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ORIGINAL ARTICLE

Topoisomerase I deregulation in laryngeal squamous cell carcinomas based on tissue microarray analysis

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Summary

Purpose: *Topoisomerases (types: I/IIa-b/IIIa-b) represent a super-family of nucleic enzymes involved in the DNA replication, transcription, recombination, and also chromosome topological formation. Topoisomerase's I (Topo I- gene location: 20q12) aberrant expression is a frequent genetic event in a variety of solid malignancies. Topo I inhibition promotes cell death due to DNA damage and for this reason it is a target for specific targeted chemotherapy (camptothecin, topotecan, irinotecan). Our aim was to investigate the role of abnormal Topo I protein expression in laryngeal squamous cell carcinomas (LSCC) in which there are very limited data regarding the influence of the marker.*

Methods: *Using tissue microarray (TMA) technology, 50 formalin-fixed, paraffin-embedded primary laryngeal SCCs were cored and re-embedded into one recipient block. Immunohistochemistry was performed using anti- Topo I antibody. Digital image analysis was also implemented for evaluating objectively the protein expression levels on the corresponding stained nuclei.*

Results: *Topo I protein overexpression (moderate to high staining intensity values) was observed in 32/50 (64%) tissue cores, whereas low expression rates were detected in 18/50 (36%) cases. Topo I overall expression was strongly associated with the differentiation grade of the examined tumors (p=0.021). No other statistical correlations were identified.*

Conclusions: *Topo I overexpression is observed in a significant subset of LSCCs affecting the level of differentiation in them. Additional molecular studies focused on the mechanism of Topo I gene/protein deregulation (i.e. amplification, abnormal epigenetic promoter methylation, mRNA aberrant expression) are necessary discriminating the eligible patients for applying specific chemotherapeutic strategies based on anti-Topo I agents.*

Key words: *carcinoma, larynx, microarrays, topoisomerases*

Introduction

Topoisomerases is a class of nucleic enzymes that affect the topological structure of 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) [1]. Topo I, Topo IIa and b isomers' combined action of temporarily cutting and rejoining the DNA double helix, al-

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Table 1. Clinicopathological parameters and total Topo I expression results

Clinicopathological parameters		*Topo I*		*p value*
LSCC (n=50)		*OE*	*LE*	
		32/50 (64%)	*18/50 (30%)*	
	n (%)	*n*	*n*	
Gender				0.342
Male	47 (94)	31/50	16/50	
Female	3(6)	1/50	2/50	
Anatomical region				0.131
Supraglotis	14 (28)	9/50	5/50	
Glotis	32(64)	21/50	11/50	
Subglotis	4 (8)	2/50	2/50	
Grade				0.021
1	9(18)	2/50	7/50	
2	29 (58)	19/50	10/50	
3	12 (24)	11/50	1/50	
Stage				0.215
I	4(8)	2/50	2/50	
II	17(34)	11/50	6/50	
III	26(52)	18/50	8/50	
IV	3(6)	1/50	2/50	
Smoking status				0.092
Current	42(84)	27/50	15/50	
Former	8(16)	5/50	3/50	

LSCC: laryngeal squamous cell carcinomas, OE: overexpression (moderate to high expression) staining intensity values ≤140 at ≥50% stained nuclei, LE: low expression staining intensity values >141 at ≥50% stained nuclei

lowing also winding and unwinding of the DNA double strand, is a critically important molecular mechanism for replication, transcription and repair of chromosome structure [2]. 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 [3]. Topo I with a molecular weight of 91 kDa consists of 765 amino acids. The molecule unwinds and uncoils the DNA supercoiled double helix by transiently cleaving one of the two strands and allowing rotation over the other, mediating finally the re-seal of the cleaved strand [4]. Interestingly, Topo I does not have an ATP-depended protein for its action, in contrast to Topo II [5].

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 podophyllotoxins (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 [6-8]. Similarly, Topo I inhibition promotes cell death due to DNA damage and for this reason it is a target for specific targeted chemotherapy (camptothecin, topotecan, irinotecan) [9-11]. Our aim was to investigate the role of abnormal Topo I protein expression in LSCC for which there are very limited data regarding the influence of the marker in its biological behavior.

