Research Article

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Investigation of effect of vitamin D receptor, calcium-sensing receptor and β -catenin on cutaneous squamous cell carcinoma

D vitamini reseptörü, kalsiyuma duyarlı reseptör ve β -katenin'in skuamöz hücreli deri kanseri üzerine etkisinin araştırılması

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Abstract

Background: Cutaneous squamous cell carcinoma (cSCC) is a malignant and invasive tumor which is originated from epidermis with a high incidence among non-melanoma skin cancers. The aim of this study was to determine whether vitamin D receptor (VDR), calcium-sensing receptor (CaSR) and beta catenin (β -catenin) proteins have an effect on cSCC.

Materials and methods: VDR, CaSR and β -catenin proteins in tissue samples of cSCC and control group were analyzed by immunohistochemistry (IHC) and Western blotting (WB) method. IHC findings were statistically evaluated.

Results: IHC staining density of VDR and β -catenin were higher in cSCC tissue samples than control. The difference between IHC staining density of VDR and β -catenin in the patient and the control groups were statistically significant ($p=0.021$, $p=0.021$, respectively), but not for CaSR

($p=0.237$). While the VDR and β -catenin staining rates obtained by the IHC method could be supported by WB results, the WB bands for CaSR could not be shown.

Conclusion: The findings suggest that VDR and β -catenin may have an effect on the disease. Further research is required to better understand the role of VDR and β -catenin together on cSCC.

Keywords: VDR; CaSR; β -catenin; Cutaneous squamous cell carcinoma; Immunohistochemistry; Western blotting.

Öz

Amaç: Skuamöz hücreli deri kanseri (SHK), melanom dışı deri kanserleri arasında görülme sıklığı artan, epidermisten köken alan malign, invaziv bir tümördür. Bu çalışmanın amacı D vitamini reseptörü (VDR), kalsiyuma duyarlı reseptör (CaSR) ve beta katenin (β -katenin) proteinlerinin skuamöz hücreli deri kanseri (cSCC) üzerinde etkili olup olmadığını belirlemektir.

Gereç ve Yöntem: cSCC ve kontrol grubuna ait doku örneklerindeki VDR, CaSR ve β -katenin proteinleri immünohistokimya (IHC) ve Western blot (WB) yöntemi ile analiz edildi. IHC bulguları istatistiksel olarak değerlendirildi.

Bulgular: VDR ve β -catenin IHC boyama yoğunluğu, cSCC doku örneklerinde kontrol grubundan daha yüksekti. Hasta ve kontrol gruplarında, VDR ve β -katenin IHC boyama yoğunluğu arasındaki fark istatistiksel olarak anlamlıydı (sırasıyla $p=0,021$, $p=0,021$), fakat CaSR için anlamlı değildi ($p=0,237$). IHC yöntemiyle elde edilen VDR ve β -catenin boyama oranları WB sonuçları ile desteklenebilirken, CaSR için WB bantları elde edilememiştir.

Sonuç: Bulgular, VDR ve β -katenin'in hastalık üzerinde etkili olabileceğini düşündürmektedir. VDR ve

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β -catenin'in birlikte cSCC üzerindeki rolünü daha iyi anlamak için ileri araştırmaların yapılması gerekmektedir.

Anahtar Kelimeler: VDR; CaSR; β -catenin; skuamöz hücreli deri kanseri; immunohistokimya; western blot.

Introduction

Cutaneous squamous cell carcinoma (cSCC) is a malignant, invasive tumor originating from the epidermis. It is the second most common type of skin cancer [1, 2]. These tumors are defined as keratinocyte neoplasms occurring in sun-damaged skin. Cutaneous SCCs begin with impaired keratinocytes progressing to precancerous lesions [3]. Keratinocyte proliferation and differentiation are regulated by several factors including vitamin D, Wnt/ β -catenin signaling pathway. 1,25-Dihydroxyvitamin D3 [1.25 (OH) 2D3], which is the active metabolite of Vitamin D, suppresses proliferation of keratinocytes, while stimulating the differentiation of keratinocytes through vitamin D receptor (VDR). Recent studies have reported that VDR binds to β -catenin and suppresses its proliferative effect. Therefore, VDR and β -catenin are co-regulated [4].

