EGYPTIAN DENTAL JOURNAL

VOL. 64, 2121:2129, JULY, 2018

I.S.S.N 0070-9484

ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY

www.eda-egypt.org • Codex : 30/1807

THE EVALUATION OF THE CHEMOPREVENTIVE EFFECT OF β-CAROTENE (A-VITON®) ON ORAL PREMALIGNANT LESIONS THROUGH THE ESTIMATION OF THE EXPRESSION OF P53 LEVELS

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ABSTRACT

Background: Oral premalignant lesions (OPL) include a wide range of lesions including leukoplakia, dysplastic leukoplakia, erythroplakia, oral submucous fibrosis, dysplastic lichenoid lesions, and oral lichen planus. Proper diagnosis and management of oral premalignant lesions (OPL) are very important; as 17% of these lesions have been reported to be transformed into malignant lesions within first 7 years of their diagnosis. Vitamin A has always been known for its chemopreventive effect in several diseases through the modulation of cell proliferation and, it is also known for being a potent antioxidant and anti-inflammatory agent. Any alterations in p53 protein results in its accumulation in the cell nuclei; may play a critical role in carcinogenesis, including oral premalignant lesions and oral malignant lesions, i.e., p53 protein is one of the determinants in the progression of oral dysplasia to invasive malignancy.

Methods: Fifteen patients having oral premalignant lesions of mild and moderate dysplasia; diagnosed both clinically and histologically, were given a chemopreventive therapy in the form of βeta- carotene (Vit. A) for four months. The levels of p53 protein immunostaining were measured pre- and post-treatment.

Results: The mean value of optical staining density of P53 in pre-treatment specimen was (59.78 ± 10.22) compared to (39.74 ± 3.36) in post-treatment cases; this value was higher in pre-treatment cases than post-treatment cases. The p-value was 0.0003.Thus when statistically compared the difference is considered statistically significant.

Conclusion: The expression of P53 protein in (OPL) is found to be inversely related to the clinical as well as histopathological response to beta-carotene supplementation.

Keywords: Oral premalignant lesions, Oral dysplasia, Beta-carotene, Vit A, P53 protein.

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INTRODUCTION

A Premalignant lesion is considered to be a benign lesion with changed clinical and/or histopathological tissue and having a higher risk of finding microscopic foci of cancer or transforming into malignancy later on after being diagnosed [1]. In 2005, the World Health Organization (WHO) held a workshop to discuss different definitions and terminologies to distinguish between the term precancerous lesions and premalignant conditions. It concluded the use of the term,"potentially malignant disorders (PMD)," i.e., lesions having a risk to malignant transformation either directly after diagnosis or later at a future date. [2]. Potentially malignant disorders (PMD) affect the population of ranging from 50-69years with a ratio of 1:1 male to female incidence. However, some studies have shown that younger group can be affected by 1-5%.[3,4].

Etiology of premalignant lesions have always been difficult to enumerate; risk factors have been related to the lesions though. These risk factors include smoking, alcohol drinking, and tobacco chewing. In a study carried out by Thomas *et al.,* it has shown that multiple precancerous lesions were seen in patients drinking alcohol and chewing tobacco, while no lesions appeared in tobacco smoking patients. Also one of the most important risk factors is viral infection with Human papillomavirus (HPV), together with the presence of immunodeficiency or genetic diseases. [5,6].

Premalignant lesions can be presented in various forms: A) Oral Leukoplakia: which is defined as "A white plaque of questionable risk excluding other known disorders that carry no increased risk of cancer." Leukoplakia can be homogenous or non-homogenous, it can also take place in non- smokers [2]. B) Oral Erythroplakia: It is defined as "A fiery red patch that can't be characterized clinically or pathologically as any other definable disease." It has a very high malignant transformation that ranges from 14-50%[7]. C) Oral submucous fibrosis: It was first described in 1952 by Schwartz, as a chronic and PMD that causes juxta epithelial inflammatory reactions in oral cavity characterized by fibrosis of the lamina propria followed by stiffness of the oral mucosa. It also has a great malignant transformation ranging from 7-30% and is usually seen in the Asian population especially Indians [8]. D) Actinic Cheilitis: It is considered as PMD usually affecting the lower lip caused by exposure to solar radiation, it usually occurs in the form of erythema, edema together with scaling and thickening of epithelium with greyish –white plaques. It shows a high risk of malignant transformation ranging from 1.4-36% [9,10].