Methods

Study group

For the purposes of our study, 50 archival, formalin-fixed and paraffin-embedded tissue specimens of histologically confirmed primary LSCC were used. The 417VA Hospital (NIMTS) ethics committee consented to the use of these tissues in the Department of Pathology for research purposes, according to World Medical Association Declaration of Helsinki guidelines. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO). Clinicopathological data of the examined cases are summarized in Table 1.

TMA construction

Areas of interest were identified in H&E-stained slides by a conventional microscope (Olympus BX-

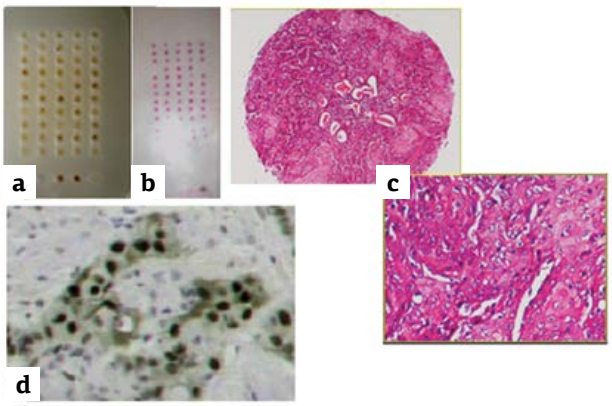

Figure 1. LSCC tissue microarray (TMA) analysis. **a:** TMA tissue cores embedded in the final block; **b:** Tissue cores on the corresponding slide (H&E stain); **c:** A case of LSCC based on a tissue core at a diameter of 0.6mm (H&E stain, original magnification: upper 4x, down 40x); **d:** Topo I overexpression in a LSCC tissue core. Note strong, dark nuclear predominantly and per-nuclear staining pattern (anti-Topo I, DAB stain, original magnification: 40x).

50, Melville, NY, USA). Selection of those areas was performed at the basis of tumor sufficiency, avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice. In order to secure the presence of each case, 0.6-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the two recipient blocks. After 4 μm microtome sectioning and H&E staining, the finally constructed TMA blocks contained 45/50 (90%) and 47/50 (94%) cores of tissue cylindrical specimens. Five cores of microscopically normal laryngeal squamous epithelia (controls) were also embedded in the blocks. We observed microscopically that all examined LSCC cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens) (Figure 1a-c).

Antibodies and immunohistochemistry (IHC) assay

For the purposes of this study, we selected and applied the anti-Topo I monoclonal antibody (clone 1D6, Novocastra, Newcastle, UK), at dilution of 1:50. IHC protocol for the antigen detection was carried out on a 4 μm-thick paraffin section of the current TMA block. Tissue section - initially deparaffinized in xylene and rehydrated via graded ethanol - was immunostained according to the EN Vision+ (DAKO, Denmark) assay using an automated staining system (I 6000-Biogenex, CA, USA) and according to the corresponding antibody manufacturer's instructions. 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 TBS was performed. The antigen-antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate (8 min at room temperature). Finally, the TMA tissue section was slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted. For negative control slides, the primary antibody was omitted. Nuclear and peri-nuclear staining pattern was considered to be acceptable for the protein and breast cancer tissue sections demonstrating Topo I expression were used as positive markers for the immunostaining pattern (Figure 1d).

Computerized image analysis (CIA)

Topo I protein expression was evaluated by measuring the corresponding staining intensity levels provided by digital image analysis, combined also with the percentage of stained nuclei (Nuclear Labeling Index-NLI). CIA was performed based on a semi-automated system (Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Combined Topo I staining intensity and NLI was estimated by conventional microscopy (Microscope Olympus BX-50) (Figure 2). Measurements were performed in 5 optical fields per case and at a magnification of ×40. Using normal epithelia as control group, we characterized Topo I expression as low, moderate and high. Cumulative results are demonstrated in Table 1.

