ASSESSMENT OF DNA PLOIDY ON EXPERIMENTALLY INDUCED HAMSTER BUCCAL POUCH CARCINOMA (HISTOLOGICAL AND FLOW CYTOMETRIC STUDIES)

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ABSTRACT

Objective: The current study's target is directed to assessment of DNA ploidy on experimentally induced hamster buccal pouch carcinoma. **Subjects and methods:** five weeks old thirty male Syrian hamsters, weighing 80-120g. Three distinct groups of experimental animals were used (10 in each). GI (negative control): animals left untreated. GII (DMBA): animals were painted with 0.5 percent 7, 12-dimethylbenz (a) anthracene (DMBA) (3times/week/14 weeks). GIII (Paclitaxel-metformin): following DMBA application, animals injected intraperitoneally (IP) with paclitaxel (IP) (20mg/kg/ twice a week /3 weeks) and metformin (IP) (10 mg/kg/ 3 a week /3 weeks). After euthanization, all right pouches were surgically excised, bisected with one section fixed and processed for H&E and other section of the fresh tissue were used for flow cytometric (FCM) assessment. **Results:** Gross observation and histopathological changes were recorded. By FCM, the data exhibited that in GII DNA diploid was detected in (20%) of cases and DNA aneuploidy was detected in (80%) of cases while in GIII DNA diploid was detected in (51.3%) of cases. While DNA aneuploidy was detected in (48.7%) of cases. The SPF values of the diploid lesions in GII and GIII, ranged between (1.93% and 25.94%, 13.40%and 42.30%,) respectively. The SPF values of the aneuploid lesions in GII and GIII ranged between (10.25% and 51.50%, 5.50% and 42.50%,) respectively. **Conclusion:** There is a substantial association between tumor DNA ploidy or SPF and responsiveness to metformin in combination with standard chemotherapeutic drugs.

KEYWORDS: HBP carcinoma, Paclitaxel, DNA ploidy.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC), which accounts for roughly 40% of head and neck squamous cell carcinoma (HNSCC), is a diverse neoplasm that arises from the epithelial layer of the oral cavity, which includes the tongue, gingiva, oral floor, and buccal mucosa [1, 2]. Because of the low rate of early detection, most patients are in advanced stages by the time they are diagnosed. Surgery, chemotherapy, radiotherapy, or combinations of these modalities are currently the primary treatment options for advanced OSCC. Regardless of the utilization of different treatment modalities throughout the past few years, the 5 -year overall survival rate of OSCC remains constant at 50% [3].

The 7, 12-dimethylbenz (a) anthracene (DMBA) caused oral carcinogenesis model is the most widely recognized model for researching biochemical abnormalities in oral carcinogenesis as well as the chemopreventive potential of natural products and synthesized therapies. Precancerous lesions such as carcinoma in site and dysplasia (6-8 weeks) precede DMBA-induced oral carcinogenesis and eventually evolve into malignant tumors (10-14 weeks). In hamsters, DMBA induced oral tumour. Is morphologically, histopathologically, and molecularly similar to human oral tumor [4].

Paclitaxel is a tricyclic diterpenoid compound isolated naturally from the bark of Taxus brevifolia Nutt. It is already one of the most popular, successful and frequently utilized natural antitumor medications because of its unique anticancer mechanism [5]. It is also used to treat heart disease, inflammation, skin disorders, renal and axon regeneration, and clinical studies for degenerative brain disorders are also being conducted [5]. Paclitaxel has been widely used to treat breast cancer, colorectal cancer, and urinary bladder squamous cell carcinoma. It has also been used to treat diseases such as nonsmall-cell lung cancer, head and neck cancer, and acquired immunodeficiency syndrome (AIDS) [6].

Metformin (1,1-dimethylbiguanide hydrochloride), the most often mandated antihyperglycemic medicine and an FDA-approved biguanide derivative, is used as a first-line prescription for type 2 diabetes. Metformin has piqued the interest of researchers in recent years because of its anticancer properties[7]. The medication has been shown to slow the progression of lung cancer [8], prostate cancer[9], breast cancer[10], esophageal cancer[11], colon cancer[12], and melanoma [13].