It is very crucial to early detect the presence of a premalignant lesion. One of the most reliable assessments used is the hematoxylin and eosin staining followed by microscopic examination and detection of cytological and architectural alterations, which are known as epithelial dysplasia. Epithelial dysplasia can be categorized from hyperplasia, mild, moderate, severe and carcinoma in situ; depending on the level of both cytological and architectural alterations are taking place, either from the lower third of epithelium up to the full thickness consecutively [1].

Carotenoids are well known fat-soluble compounds of variable biological properties [11]. The most important function of carotenoids is their activity as provitamin A. The most recognized provitamin carotenoids are β carotene, β cryptoxanthin, and α carotene; where they represent 35% of vitamin A content in humans [12].

Vitamin A together with its derivatives; have a major role in cell proliferation and growth, some studies showed their ability to target an immune-potentiation of T cell population resulting in T cell-mediated cytotoxicity, also have the ability of potentiation of natural killer cells activation, and macrophages stimulation. They also play

an important role in clinical oncology through their inhibition of tumor cell proliferation, and suppression of angiogenesis, That's why carotenoids as beta-carotene and other derivatives have been used in treatment and prevention of precancerous and cancerous lesions [13,14].

The antioxidant effect of beta-carotene have also shown through its counteracting the cellular damages of reactive oxygen species; they develop their antioxidant effect by their lipophilic characteristics mostly in cellular membranes and lipoproteins[15].

P53 is a unique protein belongs to a family of three protein members who are p53, p63 and p73. P53 is involved in the prevention of tumor development in higher organisms; whereas the other two proteins role in normal biological conditions [16].

Over the years, p53 have proved to be an accurate marker that was used in the diagnosis and also prognosis of malignant lesions. This accuracy was gained by the fact that 50% of human tumors show unusual presentation of p53 even in early lesions; where these transitional alterations can determine the magnitude and sort of cell stress [17]. The anti-cancer effect of p53 results from its apoptotic ability to induce tumor suppression [18].

Upon the previously presented data in this context, this research was designed to evaluate the anti-tumor effect of one of the available chemopreventive therapy; beta-carotene, and trying to prove its role in inhibition of premalignant lesions vascularization and epithelial dysplasia, also using p53 as a strong determinant of the outcome of this biological treatment.

SUBJECTS AND METHODS

Exclusion Criteria: Smokers, patients suffering from any systemic diseases, or patients taking any medications for the last three months before the study were excluded.

Inclusion Criteria: Fifteen patients with different oral premalignant lesions participated in this research. They were medically free. They were selected from the outpatient clinic of oncology department Faculty of Medicine, Cairo University. All the selected patients underwent the following procedures:

- **Therapy intake:**

- This was in the form of carotenoid (βeta -carotene) A- viton® capsules of 50000 I.U.
- Each patient was advised to take two capsules once daily during or after meal time.
- This regimen was given for four months.

Photographs of the lesions were taken before and after treatment

Also, biopsies were taken before and after treatment first to confirm diagnosis and presence of epithelial dysplasia, second to evaluate the progress of each case by measuring the intensity of p53 protein in the tissues pre and post-therapy.

- **Biopsy procedure**:

The affected area was selected, and a ring block anesthesia was given around the lesion with no direct infiltration of the anesthesia to the tissues. An Incisional biopsy; double wedge to a width approximately 2m.m was performed. The biopsy was rapidly fixed in 10% neutral formalin to be processed in paraffin blocks by the usual known way. Each paraffin block was cut into five microns thick sections to be stained with H&E, and other sections were immunostained.

