

## Antitumor Effects of Vitamin D3 (1 $\alpha$ , 25(OH) 2D3) On Oral Squamous Cell Carcinoma in Hamsters

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### Abstract:

**background:** Squamous cell carcinoma makes 90% of malignancies affecting the oral cavity and pharynx. Active Metabolite of vitamin D3 (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>) is thought to have an anti -oncogenic effect . This research is a study of the effectiveness of vitamin D in protecting against Squamous cell carcinoma and its role of curbing the tumor growth by analyzing the expression of tumor curbing protein P53.

**Materials and methods :** Incubated 32 Syrian Hamsters of ( 2-3) months age weighing 50 -80 Grams, were divided into two groups, and the carcinogen DMPA was applied to one of them on the buccal pouch three days weekly , while in the second group was given the active form of vitamin D<sub>3</sub> in addition to the Carcinogen three days weekly too. In the second week of the experiment , samples were taken for histoanalysis and immunohisto analysis of protein P53. This procedure was repeated systematically in the sixth, tenth, and fourteenth weeks respectively. A statistical analysis was performed using CHI-SQUARE test at a level  $P < 0.05$  in order to detect expression differences between the two groups.

**Results:** 28.7 % of the mutant P53 expression when comparing all periods of the first arcinogen group, and 18.7 % for the Vitamin group (the second group) . Using Chi-Square test showed that the difference was statistically significant at a level of  $P < 0.05$ .

**Conclusions :** Vitamin D<sub>3</sub> contributed by curbing tumor-growth and helped commencement and stimulation of programmed cellular death of genetically damaged cells and limiting damaged cells growth and multiplication.

**Keywords :** Squamous cell carcinoma – D3 active form – tumor growth curbing Protein P53.

### I. Introduction

Squamous cell carcinoma makes 90% of malignancies affecting oral cavity and pharynx<sup>1</sup>. This tumor especially affects older people, men rather than women. Smoking and Alcohol are considered the most likely predisposing factors for this tumor, which is detriment to squamous cells in epidermis and may extend to the lymph nodes or other far organs. This tumor could be seen in any part of the oral cavity but the most common are : tongue, floor of the mouth, it also may affect vestibular mucosa, gingivae and palate. Outside the oral cavity it mostly affects the lower lip<sup>2</sup>. Histologically, the tumor appears by destroying epidermis basement membrane and invading stratum Squamous epidermis. These cells produce Keratin and form inverted keratinization balls<sup>3</sup>. P53 gene is called Guardian of the Genome, it's a tumor curbing gene that disables and stops neoplastic transformation using P53 Protein. Somatic mutation of P53 is frequent in the development of human oncologic transformations<sup>4</sup>. Accumulation of the mutant P53 is noticeable in 76% of 212 human malignancies including breast, colon, stomach cancers, melanoma, bladder transitional cancer, uterus cancer, and other soft tissue cancers. Healthy form of P53 is considered as a duplicating factor due to its ability to create crucial genetic changes whether in activating or terminating cell's life cycle by curbing DNA multiplication, and it is a controlling observer for cellular life cycle and participates in programmed cellular death<sup>5</sup>. Healthy P53 Protein works in cancerous lesions as tumor curbing gene. In contrast, the mutant form works as a dominant oncogenic gene<sup>6</sup>.

Studies indicate that the type of food has an important role in initiating or preventing the tumor. Many researches were made to prove vitamin D role as a prophylactic and curative factor of oncologic genesis<sup>7,8</sup>.

The main function of vitamin D is to maintain normal plasma concentrations of calcium and phosphorus. On the other hand, 1,25- dihydroxy vitamin D<sub>3</sub>, the active form of vitamin D, is a steroid hormone, that probably has an anti-reproducing effects that can activate programmed cellular death passages and repress blood vessels formation<sup>9</sup>. Many studies were made to specify vitamin D effect on cancerous cell in different

tumor types, and found a preventive relationship between possession of the required amount of vitamin D and neutralizing cancer hazardousness.

In a study of the vitamin D analogs effects on cancerous cells in dermal Squamous cell carcinoma; it was found that these analogs caused curbing of cancerous cells growth by terminating cellular life cycle and encouraging programmed cellular death by increasing some mortifying encouraging proteins expression<sup>11</sup>.