The term DNA ploidy refers to the contents of nuclear DNA. Aneuploidy is defined as a difference in DNA content from the usual diploid value, and it is commonly acknowledged as a marker of malignancy in human cancers. The number of chromosomes in a cell is indicated by DNA ploidy. Some cell populations, such as cancer cells, can have abnormal DNA content and thus different ploidy due to errors in DNA replication. Flow cytometry can determine cell DNA content, showing not only cell cycle phase but also ploidy and DNA content of a specific cell population. [14]. Measurements of DNA are represented as a DNA index (DI) based on the sample DNA peak channel to peak channel ratio.[14].

The 'S-phase fraction' (SPF) is a concept used to denote the proportion of nuclei in the S-phase relative to the total number of nuclei, as evidenced by the number of nuclei located between the G0/G1 and G2/M histogram peaks. The percentage of cells in the S phase reflects proliferative activity. SPF has been reported as a prognostic factor in a number of malignancies, including ovarian cancer, breast cancer, non-small cell lung cancer, endometrial cancer, colorectal cancer and prostate cancer [15].

MATERIALS AND METHODS

The current study employed golden Syrian hamsters as experimental animals. They were used as a model for inducing carcinomas with the carcinogen DMBA. The paclitaxel treatment was then started via intraperitoneal injection (IP). Following that, several investigations were carried out, including staining with haematoxylin and eosin (H&E) and assessing DNA ploidy using flow cytometry (FCM).

Animals:

Thirty male Syrian hamsters, five weeks old and weighing 80-120g, were purchased from Cairo University's animal department (Cairo, Egypt). The animals were kept in conventional cages with sawdust bedding under regulated humidity (30-40%), temperature (20 ± 2°C), and light (12-hour light/12-hour dark). All of the hamsters in the study were fed a regular meal and had full access to water.

Sample size:

According to Duzgun et al [16] in the current investigation, a sample size of ten in each group will have 80% power to detect a difference in means of 0.53 with a significance threshold (alpha) of 0.05 (two-tailed) at 95% confidence intervals, and the p value will be less than 0.05 in 80% of those experiments (power) (two-tailed), indicating that the results are "statistically significant."" The remaining 20% of experiments will result in a change in means that is considered "not statistically significant. "The report has been prepared with GraphPad Stat Mate. 2.00.

Chemicals:

DMBA (0.5 percent) was purchased from the (Sigma-Aldrich) company and dissolved in paraffin oil. Paclitaxel was acquired from Squibb, a division of Bristol-Myers Squibb, while metformin was acquired from Sigma Aldrich (St. Louis, MO, USA). Metformin was made by immediately dissolving it in a solution of 1% normal saline sodium chloride.

Experimental design:

After adaption for one week, the animals had been split randomly into 3 groups, each with ten hamsters: GI (normal group) animals had been fed and watered only and will act as negative controls [17]. GII (DMBA), in which positive controls had been painted three times a week utilizing a camel's hair brush and 0.5 percent DMBA in liquid paraffin for 14 weeks[18]. GIII (Paclitaxel-metformin) animals injected (IP) 20 mg/kg of paclitaxel twice

a weekly for 3 weeks [19] and 10 mg/kg metformin 3 times weekly for 3 weeks [20].

Investigations:

Animals had been euthanized at the end of the experiment, and the (HBP) had been removed and separated into two specimens. One specimen had been preserved in 10% neutral buffered formalin, normally managed, and located in blocks of paraffin before being histologically evaluated. Other fresh tissue samples had been mechanically digested, suspended, and combined with a DNA kite for detection via fluorescence activated cell sorting (FACS).

Steps of cell cycle analysis

Physical dissociation of OSCC:

Petri dish 35 mm containing 5 mL of phosphate buffer saline (PBS) was filled with the tumor mass. To remove blood and debris from the tumor sample, it was cleaned regularly (2 to 3 times) in PBS. Tissue forceps were used to hold the specimen in place, and the back of a number 22 scalpel blade was used to drag the material downward and away, pulling cells from the tumor mass into the dish. Strands of connective tissue fibers were separated and removed from the sample as cells were torn free from the tumor bulk. The scraping continued until the specimen was too small to grip and the population in the dish had grown to a large size. A 5-ml disposable pipet was used to pipet the tumor solution up and down for 3 to 5 minutes. The solution was then transferred to a conical tube. The residual suspension was centrifuged for 2 minutes at 4°C at 2500 rpm. The solution was aspirated and the pelleted cells were resuspended in PBS to the required amount for FACS analysis.