The immunostaining detection kit used was"* *Dako Envision System Anti-Polyvalent, HRP/ DAB (Ready- To- Use) Code No. K4010, and specific antibody *Mouse-Monoclonal anti-human P53(DO-7)"*. It was provided in a liquid form: a cell culture that is supernatant and dialyzed by 0.05 mol/L Tris/HCl, of a pH 7.2 and contained 15 mmol/L NaN 3 ready for staining procedures.

These procedures were carried out twice for each case .once before therapy, the other post therapy.

Biopsy Examination:

a) H& E sections were examined microscopically to detect and confirm the presence of epithelial dysplasia, together with its grade; whether mild, moderate or severe. The study included 15 cases (7 males and 8 females). They were also examined post-therapy to evaluate the prognosis and the effect of beta-carotene on the dysplastic lesions.

b) Immunostained sections were examined by the computer system image analyzer with the known software " Lecia Q Win 500" (England). The immunoreactivity of P53 was evaluated by area optical density in the affected epithelial layers using a measuring frame per 10 fields in each specimen, by the help of a magnifying objective lens size 40, meaning producing a total magnification of 400 following grey calibration. The areas showing the most intensely and uniformly nuclear or perinuclear staining; were the areas that were calculated, any other cytoplasmic staining was ignored.

Data analysis :

Data analysis included "Descriptive Data" (standard deviation and mean were obtained). *Paired student's T-test* was used to determine and compare the significance of p53 protein optical density in both stages; pre and post-treatment. The *P* value was 0.0003; this value was extremely statistically significant.

Also *Paired student's Test* was also used in evaluating the mean value of p53 positive nuclei in both pre and post-therapy biopsies. This value; the p-value scored 0.0197; this value was extremely statistically significant.

Also, *Pearson's correlation coefficient* was used to correlate between the mean value of p53 optical density and the number of p53 nuclei in all specimens. (The correlation coefficient r=1).

RESULTS

This research included 7 males and 8 females, their ages ranging from 35-68 years. The cases were presented clinically as the follows: 7cases of mild dysplasia in the form of leukoplakia, speckled leukoplakia and erythroplakia. Three of these mild cases i.e..(37.5%) showed an obvious clinical improvement, represented by the decrease in lesion size, diminished keratinization and even the resolution of white patches in parts of the lesion. On the other hand, four of the cases (67.5%) did not show any improvement clinically. The other 8 cases; were of moderate dysplasia in the form of erythroplakia and verrucous leukoplakia; seven out of the 8 cases showed clear clinical improvement after given the medication.

Photographs that were taken for each case helped a great deal in revealing the clinical improvement of each case before and after treatment, as well as they, were very helpful in gaining the satisfaction of the patients and proving the success of the treatment. Fig. (1A,1B).

Upon microscopic examination of H & E specimen pretreatment, mild dysplasia was presented as basal and parabasal epithelial hyperplasia that was confined to the lower third. On the cytological level; cellular atypia was minimal, with mild nuclear pleomorphism. Mitosis scarcely existed and when found were also present in the basal one third (Fig.2A). The positive immunostaining of p53 in the pre -treatment specimen of the same mild dysplasia case, was revealed as intense nuclear staining which was confined to the lower one-third part of the epithelium. (Fig.2B).

Upon the microscopic examination of the H&E specimens post-treatment of the same mild case; epithelial layers were thinner, and the rete pegs were less board and even shorter. The basal hyperplasia in the basal 1/3 layer was less prominent with diminished mitotic figures (Fig.3A). on the other hand, the positive optical density of p53 was almost negligible, where tiny cells showed p53 staining (Fig.3B).

