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Chromosome 17 *In Situ* Hybridization Grid-based Analysis in Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: Oral squamous cell carcinoma (OSCC) is an aggressive malignancy due to its increased ability for local metastases and distant lymph node metastases. Extensive cytogenetic analyses have detected chromosome instability (CI) patterns in OSCC including gross chromosome numerical alterations, such as polysomy and sporadically monosomy that negatively affect the biological behaviour of the malignancy. Our aim was to investigate the frequency and impact of chromosome 17 (Chr 17) numerical imbalances in OSCC. Materials and Methods: Fifty (n=50) formalin-fixed, paraffin-embedded primary OSCCs tissue sections were used. Chromogenic in situ hybridization (CISH) was implemented for detecting Chr 17 centromeric numerical imbalances. Concerning the screening process in CISH slides, a novel real-time reference and calibration grid platform was implemented. Results: Chr 17 multiple copies were observed in 12/50 (24%) of the examined cases. Polysomy was observed in 10/50 (20%) tissue sections, monosomy in 2/50 (4%), whereas the rest of them demonstrated a normal, diploid pattern (38/50-76%). Chr 17 numerical differences were associated with the grade of differentiation of the examined tumors (p=0.001). Conclusion: Chr 17 numerical imbalances (polysomy predominantly and monosomy) are observed in sub-groups of OSCCs correlating with a progressive dedifferentiation of malignant tissues. The proposed grid-based platform on CISH slides provides a novel, fast and accurate screening-mapping mechanism for detecting chromosome numerical aberrations.*

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Oral squamous cell carcinoma (OSCC) represents the prominent malignancy in the corresponding anatomical region (oral cavity). Interestingly, this pathological entity is frequently characterized by an aggressive phenotype due to increased tendency for local metastases and distant lymph node metastases, as a result of severe genetic alterations (1). Etiopathogenetic factors that lead to OSCC development and progression include chronic tobacco and alcohol consumption and viral infection (2, 3). In fact, persistent human papilloma virus (HPV) infection is responsible for malignant transformation of the affected oral/oropharyngeal epithelia modifying the host cell genome (4). According to genetic analyses, affected oral epithelia are characterized by increased mitotic rates, accumulation of gross numerical and structural chromosome (polysomy/aneuploidy) and specific gene deregulations (deletions, amplifications, point mutations, translocations) that lead to their progressive neoplastic and finally malignant transformation (5, 6). In OSCC, chromosome 17 (Chr 17) instability is under investigation regarding its impact on the corresponding

Table I. *Clinicopathological parameters and total CISH Chromosome 17 results.*

Clinicopathological parameters		Chromosome 17 (CEN)			*p*-Value
OSCC (n=50)		N/D 38/50 (76%)	P 10/50 (20%)	M 2/50 (4%)	
Gender					0.643
Male	44 (88%)	34/50 (68%)	8/50 (16%)	2/50 (4%)	
Female	6 (12%)	4/50 (8%)	2/50 (4%)	0/50 (0%)	
HPV history					0.181
Positive	18 (36%)	12/50 (24%)	5/50 (10%)	1/50 (2%)	
Negative	32 (64%)	26/50 (52%)	5/50 (10%)	1/50 (2%)	
Grade					**0.001**
1	18 (36%)	17/50 (34%)	1/50 (2%)	0/50 (0%)	
2	21 (42%)	18/50 (36%)	3/50 (6%)	0/50 (0%)	
3	11 (22%)	3/(6%)	6/50 (12%)	2/50 (4%)	
Stage					0.371
I	9 (18%)	9/50 (18%)	0/50 (0%)	0/50 (0%)	
II	26 (52%)	20/50 (40%)	6/50 (12%)	0/50 (0%)	
III	15 (30%)	9/50 (18%)	4/50 (8%)	2/50 (4%)	
Smoking status					0.707
Current	38 (76%)	31/50 (62%)	5/50 (10%)	2/50 (4%)	
Former/Non	12 (24%)	7/50 (14%)	5/50 (10%)	0/50 (0%)	

