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The ultrastructural features of the premalignant oral lesions

Doiniţa Olinici[1], Carmen Elena Cotrutz[1], Ciprian Valentin Mihali[2], Vasile Bogdan Grecu[1], Emanuela Ana Botez[1], Laura Stoica[1], Pavel Onofrei[3], Oana Condurache[4], Daniela Cristina Dimitriu[5]

[1]Department of Cell and Molecular Biology, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania

[2]Institute of Life Sciences, "Vasile Goldiş" Western University of Arad, Romania

[3]Department of Anatomy, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania

[4]Department of Dermatology, University Clinical Railways Hospital, Iaşi, Romania

[5]Department of Biochemistry, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania

Abstract

Premalignant oral lesions are among the most important risk factors for the development of oral squamocellular carcinoma. Recent population studies indicate a significant rise in the prevalence of leukoplakia, erythroplakia/erythroleukoplakia, actinic cheilitis, submucous fibrosis and erosive lichen planus. Since standard histopathological examination has numerous limitations regarding the accurate appreciation of potential malignant transformation, the present study aims to aid these evaluations using the transmission electron microscopy (TEM) technique, which emphasizes ultrastructural changes pertaining to this pathology. Oral mucosa fragments collected from 43 patients that were clinically and histopathologically diagnosed with leukoplakia, erosive actinic cheilitis and erosive lichen planus have been processed through the classic technique for the examination using TEM and were examined using a Philips CM100 transmission electron microscope. The electron microscopy study has confirmed the histopathological diagnosis of the tissue samples examined using photonic microscopy and has furthermore revealed a series of ultrastructural details that on the one hand indicate the tendency for malignant transformation, and on the other reveal characteristic features of tumor development. All the details furnished by TEM complete the overall picture of morphological changes, specific to these lesions, indicating the importance of using these techniques in establishing both a correct diagnosis and prognosis.

Keywords: oral premalignant lesions, transmission electron microscopy, ultrastructure.

⊟ Introduction

Compared to cutaneous premalignant and malignant lesions, the absence of the clinical manifestations encountered in most oral mucosa lesions hinders a fast diagnosis, negatively influencing the prognosis and survival [1].

Most frequently, the development of oral squamocellular carcinoma undergoes a "multi-step" process in which the epithelial cells undergo transformations, first into pre-neoplastic, then in tumor cells, with the sequential accumulation of multiple epigenetic and molecular changes. More than 50% of the cases of this type of carcinoma develop upon the premalignant lesions, among which the most frequent are leukoplakia, actinic cheilitis, erosive lichen planus and submucous fibrosis [2]. Numerous clinical studies indicate this pathology as being characteristic to elderly men, past the age of 75 years, smokers and chronic ethanol consumers. Presently noticed is a worldwide increase in the prevalence among women and youth who are not smokers and have no specific history with regard to the alcohol consumption [3, 4]. The clinical diagnosis is often difficult because the subjective and objective manifestations are not specific and do not correctly reflect the degree of affected epithelial and conjunctive tissues. Moreover, the referral to a dermatologist and/or dentist is unfortunately done only in advanced, transformed stages [5, 6].

The rate of malignant transformation of these lesions is approximately 17%, during a period of seven years from the time of the diagnosis. The highest malignant transformation rate is identified in heterogeneous erythroplakia/erythroleukoplakia with dysplastic changes.

In the last three decades, despite the progress achieved in the diagnosis and therapeutic management, the 5-year survival rate of patients with oral squamocellular carcinoma lesions has not improved significantly. Thus, in the USA, this rate is of approximately 62%, and in Europe, it is less than 50%, in contrast with other recorded 5-year survival rates for example, in the case of breast cancer (89%) and prostate cancer (99%). One of the main reasons is the inability to identify a characteristic biological marker, with the help of which, the aggressiveness of the disease could be evaluated and a specific, directed and personalized treatment be established [7–13].

Specialty literature about oral mucosa lesions describes a series of specifications concerning their clinical and histopathological stages, from the *in situ* carcinoma to the invasive and metastatic carcinoma. Although some studies consider the anatomopathological examination as being the "gold standard" in the evaluation of the potential for malignant transformation, this method presents numerous limitations given the fact that the risk assessment is based on whether dysplasia is present or absent, which is in turn indicated only by a few architectural and cytological changes [7, 10, 13, 14].