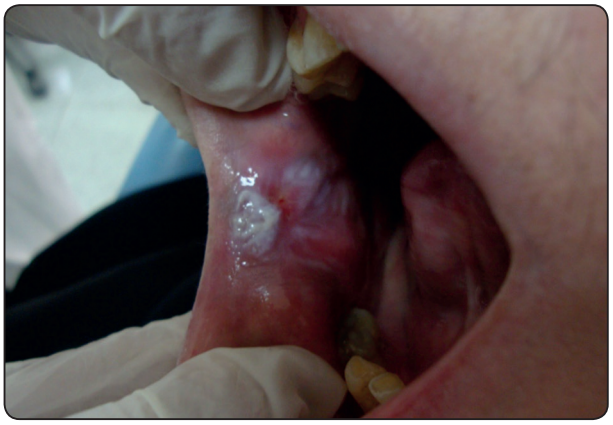

Fig. (1A) A Photograph showing a speckled leukoplakia of the buccal mucosa which was asymptomatic (pre-treatment).

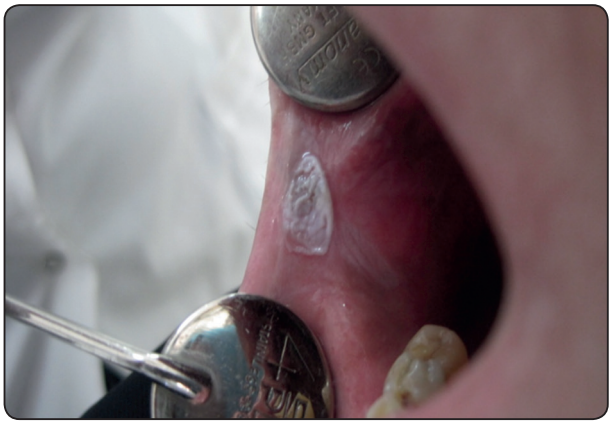

Fig. (1B) A photograph of the same patient where there is complete resolution of the atrophic part, reduction in the size of the lesion and comfort expressed by the patient.(Post-treatment).

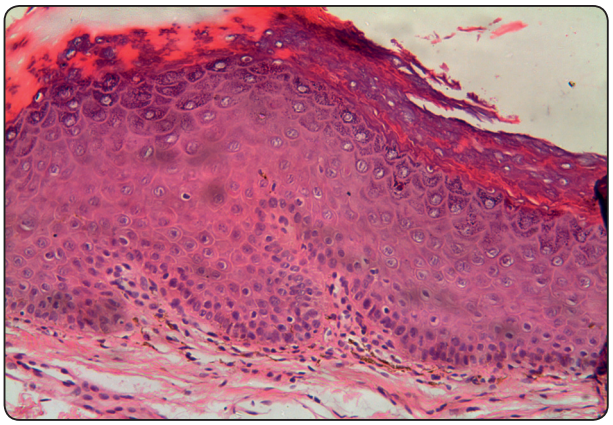

Fig. (2A) A photomicrograph of a mild dysplasia showing an increase in hyperkeratosis, prominent basal nuclei (Hyperchromatic), and slightly elongated rete pegs; all in the basal 1/3 of the epithelium. (H&E×200).

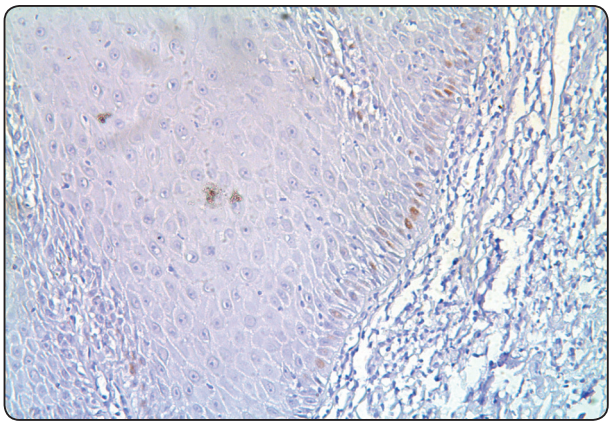

Fig. (2B) A photomicrograph of a mild dysplasia section showing:1 the positive nuclear expression of p53 which is related to basal 1/3 layer of the epithelium (DAB×200).