Statistics

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

Results

According to the protein analysis, all of the examined cases demonstrated Topo I expression in different levels. Overexpression of the marker was observed in 32/50 (64%) tissue cores. High values were detected in 21/32 (65.5%) cases, whereas moderate levels of expression were observed in 11/32 (34.5%). Additionally, low expression rates were detected in 18/50 (36%) cases. Topo I overall expression was strongly associated with the grade of differentiation of the examined tumors (p=0.021). No other statistical correlations were identified concerning the other clinicopathological parameters (gender:p=0.342, stage:p=0.215, anatomical region:p=0.114, smoking status:p=0.092, anatomic region of the malignancy:p=0.131).

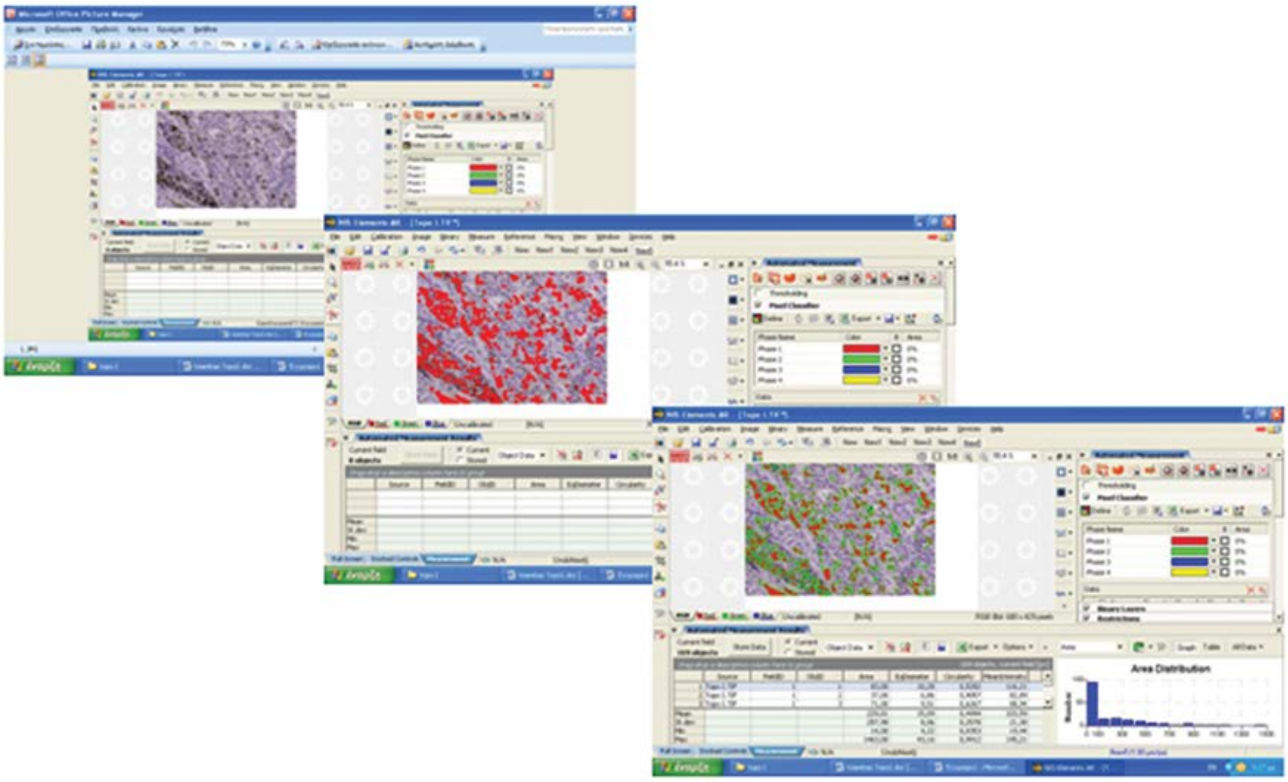

Figure 2. Topo I expression analysis based on digital imaging evaluation. Red and green signals represent nuclear staining intensity values inside the corresponding stained nuclei (original inside magnification: 40x).