Among the morphological features of dysplasia revealed by the anatomopathological examination, a high cellular density with cells similar to basal ones with hyperplasic and hyperchromatic nuclei is described, as well as an inverted nucleus/cytoplasmic ratio in favor of the nucleus, with an increased and abnormal mitotic activity also present in the superior layers.

In the case of oral leukoplakia, the photonic microscopy can emphasize the keratinocyte hyperplasia, hyperortho-keratosis, hyperparakeratosis, acanthosis or cellular atypias in various degrees that may indicate the presence of a mild, moderate or severe dysplasia.

Until recently, the oral epithelial dysplasia represented the most important prognosis factor for the malignant transformation of the oral leukoplakias, taking into account the fact that between 15.6% and 39.2% of them presented carcinomatous changes. Presently, the association between the degree of the leukoplakia lesion dysplasia and the malignant transformation risk represents a controversial subject [15–18].

In 2001, Barnes *et al.* mentioned that, as opposed to mild dysplasia, severe dysplasia is correlated with five times higher a risk for the transformation in squamocellular carcinoma, emphasizing the necessity of a more precise histological classification [19].

With regard to the electron microscopic examination, ever since 1984 Dourov *et al.* mentioned that transmission electron microscopy (TEM), having a superior resolution to photonic microscopy, can identify a suggestive aspect of malignant transformation from very early on, which may have otherwise "slipped through" an anatomical and pathological routine examination, thus significantly improving the management of these patients [20].

Materials and Methods

For the present study, fragments of the oral mucosa were collected, using the incisional/excisional scalpel biopsy, from 43 female patients, non-smokers, without chronic alcohol consumption, with an age between 45 and 60 years old, clinically and histopathologically diagnosed with *leukoplakia* – 28 cases, of which 10 cases with a mild–moderate degree of dysplasia and eight cases with moderate–severe degree of dysplasia, *erosive actinic cheilitis* – 10 cases, and *erosive lichen planus* – five cases. The normal oral mucosa fragments were obtained through the same sampling technique from the perilesional areas in order to constitute the control group.

Informed consent was obtained from each patient prior to the surgical intervention.

In aiding the anatomopathological investigation, some fragments obtained from the gathered tissue samples were processed through the immersion in paraffin technique, sectioned and stained with Hematoxylin–Eosin (HE), and a diagnosis was established within the Service of Pathological Anatomy and Morgue, University Clinical Railways Hospital (Iaşi, Romania) and in the Laboratory of Pathological Anatomy, "Dr. Iacob Czihac" Emergency Clinical Military Hospital (Iaşi).

In order to avoid any type of tissue degradation, samples were gathered on ice and a few drops of 2% glutar-aldehyde were added. Several fragments were obtained and were processed through the standard technique for the electronic transmission microscopy: double fixing, dehydration – with 30% alcohol, at 4°C, for 15 minutes, stained all together – with 2% uranyl acetate, in 30% alcohol, at 4°C, for 45 minutes, dehydration – consecutive alcohol baths of increasing concentrations, followed by absolute acetone, at room temperature (RT), for 20 minutes.

The impregnation was done with EPON 812 and absolute acetone in equal parts, at RT, overnight, and afterwards EPON 812, at RT, for two hours, followed by the immersion in EPON 812, in gelatin capsules, polymerization and cutting. Processing of the samples up to this stage was carried out in the Laboratory of Electron Microscopy within the Department of Cell and Molecular Biology ("Grigore T. Popa" University of Medicine and Pharmacy, Iaşi), while the later stages were carried on within the Laboratory of Electron Microscopy, Institute of Life Sciences ("Vasile Goldiş" Western University of Arad, Romania) and the Laboratory of Electron Microscopy ("Alexandru Ioan Cuza" University, Iaşi). The samples were examined with a Philips CM100 transmission electron microscope, equipped with a digital video camera with dedicated sensor and photo-taking system.