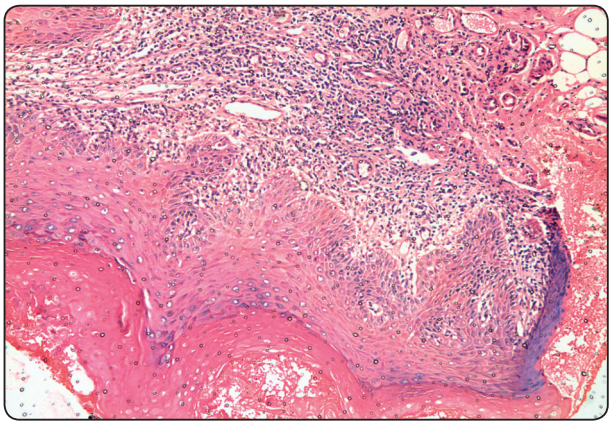

Fig. (3A) A photomicrograph of a mild dysplasia field after treatment showing thinning in the epithelial layer with shorter and less broad rete pegs with little basilar hyperplasia (H&E×100).

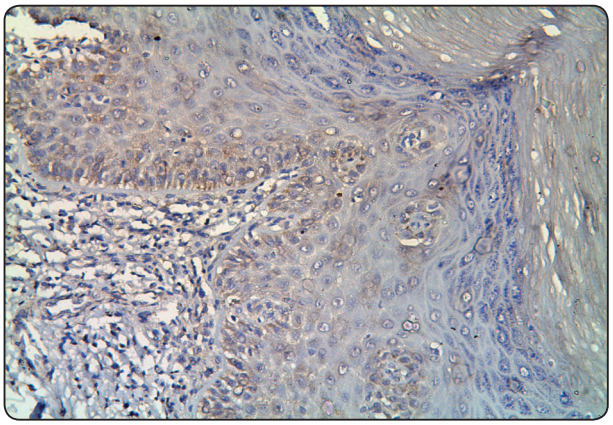

Fig. (3B) A photomicrograph of a mild dysplasia field post-treatment showing slight positive p53 expression in some of the basal cell layer almost negligible (DAB×200).

The statistical analysis of the immunoreactivity of P53 antibody in pre- and post-treatment were evaluated and showed the following: before treatment, the mean value of optical staining density of P53 was (59.78 ± 10.22) compared to (39.74 ±3.36) in after treatment cases; this value was higher in before treatment cases than after treatment cases. The p-value was 0.0003.Thus when statistically compared the difference is considered statistically significant. Table (1) (Figure 4).

TABLE (1) Mean, minimum and maximum optical density of p53 immunoreactivity before and after treatment.

	Before treatment	**after treatment**
Mean optical density ± SD	59.78 ± 10.22	39.74 ± 3.36
Minimum optical density	48.59	34.88
Maximum optical density	73.68	46.04

This table was also represented by a histogram in Fig (4):

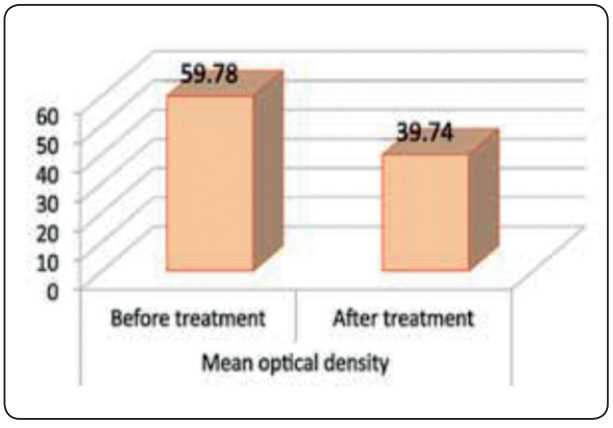

Fig. (4) : Histogram showing mean optical density of p53 immunoexpression before and after treatment.