Cell cycle analysis for cancer cells:

Suspensions of tumor cell were centrifuged before cell pellets were resuspended in 1 mL propidium iodide (PI) solution for 35 minutes in the dark before being examined by FCM. MODFIT,

a DNA analysis tool, was used to analyze the data (Verity Software House, Inc., Topsham, ME, USA). For each sample, computer software computed the coefficient of variation around the G0/G1 peak as well as the proportion of cells in each phase of the DNA cell cycle (G0/G1, S, and G2/M). An aneuploidy cell population was regarded present if a separate peak varied more than 10% from the diploid internal standard in addition to the G1 diploid peak, or if the G1 itself diverged more than 10% from a comparable G2/M peak. The suspended cellular pellet was stained and treated with a DNA PREP kit solution for cell cycle analysis (Beckman Coulter, Fullerton, CA). A flow cytometer (BD FACS Calibur, San Jose, CA, USA) coupled with ModFit software was used to evaluate the data. At least 50,000 nuclei's DNA histograms were examined. This was done at Assiut University's South Egypt Cancer Institute's FCM Unit, Clinical Pathology Department, and FCM Unit.

RESULTS

Gross finding GI (Normal group) Animals didn't show any gross changes, with healthy and active behavior; and both buccal pouches' length was about 5 cm. Gross observation of the right HBP mucosa appears pink in colour with smooth surface and no observable abnormalities (Fig.1A). GII (DMBA group) animals revealed a bad smell and whitish debris coming out from their mouth. Marked perioral hair loss in all hamsters was noted up to the abdomen in some animals, significant body weight which may have been caused by poor food intake due to DMBA-induced inflammation in the oral cavity. The pouch depth began to decrease up to 2 cm and remained fixed until end of the experiment. Gross observation of the right HBP mucosa showed whitish membrane and roughened granular surface on the pouch mucosa, with varying degrees of erythema and multiple raised nodules (Fig.1B). GIII (Paclitaxel and metformin treated group) Gross observation of the right HBP mucosa presented with visible rough granular surface.

Visible oral tumor incidences were detected and some tumors gradually decreased in volume and number of nodules and absence of erosive and bleeding surfaces (Fig.1C).

Histopathologic disclosures:

GI H&E stain revealed that the covering epithelium of HBP mucosa showing well-defined stratified squamous epithelium with flattened rete ridges, delicate and loose connective tissue, and layer of striated muscle fibers (Fig. 1D). In GII H&E stain showed that, 8 cases showed well differentiated SCC and 2 cases showed moderate SCC. Histologically, particularly well differentiated SCC showing epithelial pearls with keratin foci or cell nests or in the form of detached and scattered cell with evidence of keratin formation. Tumor cells consisting of pleomorphic, hyperchromatic nuclei exhibited altered nuclear/cytoplasmic ratio. Dysplastic features in multiple areas, destruction of basement membrane (Fig.1E). In GIII H&E stain showed that, 7 cases showed early superficially invasive SCC and 3 cases showed severe epithelial dysplasia (Fig.1F).

DNA Content Analysis:

FACS analysis was employed to provide a quick calculation of the neoplasm's ploidy status and cell proliferation by determining the contents of nuclear DNA and offer two functional parameters associated with neoplastic development, DNA ploidy and cell proliferation (SPF). A sample was termed diploid if the histogram showed a single peak in the G0/G1 phase with DI ranging from 0.95 to 1.05. If there was even one distinct second G0/G1 population to the right of the initial G0/G1 peak, and DI was less than 0.95 (Hypodiploid) or greater than 1.05, DNA aneuploidy was present (Hyperdiploid). In the GI, normal HBPs mucosa was used as a control, with a single diploid peak (reference peak) signifying G0/G1 cells (2N). The majority of the cases in GI were diploid, with DI = 0.95< DI<1.05. The SPF values calculated for the GI cell cycles varied
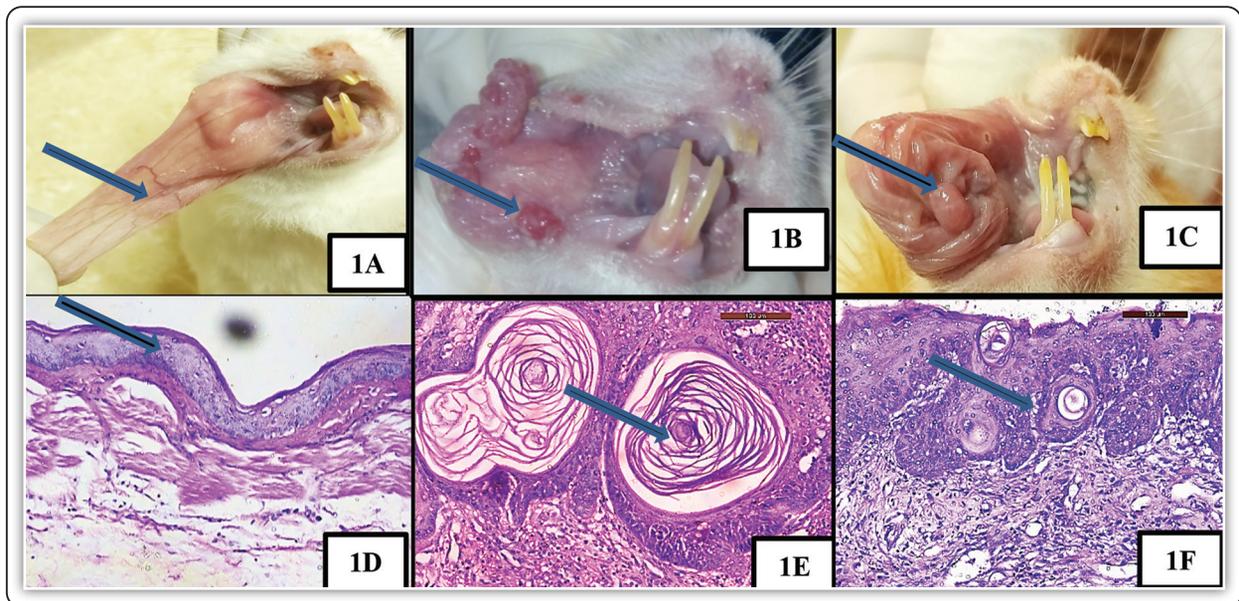
FIG (1) **(Fig.1A):** Photograph of GI (normal). displaying a rosy, smooth buccal pouch mucosa (arrow). **(Fig.1B):** Photograph of GII (DMBA) a number of polypoid papillary masses surrounded by bleeding zones (arrow). **(Fig.1C):** Photograph of GIII (Paclitaxel and metformin) indicated a little mass without ulceration or hemorrhage. (arrow). **(Fig.1D):** Photomicrograph of GI (normal) showing: HBP mucosa showing well-defined stratified squamous epithelium with flattened rete ridges, delicate and loose connective tissue, and layer of striated muscle fibers. (arrow). (H&E stain X200) **(Fig.1E):** Photomicrograph of GII (DMBA) showing well differentiated SCC with deeply invasive tumor islands into the connective tissue. (arrow). (H&E stain X200). **(Fig.1F):** Photomicrograph of GIII (Paclitaxel and metformin) showing well differentiated SCC (early invasion). (arrow). (H&E stain X200).

from 0% to 5.62 percent, with a mean of 2.32 percent. DNA diploid was identified in 20% of GII (DMBA group) cases. The diploid lesions had a single diploid peak at (2N), as the reference peak. While DNA aneuploidy was found in (80%) of the instances. The existence of extra stem lines to the right of the G0/G1 diploid peak indicated that all aneuploid instances were hyperdiploid, with DI ranging from 1.06 to 1.12, with a mean of 1.1 0.0003. The difference in ploidy status (diploid vs. aneuploid DNA pattern) was statistically significant (P-value 0.001). (Fig. 2). The SPF value of diploid lesions varied from 1.93 percent to 25.94 percent, with a mean of 15.07 percent, whereas the SPF of aneuploid lesions ranged from 10.25 percent to 51.50 percent, with a mean of 26.6 percent. As demonstrated in, the difference in SPF values of