Another study was conducted on Hamsters to determine vitamin D role on the developed Squamous cell carcinoma in Hamster buccal pouch after applying DMBA (Dimethyl benzanthracene) mixed with mineral oil 0.5% . It was found that vitamin D treatment delayed appearance of the cancerous lesions by performing a prophylactic action<sup>12</sup>

## **II. Research Objective**

This research was conducted in order to inspect vitamin D3 ability in its active form ,to prevent epidermal Squamous cell carcinoma, and verify its suggested role in tumor growth curbing by examining mutant P53 expression.

## **III. Materials And Methods**

### **Research sample :**

**Experiment animals:** hamster.

An approval was gained from the ethics committee at Damascus university to conduct the study. 32 experimental animals (Syrian hamster) were incubated in scientific research equipped incubators, which belong to the Faculty of Pharmacy at Damascus University, in prescribed conditions of ventilation, diet, and light, while taking into consideration all the animals to be males to avoid feminine hormonal effects, and to approximately have weight in the range of 50 – 80 grams in the first week .

These hamsters were divided into two main groups ,each group consisted of 16 hamsters ; the first is carcinogen group , in which the carcinogen was applied on the buccal pouch thrice a week using carcinogen DMBA manufactured by SEGMA company 7.12 Dimethyl benz [a] anthracene . After mixing 1 gram of DMBA with 200 ml of mineral oil , a size 4 wooden coloring brush was used to apply the substance on the buccal pouch -left side- to motivate the formation of Squamous cell carcinoma.

Whereas in the second group – vitamin group – the Carcinogen was applied three days weekly along with the active form of vitamin D3 (used in its pharmaceutical oral solution form manufactured by Roxana laboratories U.S ( Calcitriol)Roxana -according to used dosage, where 0.25 microgram for each kilo of body weight<sup>12</sup>).

### **Sacrificing hamsters, taking and preparing samples:**

Hamsters intended for sacrificing were chloroformed. Death happened by suppression of respiration. Sacrifice was according to a timetable with equal numbers of the two main groups (Carcinogen group, and Carcinogen with the active form of D3 group). Following hamster death, left buccal pouch on which the Carcinogen applied, was enucleated from the two groups.

The samples were stabilized using (Formalin 10%) and then laboratorial phases started by stabilizing , dehydration, slicing, consolidating with Paraffin, preparing and slicing the samples at 4 microns thickness

### **P53 portion Immuno-staining method:**

1 - Removing paraffin, rehydration (after slicing). Afterwards, applying immuno-staining set according to instructions of the manufacturing company (EPITOMICS- California U.S ), also using immuno-stain for P53 from (DAKO com. Denmark) ,and adding Hematoxylin normally for 10 seconds.

2 –Washing slides with water and drying by placing them in two 3 minute ethanol showers (100% concentration), followed by two 3 minute xylol showers, then shrouding slides in permanent incubating medium.

3 – Examining and counting slides by studying four areas of each specimen, and calculating positive and negative cells in 400 cell of each specimen.

### **Statistical analysis:**

Research statistical calculation was executed using SPSS 13.0 software; and using Chi-square test to analyze differences significance of the analyzed proteins over-expression, at a level  $P < 0.5$ .



Figure (1) A – Buccal pouch in the fourteenth week (Vitamin group).

B – Buccal pouch in the fourteenth week (Group without vitamin) images show cancerous lumps in hamster buccal pouch.

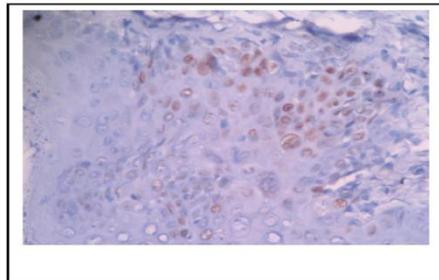


Figure ( 2 )(× 40 )

Shows P53 mutant form expression in nucleus with brown (group 1, week 6)

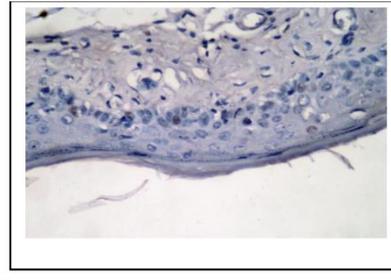


Figure ( 3 )(× 40 )

Shows P53 mutant form expression in nucleus with brown (group 2, Vitamin Group, week 6).

