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ORIGINAL ARTICLE

Topoisomerase IIa protein expression analysis in oral squamous cell carcinoma

Vasileios Papanikolaou[1], Efthymios Kyrodimos[1], Nicholas Mastronikolis[2], Evangelos Tsiambas[3], Vasileios Ragos[4], Aristeidis Chrysovergis[1]

[1]1st ENT Department, Hippocration Hospital, University of Athens, Athens, Greece. [2]Department of Otorhinolaryngology, Head and Neck Surgery, Medical School, University of Patras, Patras, Greece. [3]Department of Pathology-Cytology, 401 GAH, Athens, Greece. [4]Department of Maxillofacial Surgery, Department of Medicine, School of Health Sciences, University of Ioannina, Ioannina, Greece.

Summary

Purpose: *Topoisomerases represent a super-family of nucleic enzymes involved in the DNA replication, transcription, recombination, and also chromosome topological formation. Topoisomerase II alpha (Topo IIa-gene location 17q21) is a critical gene associated with response to chemotherapeutic agents such as anthracyclines especially in breast adenocarcinoma. Our aim was to investigate the role of aberrant Topo IIa protein expression in oral squamous cell carcinoma (OSCC).*

Methods: *Fifty formalin-fixed, paraffin-embedded primary OSCCs tissue sections were used. Immunohistochemistry was performed using an anti- Topo IIa antibody. Digital image analysis was implemented for evaluating objectively the protein expression levels on the corresponding stained nuclei.*

Results: *Topo IIa protein overexpression (moderate to high immunostaining intensity values) was observed in 29/50 (58%) tissue cores, whereas low expression rates were detected in the remaining cases (21/50;42%). Topo IIa overall expression was strongly associated with the differentiation grade of the examined tumors (p=0.037) and also with human papillomavirus (HPV) positivity (p=0.029). No other statistical correlations were identified.*

Conclusions: *Topo IIa overexpression is observed in significant subsets of OSCCs correlated with the grade of differentiation. Additionally, HPV persistent infection is associated with increased Topo IIa protein expression levels. Topo IIa expression analysis should be critical for identifying patients eligible for applying specific chemotherapeutic strategies based on anti-Topo IIa agents.*

Key words: *oral, carcinoma, topoisomerase, protein*

Introduction

Aberrant cell proliferation is a major cause in the development and progression of carcinogenic process [1]. Molecules that are critical for evaluating the proliferation status of the corresponding tissues include mainly ki-67 (cytogenetic band: 10q26.2), and also Topoisomerase IIa/Topo IIa (cytogenetic band: 17q21.2). ki-67 gene located on chromosome 10 (cytogenetic band: 10q26.2) encodes a protein which is expressed in the nucleolus in all cell cycle phases except G0 (arrest phase) [2]. In fact, ki-67 expression increases as a cell progresses through the cell cycle, with highest expression being seen in G2/M phase cells [3]. Topo IIa and b isomers' combined action promotes temporarily cutting and rejoining the DNA double helix. Winding and unwinding of the DNA double strand is a critically important molecular mechanism for replication, transcription and repair of chromosome structure. Topo IIa, with a molecular mass of 170 kDa, is expressed in proliferating cells

Corresponding author: Evangelos Tsiambas, MD, MSc, PhD. 17 Patriarchou Grigoriou E´ Street, Ag. Paraskevi, 153 41 Athens, Greece.
Fax: +30 210 6526259, Email: tsiambasecyto@yahoo.gr
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in late S phase with a peak in G2/M phases, where it is believed to be the primary mediator of chromosome condensation [4].

Oral squamous cell carcinoma (OSCC) is characterized by a broad spectrum of genomic imbalances, including gross chromosomal alterations, such as polysomy/aneuploidy and specific gene aberrations. Concerning the development of OCSSC, main factors are chronic tobacco, alcohol and also betel quid consumption combined or not with persistent viral infections, especially high risk human papilloma virus (HR-HPV) related [5]. In the current experimental study we measured Topo IIa protein expression levels in a series of OSCCs exploring its significance in the biological behavior of the malignancy.