Results

The sample examination of the HE-stained mixtures using the photonic Olympus BX40 microscope with the Olympus E330 camera, lead to the identification of suggestive aspects for *leukoplakia with a mild–moderate degree of dysplasia* – hyperplastic epithelial tissue with the marked hyper- and parakeratosis and acanthosis, the enlargement of the granular layer and rare typical mitoses in the basal layer, edema, vascular ectasia and poly-morphous inflammatory infiltrate in the stroma (Figure 1); *leukoplakia with a moderate–severe degree of dysplasia*: epithelium with hyperkeratosis, marked hypergranulosis, moderate and focal acanthosis, focal acanthosis in the basal layer (Figure 2); *erosive lichen planus*: hydropic degeneration of the basal layer, acanthosis with the formation of sharp papillary buds; discontinuous hyper-trophy of the granulate layer, hyperkeratosis, subepidermal bubbles, fascia with infiltration of lymphohistiocytes and in derma, Civatte bodies (Figure 3); *erosive actinic cheilitis*: hyperkeratosis and mild acanthosis and peri-vascular lymphocytic infiltrate around the glandular ducts (Figure 4).

Concerning the electron microscopy examination, in the case of the oral premalignant lesions of the *mild–moderate dysplastic leukoplakia and the erosive actinic cheilitis*, a series of important changes is identified, in addition to the ones revealed by the photonic microscopy, as is emphasized in Figures 5–7:

▪ discontinuities at the basal membrane level and variations in its thickness, which can be determined by the presence of the inflammatory process associated with these lesions;

▪ numerous macrophages in the conjunctive tissue and in the neighboring areas of the capillaries on which hyper-trophic rough endoplasmic reticulum (RER) is observed, suggesting an intense synthesis and cellular secretion activity, including that of the pro-inflammatory cytokines;

▪ the presence, at the suprabasal level, of mitochondria with modified structure (irregular crests, vacuolization), while in the rest of the mitochondria layers these are found in reduced numbers and have a quasi-normal structure;

▪ the presence of the dentate nuclei with the wavy nuclear membrane in the basal and suprabasal layers;

▪ the presence of some desmosomes and hemidesmosomes (HD) with disorganized structure in the basal layer;

▪ vacuolization of the cytoplasm, discontinuities and invaginations in the plasmalemma; in several cases, caveolae and pinocytosis vesicles of various sizes is observed;

▪ the enlargement of intercellular spaces, acanthosis-type changes in the basal layer;

▪ disorganization and the numerical reduction of filaments located at a greater distance from the plasmalemma and having various orientations.

In the case of *leukoplakia with moderate–severe dysplasia and erosive lichen planus*, the electron microscopy examination emphasized the presence of the ultrastructural changes enumerated above, which are even more common, to which is added (Figures 8–11):

▪ highly thickened tonofilaments retreating from the desmosomes and HD, to a large degree in disorganized fashion, sometimes in electron-dense bunches present up to the vicinity of the nucleus;

▪ powerfully edentate nucleuses, a characteristic of dyskaryosis changes, present not only in the basal and suprabasal layers, but also in the stratum spinosum, with the enlargement of the perinuclear cisterna and the occasional presence of some vacuolization, dilated nuclear pores; a sizeable increase of the nuclear volume;

▪ multiple nucleoli (2–3/nucleus) with an increased volume and peripherally located towards the internal leaf of the nuclear membrane, which are important features of malignant transformation;

▪ frequent nuclear cytoplasmic invaginations, sometimes associated with a filamentous material;

▪ the occasional presence of some cytoplasmic perinuclear vacuolization together with the constant presence of a number of ribosomes and mitochondria with the degenerative changes in all the epithelial layers; these features are suggestive for the evolution of these lesions towards malignant transformation;

▪ similar changes to those described above are identified in the basal membrane, though with much more frequent discontinuity zones, as well as thickening and ramification of the conjunctive tissue fibers.
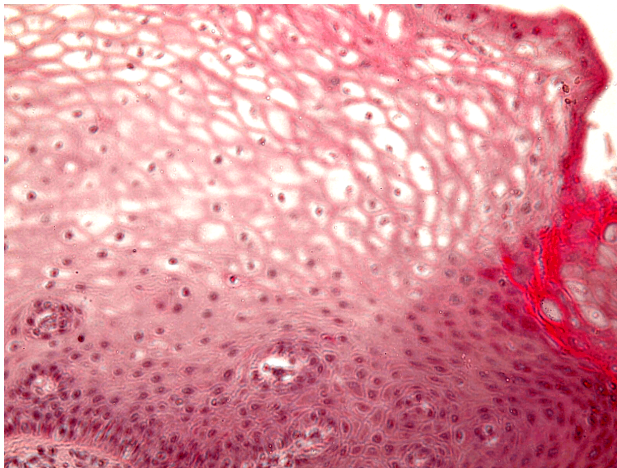