(diploid and aneuploid) was statistically significant (P-value 0.05). (Fig. 3). Diploid DNA was found in 51.3 percent of GIII cases. The diploid lesions had a single diploid peak at (2N), It corresponded to the reference peak. While DNA aneuploidy was found in 48.7 percent of the cases. The DI varied from 1.01 to 1.08 with a mean of 1.05 0.0002 in all aneuploid instances. As demonstrated in (Fig. 2), the ploidy state difference (diploid versus aneuploid DNA pattern) was not statistically significant (P-value > 0.05). The diploid SPF values varied between 13.40% and 42.30%, with a mean of 16.4 %, whereas aneuploid SPF values varying between 5.50% and 42.50%, with an average of 16.2%. As demonstrated in, the difference in SPF values of (diploid and aneuploid) lacked statistical significance (P-value > 0.05). (Fig. 3).
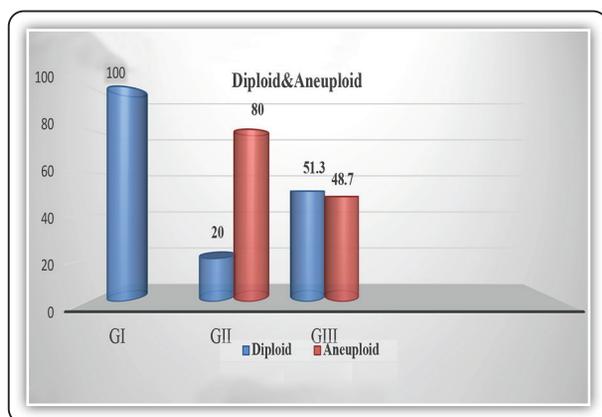
FIG (2) Bar chart representing mean results between diploid and aneuploid in studied groups
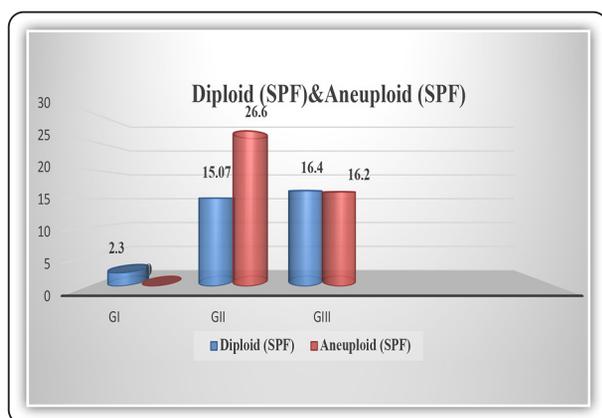

FIG (3) Bar chart representing mean results between diploid (SPF) and aneuploid (SPF) in studied groups.

DISCUSSION

OSCC is the most frequent kind of cancer in humans, accounting for 2% of all cancers. Although advances in treatment techniques such as surgery, radiation, chemotherapy, as well as interdisciplinary full-sequence treatment have improved the OSCC patients' quality of life slightly, the 5-year survival rate stays stable unchanged [21,22].

The gross observation findings in GI in the current study revealed no detectable abnormalities in the HBP. There were no skin eruptions and the hair appeared normal. All hamsters with normal histological features had buccal pouches that measured around 5cm in length after sacrifice. These findings are similar with those reported in previous investigations [23,24,25]. This finding was supported by H&E staining, it demonstrated that everything was as it should be. Two to four cell layers of stratified squamous epithelium, no rete ridges, and a fine keratin surface layer. The underlying connective tissue was discovered to be uninflamed and loose, with few vascular gaps. Larger vessels and a layer of longitudinally striated muscle created the deeper CT layer. Grawish M et al. and Samah K et al. published their findings [26,27]. These results may be due to the hamsters not exposed to any carcinogenic agent.

In the current study, normal HBPs mucosa was used as a standard, with a G0/G1 cells are represented by a single diploid peak (reference peak) (2N). This result is in line with the findings of another study, conducted by Hussein et al., who discovered that all animals in the normal group were diploid due to the normal diploid peak [28]. This result was attributed to the fact that the hamsters were not exposed to any carcinogenic agent.

In the present study, the gross observation findings in GII (DMBA treated group) showed multiple exophytic masses of variable size surrounded by area of ulceration and bleeding. The present clinical findings revealed general debilitation of the DMBA treated animals, significant reduction of the pouches' length, large exophytic masses on the painted pouches, hair loss, and skin lesions, corresponding with results of studies using the same model [29,30,25]. These results are due to the strong toxic DMBA effect. In the present study, throughout the study period, several grades of oral tumors were established in animals. In accordance with the findings of this study, Mariadoss et al.,[31] discovered that 100% of the tumors had formed after 14 weeks of applying DMBA alone on the HBP. In contrast to the current study, Yang et al.,[32], Li et al.,[33] and Hussein et al.,[34] reported that OSCC induced by DMBA was seen in the examined HBPs

was 53.5%,76.9% and 66.67% respectively. This discrepancy may be due to the using of different type of carcinogenic agent or technique or using old hamsters rather than the young ones.

