The Egyptian Journal of Hospital Medicine (Oct. 2015) Vol. 61, Page 459- 478

In Situ Hybridization and Light and Electron Microscopes of Cervical Squamous Cell Carcinoma Infected with Human Papillomavirus

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ABSTRACT

Aim of the work-In Egypt cervical carcinoma is ranked as a number 6 of all malignant tumors. There is growing evidence that the human papillomavirus (HPV) is associated with the development of cervical cancer. **Patients and Methods**-The present study hunted HPV in Egyptian biopsies of cervical cancer by using in situ hybridization (ISH) technique .Light microscopy and ultra-structural features accompanied by squamous cell carcinoma (SCC) of the cervix were monitored. **Results**-The ultra structural study revealed viral particles in some epithelial cell nuclei and cytoplasm in the moderately-differentiated squamous cell carcinoma. These were suggested to be Herpes Simplex Virus (HSV**). Conclusion**-The potential relation between HPV and HSV in the incidence of Cervical Squamous Cell Carcinoma is discussed.

Key words: Hybridization, **Cervical Squamous**, **Carcinoma**, **Papillomavirus**

INTRODUCTION

Cervical carcinoma is the second most common malignancy among women worldwide [(1,2,3)], with an estimated 465,000 new cases every year; this accounts for 15 percent of all malignant tumors diagnosed in women[(2)].

Reports of developing countries showed that cervical carcinoma ranked as one of the most frequent malignancies. In Egypt, cervical carcinoma is ranked as number 6 of all malignant tumors, where it accounts for 3.6% of all female malignancies [(4)].

Over the past decade, convincing evidence has accumulated to support an association of human papillomaviruses (HPVs) with the development of cervical cancer and precancerous cervical intraepithelial neoplasia (CIN). It is widely accepted that HPV is the primary causative agent for the development of this type of cancer and its precursor lesions [(5,6,7)].

Previous studies suggested that HPV has also some prognostic implications in invasive cervical carcinoma since patients with HPV had a higher recurrence rate with worse prognosis than in HPV negative patients [(8)].

It was proposed the most widely used histological grading system of squamous cell carcinoma that is based on the degree of differentiation. Using this method, squamous cell carcinomas are graded as well-differentiated (grade 1), moderately-differentiated (grade 2) and poorly-differentiated (grade 3) [(9)].

- ✓ **In well-differentiated (grade 1) squamous cell carcinoma,** the most striking feature is abundant keratin, which is deposited as concentric whorls (keratin pearls) in the centers of neoplastic epithelial nests. The cells appear mature, with abundant cytoplasm.
- ✓ **In moderately-differentiated (grade 2) squamous cell carcinoma,** the neoplastic cells are more pleomorphic than in grade 1 tumor and characterized by having large irregular nuclei and less abundant cytoplasm. Keratin pearls formation is virtually non existent, but individual cell keratinization is seen in the centre of nests of tumor cells
- ✓ **In poorly-differentiated (grade 3) squamous cell carcinoma**, there are large or small, pleomorphic cells with hyperchromatic oval nuclei and scant indistinct cytoplasm. Abnormal mitotic figures are frequent and necrosis is common.

Papillomaviruses are DNA viruses with double-stranded circular DNA and icosahedral structure with 72 capsomers. They cannot be cultivated in vitro [(10)].

Papilloma viruses are classified as members of the family Papovaviridae which includes Simian Virus 40 (SV40) and polyoma virus as well as the papillomaviruses. Papillomaviruses

Received:8/9/2015
Accepted:22/9/2015 DOI: 10.12816/0018751

are widely distributed throughout nature; they infect man and many other higher vertebrates (e.g. bovine, canine, rabbit, elk and deer papillomaviruses have been identified). Although human and animal papillomaviruses share a similar genomic organization, they are highly species specific and don't cross-infect other species.

Papillomaviruses are epitheliotrophic viruses that predominantly infect skin and mucous membranes and produce characteristic epithelial proliferations at the site of infections. These benign proliferations or papillomas have the capacity to undergo malignant transformation under certain circumstances [(11)]. In humans, HPV infections occur on the skin and mucous membrane in the conjunctiva, oral cavity, larynx, tracheobronchial tree, esophagus, bladder, anus and genital tracts of both sexes. HPVs appear to be fastidious in their growth requirements and replicate only in the nucleus of infected cells [(12)].

The aim of the present study is to evaluate human papillomavirus (HPV) in Egyptian biopsies of cervical cancer by using in situ hybridization (ISH) technique. Electron microscopic technique is also employed as a trial for the detection of HPV and for revealing the main ultra structural features accompanied by squamous cell carcinoma (SCC) of the cervix.

This study sheds light on the etiological role of HPV in the pathogenesis of cervical cancer in Egypt.

MATERIAL AND METHODS

Material:

The material of the present study consisted of 37 cases of carcinoma of the cervix and 2 normal cervical specimens, taken as a control from patients who underwent hysterectomy for leiomyomata. Both types of specimens were collected from the National Cancer Institute, Cairo, Egypt.

Methods of the Study:

The tumor tissues were processed as follows:

I. In Situ Hybridization Method:

In Situ Hybridization (ISH) technique was performed to detect and localize HPV DNA sequences within tissue sections. This was achieved by applying HPV Types 16/18 Biotinylated DNA Probe, provided by **DAKO (Denmark)** and the Supersensitive ISH Detection System, provided by **BioGenex (San Ramon, USA)**.

The steps of ISH can be summarized as follows [(13)]:

1. Several tissue sections, 5 µm thick, from each of 37 cases of cervical carcinoma were cut and mounted on positively charged microscopic slides. One of these sections was treated as negative control, while the positive control slides were prepared as separate slides, including one in each run.

2. Deparaffinization:

- The slides were warmed in an oven at 37˚C overnight.
- The paraffin tissue sections were dewaxed in xylol and rehydrated through descending series of ethanol.

3. Protease Digestion:

- The tissue sections were digested with freshly diluted proteinase K (200 µl of the proteinase K for each slide) and incubated for 15 minutes at 37˚C.
- Slides were washed in 1xPBS (phosphate buffered saline) for 5 minutes and then dehydrated through ascending grades of ethanol and air dried.

4. Prehybridization:

- 2 drops of the prehybridization solution were added to tissue sections, covered with the avoidance of air bubbles trapping and incubated at 37˚C for 60 minutes.
- Slides were washed twice in absolute ethanol for 3 minutes each then air dried at room temperature for 5 minutes.

5. Denaturation and Hybridization:

- 40 µl of the probe working solution was added on the tissue sections, covered with parafilm with the avoidance of air bubbles trapping, placed on a heating block, previously heated, at 95˚C for 10 minutes and incubated at 37˚C overnight.
- The slides were then soaked in 2x SSC (sodium saline citrate) until the parafilm fall then left in the buffer for 5 minutes.
- The slides were washed twice in 2xSSC for 5 minutes followed by one wash using 1xSSC for 5 minutes.
- 2 drops of protein block were added to the tissue sections then incubated at room temperature for 20 minutes.
- The slides were then washed in 3 changes of 1xPBS for 5 minutes each.

6. Detection:

- Link 1 (mouse antibiotin antibody in PBS) was added to tissue sections and incubated at room temperature for 20 minutes.
- The slides were washed in 3 changes of 1xPBS for 5 minutes each.
- Link 2 (biotinylated F (ab) 2 fragments of antimouse immunoglobulins in PBS) was added on tissue sections and incubated at room temperature for 20 minutes.
- The slides were washed in 3 changes of 1xPBS for 5 minutes each.
- Label (horseradish peroxidase-labelled streptavidin in PBS) was added to tissue sections and incubated at room temperature for 20 minutes.
- The slides were then washed in 3 changes of 1xPBS for 5 minutes each.
- The hybrid was detected by adding the substrate solution (2.5 ml of substrate buffer +2 drops of diaminobenzidine (DAB) chromogen solution +1 drop of hydrogen peroxide substrate solution) and incubated at room temperature for 30 minutes.
- The slides were then rinsed in 3 changes of distilled water.

❋❋Colour Development

It is worth mentioning that the colour development results from an oxidation-reduction reaction occurring between DAB and hydrogen peroxide at certain pH which is modulated by the substrate buffer. The peroxidase enzyme reduces $2H_2O_2$ to O_2 and $2H_2O$ and then the liberated oxygen oxidizes DAB (electron donor) to insoluble brown product which is detectable in the tissue.

7. Counterstaining and Mounting:

- The slides were counterstained with Mayer's hematoxylin for 1 minute, rinsed in running tap water, dehydrated with ascending series of ethanol, cleared in 3 changes of xylol for 5 minutes each and then mounted with DPX and cover slipped.

II. Light Microscopic Studies:

Hx & E-stained tissue sections as well as toluidine blue-stained semi-thin sections, prepared from the 37 cases of cervical carcinoma and the 2 normal exocervical specimens were examined.

III. Electron Microscopic Studies:

Electron microscopic technique was performed on:

a) Five of the 37 cases of the cervical carcinoma to show the ultra structural features characteristic for cervical squamous carcinoma.

b) Two normal cervical specimens to serve as a control by showing the normal ultrastructure of the transformation zone which is the starting point of the squamous carcinoma of the cervix in the diseased women.

The steps of the Electron Microscopic Technique can be summarized as follows:

1. Primary Fixation:

- Small pieces of fresh tumor tissues of dimensions of about 1 mm were fixed overnight in 3% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate buffer pH 7.4 at 4˚C.
- Following primary fixation, tissues were rinsed in 0.1M phosphate buffer, 3 times for 10 minutes each at 4˚C.

2. Post-fixation:

- Tissues were then post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 hours at 4˚C.
- Tissues were then rinsed in 0.1M phosphate buffer, 3 times for 10 minutes each at 4˚C.

3. Dehydration:

- Tissues were then dehydrated in ascending grades of ethanol for 15 minutes each at 4˚C followed by two changes of acetone for 30 minutes at room temperature.

4. Infiltration and Embedding in Epoxy:

- Tissues were infiltrated in 3 dilutions of epoxy resin for 45 minutes each, then in 100% epoxy resin overnight at room temperature. They were then embedded in cylindrical molds and polymerized for 48 hours at 65˚C.