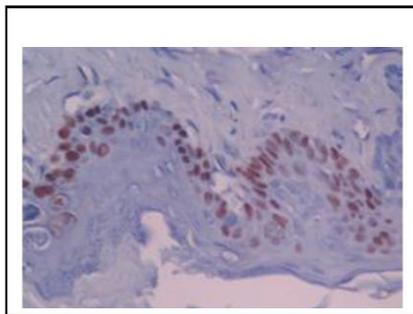


Figure ( 4 )(× 40 )

Shows P53 mutant form expression in nucleus with brown (group 1, week 10).

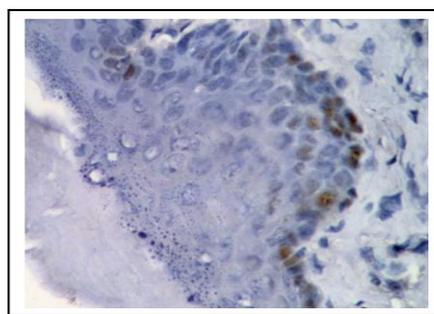


Figure ( 5 )(× 40 )

Shows P53 mutant form expression in nucleus with brown (group 2, Vitamin Group, week 10).

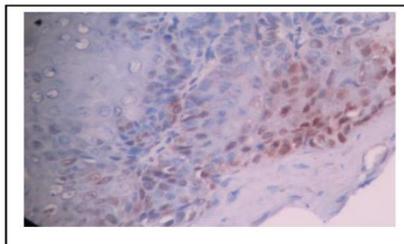


Figure ( 6 )(× 40 )

Shows P53 mutant form expression in nucleus with brown (group 1, week 14).

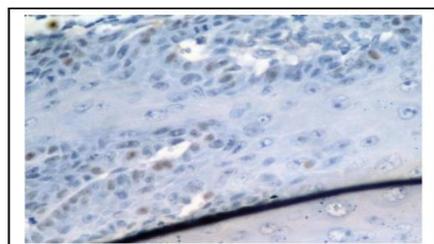


Figure ( 7 )(× 40 )

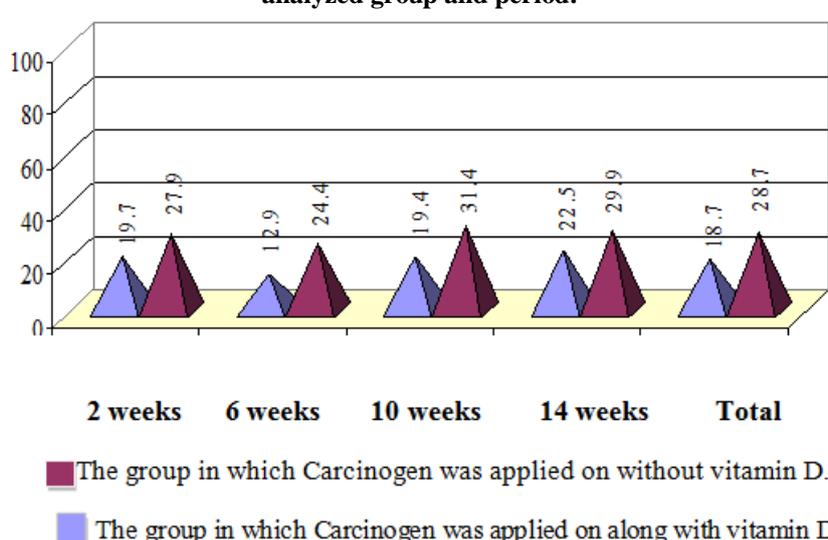
Shows P53 mutant form expression in nucleus with brown (group 2, Vitamin Group, week 14).

IV. Results

• P53 protein expression analysis :

P53 hyper-expression observing results depending on the analyzed group and period:

Graph (1) resembling P53 hyper-expression percentage in the research specimen depending on the analyzed group and period:



• Analysis of analyzed group impact on P53 hyper-expression frequencies in research specimen in compliance with study period:

Chi-square test was made to analyze differences in P53 hyper-expression frequencies between the group in which carcinogen was applied on along with vitamin D, and the group without vitamin D; in research specimen in compliance with analyzed period as follows :

Chart (1) Chi-square test results:

Shows the results of Chi-square test to analyze differences in P53 hyper-expression frequencies between the group in which Carcinogen was applied on along with vitamin D, and the group without vitamin D; in research specimen.