7-Dehydrocholesterol in the epidermis is converted to vitamin D by the effect of ultraviolet B (UVB) in sunlight. Keratinocyte, the dominant cell of the epidermis, metabolizes vitamin D to its active form [1.25 (OH) 2D3]. Keratinocytes also express VDR for production of 1.25 (OH) 2D3. 1.25 (OH) 2D3/VDR promotes the formation of skin layers by regulating cell proliferation in the basal layer and stimulating successive keratinocyte cell differentiation [5]. It has been indicated that keratinocyte differentiation is destroyed with impaired calcium absorption and hypocalcemia in VDR-deficient mice [6]. The anti-proliferative effect of 1.25 (OH) 2D3 in basal keratinocytes involves inhibition of the β -catenin and hedgehog (HH) signaling pathway through VDR [7].

Calcium-sensing receptor's (CaSR) function in cancer formation has been demonstrated in colon, parathyroid, stomach, neuroblastoma, breast, prostate, ovarian, and kidney cancers [8]. The studies reinforce the idea that calcium and vitamin D cooperate for keratinocyte differentiation and proliferation [4]. It has been shown that both calcium and 1.25 (OH) 2D3 stimulate differentiation in keratinocyte cultures and have a synergistic effect. In animal studies, VDR and CaSR gene mutant mice have been reported to develop spontaneous squamous skin cancer without UVB and chemical effect [5]. Colorectal cancer has been a good model for calcium abnormalities in skin tumors. Vitamin D and β -catenin signaling have

been demonstrated in colorectal cancer development and in human colorectal cancer cell lines [9].

It is known that VDR, CaSR and Wnt/ β -catenin play a role in tumor formation in various cancer types. The Ca^{2+} /CaSR signaling pathway, which is functionally associated with VDR-activated pathways, has been shown to suppress proliferation and promote differentiation in colorectal cancer by targeting the effectors at the lower stage in the Wnt/ β -catenin pathway. Ca^{2+} /CaSR and 1.25-(OH) 2D3/VDR inhibit Wnt signaling pathway, regulating the proliferation and differentiation of colorectal cancer cells and colonocytes [9]. In the literature, there was no study about the effects of VDR, CaSR and Wnt/ β -catenin on the tumor formation in skin cancer types. In this study, we investigated the relationship between VDR, CaSR and β -catenin proteins in cutaneous squamous cell cancer by using immunohistochemical and Western blotting methods.

Materials and methods

Patients group

The skin materials were obtained from four patients applying to the Department of Dermatology of Hafs Sultan Hospital in Manisa Celal Bayar University and being diagnosed with cutaneous squamous skin cancer. Samples were taken at a thickness of 6 mm with punch biopsy.

Control group

The skin materials were obtained from four healthy individuals who applied to the Department of Aesthetic Surgery of Hafs Sultan Hospital in Manisa Celal Bayar University.

The ethics committee permission was obtained for this research. The persons who participated in the study received permission from the volunteer consent form.

Paraffine embedding and sectioning

The tissue materials fixed with 10% formalin were dehydrated by increasing the alcohol series and the paraffin block preparation protocol was applied. The sections of 3.5 μm thickness of the paraffin blocks were taken to the polylysine slides using microtome (ThermoScientific, USA, Microm HM-325).

Hematoxylin and eosin staining

The preparations were rehydrated by increasing alcohol series and stained according to hematoxylin-eosin staining protocol [10] and examined with light microscope (Olympus BX43).