OSCC: Oral squamous cell carcinomas; N/D: normal/diploid pattern; P: polysomy; M: monosomy; CEN: centromere enumeration. Bold value indicates statistical significance.

patients' clinico-pathological features (7, 8). In the current study, we analyzed Chr 17 numerical status in OSCC tissues by implementing a chromogenic *in situ* hybridization (CISH) assay in order to identify sub-groups of patients with specific chromosome instability (CI) patterns. In this study we also applied a novel grid-based coverslip platform for a systematic and accurate CISH slide screening process.

Materials and Methods

Study group. For the purposes of our study, fifty (n=50) archived, formalin-fixed and paraffin-embedded tissue specimens of histologically confirmed primary OSCC were used. The hospital ethics committee consented to the use of these tissues in the Department of Pathology, Hippocration Hospital, University of Athens, Greece for research purposes, according to World Medical Association Declaration of Helsinki guidelines (2008, revised in 2014). The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the cor¬responding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of the World Health Organization (WHO) Pathology Series (9). The information regarding HPV DNA status (positivity or not) was derived from the patients' medical file records. Among them, eighteen (n=18) HPV DNA positive cases were recorded. HPV 16/31/53 High Risk (HR) subtypes were detected mainly by analyzing the corresponding cases. Clinicopathological data of the examined cases are demonstrated in Table I.

Chromogenic in situ hybridization (CISH) assay. For the purposes of our study, we selected and applied the SPOT LIGHT CISH assay (Zymed/InVitrogen, San Fransisco, CA, USA) based on Centromere Enumeration Probe 17 (CEP17). In brief, the sections were deparaffinized and incubated in pre-treatment buffer in a temperature-controlled microwave oven at 92˚C for 10 min using a Spot-Light formalin-fixed, paraffin-embedded reagent kit (Zymed Inc. San Francisco, CA, USA). The sections were allowed to cool at room temperature for 20 min and then washed with phosphate-buffered saline. Enzymatic digestion was carried out by applying 100 ml of formalin-fixed, paraffin-embedded digestion enzyme onto the slides for 10 to 15 min at room temperature. The slides were washed with phosphate-buffered saline and dehydrated with graded ethanol. The ready-to-use biotin-labelled probe was applied onto the slides, which were covered with a coverslip. The slides were denatured on a hot plate at 94˚C for 3 min, and hybridization was performed overnight at 37˚C. After hybridization, the slides were washed with 0.5 ml standard saline citrate for 5 min at 75˚C, followed by 3 washes in phosphate-buffered saline 0.2% concentration at room temperature. The probe was detected with sequential incubations with avidin-peroxidase and 3,3 - diaminobenzidine (3,3-DAB) according to the manufacturer's instructions (Zymed Inc., San Francisco, CA, USA). Tissue sections were lightly counterstained with hematoxylin, dehydrated in graded ethanol and embedded. At the end of the process, CISH CEP17 signals were easily visualized as dark brown scattered dots, using a conventional, bright-field microscope (Figure 1). Interpretation of Chr 17 signals was based on Zymed's Evaluation Chart for CISH. According to these guidelines, two centromere copies per nucleus demonstrate normal, diploid pattern, whereas three and more centromere copies per nucleus show chromosome polysomy in ≥50% of isolated nuclei or nuclei in small