Methods

Study group

For the purposes of our study, 50 archival, for¬malin-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, Athens, Greece for research purposes, according to World Medical Association Declaration of Helsinki guidelines (2008, revised 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 World Health Organization (WHO). Concerning HPV DNA positivity or not, the corresponding information was derived from patients' medical files. Clinicopathological data of the examined cases are demonstrated in Table 1.

Antibodies and immunohistochemistry assay (IHC)

For the purposes of our study, we selected and applied the mouse monoclonal anti- Topoisomerase IIa (clone KiS1-DAKO,UK/DN dilution 1:50). IHC protocol for the antigen detection was carried out on a 4 μm-thick paraffin sections of the current blocks. Tissue sections initially deparaf¬finized in xylene and rehydrated via graded ethanol – were immunostained according to the EN Vision+ (DAKO, Denmark) assay using an automated staining system (I 6000 - Biogenex, CA, USA) and according to the corresponding antibodies manufacturer's instruc¬tions. This specific assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and increasing the quality of the stained slides. Briefly, the sections, after peroxidase blocking, were incubated with primary antibody for 30 min at room temperature and then incubated with horseradish peroxidase labeled polymer-HRP LP for 30 min. A wash with tris buffered saline (TBS) was performed. The

Table 1. Clinicopathological parameters and total Topo IIa expression results

Clinicopathological parameters	*n (%)*	*Topo IIa*		*p value*
OSCC (n=50)		*OE 29/50 (64%) n*	*LE 21/50 (30%) n*	
Gender				0.432
Male	44 (88)	26/50 (52)	18/50 (36)	
Female	6 (12)	3/50 (6)	3/50 (6)	
HPV history			0.029	
Positive	18 (36)	14/50 (28)	4/50 (8)	
Negative	32 (64)	15/50 (38)	17/50 (26)	
Grade				0.037
1	18 (18)	7/50 (14)	11/50 (22)	
2	21 (58)	14/50 (28)	7/50 (14)	
3	11 (24)	8/50 (16)	3/50 (6)	
Stage				0.215
I	9 (18)	5/50 (10)	4/50 (8)	
II	26 (52)	13/50 (26)	13/50 (26)	
III	15 (30)	11/50 (22)	4/50 (8)	
Smoking status			0.119	
Current	38 (74)	21/50 (42)	17/50 (34)	
Former	12 (26)	8/50 (16)	4/50 (8)	

OSCC: laryngeal squamous cell carcinomas, OE: overexpression (moderate to high expression) staining intensity. Values ≤147 at ≥50% stained nuclei, LE: low expression staining intensity values >156 at ≥50% stained nuclei.

