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ORIGINAL ARTICLE

Comparative topoisomerase IIa and ki 67 protein expression in papillary thyroid carcinoma based on tissue microarrays and image analysis

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Summary

Purpose: *Topoisomerase II alpha (Topo IIa gene location 17q21) is a nucleic enzyme involved in the DNA replication, transcription and chromosome topological formation. Topo IIa inhibition strategies include specific chemotherapeutic agents such as anthracyclines. Our aim was to investigate potential protein alterations of the enzyme comparing them to ki 67 proliferation marker expression in papillary thyroid carcinoma (PTC).*

Materials and methods: *Using tissue microarray (TMA) technology, 50 specimens consisting of histologically confirmed PTCs (n=20), multi-nodular goiters (n=20) and also normal thyroid epithelia (n=10) were cored and re-embedded in the final paraffin block. Immunohistochemical analysis was performed using monoclonal anti-Topo IIa and anti-ki 67 (MIB-1) antibodies. Digital image analysis assay was also applied for the evaluation of the protein expression results (Nuclear Labeling Index-NLI).*

Results: *Topo IIa and ki 67 proteins were overexpressed in 4/20 (20%) and 14/20 (70%) cases, respectively. Concerning multi-nodular goiters, overexpression was observed in 2/20 and 4/20 specimens, respectively. Statistical association was assessed correlating ki 67 expression to pathology type, capsular invasion and also to vascular infiltration (p=0.001, p=0.008, and p=0.012, respectively). Topo IIa protein expression was strongly correlated only to capsular invasion (p=0.004). Overall expression of the examined markers demonstrated a medium concordance (kappa=0.27), but a strong association (p=0.001).*

Conclusion: *Topo IIa and also ki 67 overexpression are correlated to an aggressive phenotype in PTC. Topo IIa overexpression maybe is a reliable marker for a rational application of targeted chemotherapeutic strategies in some subgroups of patients.*

Key words: image analysis, immunohistochemistry, thyroid cancer, topoisomerase

Introduction

PTC represents the most common thyroid malignant neoplasm [1]. Although the majority of PTCs is characterized by good biological behavior and prognosis, cytogenetic analyses have shown that specific gene alterations are responsible for the development of aggressive anaplastic carcinomas arising from them [2].

Excessive proliferation is correlated to carcinogenesis and affects the biological behavior in a variety of cancers [3]. ki 67 gene located on chromosome 10 (10q25) encodes a protein which is expressed in the nucleolus in all cell cycle phases except G0 (arrest phase) [4]. In fact, ki 67 expression increases as a cell progresses through the cell cycle, with the highest expression seen in G2/M phase cells [5]. Concerning PTC, ki 67 is frequently overexpressed but its prognostic significance is under investigation [6]. Furthermore,

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molecules, such as topoisomerases, appear to be useful markers for the evaluation of proliferation activity in tumors of different histological origin [7].

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) [8]. Topo IIa and b isomers' combined action of temporarily cutting and rejoining the DNA double helix, allowing also winding and unwinding of the DNA double strand, is a critically important molecular mechanism for replication, transcription and repair of chromosome structure [9]. 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 [10]. Correlating ki 67 to Topo IIa duration of expression, Topo IIa protein level seems to provide a better estimation of the number of actively proliferating cells and for this reason it could be used as a reliable marker of proliferation [11]. Furthermore, topoisomerases' inhibition promotes cell death and for this reason they are targets for specific chemotherapy. 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 [12,13]. Concerning PTCs, there are controversial results based on *in vitro* studies regarding the potential efficacy of anti-Topo IIa chemotherapeutic drugs [14,15].

In the current study, we analyzed Topo IIa and ki 67 proliferation markers at the protein level in order to evaluate their potential correlation with clinicopathological parameters in PTCs.