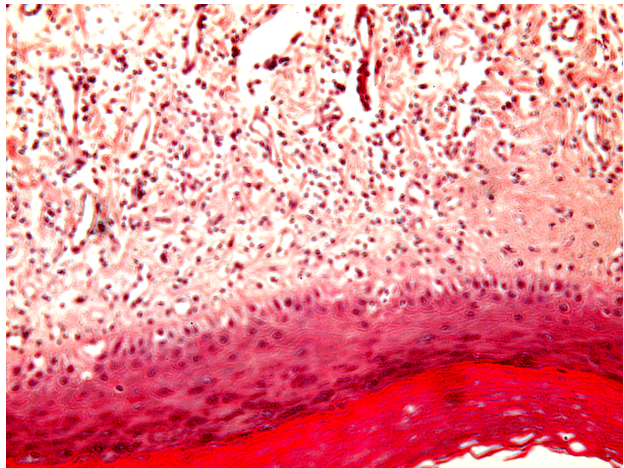**Figure 1 – *Oral mucosa: leukoplakia with mild–moderate dysplasia (HE staining, ×400).***

Figure 2 – *Oral mucosa: leukoplakia with moderate–severe dysplasia (HE staining, ×200).*
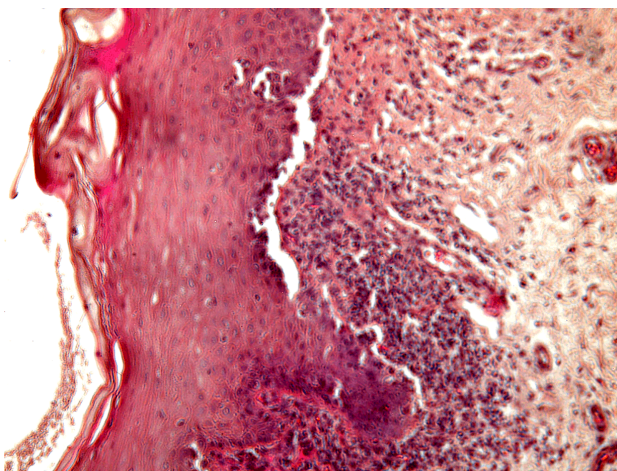

Figure 3 – *Oral mucosa: erosive lichen planus – degeneration of the basal layer, acanthosis, discontinuous hypertrophy of the granulate layer, hyperkeratosis (HE staining, ×200).*
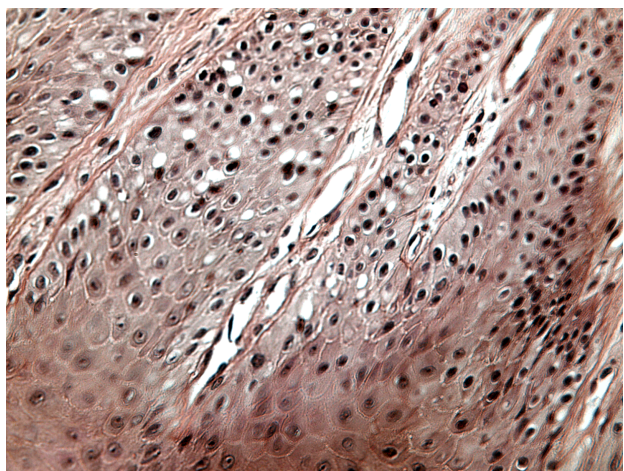

Figure 4 – *Oral mucosa: actinic cheilitis – mild acanthosis and perivascular lymphocytic infiltrate around the glandular ducts (HE staining, ×400).*
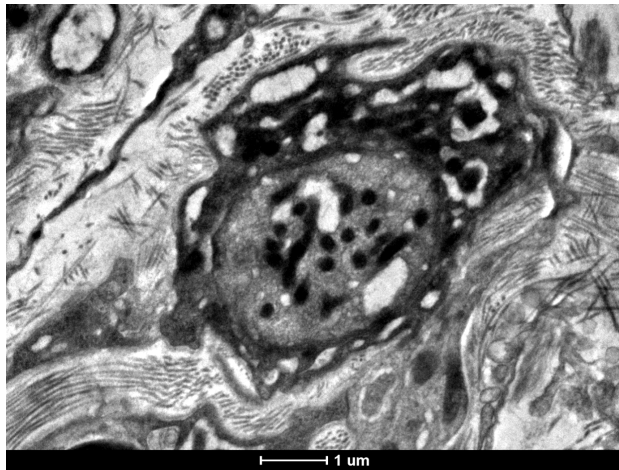
Figure 5 – *Oral mucosa: leukoplakia – vacuolization of the cytoplasm, discontinuities and invaginations in the plasmalemma, dyskaryosis changes of nucleus with the enlargement of the perinuclear cisterna, presence of some vacuolization in nuclear membrane and dilated nuclear pores (TEM).*