Immunohistochemical method

The sections were incubated in xylene for 1 h. Then, they were kept in the absolute alcohol twice for 15 min and in decreasing alcohol series (96%, 80%, 75%) for 12 min, respectively. Thus, rehydration occurred.

The sections were washed twice in distilled water for 3 min. They were incubated at room temperature for 15 min with 3% hydrogen peroxide (H_2O_2) solution. They were washed twice for 5 min with phosphate buffer saline (PBS). Citrate buffer was applied at 96°C for 30 min. The sections were washed 3 times for 5 min with PBS. Then they were incubated in the blocking solution (EMD Millipore Corporation IHC select Blocking Reagent Lot: 2838287) at room temperature for 30 min. The sections were waited for 20 min with avidin (VectorLabs, USA, Cat # SP-2001) and washed for 5 min with PBS. They were incubated for 20 min with biotin (VectorLabs, USA, Cat # SP-2001) and washed for 5 min with PBS. The primary antibodies which are β -catenin (Abcam, USA, Cat, ab22656), VDR (Santa Cruz, USA, Cat, sc-13133) and CaSR (Abcam, USA, Cat, 19347) were applied to the sections at +4°C. for a night. After incubation, the sections were washed for 5 min with PBS. Secondary antibody [biotin chicken anti-mouse IgG H&L (Abcam, USA, cat Ab6813)] was diluted with PBS at the rate of 1:200. The sections were kept at room temperature for 1 h 45 min with secondary antibody.

The washing process was repeated with PBS. The sections were left in HRP-streptavidin diluted with 1:200 ratio of PBS for 1 h and 30 min. After washing, they were stained for 2 min with diaminobenzidine (DAB) solution to make the immune activities visible.

The sections were washed three times for 5 min with distilled water. They were stained for 4 min with Harris haematoxylin-eosin (Facepath, Cat # 0231) and washed twice for 5 min with tap water. They were kept in absolute alcohol for 5 min and in the xylene for 10 min, respectively. Prepared preparations were examined in the light microscope (Olympus BX43, Japan).

The density of IHC staining between control and patient groups were analysed by using the ImageJ program (version 1.46c, NIH, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>).

Tissue homogenization and protein determination

The weight of the tissues of the patient and control group were determined. Three milliliter/grams of RIPA buffer (Sigma-Aldrich Cat # R0278) was added to each tissue. The tissues were homogenized with bead homogenizer (Mini bead Beer-16, Biospec, USA). After homogenization, proteolytic enzymes were blocked. For this purpose, 10 mg/mL phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA) and 30 μ g protease inhibitor cocktail (Sigma, Cat # 8340) were added to each gram tissue and the tissues were incubated in ice for 1 h. After incubation, the tubes were centrifuged at 8000 g and +4°C for 20 min. Supernatant was aliquotated in volumes of 15 μ L and hid at -80°C. In order to determine the total protein concentration in stored tissue lysates, the technique of bikinininikacid (BCA) (Thermo Scientific, USA, Catg 23225) was used.

SDS-PAGE method

The SDS-PAGE method was performed by using the Mini-protean III electrophoresis system (Bio-RadLabs, USA, Cat # 165-3301) and the Laemmli method [11]. For the process of executing the proteins, the lower (resolving-separation) gel and the upper (stacking) gel were prepared to be 12% and 4%, respectively. Protein marker (Thermo Scientific, USA, cat # 10747012) and samples were carried out at 90 V – 15 mA for 2 h and 15 min.

Western blotting assay

After gel electrophoresis, gel was waited in transfer buffer for 30 min. It was transferred to 0.2 μ m polivinylidendifloride (PVDF) membranes (Immun-Blotting®, Bio-RadLabs, USA, Cat # 1620174) at 100 V 350 mA for 2 h by using a blotting unit (Mini Trans-BlotElektrophoretic Transfer Cell, Bio-RadLabs, USA, Cat # 170-3930). After the transfer, the membranes were blocked in the 1X casein solution diluted with Tris Buffered Saline with Tween 20 (TBST) at room temperature for 30 min. After blocking, they were washed three times for 10 min with TBST. The membranes were incubated at +4°C overnight with the primary antibody diluted with TBST at the rate of 1:1000. They were washed three times for 10 min with TBST. Then they were incubated for 1 h at room temperature by applying the secondary antibody diluted with TBST (1:1000 ratio). The membranes were washed three times for 10 min with TBST.