Materials and methods

Study group

For the purposes of our study, 50 archival, formalin-fixed and paraffin-embedded tissue specimens including 20 histologically confirmed primary PTCs (14 classical papillary and 6 micropapillary), 20 multinodular goiters and also 10 normal thyroid epithelia - obtained by surgical resection (thyroidectomy) - were used. All of the patients were female with average age 55.25 years. The initial diagnosis was performed by fine needle aspiration biopsy (FNAB) (Figure 1a). The hospital ethics committee consented to the use of these tissues in the Department of Pathology, 417VA Hospital (NIMTS) for research purposes, according to World Medical Association Declaration of Helsinki. 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. Similarly, FNAB samples were fixed by liquid based cytology (ThinPrep™) and stained using Papanicolaou (PAP) staining procedure. All lesions were classified according to the histological typing criteria of World Health Organization (WHO). Clinicopathological data of the examined cases are demonstrated on Table 1.

Table 1. Immunohistochemistry results and correlations (n=50)

	Topo IIa			*Ki 67*		
	LL	*OE*	*p-value*	*LL*	*OE*	*p-value*
Histology			0.089			0.001
Papillary Ca (n=14)	1/14	3/14		4/14	10/14	
Micropapillary Ca (n=6)	5/6	1/6		2/6	4/6	
Nodular goiter (n=20)	18/20	2/20		16/20	4/20	
Normal epithelia (n=10)	10/10	0/10		0/10	0/10	
Capsular invasion*			0.004			0.008
Positive (n=7)	4/7	3/7		2/7	5/7	
Negative (n=13)	12/13	1/13		7/13	6/13	
Vascular infiltration*			0.639			0.012
Positive (n=6)	1/6	5/6		1/6	5/6	
Negative (n=14)	11/14	3/14		5/14	9/14	

LL: Reduced expression (low nuclear labeling index/NLI), OE: overexpression (combined high and moderate NLI)
p-value: derived from Spearman's rank correlation coefficient
*Interpretation of immunohistochemistry expression: Topo IIa & ki 67 (NLI-immunostained nuclei per optical field) High ≥10, Moderate 5-9, Low 0-4
*Referred only to carcinoma cases

TMA construction

Areas of interest were identified in H&E-stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on 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 two times (in order to secure the presence of each case in the final blocks) and 1-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient blocks. After 3 mm microtome sectioning and H&E staining, the finally constructed TMA blocks contained 47/50 (94%) and 46/50 (92%) cores of tissue cylindrical specimens. We observed microscopically that all examined PTCs and nodular goiter cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens) (Figure 1a).

Antibodies and immunohistochemistry assay (IHC)

For the purposes of our study, we selected and applied the monoclonal antibodies anti-Topoisomerase IIa (clone KiS1-DAKO, Denmark) at dilution of 1:70 and anti-ki 67 (clone MIB-1 –DAKO, Denmark) at dilution of 1:50.

IHC protocol for the antigens was carried out on 3 μm-thick paraffin serial sections of the TMA blocks. Two tissue sections for each of them- initially deparaffinized 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 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. The antigen-antibody reaction was visualized using 3-3, diaminobenzidine tetrahydrocloride (DAB) as a chromogen substrate. Finally, tissue sections were slightly counterstained with hematoxylin for 30 sec, dehydrated and mounted.