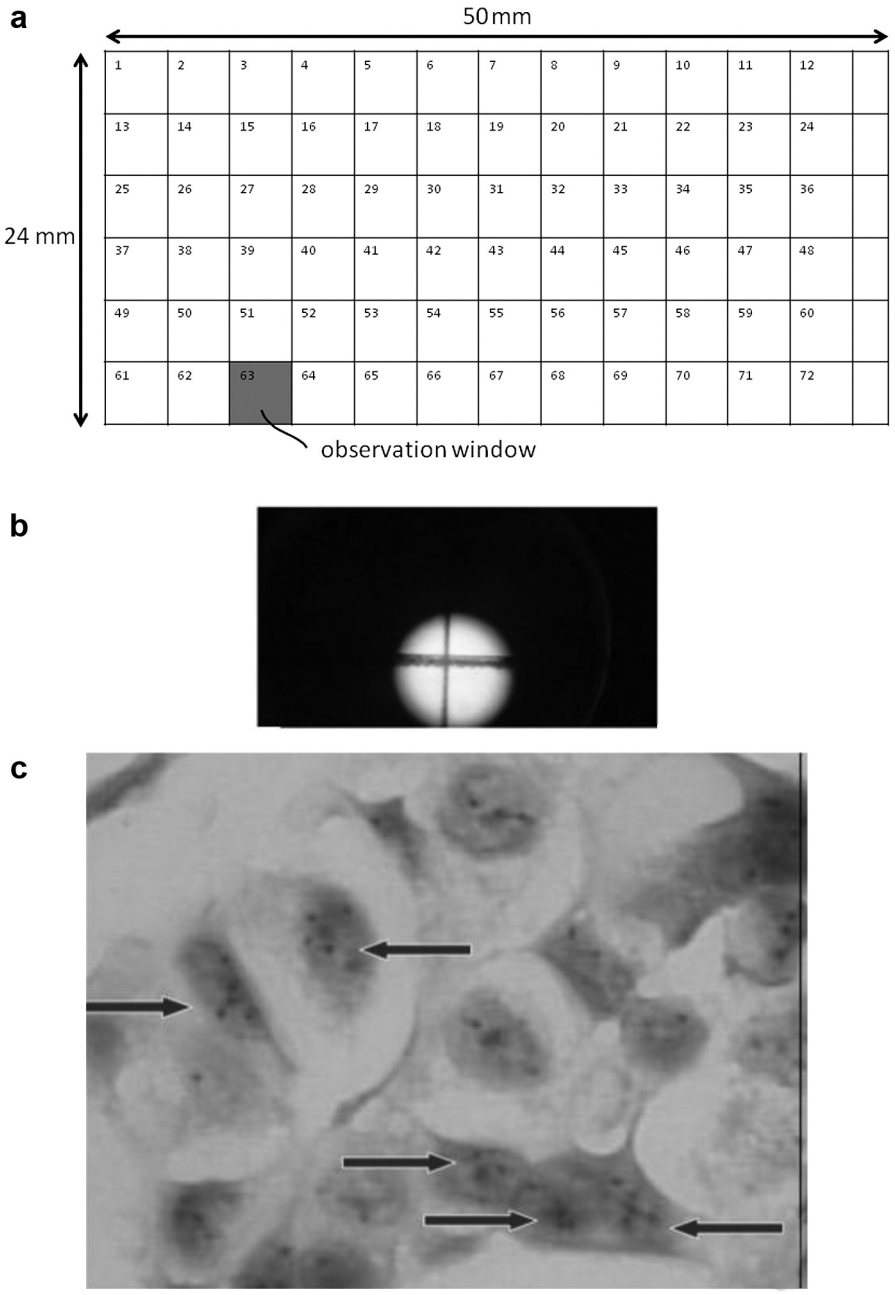

Figure 1. *Chromosome 17 CISH analysis in OSCC a. Schematic presentation of the grid-based cover slip that was implemented in CISH slide screening-mapping. Note this prototype grid consisting of seventy- two (n=72) rectangular square areas arranged in 12 columns and 6 rows. The dark stained rectangular square represents a potential field of interest under bright-field microscope b. A snapshot of the grid-based coverslip demonstrating a fine cross made by laser inscription that splits its surface in four square segments c. Chromosome 17 polysomy in a malignant tissue section. Note 3 to 5 isolated dark centromeric signals per nucleus in a sub-group of cancerous stained nuclei (black arrows; DAB chromogen; original magnification 400×).*

clusters. These measurements refer to intact, non-overlapped cancerous nuclei on every stained slide (Figure 1c). Additionally, one centromeric signal per nucleus represents chromosome monosomy (loss of one chromosome).

