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Topoisomerase IIa Protein Expression Patterns in Laryngeal Squamous Cell Carcinoma

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Abstract. *Background/Aim: Topoisomerase II alpha (TopoIIa) is a critical gene associated with response to chemo-therapeutic agents, such as anthracyclines, especially in breast adenocarcinoma. The aim of this study was to investigate the role of aberrant TopoIIa protein expression in laryngeal squamous cell carcinoma (LSCC). Materials and Methods: Fifty (n=50) LSCC cases were enrolled in the study. Immunohistochemistry and a digital image analysis assay were implemented. Results: TopoIIa protein overexpression was observed in 32/50 (64%) cases, whereas low expression rates were detected in 18/50 (36%). TopoIIa overall expression presented strong association with the grade of the examined malignant tissues and borderline association with stage. TopoIIa overexpression correlated also with Human papillomavirus (HPV) positivity. Conclusion: TopoIIa overexpression was observed in significant subsets of LSCCs, and correlated predominantly with the grade of differentiation. HPV persistent infection seems to be associated with increased TopoIIa protein expression. TopoIIa expression analysis appears to be critical in identifying sub-groups of patients eligible for specific chemotherapy.*

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Head and neck squamous cell carcinoma (HNSCC) exhibits an increasing incidence worldwide, especially in men (1). Laryngeal squamous cell carcinoma (LSCC) represents the most frequent malignancy of this anatomical region. A broad spectrum of genomic imbalances, including chromosome polysomy/aneuploidy, or specific gene deregulation mechanisms, such as point mutations, deletions and amplification have been identified in LSCC patients, which appear to modify their response to novel targeted therapeutic regimens affecting their survival status (2). Oncogenic high-risk human papillomavirus (HR-HPV) persistent infections, combined or not with chronic alcohol and tobacco consumption, are well established significant pathogenetic factors for LSCC development and progression (3).

Topoisomerases is a class of nucleic enzymes that affects the topological structure of the DNA. The main members of the family are Topoisomerase I (gene location 20q11), Topoisomerase II alpha (Topo IIa-gene, location 17q21) and Topoisomerase IIb (gene location 3p24) (4). The combined action of Topo I, Topo IIa and b isomers involving the temporarily cutting and rejoining of the DNA helix, allowing also winding and unwinding of the DNA double strand, is a critical molecular mechanism for the replication, transcription and repair of chromosome structure (5). Topo IIa, with a molecular weight of 170 kDa, is expressed in proliferating cells in late S phase with a peak in G2-M phases, where it is believed to be the primary mediator of chromosome condensation (6). The inhibition of topoisomerases promotes cell death and therefore, they may be good targets for chemotherapeutic agents. Concerning breast adenocarcinoma, anthracyclines act as critical inhibitors of Topo IIa aberrant expression, especially in gene amplified cases (7). Additionally,

Table I. *Topo IIa IHC results and statistics.*

Clinicopathological parameters		Topo IIa		*p*-Value
LSCC (n=50)		OE	LE	
		32/50 (64%)	18/50 (36%)	
	n (%)	N (%)	N (%)	
Gender				
Male	45 (90%)	27/50 (54%)	18/50 (36%)	0.127
Female	5 (10%)	3/50 (6%)	2/50 (4%)	
HPV history				**0.036**
Positive	11 (22%)	10/50 (20%)	1/50 (2%)	
Negative	39 (78%)	21/50 (42%)	18/50 (36%)	
Grade				**0.002**
1	4 (8%)	1/50 (2%)	3/50 (6%)	
2	19 (38%)	8/50 (16%)	11/50 (22%)	
3	27 (54%)	23/50 (46%)	4/50 (8%)	
Stage				0.053
II	5(10%)	2/50 (4%)	3/50 (6%)	
III	24 (48%)	20/50 (40%)	4/50 (8%)	
IV	21 (42%)	9/50 (18%)	12/50 (24%)	
Alcohol status				0.505
Yes	43 (86%)	26/50 (52%)	17/50 (34%)	
No	7 (14%)	5/50 (10%)	2/50 (4%)	

LSCC: Laryngeal squamous cell carcinomas; OE: overexpression (moderate to high expression) staining intensity values ≤139 at ≥50% stained nuclei; LE: low expression staining intensity values >148 at ≥50% stained nuclei. Bold values show significance.

Topo IIa gene amplification or deletion modifies the response rates of patients suffering from solid malignancies, such as endometrial or pancreatic cancer, and also correlates with poor prognosis (8, 9). In the current study, we analyzed Topo IIa at the protein expression level in order to determine its impact in LSCCs clinicopathological features.