Figure 6 – *Oral mucosa: leukoplakia – edentate nucleus, discontinuities and invaginations in the plasmalemma, a sizeable increase of the nuclear volume (TEM).*
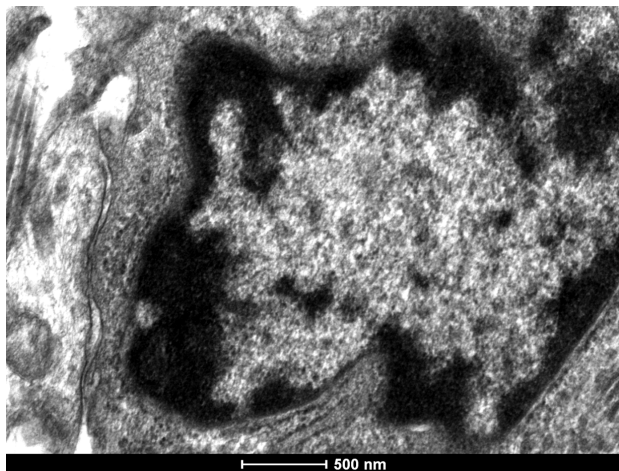

Figure 7 – *Oral mucosa: erosive actinic cheilitis – presence of dentate nucleus, a sizeable increase of the nuclear volume, vacuolization of the cytoplasm, discontinuities and invaginations in the plasmalemma, the enlargement of intercellular spaces (TEM).*
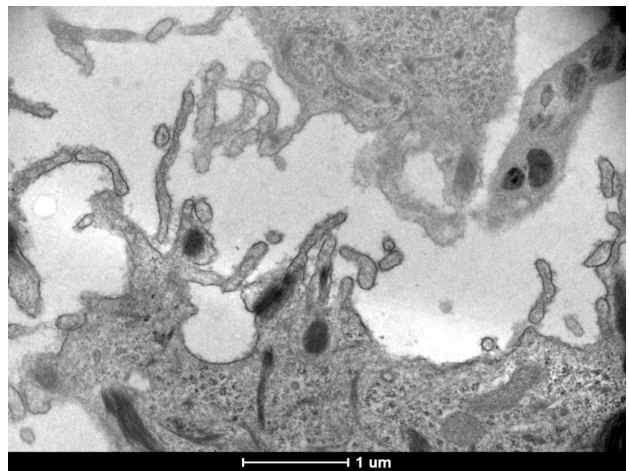

Figure 8 – *Oral mucosa: erosive lichen planus – some desmosomes and hemidesmosomes with disorganized structure, discontinuities at the basal membrane level (TEM).*
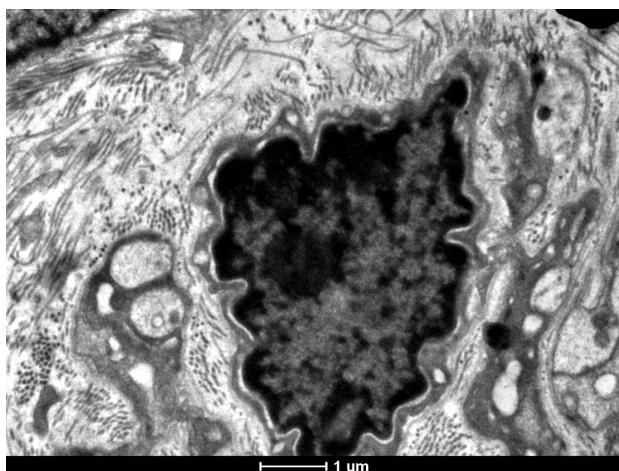