The membranes were then incubated at room temperature for 30 min with streptavidin peroxidase (Vector, Cat $\neq$ SA-5004) diluted with TBST at the rate of 1:500. They were washed twice for 10 min with TBST. They were incubated for 15 min with DAB substrate (RocheAppliedScience, Cat $\neq$ 11718096001) and reactions were made visible. After the bands were observed, the reaction was stopped with pure water and the membranes were scanned with a scanner.

Statistical analysis

The SPSS (statistical package software, Windows 23.0) was used to analyze the significancies of differences observed in between the groups. The obtained IHC data were tested between the two groups (control and patient) by Mann-Whitney U test. The p-values smaller than 0.05 was considered as significant. Data were presented as a mean $\pm$ standard deviation (sd).

Results

Histomorphology

According to results of hematoxylin eosin (HE) staining, the epidermis stratification is regular in the control group. The basal cell layer is composed of single-row and cuboid cells. These cells have dark stained and oval nuclei (Figure 1).

According to HE staining, thickening of the epidermis and dermis layers and deterioration in cellular alignment in cSCC tissues were observed. Neoplastic keratinocyte islands and keratin pearls were observed in cSCC tissues (Figure 1).

Immunohistochemistry (IHC)

In the tissue sections of the control group samples, it was observed that VDR staining (Figure 2) was high throughout the epidermis as a result of being marked with 3.3'-DAB in the presence of the VDR antibody. In some parts of the dermis layer, the IHC staining was observed even though it was very low (Figure 2A).

While VDR staining was not observed in the thickened keratin layer as a result of DAB marking in the presence of VDR antibody in the tissue sections of the samples who received cSCC diagnosis, the IHC staining was observed in epidermis, dermis and tumor cells at a high rate. The integrity between epidermis and dermis was lost in tissue sections of cSCC patient samples (Figure 2A).

In the samples belonging to the control group, CaSR staining was observed in the cells in the epidermis as a result of marking with DAB in the presence of the CaSR antibody. In some parts of the dermis layer, there is a very low rate of staining (Figure 2B).

In the cases that have been diagnosed with cSCC, CaSR staining was observed at very low rate in both epidermis and dermis layer as a result of marking with DAB in the presence of CaSR anticorrosion. The integrity between epidermis and dermis was lost in tissue sections of cSCC patient samples (Figure 2B).

In the tissue sections of the control group samples, it was observed that the β -catenin staining was limited to epidermis. The staining density of the dermis layer was very low (Figure 2C).

It was observed that β -catenin staining was significantly high in both the epidermis and dermis in the sections of cSCC. The integrity between epidermis and dermis was lost in tissue sections of cSCC patient samples. While more intensive cytoplasmic staining was observed on peripheral areas in the keratinocyte islands, weak staining was observed in central regions (Figure 2C).

Figure 1: HE staining of the control and patient groups (x4). Epidermis papiller dermis (stars) in the section of control tissue. Keratinocyte islands (dark arrow) and keratin pearls (white arrow) in the section of patient tissue.

Figure 2: Results of IHC staining for investigated proteins in the control and cSCC groups. (A) VDR, (B) CaSR and (C) β -catenin (X20).

The results of IHC staining rates of VDR, CaSR, β -catenin proteins determined with Image J analysis programme were given in Table 1. The staining rates of VDR and β -catenin were higher in the patient group (62%, 72%, respectively) than control group (21.2%, 12.46%, respectively). IHC staining rate of CaSR was higher in the control group (5.4%) than patient group (4.3%).