Chart ( 1)

The two analyzed variables = analyzed group × P53 protein hyper-expression frequencies						
Analyzed Protein	Analyzed period	Cells number	Chi-square value	Liberty levels	P estimated value	Differences significance
P53	2 weeks	3496	32.30	1	0.000	Significant differences exist
	6 weeks	3627	78.59	1	0.000	Significant differences exist
	10 weeks	4871	89.65	1	0.000	Significant differences exist
	14 weeks	4229	28.41	1	0.000	Significant differences exist
	Total period	16223	220.76	1	0.000	Significant differences exist

Chart (1) exposes that P value is much smaller than 0.05 value regardless of the analyzed period ; which means at reliability level 95% , there is statistically significant differences in P53 over-expression frequencies of research specimen, between the two groups : ( Carcinogen + vitamin D group) and ( without vitamin D).

By analyzing frequencies chart and subordinate percentages:

It is noticeable that P53 hyper-expression percentage of (Carcinogen + **vitamin D group**) was smaller than percentage of (Carcinogen without vitamin D group).

• Analysis of analyzed period impact on P53 hyper-expression frequencies in research specimen in compliance with analyzed group:

Chi-square test was performed to analyze the differences in P53 over-expression frequencies between analyzed period groups ( 2 , 6 , 10 , and 14 weeks) in research specimen in compliance with analyzed groups as follows :

Chai-square test results Chart (2)

- Shows the results of Chi-square test to analyze differences in P53 hyper-expression frequencies between analyzed period groups( 2 weeks, 6, 10, and 14 weeks ) in research specimen in compliance with analyzed groups as follows :

Chart ( 2)

Analyzed Protein	Analyzed group	Period (A)	Period (B)	Cells number	Chi-square value	Liberty levels	P estimated value	Differences significance
P53	Carcinogen + Vitamin D Group	2 weeks	6 weeks	3394	32.30	1	0.000	No significant differences
			10 weeks	3906	78.59	1	0.820	No significant differences
			14 weeks	3450	89.65	1	0.045	significant differences exist
			10 weeks	3944	28.41	1	0.000	No significant differences
		6 weeks	14 weeks	3488	220.76	1	0.000	significant differences exist
		10 weeks	14 weeks	4000	220.76	1	0.017	significant differences exist
	Carcinogen <u>without</u> Vitamin D Group	2 weeks	6 weeks	3729	32.30	1	0.015	significant differences exist
			10 weeks	4461	78.59	1	0.014	significant differences exist
			14 weeks	4275	89.65	1	0.169	No significant differences
			10 weeks	4554	28.41	1	0.000	significant differences exist
		6 weeks	14 weeks	4368	220.76	1	0.000	significant differences exist
		10 weeks	14 weeks	5100	220.76	1	0.248	No significant differences

Chart (2) shows that P value is smaller than 0.05 by comparing the period (14 weeks) with other analyzed periods of (Carcinogen plus vitamin D).

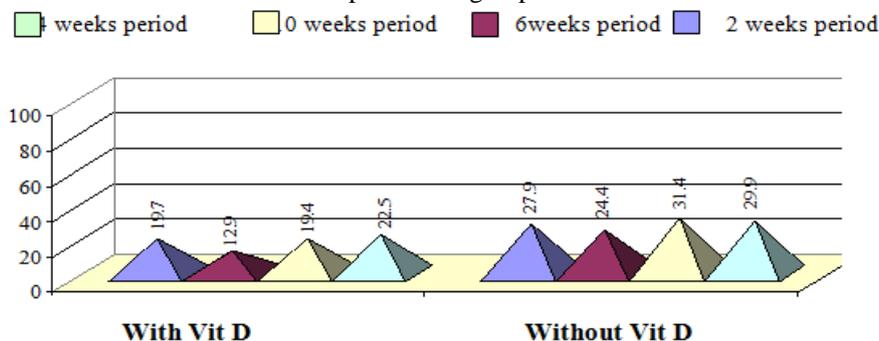
Comparing the period (6 weeks) with the remaining analyzed periods, and the comparison between the periods (2 weeks, and 10 weeks) in the carcinogen without vitamin D group; at reliability level 95%, there is a statistically significant differences in P53 over expression- frequencies of research specimen, between the analyzed periods.