5. Sectioning and Examining:

- Semithin sections (0.7−1μm) were cut on RMC, MT 6000-XL ultratome and stained with toluidine blue stain.
- Ultrathin sections were then cut, mounted on copper grids and stained with uranyl acetate and 0.4% lead citrate.
- Examination of the ultrathin sections was done in the Electron Microscope Unit of **Faculty of Science, Ain Shams University**.

RESULTS:

In Situ Hybridization (ISH) Results:-

Thirteen cases out of the 37 cases (35%) showed positive staining affinity for ISH labelling for HPV which appeared in the form of brownish nuclear staining **(Fig. 1)**.

All of the 3 non-invasive cases were positive (100%) and only 10 (33.3%) of the 30 invasive cases of the squamous cell carcinoma showed positive staining affinity for ISH labelling. This difference was statistically significant (P=0.052) **(Table 1)**.

II.A Normal Structure of the Cervix

1. Histological Observations:-

- Histological examinations of both Hx & E-stained tissue sections and toluidine blue-stained semi-thin sections have revealed the presence of a typical normal stratified squamous epithelium in the transformation zone of the cervix (**Figs. 2, 3**). Basal layer, in contact with the basement membrane and the stroma, is occupied by small, uniform, elongated darkly-stained cells with high nuclear to cytoplasmic ratio (**Fig. 4**) followed by polygonal cells with lower nuclear to cytoplasmic ratio, occupying the above middle layers. As the cells move towards the surface, the cytoplasm expands and the cells become progressively flattened with small pyknotic nuclei.

- The semi-thin sections have also revealed that the polygonal cells of the middle layers display numerous long cytoplasmic extensions in widened intercellular spaces and that the flattened surface cells displayed similar cytoplasmic extensions, but shorter ones are extending into narrow intercellular spaces.

II-B Histopathological Findings:-

- Histopathological diagnosis of the 37 cases of cervical carcinoma is classified as follows:

Non-Invasive Squamous Cell Carcinoma (Carcinoma in situ, CIS)

Three cases [8.11%] **(Fig., 5).**

Invasive Squamous Cell Carcinoma (SCC)

Thirty cases [81.1%] which are subtyped into well-differentiated GI (2 cases) **(Fig. 6)**, moderately-differentiated GII (11 cases) and poorly-differentiated GIII (17 cases).

Adenocarcinoma

Four cases [10.8%] which are subtyped into well-differentiated GI (3 cases) and moderately-differentiated GII (1 case).

- Histopathological diagnosis of toluidine blue-stained semi-thin sections of the 5 SCC has also revealed the presence of neoplastic cells with enlarged, irregularly-shaped nuclei in the 3 cases of moderately-differentiated SCC and the presence of neoplastic cells with lucent nuclei and degenerated cytoplasm in the 2 cases of poorly-differentiated SCC **(Fig. 7)**.

III-A Normal Ultrastructural Observations:-

- **The features revealed by the cells of the different strata of the epithelium of the normal transformation zone:**

1. Basement membrane: separates the stroma from the basal cell layer. It consists of an outer dense band, the lamina densa, which is separated from the cell surface by lamina lucida. The lamina densa usually follows the contours of the basal cell surface and appears denser and wider opposite to hemidesmosomes.

2. **Cell surface:** The basal cell surface in the basal layer varies from slightly undulating to extremely irregular with several foot processes extending to the basement membrane **(Fig. 8)**. Large numbers of cellular extensions, illustrated through the basal and the above layers, connect with those of neighbouring cells by desmosomes **(Fig. 8)**. As the superficial cell layer is approached, cellular extensions decline in number and become shorter and blunter. Some cell surfaces have very few desmosomes and others containing many desmosomes **(Fig. 10)**.
3. **Intercellular spaces:** The intercellular spaces in the basal area varies in width from moderately to extremely wide **(Fig. 9)** which narrowed towards the surface **(Fig. 10)**.
4. **Cell shape, polarity and size:** Cells in the basal layer are elongated with their long axes, oriented perpendicular to the basement membrane **(Fig. 8)**. In the above layers, cells are larger, polyhedral in shape and variable in polarity **(Fig.9)**. In the superficial layers, the cells become oblong, flattened and oriented parallel to the surface **(Fig. 10)**.
5. **Nucleus-** The size of the nucleus initially increases and then decreases from the basal to the superficial layer. The relative size of the nucleus to the cytoplasm (nucleo-cytoplasmic index) is high in the basal cells **(Fig. 8)** and decreases towards the surface **(Fig. 10)**. The nucleus of the basal cells is oval to elongate with varying numbers of slight to deep indentations. Chromatin is fine to moderately dense with some marginal or central clumping **(Fig. 8)**. Cells in the most superficial layers, contain either electron-lucent or completely pyknotic nuclei **(Fig. 10)**. Nucleoli are

observed in the cells of the basal and the above strata **(Fig. 8)**.

6. Cell components:

- The cytoplasm of the basal cells contains a large number of rod-shaped mitochondria and many ribosomes **(Fig. 9)**. Cells in the above middle layer contain many ribosomes while the most superficial layers are almost devoid of organelles **(Fig, 10)**.
- Tonofilaments are observed in the cells of the basal **(Fig. 9)** and the above middle layers**)** scattered throughout the cytoplasm and inserting into desmosomes. However, in the most superficial layers, tonofilaments are sparse or completely absent **(Fig.10)**.

III-B Electron Microscopic Results of SCC:-

A. The features revealed by the moderately-differentiated (G2) SCC are:

1. Cellular borders:

- Irregular cell borders showing finger-like intercellular pseudomicrovilli that are clearly seen in most of the neoplastic cells **(Fig. 11)**.
- Widened intercellular spaces are also seen between most of the neoplastic cells **(Fig. 11)**.
- Large numbers of desmosomes are clearly identified, bounding the neoplastic cells **(Fig. 11)**.

2. Nuclei:

- Enlarged nuclei with irregular outlines, are seen in most of the neoplastic cells **(Fig.11)**, sometimes lobulated**,** with different pattern of chromatin distribution. The majority of the nuclei have irregular dispersed clumped chromatin, mainly marginated at the nuclear envelope **(Figs. 12, 13)**, some others are pyknotic where chromatin is highly condensed **(Fig. 14)** and others are pale, showing partial loss of chromatin **(Figs. 15, 16)**.
- Widened perinuclear space is identified in a very few cells.
- Widened nuclear pores are also identified in a very few cells **(Fig. 12).**
- Enlarged nucleoli are identified in some of the neoplastic cells **(Fig., 14)**.
- Some nuclei containing inclusions of the cytoplasm are also seen **(Figs. 21, 22**).
- Some nuclei containing inclusions of the cytoplasm are also seen **(Figs. 21, 22**).
- Some nuclei enclosing virus particles of 100-150 nm in diameter are demarcated **(Figs. 17, 18)** in the 3 cases of the moderately-differentiated SCC, examined by electron microscopy. However, some of the virus particles ─ 150-200 nm in diameter ─ are also seen, surrounded by halos, in the cytoplasm close to the nuclear envelope (**Fig. 20**).

3. **The cytoplasmic organelles, structures and inclusions:**

- Swollen mitochondria with disintegrated cristae and lucent matrix are clearly seen in most of the neoplastic cells **(Figs., 12, 13, 19)**, sometimes containing dense inclusions.
- Dilated cisternae of proliferated rough endoplasmic reticulum are seen in most of the neoplastic cells **(Fig. 19).** Degenerated and fragmented cisternae are also seen in some cells **(Figs. 21).**
- Golgi complexes and their associated vesicles appear atrophic in some neoplastic cells.
- Lysosomal bodies or aggregates **(Fig. 19)** and mutivesicular bodies are seen in some neoplastic cells.
- Numerous ribosomes are also seen in some neoplastic cells **(Fig. 13**).
- Sheaves of tonofilaments are clearly identified in most of the neoplastic cells **(Fig. 12).** They may form tonofibrillar bundles that are aggregated around the nucleus forming perinuclear wreath **(Figs. 15, 16)**.
- Numerous glycogen particles or rosettes are seen in many neoplastic cells **(Figs. 21, 22)**.
- Mucoid-like substances and lipid vacuoles **(Fig. 14)** are identified in some of the neoplastic cells.

B. The features revealed by the poorly-differentiated (G3) SCC:

- Lucent or pale nuclei undergoing karyolysis are seen where the nuclear matrix is lost while the nuclear envelope is intact**.**
- The cytoplasm shows vacuolation and complete degeneration of its organelles and only some sheaves of tonofilaments are seen.

DISSCUSSION

The present study have investigated HPV in 37 biopsies of cervical carcinoma taken from Egyptian patients using in situ hybridization (ISH) technique for detection of HPV 16/18 DNA.

The results have revealed that all cases of cervical carcinoma in situ (CIS) were HPV positive by means of ISH (3/3). By means of ISH, the frequency of HPV positivity in CIS varied between 15.4% and 82.6% as reported [14,15].

The results have also revealed that the cases of invasive cervical carcinoma showed positivity in 10 cases by ISH (10/30 = 33.3%). This variability in the positivity of the ISH results is attributable to several factors, including the type of viral infection (productive or non-productive) [16,17], the number of copies of the viral genome in each cell as well as its physical state (episomic or integrated) [18], where integration of HPV DNA into the DNA of malignant cells results in lowering of the copy number of HPV DNA and detectable high copy number as well can be found in some tumour cells which can be explained by that such cells have been shown to contain HPV DNA integrated in head to tail tandem repeats as well as free unintegrated forms [19]. These free forms account for the focally distributed positive cells and the head to tail tandem repeats may produce through homologous recombination free, unintegrated copies which can replicate to a high copy number. Finally, the sensitivity of the technique and the size of the specimens are also believable to be important factors in this variation [19].

By ISH, the present study have revealed that the frequency of HPV positivity in CIS is significantly higher in non-invasive (CIS) cases than invasive ones [P= 0.04]. By searching in the literature, the reports investigating this correlation by ISH, revealed 50% in CIS and 37.7% in invasive cases [21], 64.3% in CIS and 60% in invasive cases [22] and 77% and 79% respectively [23]. However, a greater difference in the ISH results between the CIS and the invasive cases were reported [55% for CIS and 22% for SCC] and [31% and 20% respectively] [24,25].