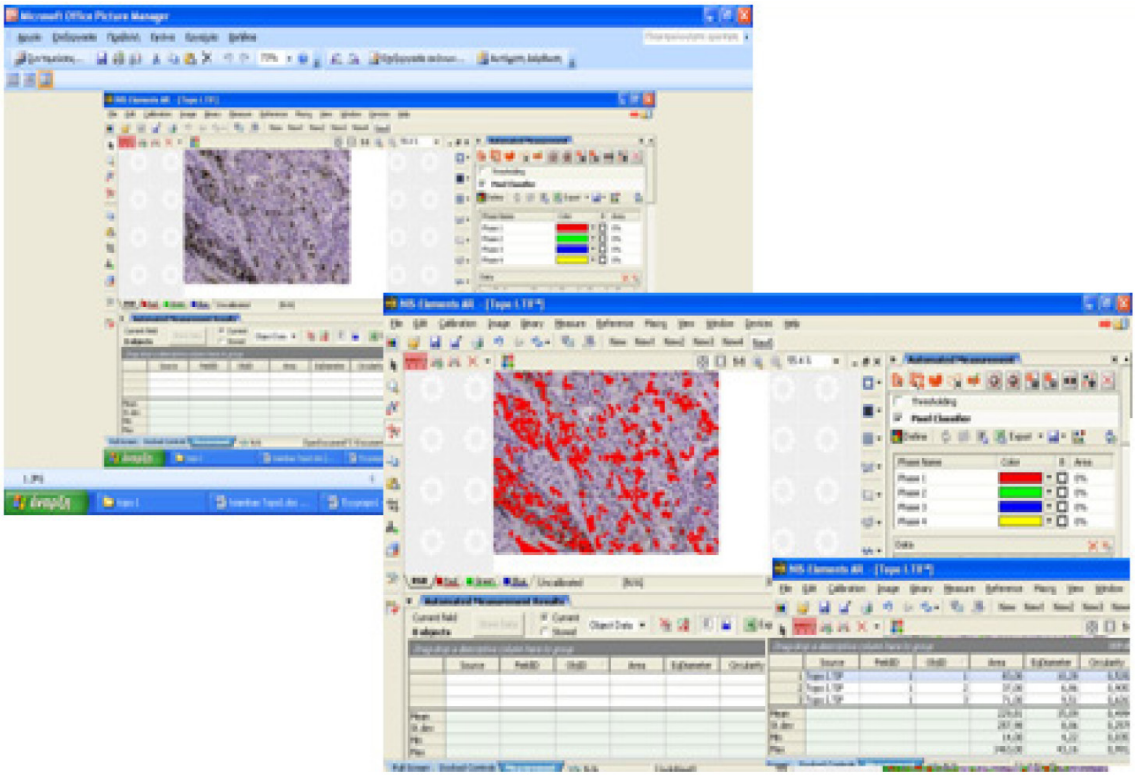

Figure 1. Topo IIa overexpression in an OSCC tissue section analyzed by digital image analysis. Reddish areas represent different Topo IIa expression levels inside the corresponding immunostained nuclei (anti-Topo IIa, DAB stain, original magnification: 40×).

antigen-antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate (8 min at room temperature). Finally, the tissue sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibody was omitted. Nuclear staining pattern was considered to be acceptable for the marker and breast cancer tissue sections demonstrating Topo IIa strong expression were used as positive markers for its immunostaining pattern.

Digital image analysis (DIA)

Topo IIa protein expression levels were evaluated by measuring the corresponding staining intensity levels provided by digital image analysis combined also with the number of stained nuclei as a percentage (% Nuclear Labelling Index- NLI) (Figure 1). We performed digital image analysis (DIA) based on a semi-automated system (Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). NLI was estimated by conventional microscopy (Microscope Olympus BX-50). Measurements were performed in 5 optical fields per case and at a magnification of ×100. Using normal epithelia as control group, we character¬ized Topo IIa expression as low, moderate and high. Total results are demonstrated in Table 1.

Statistics

Associations between Topo IIa protein expression levels and clinicopathological parameters were assessed by applying chi-square and Fischer's tests (SPSS v 20.0 statistical software- Inc Chicago IL, USA). Total IHC results and also p-values are described in Table 1.

Results

According to the digital protein analysis, all of the examined cases demonstrated Topo IIa expression in different levels. Topo IIa protein overexpression (moderate to high immunostaining intensity values) was observed in 29/50 (58%) tissue cores, whereas low expression rates were detected in the rest of the examined cases (21/50- 42%). Topo IIa overall expression was strongly associated with the differentiation grade of the examined tumors (p=0.037) and also with human papillomavirus (HPV) positivity (p=0.029). No other statistical correlations were identified concerning the other clinicopathological parameters (gender: p=0.432, stage: p=0.215, smoking status: p=0.119).