Western blotting assay

In the samples of control and patient groups, the bands of VDR (48 kDa) and β -catenin (85 kDa) were obtained

(Figure 3). There is no results for CaSR (120,6 kDa). In the findings obtained from the Western blotting method, the differences between the VDR and β -catenin were observed in the patient and control groups.

Table 1: Antigen-antibody interaction rates obtained from immunohistochemistry study in sample tissue sections according to image J analysis program.

Group	VDR	CaSR	β -catenin
Control	21.2%	5.4%	12.46%
Patient	62%	4.3%	72%

Figure 3: The Western blotting bands of VDR and β -catenin in membran filter for the control and patient groups (48 kDa, 85 kDa, respectively). β -actin was used as an internal control. 1–4: control group, 5–8: patient group.

Statistical analysis

It was determined that IHC staining for VDR and β -catenin were statistically different between the patient and control groups, respectively ($p=0.021$, $p=0.021$). There was no statistically significant difference for CaSR staining ($p=0.237$) (Table 2).

Discussion

Cutaneous squamous cell cancer is the most common non-melanoma skin cancer in the world. The cancer occurs due to excessive proliferation of keratinocytes, which has deteriorated the mechanism of differentiation in epidermis. Keratinocyte proliferation and differentiation are regulated by several factors [12]. It is suggested that there has been a common arrangement between signalling pathways of VDR and β -catenin since the molecular interactions between them were revealed [4]. In addition, it has been reported that CaSR inhibited Wnt/ β -catenin signalling in different cancers and there is an inverse relationship between them [13, 14]. It has been reported that CaSR-mediated calcium signalling may

affect the function of VDR, but these mechanisms have not been fully clarified [15].

In our study, we investigated VDR, CaSR and β -catenin proteins in cancer tissue of cSCC patients. When VDR and β -catenin protein IHC staining were compared in patient and control tissues, it was found that there was a statistically significant difference ($p=0.021$, $p=0.021$, respectively). Although CaSR staining was found to be higher in patient tissues, there was no statistically significant difference between patient and control group ($p=0.237$). Similar to the results of immunohistochemistry obtained for both β -catenin and VDR, Western blotting analysis results were also obtained. According to IHC, CaSR staining was found to be very low only in the control group samples. In WB analysis, failure to obtain CaSR bands may be due to very low levels of protein in the skin tissue.

In our study, hematoxylin eosin staining properties obtained from paraffin blocks of control and patient group are compatible with the literature [16]. VDR regulates the expression of various genes involved in calcium/phosphate homeostasis, cellular proliferation, cellular differentiation and immune response in different tissues [17]. VDR plays a role in the formation of signal for anti-proliferative and pro-differentiation. Loss of function in VDR is involved in the formation of various tumors [18]. $1.25(\text{OH})_2\text{D}_3/\text{VDR}$ interaction plays a role in the regulation of the differentiation of keratinocytes from the basal layer to the upper layers in the epidermis. Keratinocytes express VDR in response to the production of $1.25(\text{OH})_2\text{D}_3$ [5]. Impairment of the function of the VDR creates susceptibility to epidermal cancer by increasing the proliferation of keratinocytes and decreasing differentiation [19, 20].

Some studies related to breast cancer indicated that there was a higher level of VDR in tumour tissue [21]. Reichrath et al. [22] reported that there was a high level of mRNA and protein expression of VDR in basal cell cancer (BCC). According to our results with immunohistochemical method, VDR immunoreactivity was found to be higher in cSCC patients. When VDR staining was compared between patient and control groups, the difference was found to be statistically significant ($p=0.021$). Our WB results related to VDR staining also supported IHC data. Our findings are consistent with the results of the study conducted with breast cancer [21] and BCC [22].

