration of skin cancer.

## Conclusion

Blocking Wnt production ameliorated skin cancer via blocking pro-inflammatory cytokines and enhancing the anti-inflammatory cytokines. Moreover, blocking Wnt attenuated skin cancer-induced activation of fibrosis pathway (Fig. 9).

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

**Ethical approval** All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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