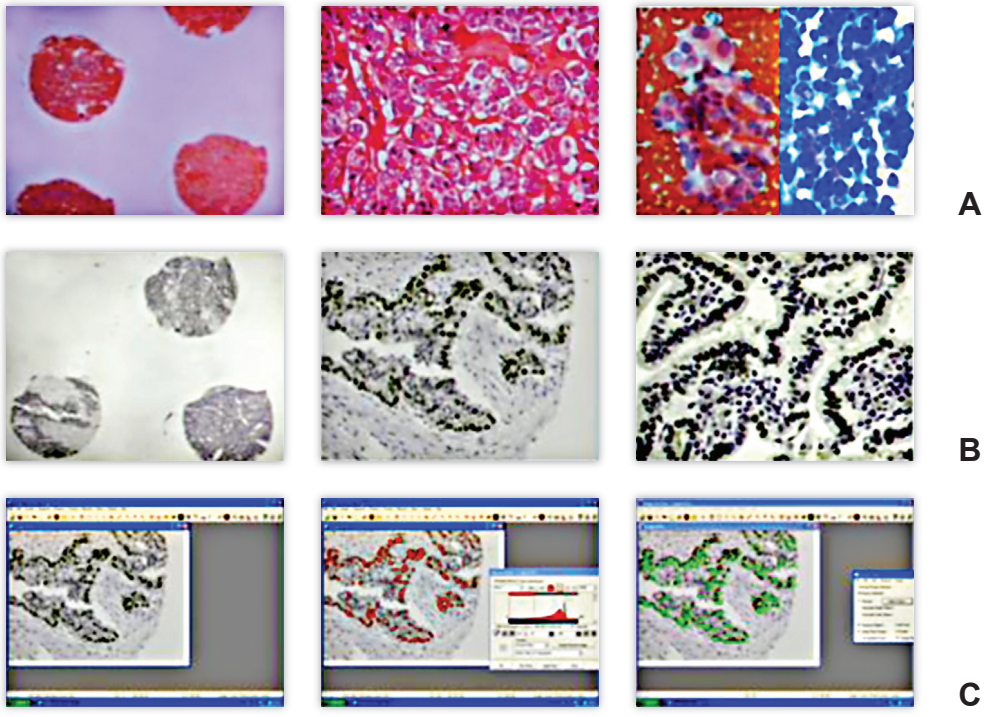

Figure 1. A. Tissue microarray cores of PTCs (left-H&E stain, original magnification ×100). A case of PTC (center-H&E stain, original magnification ×400). FNAB specimen that demonstrates classical cytomorphological characteristics of PTC. Note nuclear heterogeneity and overlapping, intranuclear pseudoinclusions (right-Pap stain on conventional and also liquid based fixed samples, original magnification ×400); **B.** Topo IIa expression in the corresponding tissue microarray cores (left- original magnification ×100). A case of Topo IIa protein overexpression. Note a significant number of stained nuclei (center- original magnification ×200). A case of ki 67 protein overexpression. Note a significant number of stained nuclei (right- original magnification ×400); **C.** Evaluation of Topo IIa expression in a case of PTC. Reddish areas represent immunostained nuclei (dark oval to circular objects), whereas green signals are referred to the automated counting procedure (original magnification ×200).

For negative control slides, the primary antibody was omitted. Nuclear staining pattern was considered to be acceptable for both proteins and breast cancer tissue sections demonstrating Topo IIa and ki 67 expression were used as positive markers for the immunostaining pattern (Figure 1b).

Computerized image analysis (CIA)

In order to evaluate the IHC results in an accurate and fast way, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium IV, MATROX II Card Frame Grabber, Digital Camera Microwave systems (800×600), Microscope Olympus BX-50, and the following software: Windows XP/Image Pro Plus, version 3.0-Media Cybernetics 1997. Measurements for Topo IIa and ki 67 Nuclear Labeling Index (NLI) were performed in 5 optical fields per case and at a magnification of ×400 (Figure 1c). For objectivity reasons, we focused on representative tissue areas demonstrating even expression of the marker. Using normal epithelia as control group, comparing them to the analyzed tumors, we characterized NLIs as Low, Moderate and High. Interpretation of NLI categorization is demonstrated on Table 1.

Statistical analysis

Associations between variables including protein expression levels (NLIs) and pathological parameters were performed by the application of Spearman's test (SPSS, Chicago Inc, USA, v. 11.0). Two-tailed p-values ≤ 0.05 were considered statistically significant. Kappa analysis was performed for the evaluation of concordance regarding those markers. Total IHC results and correlations (p-values) are described on Table 1.

Results

According to the computerized image analysis procedure, all the examined cases demonstrated Topo IIa and ki 67 protein expression in different levels. Overexpression was observed in 4/20 (20%) cases and 14/20 (70%), respectively. High NLI values were detected in 2/4 (50%) and 4/14 (28.5%) cases, whereas moderate levels of expression were observed in 2/4 (50%) and 10/14 (71.5%) cases. The rest of the examined cases were characterized by low NLIs. Concerning multi-nodular goiters, overexpression was observed in 2/20 (10%) and 4/20 (20%) specimens regarding Topo IIa kai ki 67, whereas normal thyroid epithelia (control group) demonstrated low NLIs for both of the examined markers. Statistical association was assessed correlating ki 67 expression to pathology type, capsular invasion and also to vascular infiltration (p=0.001, p=0.008, and p=0.012, respectively). Topo IIa protein expression was strongly correlated only to capsular invasion (p=0.004). Both markers' overall expression was not correlated to regional lymph node metastasis (p=0.195, p=0.179, for Topo IIa and ki 67, respectively), although that 3/20 cases were also characterized by capsular invasion and vascular infiltration (advanced diseased). Overall, protein expression of the examined markers demonstrated a medium concordance (kappa=0.27), but a strong association (p=0.001).