In the present study, the neoplastic cells of cervical squamous cell carcinoma showed great pleomorphism, where elongated, rounded, polygonal or oval cells are found. This agrees with the findings of **Foschini et al.** [26] who revealed that neoplastic cells are elongated, rounded or polygonal in shape. These neoplastic cells are also characterized by having irregular cell borders showing finger-like pseudomicrovilli extended into a widened intercellular space. This is also revealed by **Foschini et al.** [26] who reported that the neoplastic cells have irregular cytoplasmic borders with a pseudomicrovillous surface and some of these cells were adherent to each other, while others appeared separated by obvious intercellular spaces. **Wright et al.** [27] have also reported that finger-like intercellular microvilli are one of the ultra structural hallmarks of neoplastic cells of squamous origin. Finally, **Ito et al.** [28] have also ascertained that a small number of microvilli are shown on the cell surface of the malignant squamous cells with an interdigitation of a microvilli-like structure and desmosome bonds between interconnecting cells. As for the normal cells of the stratified squamous epithelium occupying the transformation zone, they can be divided into three main strata, elongated cells of variable sizes and oriented perpendicular to the basement membrane, occupy the basal layer with their surface varies from slightly undulating to extremely irregular with several foot processes extending to the basement membrane and large number of long cellular extensions as well are illustrated through the basal layer between the neighboring cells. Polyhedral cells of variable polarity, occupy the middle layer. Oblong and flattened cells, oriented parallel to the surface, occupy the superficial layers and as the superficial layers are approached, cellular extensions decline in number and become shorter and blunter. This is in concordance with what reported by **Feldman et al.** [29]. Furthermore, **Kenemans et al.** [30] have revealed that cancer cells differ in their surface morphology from normal cells, and have an extra- ordinary amount of surface activity.

The coexistance of abundant tonofibrils and desmosomes within the cancer cells is of the ultra structural hallmarks of neoplastic cells of squamous differentiation. This was also reported [31]. In the present study, most of the neoplastic cells of the cervical squamous cell carcinoma (SCC) of the moderately-differentiated type are bounded together by large number of desmosomes. This finding agrees with **De Jesus et al.** [32] who revealed that clear cytoplasmic borders with few desmosomes are from the features which are consistent with well-differentiated squamous cell carcinoma cells, and also with **Wright et al.** [27] who have also revealed that desmosome-tonofilament complexes are identified in well and moderately differentiated neoplasms and finally with **Ito et al.** [28] who have also revealed the presence of an interdigitation of microvilli-like structure and desmosome bonds between interconnecting

cells in the malignant cervical squamous cells. Tonofilaments are converging the desmosomes and are also shown in the cytoplasm as sheaves or form tonofibrillar bundles which may be aggregated around the nucleus forming perinuclear wreath. This is in accordance with the findings of **Matsuura et al.** [33] who have also reported that in well-differentiated types of tumors, tonofibrils are from the ultrastructural or cellular evidence of differentiation, also **Henderson et al.** [34] have revealed that in squamous cell carcinoma, sheaves of tonofilaments are abundant forming tonofibrillar bundles which may show considerable disarray and in some tumors, they form a distinct perinuclear wreath and also added that numerous tonofibrils are apparent converging perpendicular on to desmosome, to **Wright et al.** [27] who have also revealed intracytoplasmic bundles of tonofilaments which may be aggregated and form large globular masses and also with the findings of **Hewan-Lowe and Dardick** [35] who have revealed that squamous differentiation was defined as the presence of cells or cell groups with numerous prominent tonofilament bundles. In contrast to the poorly-differentiated squamous cell carcinoma, desmosomes are scant and only very thin sheaves of tonofilaments may be recognized in the cytoplasm of these cells. This agrees with the results of Schindler **et al.** [36] who reported that the number of desmosomes decreased gradually with increasing degrees of malignancy which might express dedifferentiation and **Wright et al.** [27] have also reported that in lesser-differentiated lesions, tonofilaments and desmosomal plates are reduced and poorly-developed which indicate that loss of desmosomal attachments and separation of desmosomal-tonofilament complexes lead to loss of cellular cohesion. As for the normal cells of the different strata of the stratified squamous epithelium, desmosomal plaques are evident in the basal and above cell strata as well as the superficial layers, inserting into desmosomes or scattered throughout the cytoplasm. However, tonofilaments are sparse and less frequently concentrated into thick bundles in the more superficial layers. This is in concordance to what reported by **Feldman et al.** [29], while **Kocher et al.** [37] have reported that the percentage of cell surface occupied by desmosomes decreases significantly between normal and squamous cell carcinoma.

In the present study, most of the neoplastic cells have shown enlarged nuclei, with an irregular outline, possessing many deep invaginations in the nuclear envelope which leads to the formation of nuclear lobules. This agrees with the findings of **González-Oliver et al.** [38], who reported that the folds in the nuclear envelope cause the formation of nuclear lobules in invasive cancer cells. These characteristic nuclear forms of the neoplastic cells could provide an increased area of contact between the nucleus and the cytoplasm which seems to denote increased nucleo-cytoplasmic exchanges and heightened metabolic activity, accompanied by tumors as reported by **Franceschi et al.** [39]. As for the normal cells of the transformation zone, the absolute size of the nucleus initially increases and then decreases from the basal to the superficial layer, where the nucleus of basal cells is oval to elongated, with varying numbers of slight to deep indentations. This is in concordance with the findings of **Feldman et al.** [29]. **González-Oliver et al.** [38] have also revealed that invaginations are more deep and complex in invasive cancer than in normal cells.

The present study showed that in case of moderately-differentiated cervical squamous cell carcinoma, most of the nuclei of the neoplastic cells of this type are hyperchromatic, where increased irregular dispersed clumped chromatin is found, mainly marginated close to the nuclear envelope and some cells are nearly pyknotic where highly condensed chromatin is found. In contrast to the neoplastic cells of the poorly-differentiated type, their nuclei are pale, showing partial loss of chromatin, a stage for undergoing karyolysis**.** This agrees with the findings of **Gonález-Oliver et al.** [38] who have reported two populations of cells: cells with compact chromatin and cells with sparse chromatin and revealed that the former type is made up of well-differentiated cells while the latter is composed of relatively undifferentiated cells. As for the normal cells of the transformation zone, chromatin is fine to moderately dense with some marginal or central clumping while cells in the most superficial layers contain either pale or completely pyknotic nuclei. This agrees with the results of **Feldman et al.** [29]. Few nuclei contained areas in which the nuclear envelope is recognized, where widened perinuclear space and widened nuclear pores

are evident. This agrees with the findings of **Franceschi et al.** [39] who reported that enlarged nuclear pores are seen in tumours thus giving access of certain inclusions and organelles into the nuclei as it is revealed in the present study where a cytoplasmic inclusion; rich in glycogen; is seen inside the nucleus of one of the neoplastic cells. The nucleolus is a sensitive indicator of the activity of the whole cell where enlarged nucleoli are seen in the nuclei of some neoplastic cells. This agrees with the findings of **Matsuura et al.** [33] who reported that enlargement of the nucleolus is evident in cultures of malignant cells and also with the findings of **Munoz et al.** [40] who have reported that a large number of active nucleoli were proved in cells taken from cervical carcinoma lesions. As for the normal cells of the transformation zone, the nucleoli are observed in the basal and superficial layers, while other investigators as **Feldman et al.** [29] have reported that nucleoli were observed in basal cells, but less frequently in cells of the more superficial layers.

In the present study, the cytoplasm of the neoplastic cells of the moderately-differentiated cervical squamous cell carcinoma has shown different organelles and inclusions which may be looked upon as an expression of tumor differentiation and functional activity, where swollen mitochondria with disintegrated cristae, lucent matrix and dense inclusions were found. Dilated cisternae of proliferated rough endoplasmic reticulum, degenerated and fragmented cisternae as well were also found. Golgi complexes with its associated vesicles, large lysosomal bodies, microbodies and multivesicular bodies were also found. Numerous ribosomes were also seen in some neoplastic cells. By searching in the literature, some investigators have only revealed that a moderate number of small mitochondria [28] and abundant ergastoplasmic reticulum and dilated cisternae [26] were found. This also ascertained by **Hewan-Lowe and Dardick** [35] who revealed the presence of numerous polyribosomes, scattered profiles of rough endoplasmic reticulum and few mitochondria. Numerous glycogen particles or rosettes have also been observed in many neoplastic cells and this is in concordance to **Strickler et al**. [41] who revealed that glycogen aggregation is one of the characteristics which are evident in the tumors with squamous traits. Mucoid-like substances and lipid vacuoles were also observed, but no reports have revealed their presence. In contrast to the neoplastic cells of the poorly-differentiated cervical squamous cell carcinoma, the cytoplasm showed vacuolation which indicates complete degeneration of its organelles except for only some thin sheaves of tonofilaments. This agrees with the findings of **Clifford et al.** [42] and **Suo et al.** [43] who revealed that the tumor cells of the poorly-differentiated SCCs had accumulations of less dense tonofilaments in the cytoplasm. The difference in the cell constituents between the moderately and poorly-differentiated tumour type is related to the tumour cell differentiation and maturation. This agrees with the findings of **Mackay et al.** [44] who reported that the number of organelles decreases with diminishing differentiation in case of tumors. As for the normal cells of the transformation zone, the cytoplasm in the basal cells contains a large number of rod-shaped mitochondria and many ribosomes. These cell organelles decrease in concentration towards the cell surface where cells in the most superficial layers are almost devoid of organelles.