This finding reflected on H&E staining in which histologically, DMBA-induced HBP tumors were shown to be well to moderately differentiated SCC with keratin pearl invasion and dysplastic epithelial nests.in the underlying connective tissue. These results are agreement with other research work [35]. This may be due to the procarcinogenic nature of DMBA, which is metabolized by phase I enzymes such cytochrome P450 to generate its ultimate dangerous metabolite, dihydrodiol epoxide, which adheres to and damages DNA, resulting in mutation and carcinogenesis [36,37].

The present study reported that 80% of cases in GII showed aneuploid DNA pattern and 20% of cases showed diploid DNA pattern. The significant difference between GI and GII were highly significant (P <0.001). All of the aneuploid cases were hyperdiploid, with DI ranging from 1.06 to 1.12 and a mean of 1.1 ±0.0003. The ploidy state difference (diploid versus aneuploid DNA pattern) was statistically highly significant (P< 0.001). In line with our findings, Das et al., [38], Oyo et al., [39] and El-Deftar M et al., [40] discovered a greater frequency of aneuploidy in OSCC tumors, as well as a rising the rate of aneuploidy decreases as the degree of tissue differentiation decreases. In oral and HNC cancers. It was shown that individuals who employed fresh OSCC tissues, such as those used in this study, had a greater aneuploidy rate than those who utilized frozen OSCC tumor tissues[41,42]. Furthermore, when numerous tumor's tissue samples were evaluated, the scientists discovered that DNA ploidy is diverse throughout SCC of the oral region, and aneuploidy was relatively common[43].

The SPF values of the diploid lesions varying from 1.93% and 25.94% with an average of 15.07%, and the aneuploid's SPF vary among 10.25% and 51.50% with a mean of 27.42%. The variation in

SPF values between (diploid and aneuploid) was statistically significant (P-value < 0.05). Our study's mean SPF is close to the reported results for FCM estimated SPF in HNC. [44, 39]. These results indicated that high SPF reflects the high proliferative activity of the tumor. According to Chen R., and Mahmood J et al., aneuploid tumors have a considerably higher SPF than diploid tumors [45,46].

In the current study, GIII (Paclitaxel and metformin treated group) gross perception of the right HBP mucosa presented with visible rough granular surface. Visible oral tumor incidences were detected and some tumors gradually decreased in volume and number of nodules and absence of erosive and bleeding surfaces. These outcomes are in concurrence with other reviews [47,48,49]. Rocha G et al., revealed that the combination of paclitaxel and metformin was found to decreased proliferation in a xenograft model in the lungs when contrasted with the singular medication medicines alone [49]. This finding reflected on H&E staining in which 7 cases exhibited early tumor cell invasion in the form of well-differentiated SCC and 3 cases exhibited severe dysplasia. These outcomes may attribute to that; metformin may sensitize the response of animals to DNA damaging agents (Taxol/chemotherapy) which a mitotic inhibitor used in cancer chemotherapy that interferes with the normal function of microtubule growth leading to mitotic arrest, prevention of cell division, and eventually apoptosis [50,51].

In GIII DNA diploid was distinguished in (51.3%) of cases. While DNA aneuploidy was distinguished in (48.7%) of cases. All aneuploid cases were hyperdiploid with DI gone from 1.01 to 1.08 with a mean of 1.05 ± 0.0002. The difference in the ploidy state (diploid and aneuploid DNA pattern) There was no statistically relevant difference. (P-value> 0.05). The SPF values of the diploid lesions varied from 13.40% and 42.30% based on an average 16.39%, and the SPF of the aneuploid lesions varied from 5.50% and 42.50% with an average of 15.85%. The distinction in the SPF value of (diploid

and aneuploid) was of no statistically significant distinction (P-value >0.05). This outcome is in concurrence with others studies [49,50,52]. Patel S et al., showed that blend of metformin and paclitaxel there was a general expansion in G2/M period of cell cycle after treatment with Taxol, a general decline in cells captured in the G0/G1 stage and a simultaneous expansion in cells captured in the G2/M period during the cell cycle was noticed [50].

CONCLUSION

In conclusion, the present study suggested that there is a substantial relationship between tumor DNA ploidy or SPF and metformin used in combination with standard chemotherapeutic drugs. Also, the evaluation of S-phase fraction and overall ploidy DNA content is a potent critical indication for tumor activity and malignancy throughout OSCC development.

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