Slide screening process. The screening procedure regarding the corresponding archived CISH stained slides was performed using bright field microscopy (microscope Olympus CX-31, Menvile, NY, USA, with ToupView image analysis software, (ProWay/ToupTek

Protonics, Hangzhou, China) with combined 100X/400X magnification. Concerning the CISH slides evaluation, screening was based on a set of novel designed cover slips with integrated spatial rectangular grid (Grid Cover Slip, now GCS) in order to provide an efficient way of slide eye-scanning ensuring the systematic inspection with full visual coverage of the slide. The grid patterns on GCSs were produced by applying a prototype home-built high precision and efficient Femtosecond Laser based Micromachining system (Femtosecond Laser Micromachining-FLM) based on a high power femtosecond fiber laser (Model: HE-1060-1μJ-fs, Fianium, Southampton, UK) Laser inscription techniques can allow direct writing and transfer of predetermined patterns by means of surface or sub-surface micromachining in a variety of materials ranging from glasses to soft polymeric materials. Adjusting the laser writing characteristics, the inscribed grid lines' width could be flexibly defined to a range from 1 μm to 500 μm. The FLM inscription technique has been applied here for the first time towards the fabrication of a visible rectangular grid in a microscope CISH slide's cover slip, for research medical purposes. Commercially available cover slips, by typical borosilicate-based glass, 50×24×0.5 mm (length × width × thick) (Menarini, Florence, Italy) were used in the study. The grid's size and observation windows' density could be modified according to researcher's needs. According to our previous experience, we selected a prototype grid consisting of seventy-two (n=72) rectangular squares, of typical surface area of 4 mm × 4 mm equal to 16 mm^2, arranged in 12 columns and 6 rows. Each square segment can have also appropriate spatial indexing marks (printed with various techniques like FLM) in a suitable way to assure minimum visual interference under microscope inspection (Figure 1b). The indexing can be provided by sequential numbering of the square cells as shown schematically in Figure 1a. In the specific example, due to the selection of grid's windows size, six residual non-rectangular cells, numbered at the figure as 73-78, appear also at the right part of the grid that do not affect the generalization of the proposed grid's architecture. The developed GCSs were used for the visual detection of Chr 17 centromeric signals on the corresponding CISH slides by perfectly covering the entire conventional coverslips.

Statistical analysis. Statistics software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Associations between variables were assessed with of Pearson Chi-Square (χ^2) test and Fisher's exact. Correlation analysis with Spearman Rank test was performed for variables with significant chi^2 associations. Two-tailed *p*-values ≤0.05 were considered statistically significant. Results and correlations (*p*-values) are described in Table I.

Results

According to CISH implementation and bright-field centromere signal grid-based screening and analysis, Chr 17 numerical imbalances were detected in a subset of the examined tissue sections. Multiple copies of Chr 17 were observed in 12/50 (24%) cases. In detail, polysomy was observed in 10/50 (20%) tissue sections, monosomy in 2/50 (4%), whereas the rest of them demonstrated a normal, diploid pattern (38/50-76%). Chr 17 numerical imbalances were associated with the grade of differentiation of the examined tumors (p=0.001), reflecting an association with a progressive dedifferentiation of the malignant tissues. No other statistical correlations were identified regarding the other clinic pathological parameters (gender: p=0.643, stage: p=0.371, smoking status: p=0.707, HPV persistent infection: p=0.181).