Figure 9 – *Oral mucosa: leukoplakia – wavy nuclear membrane, cytoplasmic perinuclear vacuolization, multiple nucleoli with an increased volume and peripherally located towards the internal leaf of the nuclear membrane (TEM).*
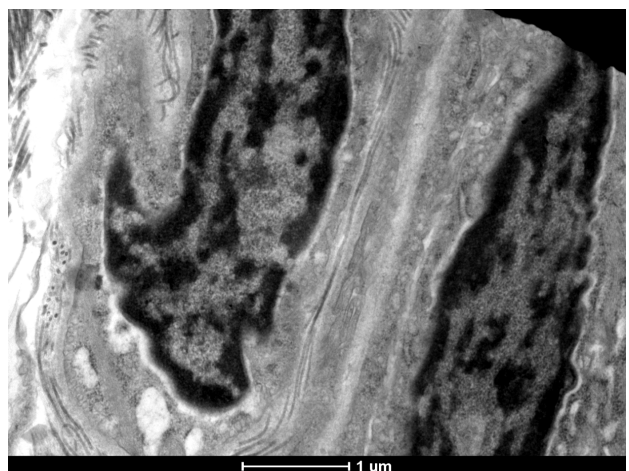

Figure 10 – *Oral mucosa: leukoplakia – vacuolization of the cytoplasm, discontinuities and invaginations in the plasmalemma, the enlargement of intercellular spaces, disorganization of cytoplasmic filaments with various orientations (TEM).*
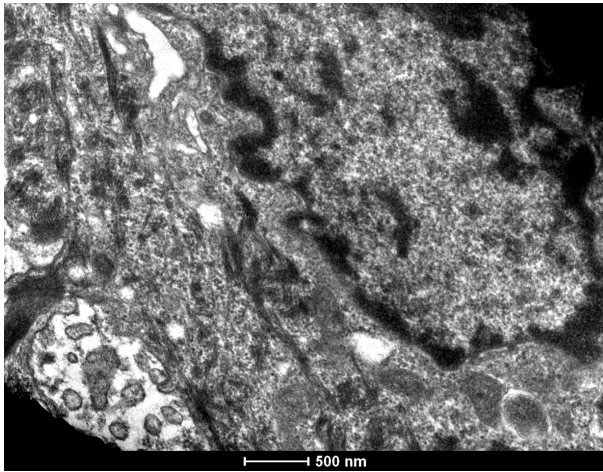
Figure 11 – Oral mucosa: leukoplakia – dentate nuclei with the wavy nuclear membrane, a sizeable increase of the nuclear volume, mitochondria with modified structure (TEM).

⯐ Discussions

The morphological aspects revealed through the photonic microscope examination of the HE-stained tissue samples (Figure 4) comprise characteristic elements based on which in current practice, the histological and pathological diagnosis of these lesions is established. Therefore, leukoplakia with keratinized cells, anucleate in the superior layer, with all forms of cellular crossing between this layer and nucleate cells, and with a thick granular layer and hyperorthokeratosis, as well as parakeratosis, seem associated with the mild or moderate dysplasia lesions, with rare mitosis in the epithelial inferior third (Figure 1). In the advanced severe forms of leukoplakia (Figure 2), associated with moderate or grave dysplastic lesions, when the tissue is made up mainly of basal-type cells, while on the surface with parakeratosis cells, the zone with the obvious dyskeratosis occupy parabasal territories.

In this case, the clinical manifestation characteristic of dysplasia lesions evidences cells with inversed nuclear/cytoplasmic ratio, with mitoses observed beyond the inferior third.

An interesting observation would be lesion manifesttations that are slightly modified from the moderate and grave forms of dysplasia associated with leukoplakia. The polarity is mostly maintained for the forms mentioned above, with less modified cell forms, so that only the presence of mitoses and the inverted nuclear/cytoplasmic ratio suggest an advanced from of dysplasia.

The morphological aspects revealed through the photonic microscopy examination of the HE-stained tissue samples were confirmed by the examination using the transmission electron microscopy, which has offered important ultrastructural details of the epithelial dysplasia changes, which characterize these premalignant lesions.

The results of the ultrastructural evaluation indicate a high degree of change in the cellular phenotype for the oral premalignant lesions compared to the cutaneous ones, suggested not only by previous literature data, but also through the results obtained by our team in a study aimed at identifying the correlation of ultrastructural aspects with clinical ones, the outcome of which has yet to be published.