Materials and Methods

Study group. For the purposes of our retrospective analytical study, fifty (n=50) archival, formalin-fixed and paraffin-embedded tissue specimens (surgical resections or biopsies) of histologically confirmed primary LSCC cases were used. Specimens were obtained from 45 males and 5 female patients, all smokers. The median age was 62 years. Concerning HPV DNA status (positivity or not), the corresponding information was derived from patients' medical file records. Eleven (n=11) were found to be positive for HR-HPV types, predominantly HPV 16/31. According to their clinical status, patients were treated with chemotherapy (cisplatin-based), radiotherapy, or the combination. The hospital Ethics Committee (reference ID Research Protocol: 2226/09.09.2018) consented to the use of these tissues at the Department of Pathology, Hippocration Hospital, University of Athens, Athens, Greece, for research purposes, according to the World Medical Association Declaration of Helsinki guidelines (2008 revised in 2014). Informed consent was obtained from all patients for the analysis of the tissue specimens.

The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of the World Health Organization (WHO) Tumor Classification (10). Demographic and clinicopathological data of the examined cases are demonstrated in Table I.

Antibodies and Immunohistochemistry (IHC). For the purposes of our study, we selected and applied the mouse monoclonal anti-Topoisomerase IIa antibody (clone KiS1-DAKO, Glostrup, Denmark, dilution 1:50). The IHC protocol for antigen detection was carried out on 4 μm-thick paraffin sections. Tissue sections were initially deparaffinized in xylene and rehydrated *via* graded ethanol. Then, sections were immunostained according to the EN Vision+ (DAKO, Glostrup) assay using an automated staining system (I 6000 - Biogenex, CA, USA) and according to the manufacturer's instructions. This specific assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and therefore increasing the quality of the stained slides. Briefly, the sections, after peroxidase blocking, were incubated with the 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 then performed. The 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 antibodies were omitted. Predominantly nuclear and slightly peri-nuclear cytoplasmic staining was accepted for the markers' positive expression pattern, according to the manufacturers' data sheet (Figure 1a). Breast cancer tissue sections demonstrating Topo IIa strong expression were used as positive markers.

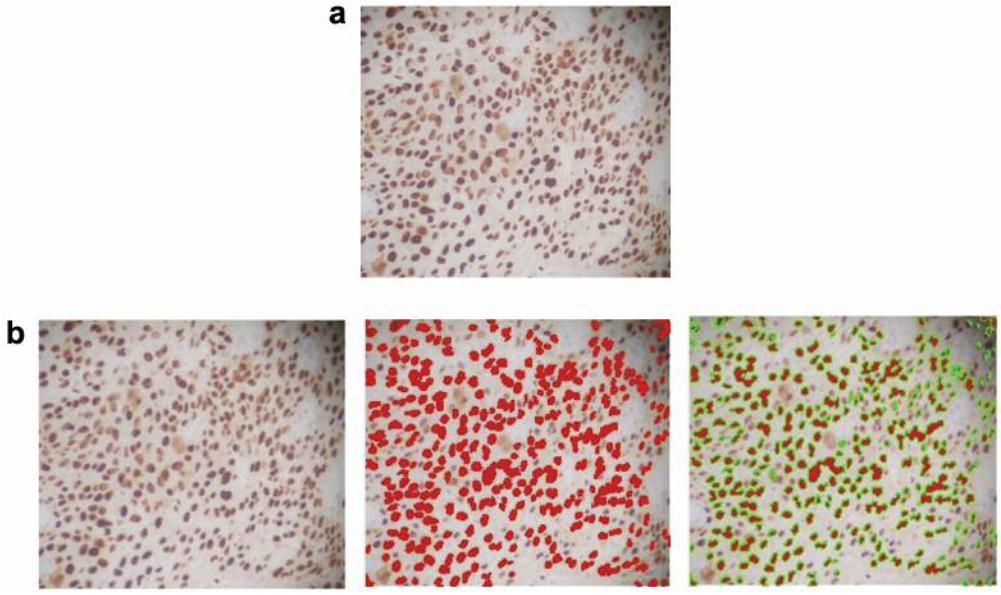

Figure 1. *Overexpression of Topo IIa and digital image analysis in laryngeal squamous cell carcinoma (LSCC). a: Topo IIa overexpression (high staining pattern). Predominantly nuclear and cytoplasmic brown staining pattern (diaminobenzidine stain-DAB, original magnification: 100×), b: Digital image analysis assay. Red spots represent different expression values of Topo IIa-stained nuclei in a case of LSCC. Green loops surrounding red spots represent the final stage of digital analysis, providing numerical data (staining intensity values).*

Digital image analysis assay (DIA). Topo IIa protein expression levels were evaluated quantitatively by calculating the corresponding staining intensity levels (densitometric evaluation in stained nuclei). DIA was performed using a semi-automated system (hardware: Microscope CX-31, Olympus, Melville, NY, USA; Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest per tissue section were identified (five optical fields at ×100 magnification) and filed in a digital database as snapshots. Measurements were performed by implementing a specific macro (focal nuclear and peri-nuclear cytoplasmic protein expression). Based on an algorithm, staining of normal tissue sections (control) was measured independently and compared to the corresponding values in malignant tissue sections. A broad spectrum of continuous grey-scale values (0-255) in the Red Green Blue (RGB) analysis was available for discriminating different protein expression levels (Figure 1b). Immunostaining intensity values decreasing to 0 represent progressive overexpression of the marker, whereas values increasing to 255 show progressive loss of its staining intensity.