In the present study, investigation for the presence of HPV particles in the biopsies of cervical squamous cell carcinoma taken from Egyptian patients were also experienced using the electron microscopy, where it has been revealed that 100-150 nm virus particles were seen in the nuclei of some neoplastic cells of the 3 cases of the moderately-differentiated SCC and not in the poorly differentiated SCC, examined by electron microscopy since the formation of viral-like particles are coupled to cellular differentiation. These nuclei are either elongated or lobulated and are surrounded by large clear zones almost completely lacking cytoplasmic components. Most of the virus particles seen are closely associated with the chromatin clumps (ranging between 100-150 nm in diameter) and were also observed in the perinuclear cytoplasm, but of greater diameter (150-200). This can be explained by that the virus particles, which consist of the core and the capsid, are about 100-150 nm in diameter and in their migration to the nuclear envelope, the inner membrane of the nuclear envelope develops a focal thickening and become evaginated and encloses the virus so the virus particles escape from the nucleus and come to lie in the perinuclear cytoplasm after acquiring envelopes (appear as halos) from the host

nuclear membrane. These virus particles are suggested to be Herpes Simplex virus 2 and not HPV particles since HPV particles range from 45-55 nm in diameter [45]. Several reports have ascertained the role of HSV in cervical carcinoma where it was revealed that HSV was detected in 8% of cervical cancer, 41% of invasive squamous cell carcinoma and 44% of cervical infections as reported by **Ibrahim et al.** [46] **and Chatterjee et al.** [47] respectively. Other investigators have also admitted that HSV are predictors of cervical carcinoma and ascertained that it can have besides other factors, a very important role or may be an initial role in the development of malignant changes of female genital tract [45, 48]. In contrast, **Murthy and Mathew** [49] have revealed that no independent effect for HSV 2 in the development of precancerous lesions of the uterine cervix is observed. However, it is worth noting that the risk factors for the prevalence of HSV as reported by **Smith et al.** [50] are independently associated with younger age at first intercourse, but more than one life-time sexual partner, a husband with other sexual partners and long-term hormonal contraceptive use were associated with increased risk.

The failure to demonstrate HPV particles by transmission electron microscopy does not rule out the possibility that this virus is the etiologic agent. This agrees with the findings of **Castellsague et al.** [51] and **De Jesus et al.** [32] who admitted that in general, the detection rate is low in random sampling of the tissues for electron microscopy. However, **Wu et al.** [52] have reported that electron microscopic studies revealed 50 nm uniform particles, consistent with HPV viral-like particles, in the nuclei of some cells in well-differentiated areas of the tumours. In all cases it is widely believed that HPV plays a central if not a critical role in the pathogenesis of most cervical cancers [53, 54].

The detection of HSV in the biopsies of cervical squamous cell carcinoma, taken from the Egyptian patients may either support the hypothesis that a multifactorial etiology is likely to be the causative agent, in such a way that HSV may act synergistically as an initiator or promoter in HPV-stimulated lesions for more efficient cellular transformation or that HSV may play the initial role for the development of invasive cervical squamous cell carcinoma in Egyptian patients.

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Table 1- Relation between ISH and the stromal invasion.

Invasion	ISH				Total	
	-ve		+ve		No	%
	No	%	No	%		
Invasive	20	66.7%	10	33.3%	30	100.0%
CIN	0	0	3	100 %	3	100.0%
	20	66.7%	13	39.4%	33	100.0%

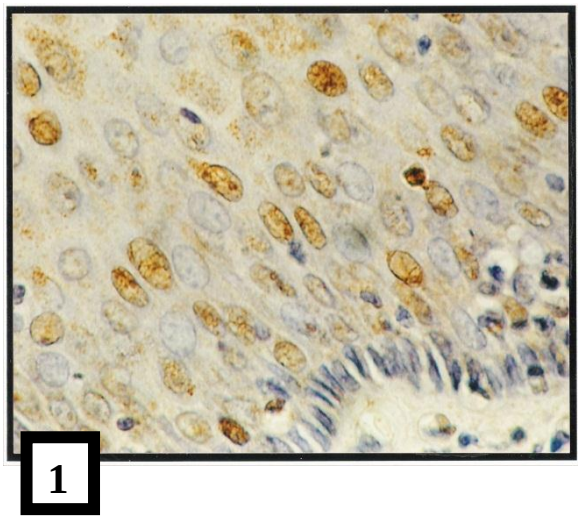


Fig. 1- A case of carcinoma *in situ* (CIS) of the uterine cervix showing positive nuclear staining affinity for HPV 16/18. **(ISH, X 400)**

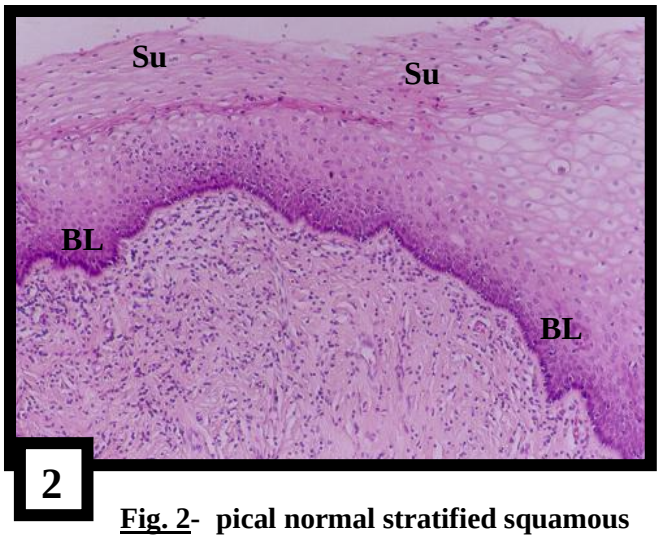


Fig. 2- pical normal stratified squamous epithelium of the cervix. Small, darkly-stained cells in the basal layers (BL) and more eosinophilic, flattened cells at the surface (Su) are shown. **(Hx &E, X 100)**

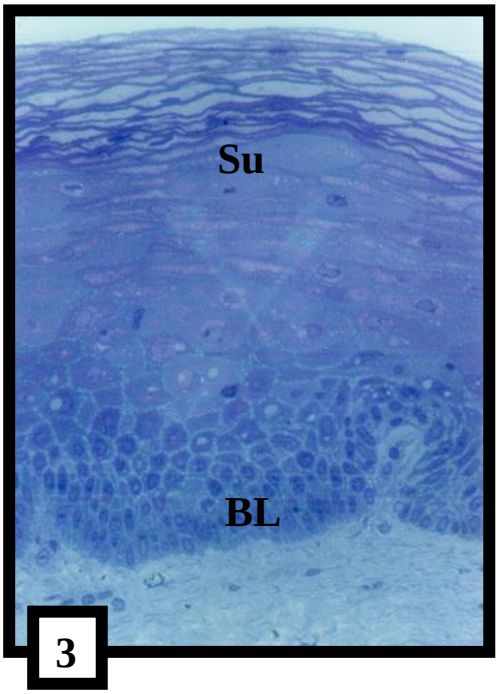


Fig. 3- Semi-thin section of the normal stratified squamous epithelium of the cervix, stained with toluidine blue. Cellular proliferation is confined to the **basal layer** (BL) where the basal cells are small and darkly-stained, while the cells become progressively flattened near the surface (Su). Notice the diminished ratio of nucleus to cytoplasm as the cells pass from the basal to the surface layers. **(Toluidine blue, X 400**

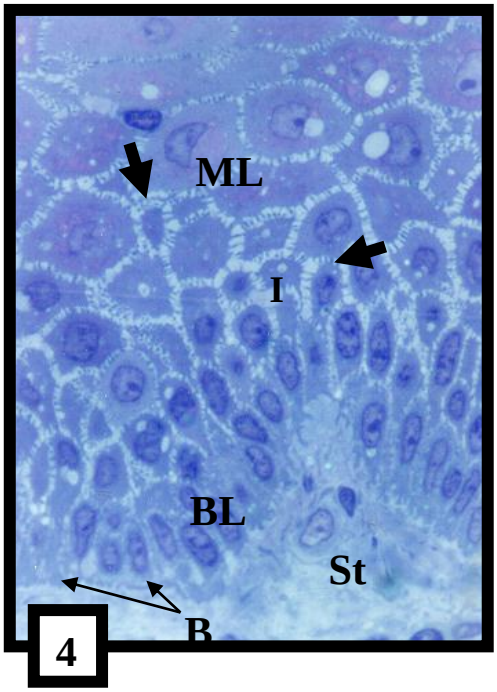


Fig. 4- Semi-thin section of a part of the normal transformation zone of the cervix, stained with toluidine blue showing elongated **basal cells** (BL) with high nuclear to cytoplasmic ratio, in contact with the basement membrane and the stroma (St) followed by polygonal cells with lower nuclear to cytoplasmic ratio, occupying the above middle layers (ML). Notice the presence of numerous long cytoplasmic extensions (arrows) in the intercellular spaces (I) between the latter cells. **(Toluidine blue, X 1000)**

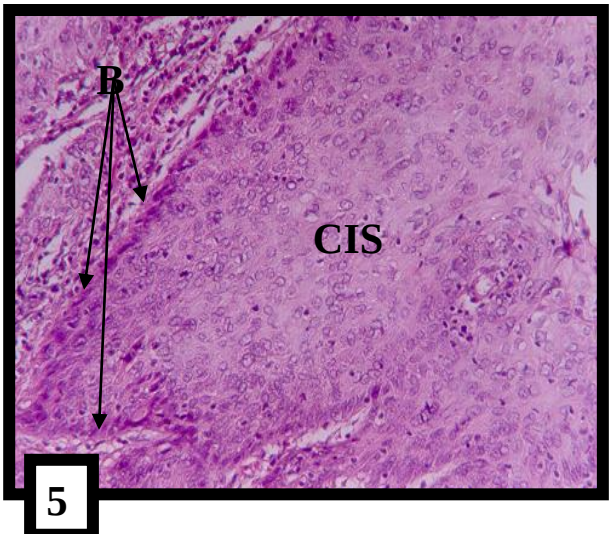


Fig. 5- Cervical carcinoma *in situ* (CIS). No orderly differentiation of squamous cells is evident where the entire thickness of the epithelium is replaced by atypical dysplastic cells with marked cellular and nuclear pleomorphism. Notice the basement membrane **(Bm)** is intact. **(Hx & E, X 160)**

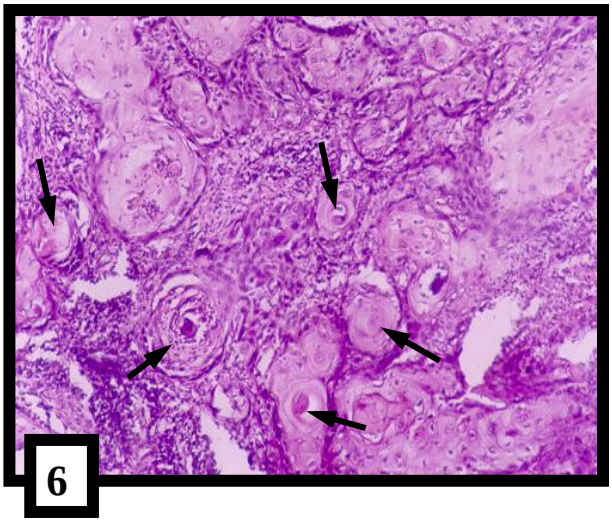


Fig. 6- Well-differentiated squamous cell carcinoma of the uterine cervix (grade 1) with abundant keratin pearls (lamellated central keratin) **(arrows)**. **(Hx & E, X 100)**