By analyzing frequencies chart and percentages :

In the research specimen, it is noticeable that P53 over-expression percentage of the period (14 weeks) was greater than percentage of the remaining analyzed periods in (Carcinogen plus vitamin D group). Furthermore, it is noticeable that P53 over-expression percentage of the period (6 weeks)- was smaller than percentage of the remaining analyzed periods, and P53 over-expression percentage of (2 weeks) period was smaller than percentage of (10 weeks) period in (carcinogen without vitamin D group).

In the rest of the analyzed comparisons, it is noticeable that P value is much greater than 0.05 value. This Means that, at reliability level 95%, there isn't any statistically significant differences in P53 over-expression frequencies between the above mentioned periods for research specimen.

Graph (2) resembling P53 hyper-expression percentage in the research specimen depending on the analyzed period and group:



### V. Discussion

After the 14-week experiment, Squamous cell carcinoma was recorded in four cases in the first group in which Carcinogen was applied. On the other hand, a case of localized cancer, two cases of malformation, and Squamous cell carcinoma case were recorded in the second group (carcinogen plus vitamin D group).

On studying mutant P53 expression, we noticed that its expression after two weeks in the group in which the Carcinogen was applied, was 27.9%, and in the second group (carcinogen plus vitamin ) was 19.7 %.

After six weeks, its expression in (carcinogen group without vitamin) was 24.4 % , and in the second group ( carcinogen plus vitamin )was 12.9% .

After ten weeks, in (carcinogen group) its expression was 31,4% , and in the (carcinogen plus vitamin ) group was 19.4% .

After fourteen weeks, in the carcinogen group its expression was 29.9%, and in the (carcinogen plus vitamin) group was 22.5% .

When comparing all periods we noticed that: P53 expression in the first group ( carcinogen without vitamin) was 28.7%, and in the second group ( carcinogen plus vitamin ) was 18.7%. This difference had a statistical value.

This value was noticeable when comparing each period separately ; thus we can say that :1) inactive mutant P 53 expression was smaller in the second group compared with the first group ,2) the effect of vitamin D3 was similar, after two, six, and ten weeks, the expression percentage was between 12. 9% and 19.7% . Nonetheless, its effect decreased in the fourteenth week when inactive mutant P53 expression increased to 22.5% , whereas in the first group (carcinogen without vitamin) expression was increasing with the time of applying the carcinogen , and cellular trauma increasing .

As a result, vitamin D3 has contributed in curbing tumor growth by P53 efficiency and assisted programmed cellular death by P53.

-We are in agreement with Hershbringer (2002) where vitamin D3 had an effect in tumor curbing and initiating programmed cellular death in infected cells . This observation was accompanied with increasing P53 expression, which acts by tumor curbing and initiating programmed cellular death.<sup>13</sup>

- likewise, we are in agreement with Prudenco.( 2001), where vitamin D53 stopped the cellular life cycle of infected cells accompanied with active P53 expression increasing, which caused tumor curbing and cancer cells reproductive efficiency decreasing .<sup>14</sup>

- Our results disagree with Mathiasen,( 1999) who noticed that P53 active form

Contributed by reducing BCL-2 expression, which indicates its role in stimulating programmed cellular death, but this result was combined with invariable P53 expression, this dissimilarity due to the fact of researcher experiments were conducted on breast cancer, and with smaller D3 dosage than our research .<sup>15</sup>

- We are also in agreement with ( Thompson , 2010) whose experiments were conducted on Leukemia, where he noticed that cancer cells cellular cycle was terminated by active P53 expression increasing, along with programmed cellular death stimulation by BCL-2 expression reduction.<sup>16</sup>

- We are also in agreement with ( William B. Grant, 2009), where he studied vitamin D3 in preventing Squamous cell carcinoma and improving body warning .<sup>7</sup>

-Our results also agree with (Cedrec 2006) study on breast, colon, and prostate cancers , where it was noticeable that vitamin D3 had an important role in preventing cancer<sup>10</sup>.

## VI. Conclusion

By analyzing an inactive mutant P53 expression, we noticed that the inactive mutant P53 expression was smaller in the vitamin group. Therefore, we reached the following conclusion: vitamin D3 active form had a role in curbing the tumor and organizing programmed cellular death, , and reached its efficiency peak after six weeks , then declined at the fourteenth week.

**Declaration:** The authors declare no financial interest

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