Discussion

Extensive molecular analyses have shown that thyroid carcinogenesis is characterized by an accumulation of chromosome and specific gene numerical and structural alterations in normal follicular cells [16]. Furthermore, alterations in proliferation/apoptotic balance are responsible for the neoplastic and finally malignant transformation of them [17]. Additionally, there is an increasing need for establishing new and reliable markers regarding the biological behavior (response to chemotherapeutic agents, prognosis) of the thyroid carcinoma histological subtypes [18].

In the current study, we explored the role of ki 67 and Topo IIa proliferation markers based on TMA immunohistochemical analysis. We observed that ki 67 was frequently overexpressed in PTCs, whereas Topo IIa overexpression was assessed only in a small subgroup of the examined cases, especially in poorly differentiated and aggressive ones. Overexpression of the markers was selectively correlated to pathological parameters demonstrating an association with advanced disease (capsular invasion, vascular infiltration, lymph node metastasis). Furthermore, although relatively rare, overexpression of the proteins in multi-nodular goiters reflects a potential progressive process in thyroid neoplasia. Similar studies have shown controversial results regarding Topo IIa protein expression levels in PTCs. Interestingly, in one of them PTCs demonstrated the lowest levels of expression compared with other types of thyroid neoplasms according to immunohistochemical analysis [19]. Additionally, another study showed that Topo IIa expression levels were correlated to the age of the examined patients, whereas only telomerase activity was strongly correlated to advanced disease [20]. Besides these observations, a recently published study based on a conventional eye-microscopy evaluation of ki 67 and Topo IIa protein expression concluded

that both of them should be recommended for the determination of thyroid tumor cell proliferation [21]. Concerning the combination of tissue microarrays and digital image analysis in thyroid neoplasia, there are encouraging results evaluating nuclear markers such as ki 67 or other proteins, such as prolactin (PRL) and its receptor (PRLR) [22,23]. We strongly support the idea of quantitative analysis regarding nuclear markers including Topo IIa based on our previous published experience [24,25].

In conclusion, Topo IIa and ki 67 expression in PTCs reflect the proliferation activity. Additionally, overexpression of the markers is correlated with aggressive phenotype in PTCs (advanced stage). Especially, Topo IIa overexpression maybe is the basis for applying targeted chemotherapeutic strategies in subgroups of patients. Furthermore, structural or numerical aberrations of the gene should provide a better knowledge and criteria for this purpose.