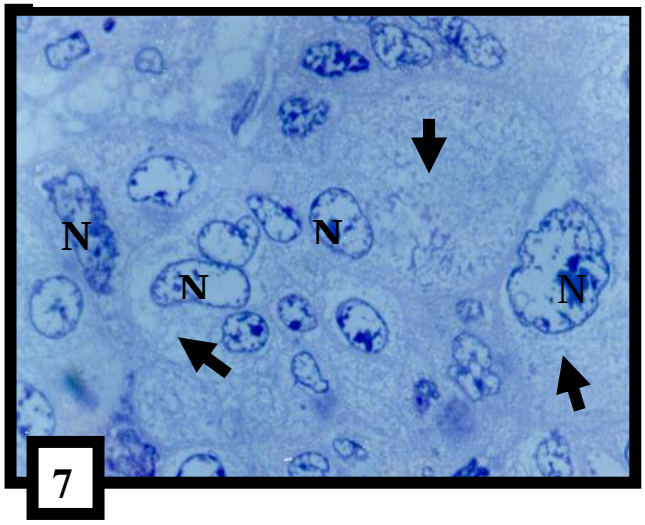


Fig. 7- Semi-thin section of poorly-differentiated squamous cell carcinoma of the cervix stained with toluidine blue. Notice the neoplastic cells with lucent nuclei **(N)** and degenerated cytoplasm **(arrows)**. **(**Toluidine blue. **X 1000)**

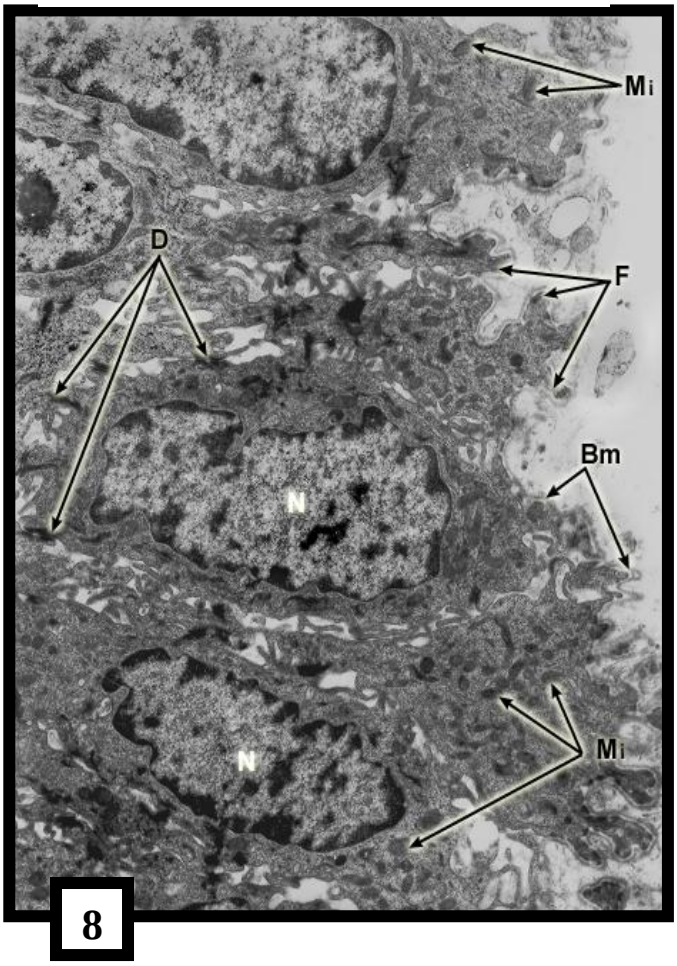


Fig. 8- Electron micrograph of a part of the basal cell layer of the stratified squamous epithelium of the normal transformation zone showing elongated cells with several foot processes **(F)** extending to the basement membrane **(Bm)**. Enlarged elongated nuclei **(N)** with varying numbers of slight to deep indentations containing moderately dense chromatin with some marginal or central clumps and dense rod-like mitochondria **(Mi)** are seen in the cytoplasm. Notice the presence of many long cytoplasmic extensions and desmosomes **(D)** between the neighboring cells. **(Original X 5000 – Mag. X 10185)**

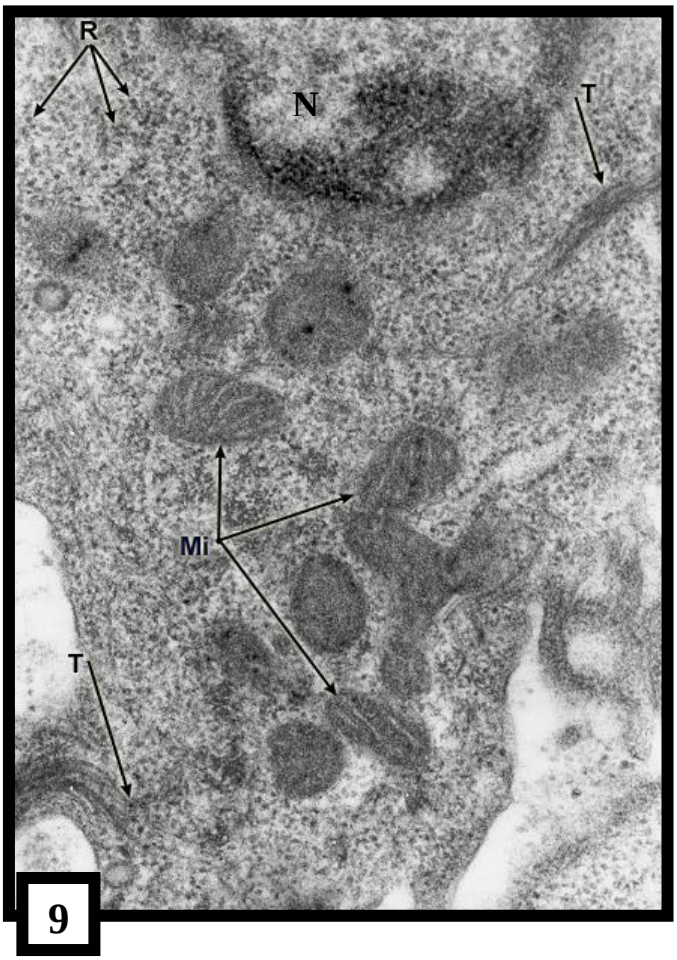
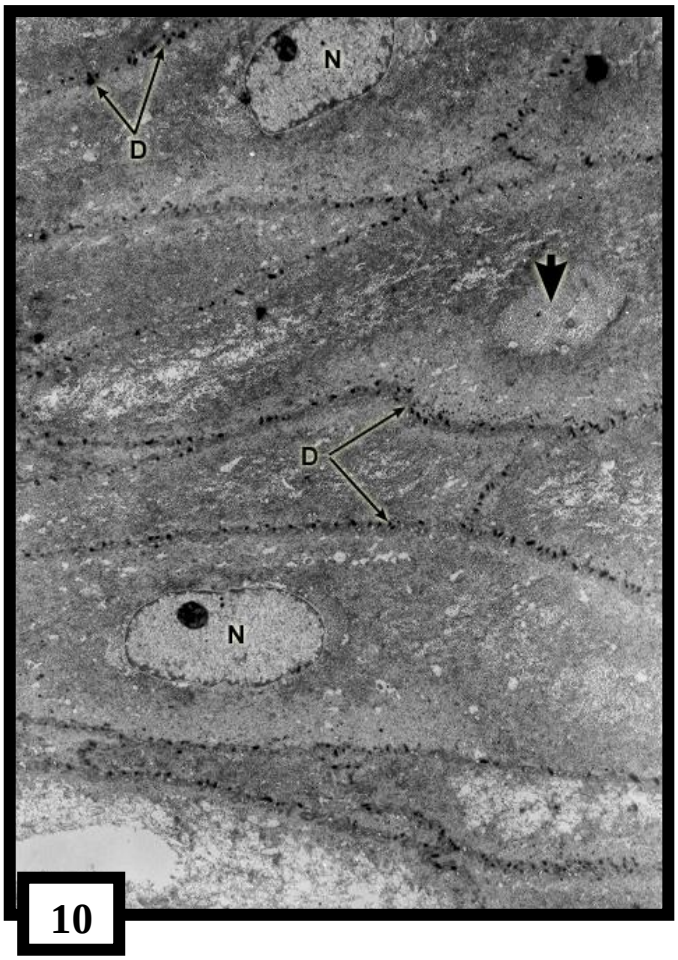


Fig. 9 - Electron micrograph of a part of one of the basal cells of the stratified squamous epithelium of the normal transformation zone showing part of its nucleus **(N)**, rod-like mitochondria **(Mi)** with longitudinal and oblique cristae, many ribosomes **(R)** and sheaves of tonofilaments **(T)**. **(Original X 30000 – Mag. X 62687**)



<u>Fig. 10</u>- Electron micrograph of the superficial cells of the normal transformation zone showing a group of flattened cells. Notice that the nuclei **(N)** display paucity of heterochromatin and that the nuclear envelope is intact. Also, notice that one of these nuclei is in its way to be completely degenerated **(arrow)**. Cellular borders, crowded with desmosomes **(D)**, are also seen. **(Original X 2000 – Mag. X 4279)**