Evans et al. [23] reported that there was no evidence of VDR expression in advanced lesions at different stages of colon cancer. A similar finding has also been reported for prostate cancer [24]. In the other study, it was found that VDR expression decreased in sun-exposed and non-sun-exposed melanomas [25]. In our study, IHC staining and WB band density were found to be higher in the patient

Table 2: Mann-Whitney U test results for control and patient groups.

Protein	Control (mean rank) (mean $\pm$ sd) (n=4)	Patient (mean rank) (mean $\pm$ sd) (n=4)	p-Value ^a
VDR	2.50 (0.090 $\pm$ 0.022)	6.50 (0.452 $\pm$ 0.200)	0.021 ^b
CaSR	5.50 (0.023 $\pm$ 0.016)	3.50 (0.015 $\pm$ 0.009)	0.237
β -catenin	2.50 (0.053 $\pm$ 0.050)	6.50 (0.052 $\pm$ 0.314)	0.021 ^b

^ap-Values were compared between control group and patient group.

^bVariables with p values less than 0.05, sd, standard deviation.

group for VDR. This finding is different from the result reported for colon [23], prostate cancer [24] and melanoma [25]. This suggests that VDR expression may differ depending on the cell type in various types of cancer.

CaSR in cancer has a role as yin-yang. CaSR regulates proliferation, differentiation, cell death, angiogenesis and migration in cancer. It acts as a tumour suppressor in colon, parathyroid, stomach and neuroblastoma and as oncogene in breast, prostate, ovarian and renal cancers [8].

CaSR expression increases during colorectal cancer progression and the mechanism is not fully understood [26]. In prostate cancer, CaSR responds to increase cell proliferation depending on high calcium levels. In contrast to this data, the loss of CaSR in parathyroid carcinoma [27] and other tumours promotes tumour cell proliferation [9]. In our study, CaSR staining was found to be higher in the control group than the patient group in immunohistochemical marking. However, there was no statistically significant difference between them ($p=0.237$). No clear result about the role of CaSR in cSCC has been obtained.

Wnt/ β -catenin protein activation and β -catenin mutations are the most common changes associated with tumour growth and migration of cancer cells or metastasis [28]. Altered expression of β -catenin have been associated with a loss of differentiation, a more aggressive phenotype, increase in tumour invasion, and poor prognosis in a number of different cancers [29]. β -catenin is highly expressed in various epithelial cancers [30]. In studies of skin cancer, the β -catenin expression level was found to be quite high in BCC from non-melanoma skin cancer types [31, 32]. In papillary thyroid carcinoma, cytoplasmic accumulation of β -catenin were associated with aggressive clinicopathologic behaviour [33]. In our study, beta catenin staining was found to be more intense in the patient group and there was a statistically significant difference between the control and patient group ($p=0.021$). The results were consistent with previous studies.

It was reported that vitamin D and VDR in the hair follicle differentiation in mice had reciprocal effects on β -catenin signalling and alopecia occurred in VDR-deficient mice [4]. It was indicated that the interaction between β -catenin and VDR contributed to some types of skin cancer. In a study in which VDR was deleted or mutated, it was reported that β -catenin was overexpressed and caused hair follicle tumours [34].

In the present study, it was observed that the IHC staining of VDR and β -catenin were close to each other in patient group. In addition, there was a statistical difference between the staining rates in VDR and β -catenin among control and patient groups. It was considered that both proteins may be associated with the development of cSCC.

Conclusions

This study has scientific value because it is the first study in terms of the relationship between VDR, CaSR and β -catenin proteins in cutaneous squamous cell cancer in humans. It was determined that there was no statistically significant difference between CaSR staining in control and patient groups. There was a difference in the Western blotting band density between the patient and control groups for both VDR and β -catenin. It has indicated that the relationship between VDR and β -catenin may have an effect on the development of cSCC. Further research is required to better understand the role of VDR and β -catenin together on cSCC.

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

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration (Decision number of MCBU Faculty of Medicine Ethics Committee of Health Sciences: 20478486-050.06.04).

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