Discussion

CI in OSCC is a relatively frequent genetic abnormality associated with an aggressive phenotype due to increased metastatic potential and elevated malignancy recurrence rates (10). It is also observed as an early genetic event in pre-malignant lesions, such as oral lichen planus and leukoplakia (11-13). Specific molecular studies analyzing oro-pharyngeal and laryngeal malignant tissue specimens have identified increased intra-tumoral chromosomal heterogeneity implicating chromosomes 9, 8, 11, and 17 (14). According to multi-chromosome probe CGH analysis in OSCC cell lines (cell cultures), a variety of gross aberrations in a chromosome spectrum were assessed. Structural and numerical abnormalities were detected in chromosomes 3, 4, 5 ,7, 8, 9, 11, 14, 19 and 20 (15, 16). 3q, 5p, 7p/q, 8q, 9q, 11q, 14q, 19q, and 20q chromosome segments were found to be multi-copied (gains) combined or not with a specific band amplification (11q13), whereas chromosomal losses were identified at 3p, 4p, 8p, 11q, and l8q regions. Among them, most lost bands harbour suppressor genes, whereas gains refer more selectively to oncogenes.

In the current study, we analyzed Chr17 numerical status in OSCC for identifying sub-groups of patients with specific CI patterns. We detected predominantly Chr17 polysomy in a sub-group of the examined specimens with specific pathological characteristics, whereas two cases demonstrating chromosome 17 monosomy were also observed. Overall Chr17 numerical imbalances were correlated to a progressive dedifferentiation of the malignant tissues. Because Chr17 is a critical chromosome altered in many solid malignancies, its numerical aberrations affect indirectly or directly the expression of the hosted genes. Concerning OSCC, some studies have focused on the combination of Chr17 instability and p53 (cytogenetic band: 17p13) aberrant expression. They have reported a strong correlation between Chr17 numerical imbalances and p53 mutations that affect also the grade of malignancy's differentiation (17). Besides this, loss of heterozygosity (LOH) on Chr17 and especially in 17p13 band leads to p53 over expression correlating with advanced stage (positive lymph node metastases) (18). Furthermore, Chr17 aneuploidy/polysomy affects the expression of another critical gene located on this chromosome. Co-analyzing the HER2/neu (cytogenetic band: 17q21), p53 protein expression levels with Chr 17 numerical imbalances, two study groups concluded that mainly its polysomy and not HER2/neu gene amplification was responsible for over expression of the protein in a subset of the

examined OSCC (19). Interestingly, the combination of Chr 17 polysomy/HER2 amplification/p53 allele deletion modifies crucially signal transduction pathways and apoptosis in specific sub-populations of OSCC patients associated with a more aggressive phenotype (20). In the current experimental study, we also applied an innovative reference and calibration grid on conventional cover slips as a pilot screening mechanism for CISH slides evaluation. We have already reported this improved technique as a tool for systematic screening in Pap test and immunocytochemically stained slides, respectively (21, 22). It is an easy to use and accurate tool for visual scanning of the slide under bright-field microscopy with a broad spectrum of applications including cytological (conventional/liquid-based) and molecularly analyzed (CISH) slides.

Conclusion

In conclusion, our study showed that Chr17 numerical imbalances (polysomy mainly and sporadic monosomy) are observed in sub-groups of OSCCs and correlate with a progressive dedifferentiation of the corresponding malignancies. Chr 17 seems to be a critical chromosome for OSCC phenotype because it hosts genes that are frequently altered in this malignancy. The proposed grid-based platform -as described above on CISH slides- it provides a novel, fast and accurate screening-mapping method for detecting chromosome numerical imbalances.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

Authors' Contributions

Aristeidis Chrysovergis, Vasileios S. Papanikolaou: Clinical advisors, researchers; Nicholas Mastronikolis: Case stratification, statistical analysis; EvangelosTsiambas: Researcher, article writing; Vasileios Ragos, Dimitrios Peschos: Academic advisors; Christos Riziotis: Laser & Photonics expert, Academic researcher; Chara Stavraka: Clinical advisor, statistical analysis; Dimitrios Roukas: Clinical advisor; Efthymios Kyrodimos: Academic advisor, article writing.