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References

1. Fischer S, Asa SL. Application of immunohistochemistry to thyroid neoplasms. Arch Pathol Lab Med 2008; 132: 359-372.
2. Kitamura Y, Shimizu K, Tanaka S, Ito K, Emi M. Allelotyping of anaplastic thyroid carcinoma: frequent allelic losses on 1q, 9p, 1, 17, 19p, and 22q. Genes Chromosomes Cancer 2000; 27: 244-251.
3. Gerdes J. Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. Semin Cancer Biol 1990; 1: 199-206.
4. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2002; 182: 311-322.
5. Duchrow M, Gerdes J, Schluter C. The proliferation-associated Ki-67 protein: definition in molecular terms. Cell Prolif 1994; 27: 235-242.
6. Saiz AD, Olvera M, Rezk S, Florentine BA, McCourty A, Brynes RK. Immunohistochemical expression of cyclin D1, E2F-1, and Ki-67 in benign and malignant thyroid lesions. J Pathol 2002; 198: 157-162.
7. Holden JA, Perkins SL, Snow GW, Kjeldsberg CR. Immunohistochemical staining for DNA topoisomerase IIa in non-Hodgkin's lymphomas. Am J Clin Pathol 1995; 104: 54-59.
8. Liu LF. DNA topoisomerases: enzymes that catalyse the breaking and rejoining of DNA. CRC Crit Rev Biochem 1983; 15: 1-24.
9. Heck MM, Earnshaw WC. Topoisomerase IIa, a specific marker for cell proliferation. J Cell Biol 1986; 103: 2569-2581.
10. Heck MMS, Hittelman WN, Earnshaw WC. Differential expression of DNA topoisomerase I and II during the eukaryotic cell cycle. Proc Natl Acad Sci 1988; 85: 1086-1090.
11. Lewy-Trenda I, Bienkiewicz M. Evaluation of MIB-1 immunoreactivity and nucleolar organizer regions in nonneoplastic and neoplastic thyroid lesions. Pol J Pathol 1999; 50: 129-138.
12. Koshiyama M, Fujii H, Kinezaki M, Morita Y, Nanno H, Yoshida M. Immunohistochemical expression of topoisomerase IIa and multidrug resistance-associated protein, plus chemosensitivity testing as chemotherapeutic indices of ovarian and endometrial carcinomas. Anticancer Res 2001; 21: 2925-2932.
13. Thigpen TJ, Aghajanian CA, Alberts DS et al. Role of pegylated liposomal doxorubicin in ovarian cancer. Gynecol Oncol 2005; 96: 10-17.
14. Osawa Y, Yoshida A, Asaga T, Kawahara S, Yanoma S. In vitro chemosensitivity test for seven undifferentiated thyroid carcinoma cell lines using MTT assay. Japan J Cancer Chemother 1996; 23: 471-476.
15. Kelsen D, Fiore J, Heelan R, Cheng E, Magill G. Phase II trial of etoposide in APUD tumours. Cancer Treat Rep 1987; 71: 305-307.
16. Várkondi E, Gyory F, Nagy A, Kiss I, Ember I, Kozma L. Oncogene amplification and overexpression of oncoproteins in thyroid papillary cancer. In Vivo 2005; 19: 465-470.
17. Saltman B, Singh B, Hedvat CV, Wreesmann VB, Ghossein R. Patterns of expression of cell cycle/apoptosis genes along the spectrum of thyroid carcinoma progression. Surgery 2006; 140: 899-905.
18. Murphy KM, Chen F, Clark DP. Identification of immunohistochemical biomarkers for papillary thyroid carcinoma using gene expression profiling. Hum Pathol 2008; 39: 420-426.
19. Lee A, LiVolsi VA, Baloch ZW. Expression of DNA topoisomerase II alpha in thyroid neoplasia. Mod Pathol 2000; 13: 396-400.
20. Karayan-Tapon L, Menet E, Guilhot J, Levillain P, Larsen CJ, Kraimps JL. Topoisomerase II alpha and telomerase expression in papillary thyroid carcinomas. Eur J Surg Oncol 2004; 30: 73-79.
21. Ludvíková M, Holubec L Jr, Ryska A, Topolcan O. Proliferative markers in diagnosis of thyroid tumors: a comparative study of MIB-1 and topoisomerase II-a immunostaining. Anticancer Res 2005; 25: 1835-1840.
22. Matos LL, Stabenow E, Tavares MR, Ferraz AR, Capelozzi VL, Pinhal MA. Immunohistochemistry quantification by a digital computer-assisted method compared to semiquantitative analysis. Clinics 2006; 61: 417-424.
23. Costa P, Catarino AL, Silva F, Sobrinho LG, Bugalho MJ. Expression of prolactin receptor and prolactin in normal and malignant thyroid: a tissue microarray study. Endocr Pathol 2006; 17: 377-386.
24. Tsiambas E, Alexopoulou D, Lambropoulou S, Gerontopoulos K, Karakitsos P, Karameris A. Targeting Topoisomerase IIa in endometrial adenocarcinoma: A combined Chromogenic In situ Hybridization and Immunohistochemistry Tissue Microarrays Study. Int J Gyn Cancer 2006; 16: 1424-1431.
25. Tsiambas E, Karameris A, Tiniakos DG, Karakitsos P. Evaluation of topoisomerase IIa expression in pancreatic ductal adenocarcinoma: a pilot study using chromogenic in situ hybridization and immunohistochemistry on tissue microarrays. Pancreatology 2007; 7: 45-52.