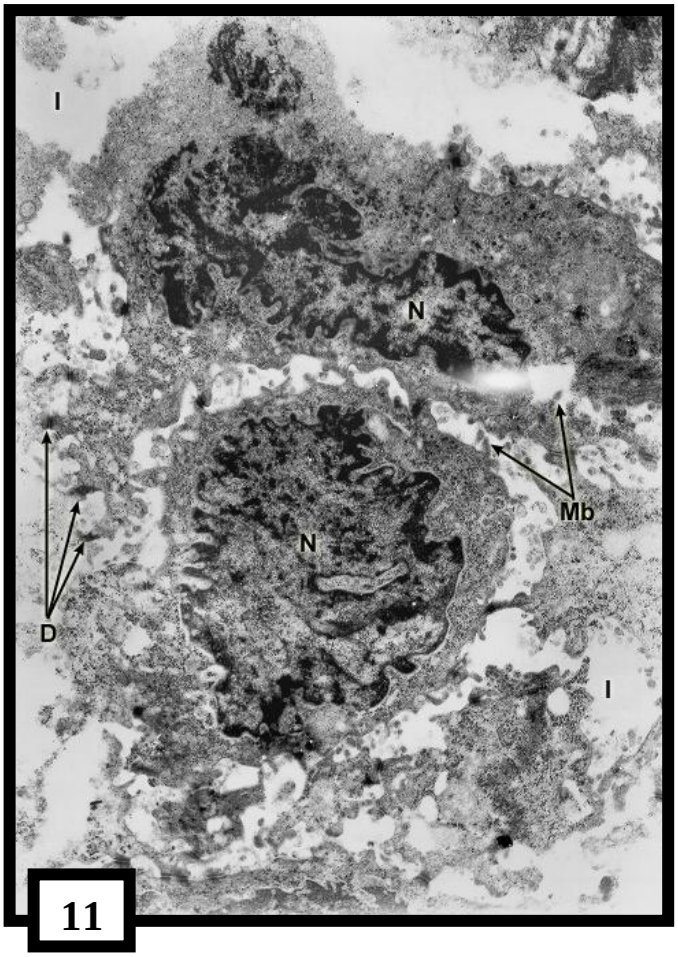


Fig. 11: Electron micrograph of moderately differentiated cervical carcinoma showing two neoplastic cells with typical malignant irregularly branched nuclei **(N)** and structureless cytoplasm. Notice the presence of irregular cell borders with finger-like intercellular pseudomicrovilli, numerous desmosomes **(D)** and widened intercellular spaces **(I)**. **(Original X 5000 – Mag. X 12000)**

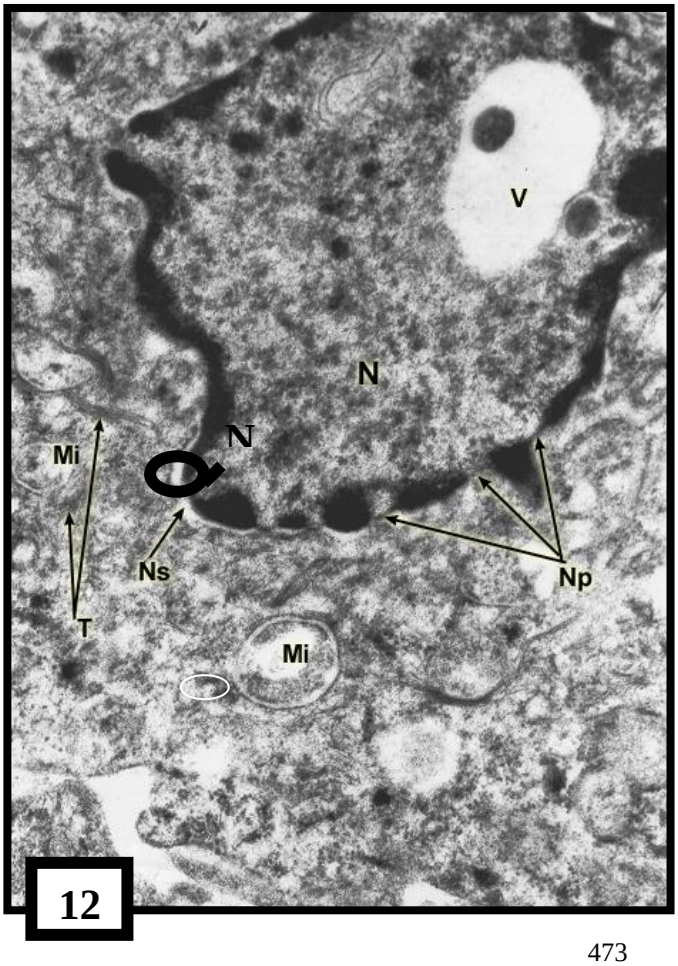


Fig. 12- Electron micrograph of moderately differentiated cervical carcinoma showing a part of neoplastic cell with an irregularly-shaped nucleus **(N)**. Coarse clumps of chromatin can be seen, mainly close to the nuclear envelope and a large vacuole **(V)** can be also seen between the chromatin clumps in the interior of the nucleus. Notice the widened nuclear pores **(Np)** and the perinuclear spaces **(Ns)**. Swollen mitochondria **(Mi)** with disintegerated cristae and scattering bundles of tonofilaments **(T)** are also evident in the cytoplasm. **(Original X 15000 – Mag. X 32260)**

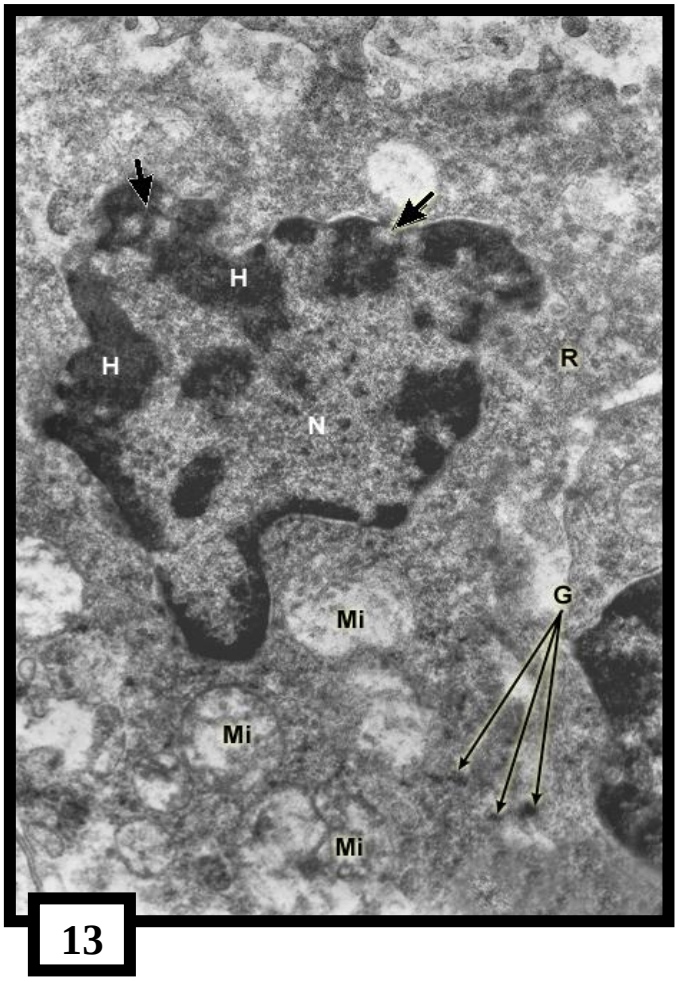


Fig. 13 - Electron micrograph of moderately differentiated cervical carcinoma showing a neoplastic cell with its an irregularly-shaped nuclear **(N)** outline where increased clumps of heterochromatin **(H)**, marginated close to the nuclear envelope, seem to contain some virus particles **(arrows)**. Swollen mitochondria **(Mi)** with disintegerated cristae, lucent matrix and dense inclusions are seen. Numerous ribosomes **(R)** and glycogen rosettes **(G)** are also seen. **(Original X 13000 – Mag. X 22588)**

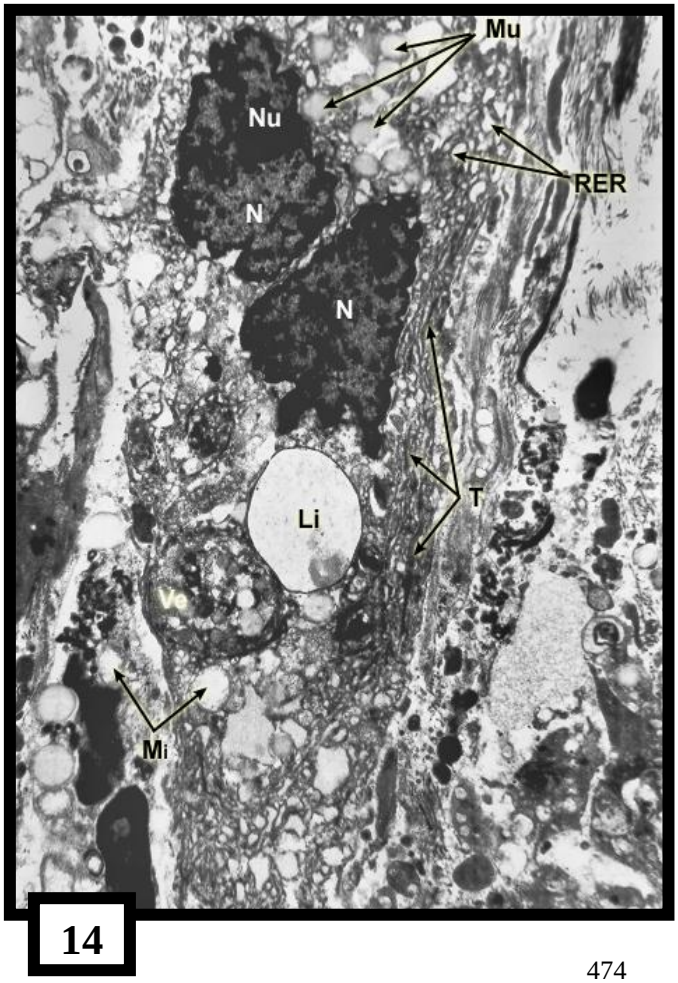


Fig. 14 - Electron micrograph of moderately differentiated cervical carcinoma showing a neoplastic cell with a pyknotic, bilobulated irregularly-shaped nucleus **(N)** and enlarged nucleolus **(Nu)** in one of these nuclei. Swollen mitochondria **(Mi)** with disintegerated cristae and lucent matrix, dilated cisternae of rough endoplasmic reticulum **(RER)**, multivesicular body **(Ve)**, a large lipid droplet **(Li)** and mucoid-like substances **(Mu)** are also seen. Notice the presence of some sheaves of tonofilaments **(T)** scattering throughout the cytoplasm. **(Original X 3000 – Mag. X 6200)**