Discussion

Structural changes regarding the topological formation of the DNA molecule are essential for its normal anatomy and function. A transient single-stranded break into DNA, combined with a passing of a single strand through the broken strand, and relegation of the break is mediated by Topo I catalytic activity [12]. Two recently published molecular studies explored a novel function of the gene and the authors concluded that Topo I-mediated cleavage at unrepaired ribonucleotides generates DNA double-strand breaks [13]. Similarly, it recruits Sir2p to the rDNA and clarifies a structural role of the molecule in the epigenetic regulation of rDNA, independent of its known catalytic activity [14]. In addition to this activity, Topo IIa is responsible for cleaving double-stranded breaks in one segment of replicated DNA. Interestingly, an interaction between Topo I and p53 proteins has been already described. According to a proposed model, p53 is activated through the c-terminus-Topo I, leading to additional DNA damage. This co-reaction affects negatively the viability of malignant cells. In cases that p53 gene harbors mutations, the corresponding cells seem to be more sensitive to Topo I inhibitors that the p53 wild-type dependent ones [15]. Some studies show also a potential role of the molecule combined with the DNA gyrase in targeting non-tumor lesions, such as tuberculosis inflammation [16]. Based on those significant actions that make DNA integrity sustainable, a variety of Topo I inhibitors has been already developed and applied in subgroups of patients suffering of solid malignancies. Agents including camptothecin, topotecan, irinotecan, their synthetic analogues and also novel ones under investigation, such as crude amaryllidaceae alkaloids, show strong antiproliferative activity [17].

Topo I abnormal expression is a frequent event in solid malignancies. Concerning colorectal carcinoma, aberrant expression of the marker -due to increased number of gene and/or not chromosome 20 copies- is associated with aggressive phenotype (advanced stage III-IV) [18]. Furthermore, low Topo I expression seems to be an independent favorable prognostic factor for longer overall survival in postoperative lung cancer patients, especially in squamous cell carcinoma [19]. The same study showed that there is a correlation between the expression of thymidylate synthase and Topo I in the examined tumor tissues. Sim-

ilarly, another study showed that Topo I mRNA analysis can predict the cisplatin outcome and prognosis [20]. In contrast to those results, Topo I overexpression in esophageal squamous cell carcinoma does not correlate with the biological behavior of the malignancy [21].

In the current study we explored the role of Topo I aberrant expression in LSCC TMA slides. Based on our results we detected moderate to high expression in a significant proportion of the analyzed tissue cores (32/50-64%). Concerning the impact of the abnormal protein expression in the corresponding cases, we showed that statistical significance rose correlating the overall expression to the grade of differentiation of the examined tumors. There are very limited published data on this field regarding LSCC. Based on mRNA analysis by a quantitative RT-PCR, a study group showed that normalized Topo I/G6PDH mRNA ratios were significantly correlated with that of Topo I/PBGD in colorectal tumors but not in pharyngeal/laryngeal tumors [22]. Similarly, another study suggested that Topo I inhibitors including camptothecin enhance the cytocidal effect of Adeno-Associated Virus (AAV)-HSVtk/ganciclovir on head and neck cancer cells. The combination of AAV-mediated suicide gene therapy and treatment with Topo I inhibitors may have synergistic therapeutic effects [23]. Additionally, CKD-602 (7-[2-(N-isopropylamino) ethyl]-(20S)-camptothecin, belotecan), a synthetic water-soluble camptothecin derivative, may inhibit the proliferation of oral SCC by increasing apoptosis in G2/M phase arrest [24]. In conjunction to the previous analysis, another study group explored the role of SN-38, an active metabolite of irinotecan. They proposed that SN-38 is highly cytotoxic to OSCC cell lines, regardless of the type of induced cell death, suggesting its future application for chemotherapy of OSCC [25]. Concerning specifically LSCC, some studies suggest that Topo IIa instead of Topo I abnormal expression may be a more useful indicator of tumor aggressiveness and poor outcome in LSCC [26,27].

In conclusion, Topo I overexpression is observed in a significant subset of LSCCs affecting their level of differentiation. Additional molecular studies focused on the mechanism of Topo I gene/protein deregulation (i.e. amplification, abnormal epigenetic promoter methylation, mRNA aberrant expression) are necessary discriminating the eligible patients for applying specific chemotherapeutic strategies based on anti-Topo I agents. Interestingly, the role of Topo expression in head and neck squamous cell carcinomas and its relationship with cancer stem cells profiles and lymph node metastasis is a very promising molecular field [28].

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Conflict of interests

The authors declare no confict of interests.

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