P53 positive nuclei demonstrating brown immunostaining were mainly observed in the basal cell layer of the mild dysplasia cases, and in the moderate dysplasia were observed in the prickle cell layer. The number of positive nuclei was calculated in ten high-power (x400) fields in each case before and after treatment, and the mean was calculated. The results obtained are summarized in table (3) and expressed in figure (5). As shown in the table (2): A higher mean number of p53 immunoreactive nuclei were recorded before treatment (14.44 ± 10.12) compared to after treatment (3.44 ± 2.51). Using Paired Student's T-test, the p-value was 0.0197. By conventional criteria, this difference is considered to be statistically significant where (P≤0.05). Also, data shown in the table (2) were also explained by a histogram in figure (5).

Using Pearson correlation test, a statistically significant correlation was detected between the mean p53 optical density and number of p53 positive nuclei both before and after treatment (correlation coefficient r= 1). This value reveals a satisfying positive linear relationship. Those values were a plot in a scatter figure (6).

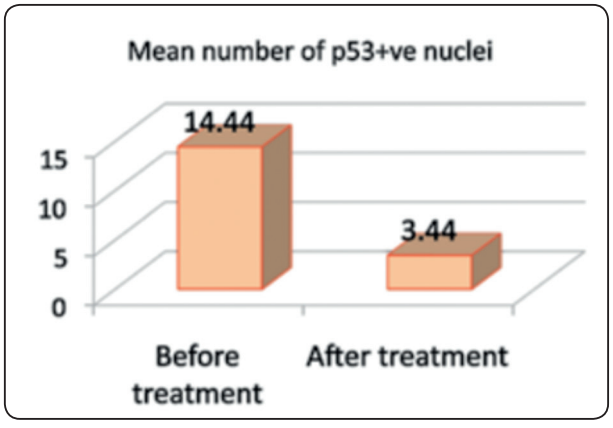

Fig. (5) Mean a number of p53 positive nuclei before and after treatment.

TABLE (2): Mean number of p53 positive nuclei before and after treatment and statistical significance of the difference (paired student's T-test)

Mean optical density ± SD		Difference Between means	95% Confidence interval	t value	Standard error of difference	P value
Before treatment	After treatment					
14.44 ± 10.12	3.44 ± 2.51	11.00	From 2.27 to 19.73	2.9055	3.786	0.0197*

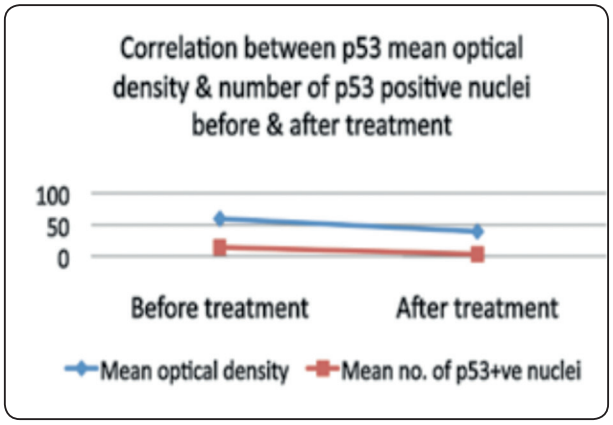

Fig. (6) Scatter showing the correlation between mean p53 optical density & number of p53 positive nuclei before & after treatment

DISCUSSION

All the previous data of this research made it worth aiming our attention towards the challenging field of diagnosing and managing intra oral premalignant lesions. Also the study of the various benefits of chemopreventive therapy that has been studied along the past years, together with their role in regression and prevention of malignant lesion transformation; by using the least toxic therapies that are available. In this study there was a clear improvement on the clinical level that was proven by the photographs that were taken before and after treatment and were examined by more than two examiners. Also, those improvements that were presented by the change in size and texture of the lesions; were observed by the patients that joined this research and were greatly satisfied with results. On the histopathological level, there was obvious change in levels of epithelial dysplasia together with cellular and nuclear pleomorphism and a reduction in the expression of p53 in the specimen examined after carotenoid therapy (A- Viton), then in pre-treatment specimens.