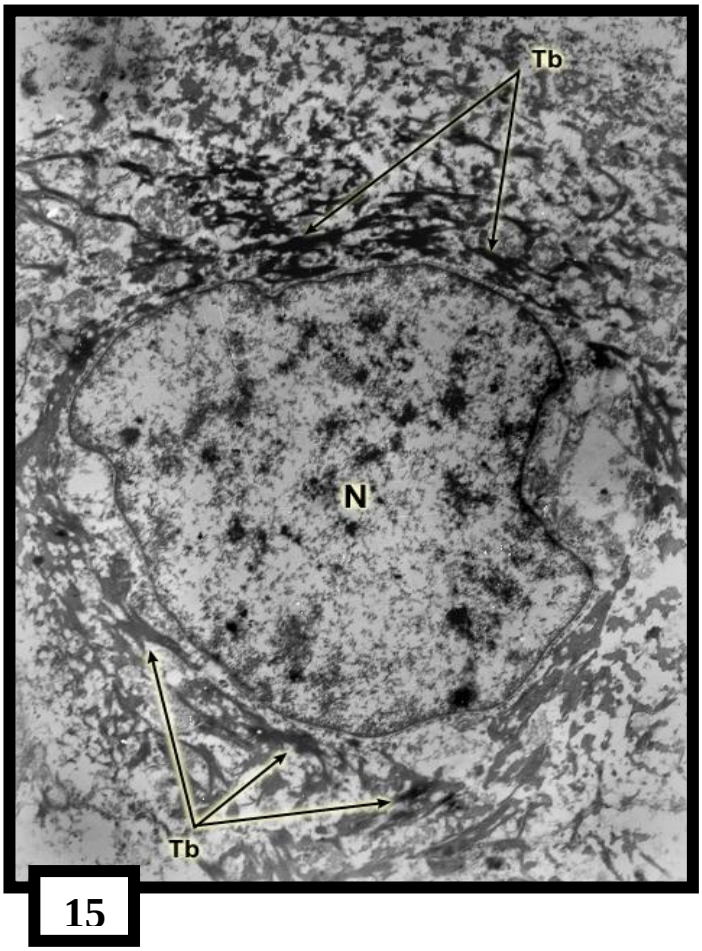


Fig. 15- Electron micrograph of moderately differentiated cervical carcinoma showing a neoplastic cell with a nucleus **(N)**, poor in chromatin. Tonofibrillar bundles **(Tb)** – consist of sheaves of tonofilaments – are seen scattered throughout the cytoplasm and are aggregated around the nucleus forming perinuclear wreath. **(Original X 5000 – Mag. X 11000)**

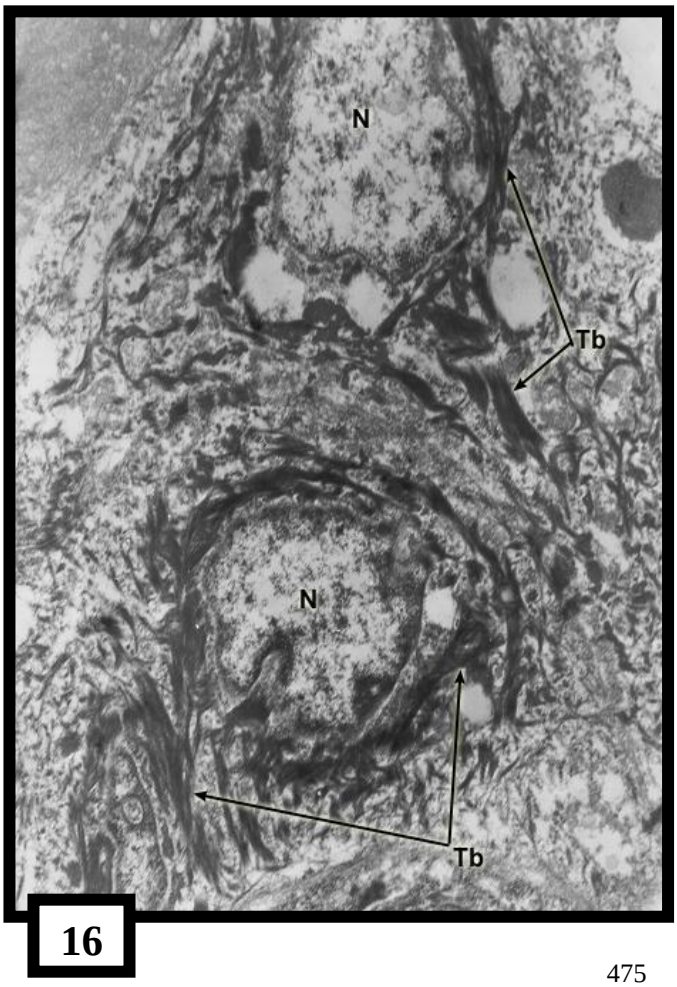


Fig. 16 - Electron micrograph of moderately differentiated cervical carcinoma showing two neoplastic cells with lucent nuclei **(N)** displaying partial loss of chromatin. Stuctureless cytoplasm is also evident except for numerous tonofibrillar bundles **(Tb)** which are seen either scattered throughout the cytoplasm or heavily aggregated around the nuclei forming perinuclear wreath. **(Original X 7500 – Mag. X 16139)**

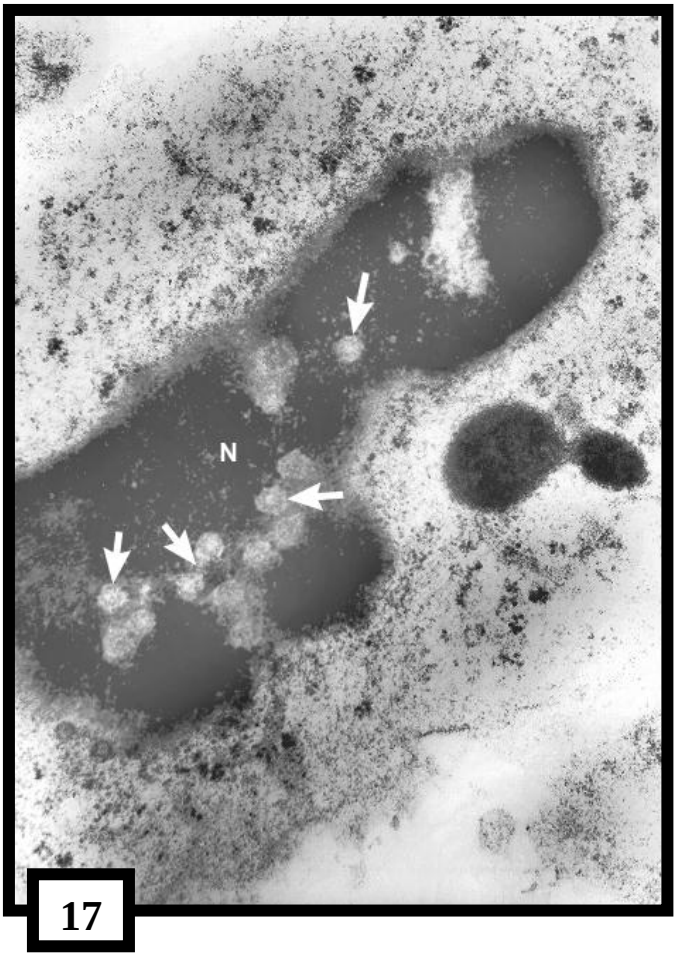


Fig. 17- Electron micrograph of moderately differentiated cervical carcinoma showing a neoplastic cell with an elongated pyknotic nucleus **(N)** where chromatin is heavily condensed and some virus particles [100-150nm] **(arrows)** are seen associated with the chromatin clumps. Structureless cytoplasm is also evident. **(Original X 20000 – Mag. X 44000)**

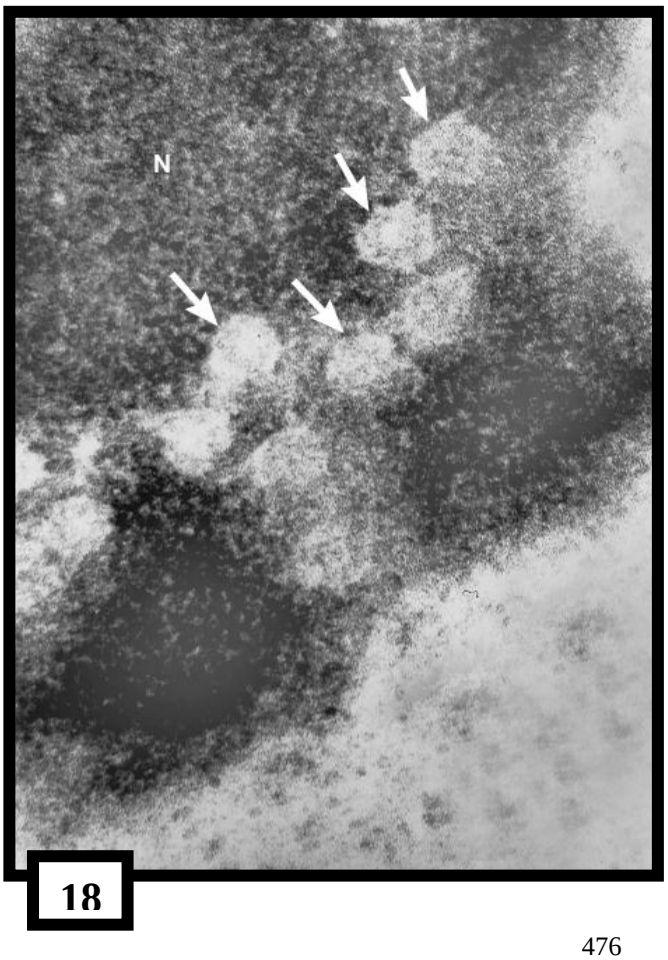


Fig. 18- Electron micrograph of moderately differentiated cervical carcinoma showing an enlarged portion of the pyknotic nucleus **(N)** of the neoplastic cell, where some virus particles [100-150] **(arrows)** are seen associated with the heavily clumped chromatin. **(Original X 50000 – Mag. X 110000)**

