The value of TOP2A, EZH2 and paxillin expression as markers of aggressive breast cancer: relationship with other prognostic factors

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Summary

Introduction: The immunocytochemical expression of topoisomerase II alpha (TOP2A), enhancer of zeste homologue 2 (EZH2) and paxillin has recently gained increasing attention. Although previous studies have commented on the clinical usefulness of these markers, their role remains controversial. *Aim:* The purpose of the study was to investigate the expression of TOP2A, EZH2 and paxillin in relation to classic prognostic parameters and their significance as prognostic markers in imprints of resected breast carcinomas. *Methods:* Imprint smears from 55 patients who underwent surgical treatment for primary carcinoma in our department between 2005 and 2006 were studied immunocytochemically with the use of TOP2A, EZH2 and paxillin antibodies. *Results:* The expression of TOP2A correlated with higher histologic grade, tumor size and negative PR expression. High intensity staining for EZH2 expression was associated with higher histologic grade, negative ER and PR expression and positive Ki-67 expression. The expression of paxillin showed no correlation with estrogen/progesterone and HER2 expression nor with tumor grade and stage. *Conclusion:* Our data indicate that TOP2A and EZH2 expression are related to a more aggressive tumor phenotype. The expression of paxillin failed to correlate with any of the studied clinicopathologic factors. Further studies are needed to verify these results.

Key words: TOP2A; EZH2; Paxillin; Tumor markers; Breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer deaths among women in Western societies. Prompt diagnosis and selection of appropriate treatment play an important role in reducing mortality [1]. In order to plan specific therapies several prognostic/predictive markers have been evaluated over the last years [2]. Although molecular profiling has shown promise in refining treatment decision making, to date, immunohistochemistry remains a validated and less expensive method for the investigation of new prognostic and predictive markers [3]. In view of this notion the immunohistochemical expression of topoisomerase II alpha (TOP2A), zeste homologue 2 (EZH2) and paxillin has recently gained increasing attention. TOP2A IIa is a key enzyme in DNA replication and the polycomb group protein enhancer of EZH2 is a major component for the maintenance of cell identity and cell cycle regulation [4, 5]. Both these factors have been proposed as potential markers for targeted therapy [6, 7]. Paxillin is a focal adhesion protein that regulates various biologic pathways such as cell migration and proliferation [8]. Although previous studies have commented on the clinical usefulness of these three markers, their role remains controversial. In this prospective study we examine the association between TOP2A, EZH2 and paxillin with other clinicohistopathological parameters and discuss the clinical implications of our findings.

Materials and Methods

Imprint samples were obtained from 55 patients who underwent surgery for breast cancer immediately after tumor removal in the operating room. Mean age of the patients was 56.7 years old at the time of diagnosis. Imprint smears were taken from different areas of macroscopically estimated breast carcinoma. We prefer to use imprint smears instead of paraffin-embedded tissue sections, as the latter present a lot of difficulties regarding immunoreactivity. Depending on the thickness of the section, there will always be a number of cells sliced or overlapped, thus leading either to false low or false high immunoreactivity, respectively [9-11]. Furthermore, tissue fixation and to a lesser degree tissue processing are potential causes of variation in the reproducibility of immunohistochemical staining [12]. Besides that, in cytologic preparations, the cells are whole, a large surface of the tumor is sampled and tissue is preserved for subsequent pathologic and molecular analyses [11, 13]. Its now firmly established that a wide variety of markers can be applied on cytologic preparations and that immunocytochemistry correlates well with immunohistochemistry [14-16]. After air drying, smears were fixed in buffer formalin 5% for 20 min and stored at –70°C until used for an immunocytochemical procedure. All histological

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diagnoses were performed using sections from the samples that were used for the imprints. The tumors were classified according to the histological typing of breast tumors by the WHO [17] and grade of tumors according to the Scourff Bloom Richardson classification modified by Elston and Elis [18, 19].

Additional informational was recorded concerning the patient's ER/PR, HER-2 expression, EGFR status, p53 and ki-67 as determined immunohistochemically. Immunostaining was performed by the Avidin-Biotin Complex immunoperoxidase method with the use of TopoIIa (Novocastra, clone 3F6) at a dilution 1:50, EZH2 (Novocastra, clone 6A10) at a dilution 1:50 and paxillin (Novocastra, clone 5H11) at a dilution 1:30.