Statistical analysis. Statistics software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was implemented. Associations between variables were assessed with Pearson Chi-Square (χ^2) test and Fisher's exact test. Correlation analysis with Spearman Rank test was performed for variables with significant χ^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 the DIA-based expression analysis, Topo IIa protein overexpression (moderate to high immunostaining intensity values) was observed in 32/50 (64%) cases, whereas low expression rates were detected in the rest of them [18/50 (36%)]. Topo IIa overall expression was strongly associated with the grade of the examined malignant tissues (p=0.002) and borderline with the stage (p=0.053). Interestingly, an association was observed between Topo IIa overexpression and HPV positivity (p=0.036), but not with systematic alcohol consumption (p=0.505) or gender (p=0.127).

Discussion

In modern oncology, optimal therapeutic management of solid malignancies requires a spherical molecular knowledge of the corresponding examined tissue. Identification of specific gene signatures and therefore expression patterns is critical in applying targeted chemotherapeutic agents that inhibit specific altered, mutated oncoproteins (11). Furthermore, aberrant cell proliferation is a major cause in the development and progression of carcinogenesis (12). Thus, the frequent observation of abnormal overexpression of Topo IIa and ki-67 (cytogenetic band: 10q26.2) - a major proliferative marker- in LSCC appears to be a very important finding (13, 14).

In the current study, IHC was used to stain LSCC tissues and measure Topo IIa protein expression levels based on a digitized image analysis protocol. We observed that a significant proportion of the examined tissues overexpressed

Topo IIa, especially in cases of moderate and poor differentiation and borderline with advanced stage. Interestingly, high expression levels were detected in HPV-positive cases. To date, there are limited data regarding the impact of Topo IIa on LSCC, especially in HPV-related cases. Similar studies that analyzed LSCC tissues by IHC and/or by fluorescence *in situ* hybridization (FISH) have shown that Topo IIa overexpression is associated predominantly with the grade of the examined tumors (15). Furthermore, gene deregulation mechanisms (amplification) and chromosome 17 status (polysomy/aneuploidy) are involved in the aggressiveness of LSCCs., Topo IIa aberrant expression seems to be associated to Chr 17 polysomy (16). Similarly, other studies have shown that Topo IIa deregulation seems to be an early genetic event in LSCC and the Topo IIa/Ki-67 ratio could be used as a sensitive proliferation marker (17). Another important published observation is the negative influence of Topo IIa overexpression in patients with LSCC that receive radiotherapy. It seems that aberrant expression of this molecule due to its hypermethylation – an epigenetic change- increases radio-resistance rates (18). For this reason, novel anti-Topo IIa agents, including the epipodophyllotoxin based F14512 drug and cisplatin, induce radio-sensitivity in sub-groups of LSCC patients (19). Similarly, the role of anti-Topo IIa drugs combined or not with radiotherapy is under investigation in HPV-positive cases. Recently, a study group analyzed the efficacy of pemetrexed and etoposide combined with cisplatin as therapeutic regimens in HPV positive patients with oral and oropharyngeal cancer. They concluded that in Topo IIa and thymidylate synthase (TS) overexpressed cases these regimens offer increased response rates, although extended clinical trials are necessary for evaluating their benefits in the corresponding patients characterized by specific molecular signatures (20).

Conclusion

In conclusion, Topo IIa overexpression is observed in significant subsets of LSCCs, and correlated predominantly with the grade of differentiation. Additionally, HPV persistent infection appears to be associated with increased Topo IIa protein expression levels. Topo IIa expression analysis combined with detection of gene deregulation mechanisms could be an optimal approach, critical for identifying patients eligible for the administration of specific chemotherapeutic agents, as practiced in breast adenocarcinoma targeted treatment.

Conflicts of Interest

The Authors declare no conflicts of interest related to this study.

Authors' Contributions

Vasileios S. Papanikolaou: Clinical advisor, researcher; Aristeidis Chrysovergis: Clinical advisor, researcher; Nicholas Mastronikolis: Case stratification, statistical analysis; Evangelos Tsiambas: Researcher, article writing; Vasileios Ragos: Academic advisor; Dimitrios Peschos: Academic advisor; Chara Stavraka: Clinical advisor, statistical analysis; Dimitrios Roukas: Clinical advisor; Efthymios Kyrodimos: Academic advisor, article writing.

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