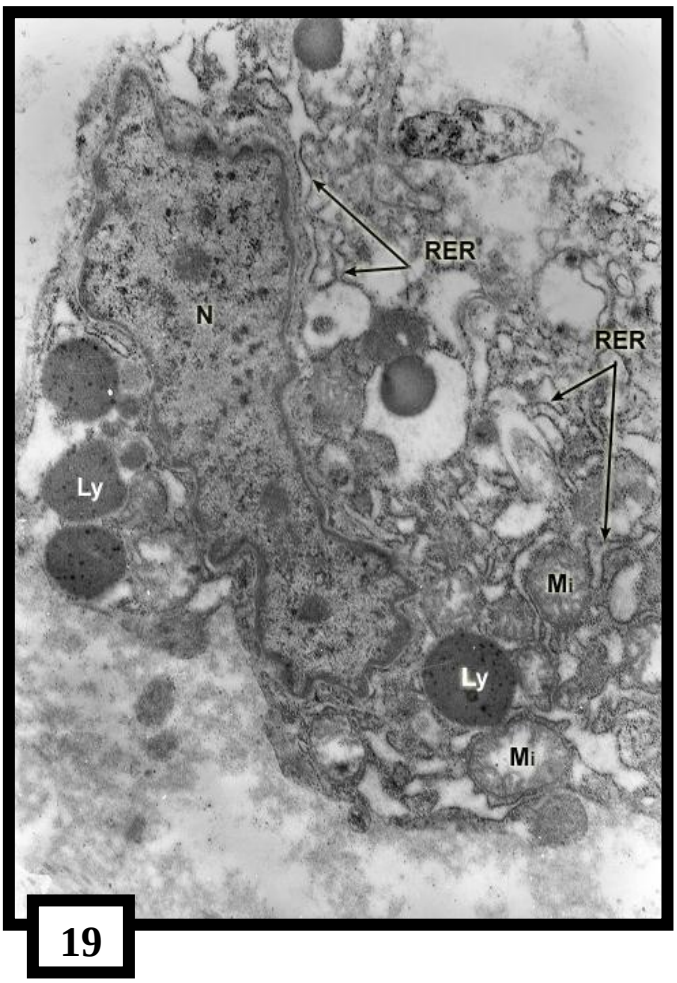


Fig. 19 - Electron micrograph of moderately differentiated cervical carcinoma showing a neoplastic cell with an elongated nucleus **(N)** which exhibiting irregular outline. Dilated cisternae of proliferated rough endoplasmic reticulum **(RER)**, swollen mitochondria **(Mi)** with disintegerated cristae and large lysosomal bodies **(Ly)** are seen. **(Original X 10000 – Mag. X 22000)**

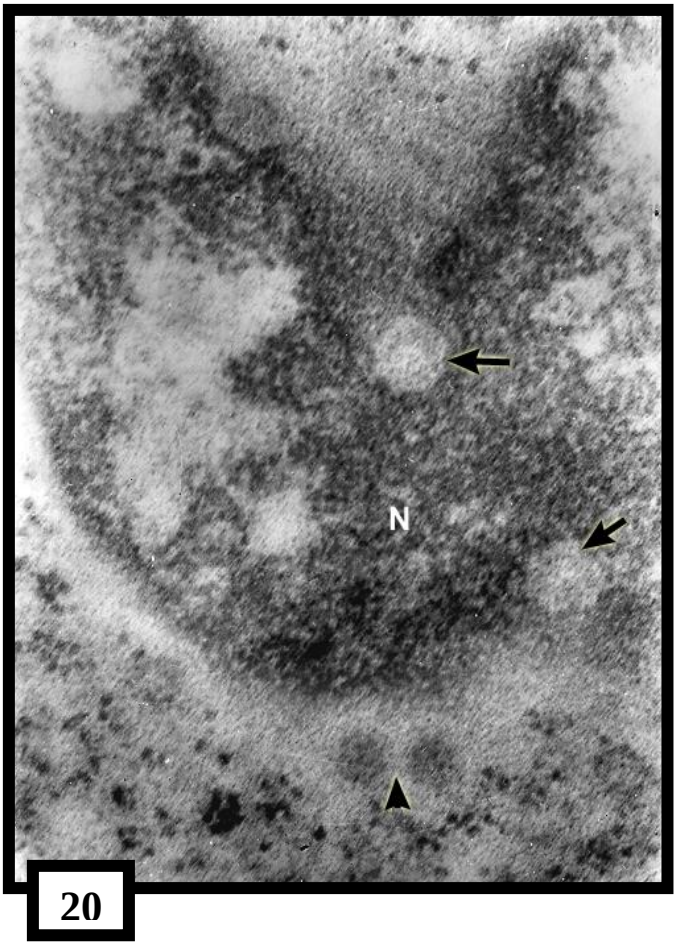


Fig. 20 - Electron micrograph of moderately differentiated cervical carcinoma showing an enlarged part of the nucleus **(N)** of a neoplastic cell with some virus particles [100-150] **(arrows)** associated with the chromatin clumps. Two virus particles [150-200] **(arrowhead)**, surrounded by halos, are also seen close the nuclear envelope. **(Original X 50000 – Mag. X 110000)**

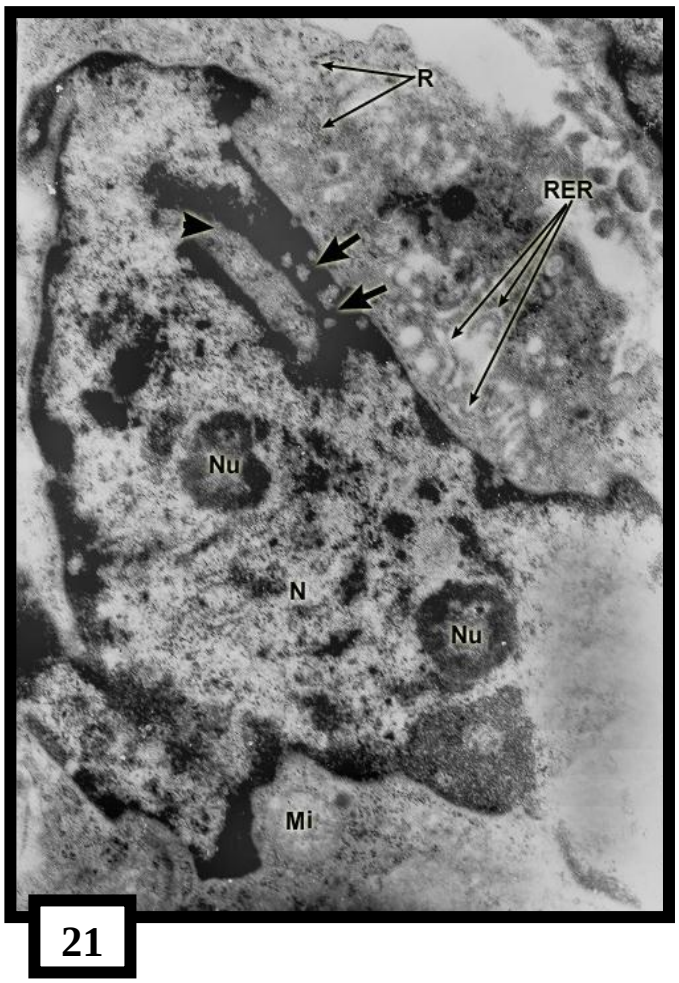


Fig. 21- Electron micrograph of moderately differentiated cervical carcinoma showing a part of a neoplastic cell with a part of its enlarged irregularly-shaped nucleus **(N)** containing two nucleoli **(Nu)**. A condensed region of chromatin, containing some virus particles **(arrows)** and an inclusion of the cytoplasm **(arrowhead)** is also seen at one side of the nucleus. The cytoplasm also shows a swollen mitochondrion **(Mi)** with disintegerated cristae, some cisternae of rough endoplasmic reticulum **(RER)**, numerous glycogen particles **(G)** and some dense bodies. **(Original X 10000– Mag. X 22000)**

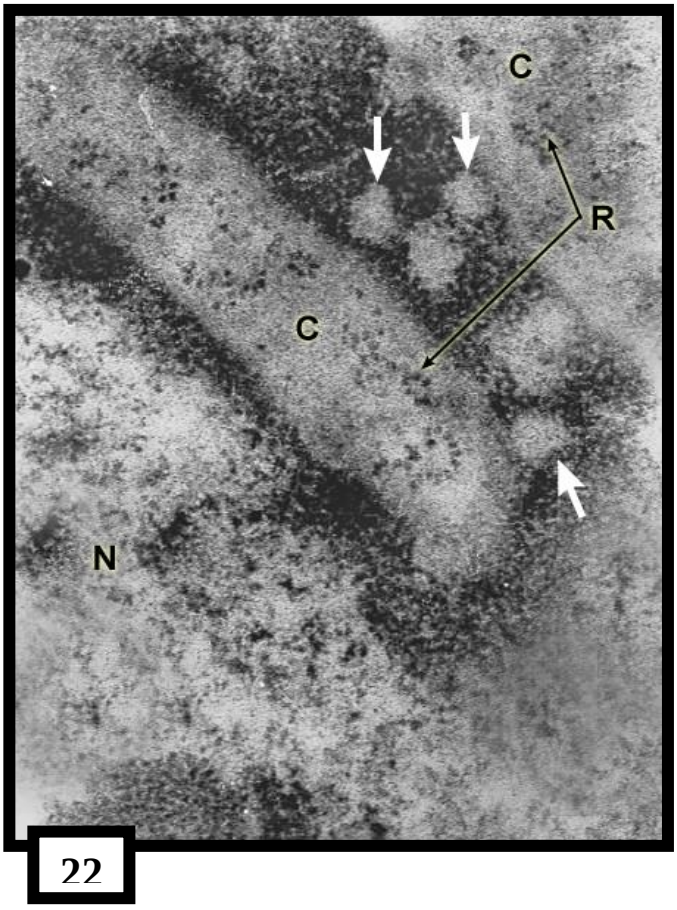


Fig. 22- Electron micrograph of moderately differentiated cervical carcinoma showing an enlarged part of the nucleus **(N)** of the neoplastic cell with condensed chromatin which contains some virus particles **(white arrows)** and an elongated part of the cytoplasm **(C)**, rich in glycogen **(G)**. Glycogen **(G)** particles are also clearly identified in the cytoplasm outside the nucleus. **(Original X 40000 – Mag. X 92308)**