Discussion

OSCC demonstrates an increasing rate due to HR-HPV persistent infection, and also to chronic cigarette and alcohol consumptions. Gross chromosomal alterations (polysomy, aneuploidy, intra-chromosome rearrangements) and specific gene ab-

errations such as amplifications, deletions, point mutations combined or not with epigenetic ones (promoter methylations and miRNA deregulations) are responsible for the progressive transformation of normal squamous cell epithelia to the corresponding malignant [6]. Novel targeted chemotherapeutic strategies are based on specific gene alterations. Topoisomerases' inhibition promotes cell death and for this reason they are targets for specific chemotherapy. Concerning Topo IIa, many clinical studies have shown that adjuvant chemotherapy strategies, which include anthracyclines (doxorubicin) and podophyllo¬toxins (etoposide) in conjunction with fluorouracil and cyclophosphamide or carboplatin/paclitaxel are most effective, especially in handling patients with breast cancer and other gynecologic malignancies, such as endometrial or ovarian cancer, respectively [7-9].

In the current study we explored the role of Topo IIa aberrant expression in OSCC tissue sections. Based on our results we detected moderate to high expression in a significant proportion of the analyzed tissues (29/50-58%). Concerning the impact of the abnormal protein expression in the corresponding cases, we showed that statistical significance was detected correlating the overall expression to the grade of differentiation of the examined tumors. Interestingly, HPV positivity (High Risk subtypes) was also associated with increased rates of Topo IIa expression in the examined cases. Similarly, another study analyzed the role of two agents, pemetrexed and etoposide, as a potential therapeutic regimen in HPV-depended OSCC and oropharyngeal carcinomas. They concluded that combined or alone pemetrexed/etoposide application accompanied by other chemotherapeutic factors, such as cisplatin, should be effective in these patients [10]. In fact, they observed that in subsets of HPV positive cases, thymidylate synthase (TS) and Topo IIa were co-overexpressed. Interestingly, HPV positivity is observed in significant proportions of premalignant lesions in oral mucosa. Oral atrophic lichen planus (OALP) is involved as a substrate in oral intraepithelial dysplasias. HPV persistent infection in these cases leads to a progressive transformation of the normal mucosa to malignant. A study group co-analyzed Topo IIa and HPV DNA in OALP tissues. They reported that HPV infection is associated with a subgroup of atrophic OLP. Topo IIα overexpression was also related to HPV positivity in these lesions [11]. Similarly, another study group explored the role of Topo IIa, Ki-67 abnormal protein and an intermediated filament protein cytokeratin-19 (CK-19) in OALP pre-malignant lesions. They showed that Topo IIa should be considered a reliable proliferation and also an apoptotic marker in OALP lesions indicating also a progressive malignant transformation of the oral mucosa [12]. Concerning the influence of Topo IIa in proliferation and apoptosis regarding oral preneoplastic lesions and OSCCs, another protein analysis showed that the molecule demonstrated progressively increased expression in hyperplastic, dysplastic and OSCCs, respectively [13]. In fact, Topo IIa and Topo I expression are targets for specific targeted chemotherapy in subsets of OSCC patients with specific molecular criteria. Based on this, experimentally developed novel anti-Topo IIa agents seem to be very effective in inhibiting molecules' proliferative function. Camptothecin, irinotecan, SN-38, etoposide, and also teniposide are important inhibitors. In particular, a study group concluded that SN-38 is highly cytotoxic to OSCC cell lines [14]. In conjunction to previously referred data regarding teniposide, another clinicomolecular study showed that the agent could significantly induce apoptotic activity in OSCC and inhibit cell growth [15]. Besides Topo IIa, another molecule seems to be critical in oral mucosa carcinogenetic process. A study group co-analyzed cyclooxygenase (COX)-2 and Topo IIa expression in precancerous and cancerous lesions of the oral mucosa and concluded that patients who demonstrated high expression of both COX-2/Topo IIa showed poor prognosis [16].

In conclusion, Topo IIa overexpression is observed in significant subsets of OSCCs correlated with the grade of differentiation. Additionally, HPV persistent infection is associated with increased Topo IIa protein expression levels. Topo IIa expression analysis should be critical for identifying patients eligible for applying specific chemotherapeutic strategies based on anti-Topo II agents. Additionally, Topo IIa is a potential reliable marker for the biological behavior of OSCC, but further molecular studies are necessary for a precise result on this quest [17].

Conflict of interests

The authors declare no conflict of interests.

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