These results were proven to agree with a previous observation in a study performed with Lippman et al.[19] that p53 expression in oral potentially malignant lesions can show variable expression when treated with carotenoids especially beta-carotene [19].Their results reported that accumulation of p53 was higher in lesions showing higher grades of dysplasia, and the relation between its accumulation was inversely related to the degree of response towards the carotenoid therapy. In another research, that studied another biomarker which is known RAR-β; it showed an obvious response towards chemopreventive therapy [20]. According to this study, it has been proposed that the use of beta-carotene led to the up-regulation of RAR-β, and acted as a useful indicator of its responsiveness to therapy [21].

Another study that was carried out by Papadimitrakopoulou et al., [22], where they used similar chemopreventive therapy; was also compared to this research results. That study enrolled 26 patients suffering from premalignant lesions of various grades of dysplasia mild, moderate, and severe affecting the oral cavity, larynx and oropharyngeal region. It has been proved that lesions with a low degree of p53 expression showed a high rate of response to the therapy when compared to the lesions of a high degree of p53 levels during the 6 months assessment [22]. When comparing the previous results to this research; there was a slight difference where the moderate dysplastic lesions that showed more p53 expression before therapy were more responsive and expressed less p53 levels after therapy. This observation was made explained by the fact that mostly dysplastic cells are inhibited during the S phase and are rushed towards the apoptotic phase [23] .

On other hand a study conducted by Liu et al.[24]; evaluated the long-term intake of beta carotene therapy in patients with lung carcinogenesis , and its effect on some biological markers; it showed that the positive up-regulation of some biological markers as p53, cyclin D1, PCNA, RAR-β was not apparently lower in patients having lung cancer (n=39) that received beta-carotene 50mg on alternative days; when compared to those(n= 20) who received placebo [24].

Retinoids including Vitamin A and provitamin A as beta-carotene, play an important role in the regulation of morphogenesis, differentiation, growth, and development of cells [25]. They also were proved to cease the transformation of premalignant lesions affecting oral cavity, cervix, and skin, they can prevent the incidence of metastasis of primary tumors of the head and neck as well [26,27].

In general, it was always reported that the overexpression of p53 is always significant and relatively high in premalignant lesions that has been transformed into malignancy. This was related to the fact that dysplastic fields were always derived from stem cells that showed mutated p53 [28]. Some speculations were inclined towards the role of chemopreventive therapy in down-regulation of these mutated doer molecules [29]. Future studies should be carried out on multiple biomarkers especially at the baseline to confirm and detect the mechanism by which chemopreventive therapy can prevent the malignant transformation of potentially malignant disorders.

CONCLUSION

Biomarkers like p53, have been used to evaluate the efficiency of chemopreventive therapies over the past years, and they have given promising results. Using p53 as a marker in this research has been of great help especially with the satisfying results of the use of A-Viton as a drug of choice in treatment, and sometimes elimination of variable premalignant lesions.

Further study of other forms of chemopreventive therapy can be of great use in some of the challenging conditions that dentists usually encounter in the dental office.

This study also showed that there was a significant decrease in expression of p53 in premalignant lesions of a higher degree of dysplasia following the expression of the therapy.

ACKNOWLEDGMENT

This research was supported by the unconditional co-operation of Professor Doctor, Hossam Hussien, Professor of General Pathology, Faculty of Medicine, Cairo University. He contributed in this work by the microscopic examination of the samples, and confirmation of the proper diagnosis, interpretation of the cellular and nuclear changes, and also different forms of p53 optical density by using the image analyzer for the pre and post-treatment samples.

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