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References

1 Ali J, Sabiha B, Jan HU, Haider SA, Khan AA and Ali SS: Genetic etiology of oral cancer. Oral Oncol *70*: 23-28, 2017. PMID: 28622887. DOI: 10.1016/j.oraloncology.2017.05.004

2 Grégoire V, Lefebvre JL, Licitra L and Felip E: Squamous cell carcinoma of the head and neck: EHNS–ESMO–ESTRO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol *21*: 184-186, 2010. PMID: 20555077. DOI: 10.1093/annonc/mdq185

3 Patel V, Leethanakul C and Gutkind JS: New approaches to the understanding of the molecular basis of oral cancer. Crit Rev Oral Biol Med *12(1)*: 55-63, 2001. PMID: 11349962. DOI: 10.1177/10454411010120010401

4 Reder H, Wagner S, Gamerdinger U, Sandmann S, Wuerdemann N, Braeuninger A, Dugas M, Gattenloehner S, Klussmann JP and Wittekindt C: Genetic alterations in human papillomavirus-associated oropharyngeal squamous cell carcinoma of patients with treatment failure. Oral Oncol *93*: 59-65, 2019. PMID: 31109697. DOI: 10.1016j.oraloncology.2019.04.013

5 Kang H, Kiess A and Chung CH: Emerging biomarkers in head and neck cancer in the era of genomics. Nat Rev Clin Oncol *12*: 11-26, 2015. PMID: 25403939. DOI: 10.1038 /nrclinonc.2014.192

6 Jin C, Jin Y, Wennerberg J, Annertz K, Enoksson J and Mertens F: Cytogenetic abnormalities in 106 oral squamous cell carcinomas. Cancer Genet. Cytogen *164*: 44-53, 2006. PMID: 16364762. DOI: 10.1016//j.cancergencyto.2005.06.008

7 Grade M, Difilippantonio MJ and Camps J: Patterns of chromosomal aberrations in solid tumors. Recent results. Cancer Res *200*: 115-142, 2015. PMID: 26376875. DOI: 10.1007/978-3-319-20291-46

8 Okafuji M, Ita M, Oga A, Hayatsu Y, Matsuo A and Shinzato Y: The relationship of genetic aberrations detected by comparative genomic hybridization to DNA ploidy and tumor size in human oral squamous cell carcinomas. J Oral Pathol Med *29(5)*: 226-231, 2000. PMID: 10801040. DOI: 10.1034/j.1600-0714.2000.290506.x

9 Barnes L, Eveson JW, Reichart P and Sidransky D: Pathology and Genetics: Head and Neck Tumours. WHO IARC Press, Lyon, France pp. 118-130, 2005.

10 Sato H, Uzawa N, Takahashi K, Myo K, Ohyama Y and Amagasa T: Prognostic utility of chromosomal instability detected by fluorescence *in situ* hybridization in fine-needle aspirates from oral squamous cell carcinomas. BMC Cancer *10*: 182-186, 2010. PMID: 20459605. DOI: 10.1186/1471-2407-10-182

11 Salahshourifar I, Vincent-Chong VK, Kallarakkal TG and Zain RB: Genomic DNA copy number alterations from precursor oral lesions to oral squamous cellcarcinoma. Oral Oncol *50(5)*: 404-412, 2014. PMID: 24613650. DOI: 10.1016/j.raloncology.2014.02.005

12 Siebers TJ, Bergshoeff VE, Otte-Höller I, Kremer B, Speel EJ, van der Laak JA, Merkx MA and Slootweg PJ: Chromosome instability predicts the progression of premalignant oral lesions. Oral Oncol *49(12)*: 1121-1128, 2013. PMID: 24613650. DOI: 10.1016/j.oraloncology.2013.09.006