In this regard, it must be taken into account that due to constant exposure and action of various internal and external environment factors, the oral mucosa is often under an inflammatory stress, a fact which explains its vulnerability to the infectious/inflammatory processes, such as the ones involved in the stages of carcinoma genesis [21]. Presently, the mechanisms through which the inflammation, both acute and chronic, induced by various bacteria, viral, mycological, chemical and physical factors trigger and maintains the cascade of oral carcinoma genesis, is not fully understood. What is clear is the capacity of the tumor cells to secrete a series of inflammatory factors, with the aim of changing the stroma and in order to facilitate invasiveness [21, 22]. Frequently, the cutaneous and oral premalignant lesions represent an important risk factor for the development of squamocellular carcinoma. The pathological genesis of this neoplasia is complex and involves the sequential progression of the normal tissue through a successive spectrum of lesions, including hyperplasia, dysplasia, *in situ* and invasive carcinoma [23]. Between 15–20% of the tumor lesions, especially the chronic ones, are associated with inflammation. The numerous laboratory trials also show that acute inflammation can stimulate the invasiveness and the metastasis. This malignity–inflammation link is controlled firstly by the cytokines, produced not only by the tumor cells but also by the activated immune cells [22, 24]. The interactions between the neoplasia and the normal cells significantly influence the evolution of epithelial cancer. In the past years, it has been suggested that the start and progression of the cancer's genesis is represented by the consequence of the interaction between the tumor cells and the neighboring tissue microenvironment.

TEM has furnished not only additional details which could not be observed with photonic microscopy, but new aspects as well which are essential for understanding the evolution of these lesions. These aspects indicate the importance of completing the classic pathological diagnosis with electron microscopic examination for a precise diagnosis and a correct prognosis of these lesions.

Thus, details such as the enlargement of the intercellular spaces observed in the case of these lesions and which do not represent artifacts of the processing technique of the samples, together with the disorganization of the cell–cell and cell–matrix coupling areas, with various degrees of mitochondrial degeneration (Figure 9), which suggest deficient cellular energetics, with nuclear and nucleolar changes (Figure 11), with the increase in the number of ribosomes, and changes at the basal membrane level (their detachment from the epithelial cells, the presence of the residual material in their own lamina) (Figure 8) are aspects revealed only by the electron microscope study, which on the one hand indicate the tendency of malignant transformation and on the other hand represent many characteristics of tumor development.

In the case of the leukoplakia associated with a moderate–severe degree of dysplasia, the presence of a large number of macrophages that evidenced a supra-dimensioned RER and Golgi complex lattice, indicating an intense synthesis activity and cellular secretion, probably for the proinflammatory cytokines that were mentioned earlier, was observed.

The more severe ultrastructural changes were generally noticed in the case of the leukoplakia associated with a moderate–severe degree of dysplasia (Figures 10 and 11) and in the oral erosive lichen planus (Figures 8 and 9), rather than in those of *erosive actinic cheilitis* (Figure 7) and the leukoplakia with mild–moderate dysplasia (Figures 5 and 6).

These results, about erosive lichen planus, disagree with the theories according to which this type of lesion presents a low risk of turning malignant.

⊟ Conclusions

The electron microscopy study of the premalignant lesions discussed reveal the increased prevalence of important ultrastructural changes, some of which are quite specific to tumor cells, thus emphasizing their severity and their major potential to turn malignant. Ultrastructural details provided by TEM, complete the information offered by photonic microscopy, sustaining the terminology of premalignant lesions for this type of pathology and emphasizing the value of the electron microscopy examination, often underestimated or ignored. Furthermore, the results of the present study add to the data comprised from the few ultrastructural studies existing in the literature on this subject, contributing to a more complete and relevant illustration of the morphological context specific to these lesions and offering important information for their clinical and therapeutic management, thus sustaining the importance of early diagnosis for these types of lesions, which represent a real provocation for the public health systems worldwide.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author
Carmen Elena Cotrutz, Professor, MD, PhD, Department of Cell and Molecular Biology, "Grigore T. Popa" University of Medicine and Pharmacy, 16 Universităţii Street, 700115 Iaşi, Romania; Phone +40730–030 302, e-mail: cotrutz@yahoo.com