13 Yarom N, Shani T, Amariglio N, Taicher S, Kaplan I, Vered M, Rechavi G, Trakhtenbrot L and Hirshberget A: Chromosomal numerical aberrations in oral lichen planus. J Dent Res *88(5)*: 427-432, 2009. PMID: 19493885. DOI: 10.1177/0022034509337089

14 Hardisson D, Alvarez-Marcos C, Salas-Bustamante A, Alonso-Guervós M, Sastre N and Sampedro A: Numerical aberrations of chromosomes 8, 9, 11, and 17 in squamous cell carcinoma of the pharynx and larynx: a fluorescence *in situ* hybridization and DNA flow cytometric analysis of 50 cases. Oral Oncol 40(4): 409-417, 2004. PMID: 14969820. DOI: 10.1016/j.oraloncology.2003.09.008

15 Martin CL, Reshmi SC, Ried T, Gottberg W, Wilson JW, Reddy JK, Khanna P, Johnson JT, Myers EN and Gollinet SM: Chromosomal imbalances in oral squamous cell carcinoma: examination of 31 cell lines and review of the literature. Oral Oncol *44(4)*: 369-382, 2008. PMID: 17681875. DOI: 10.1016/j.oraloncology.2007.05.003

16 Mastronikolis NS, Tsiambas E, Fotiades PP, Baliou E, Karameris A, Peschos D, Mastronikolis SN, Asimakopoulos AD, Giannakopoulos X and Ragos V: numerical imbalances of chromosome 7 in oral squamous cell carcinoma. Anticancer Res *38(4)*: 2339-2342, 2018. PMID: 29599358. DOI: 10.21783/anticancer.12480

17 Zedan W, Mourad MI, El-Aziz SM, Salamaa NM and Shalaby AA: Cytogenetic significance of chromosome 17 aberrations and P53 gene mutations as prognostic markers in oral squamous cell carcinoma. Diagn Pathol *10*: 2-8, 2015. PMID: 25881131. DOI: 10.1186/s13000-015-0232-1

18 Choi KY, Choi HJ, Chung EJ, Lee DJ, Kim JH and Rho YS: Loss of heterozygosity in mammary serine protease inhibitor (maspin) and p53 atchromosome 17 and 18 in oral cavity squamous cell carcinoma. Head Neck *37(9)*: 1239-1245, 2015. PMID: 24801268. DOI: 10.1002/hed.23741

19 Papavasileiou D, Tosios K, Christopoulos P, Goutas N and Vlachodimitropoulos D: Her-2 immunohistochemical expression in oral squamous cell carcinomas is associated with polysomy of chromosome 17, not Her-2 amplification. Head Neck Pathol *3(4)*: 263-270, 2009. PMID: 20596843. DOI: 10.1007/s12105-009-0134-1

20 Stoicănescu D, Andreescu N, Belengeanu A, Meszaros N and Cornianu M: Assessment of p53 and HER-2/neu genes status and protein products in oral squamous cell carcinomas. Rom J Morphol Embryol *54(4)*: 1107-1113, 2013. PMID: 24399009.

21 Tsiambas E and Riziotis C: Implementation of a real-time reference and calibration grid platform for improved screening - mapping in Pap test slides. Pathol Int *67*: 24-31, 2017. PMID: 27891686. DOI: 10.111/pin.12481

22 Tsiambas E, Riziotis C, Mastronikolis NS, Peschos D, Mortakis A, Kyroysis G, Mastronikolis SN, Batistatou A, Lazaris AC, Patsouris E and Ragos V: Comparative $p16^{IKN4A}$ expression in laryngeal carcinoma and cervical cancer precursors: A real-time grid-based immunocytochemistry analysis. Anticancer Res *38*: 5805-5810, 2018. PMID: 302753203. DOI: 10.21873/anticanres.12920

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