Smears were incubated for 45 min with a normal rabbit serum diluted 1:40 in PBS. Then the smears were rinsed in three changes of PBS for 5 min each and incubated overnight with primary antibodies. After washing in PBS the smears were incubated with rabbit anti mouse biotinylated immunoglobulin diluted 1:200 followed by the ABC/HPR. Visualization was achieved by a final incubation in 3.3-diaminobenzide tetrahydrochloride. The smears were counterstained with Mayer's hematoxylin. Smears of known positive reactivity were included as positive controls and negative controls were stained by omitting the primary antibody incubation. Results were interpreted by two independent cytologists. In cases where staining was heterogeneous in the slide examined fields included those with the highest and those with the lowest percentage of stained cells. The immunostaining for each protein was determined as positive or negative. Staining was interpreted as positive when > 10% of the tumor cells showed cytoplasmatic or nuclear staining.

Statistical analysis

A standard statistical software package SPSS (SPSS Inc, Chicago IL) was used in the analysis. Descriptive statistics were calculated for all variables. The chi-square test or Fisher's exact test' as appropriate, was used to examine the association between TOP2A, paxillin and EZH2 expression and the steroid hormone, c-erbB-2, EGFR, p53 and Ki-67 status, as well as the correlation of the former markers with tumor type, grade and stage. The one-sample Kolmogorov-Smirnov test was used to test if a variable was normally distributed. All data were normally distributed, and the association between TOP2A, paxillin and EZH2 expression and patient age and tumor size was analyzed with the Student's t-test and with the one-way ANOVA with post-hoc LSD analysis; p values less than 0.05 were considered statistically significant.

Results

Mean age of the patients was 56.7 years old (SD ± 13.4 years) during the time of diagnosis. Tumor characteristics are presented in Table 1. Immunocytochemical analysis revealed that 32 (58.2%) patients were positive for paxillin expression, 35 (74.5%) out of 47 tested patients were positive for TOP2A expression and nine (16.4%), 11 (20%) and 14 (25.5%) patients were categorized as having high, moderate and weak staining, respectively, for EZH2 expression.

Statistical analyses showed that TOP2A positive tumors correlated with higher histological grade (57.1% of the TOP2A positive tumors had grade 3 malignancies compared to 41.7% of the TOP2A negative tumors, $p < 0.05$), higher tumor size (2.5 cm vs 1.6 cm, $p < 0.05$) and negative PR expression (48.6% vs 8.3%, $p < 0.05$). High intensity staining for EZH2 expression was associated with higher histologic grade (88.9% vs 21.4%, $p < 0.05$), negative ER and PR expression (55.6% vs 7.1% and 66.7% vs 7.1% respectively, $p < 0.05$) and positive Ki-67 expression (77.8% vs 7.1%, $p < 0.05$) (Table 2). The expression of paxillin failed to correlate with estrogen/progesterone and HER2 expression as well as with tumor grade and stage.

Table 1. — *Tumor characteristics*

Tumor size (mean ± SD)	2.3 ± 1.4
Histological type I, number (%)	
Invasive	46 (83.6%)
Invasive/In situ	9 (16.4%)
Histological type II, number (%)	
Ductal	50 (90.9%)
Lobular	5 (9.1%)
N stage, number (%)	
N0	30 (54.5%)
N1	14 (25.5%)
N2	6 (10.9%)
N3	5 (9.1%)
Tumor grade, number (%)	
1	3 (5.5%)
2	25 (45.5%)
3	27 (49.1%)
TNM stage, number (%)	
I	19 (34.5%)
IIA	19 (34.5%)
IIB	6 (10.9%)
IIIA	6 (10.9%)
IIIC	5 (9.1%)
Positive tumors for [number (%)]	
ER	43 (78.2%)
PR	37 (67.3%)
HER2	13 (23.6%)
EGFR	4 (7.3%)
p53	36 (65.5%)
Ki-67	23 (41.8%)

Discussion

This prospective study evaluated the role of the immunocytochemical expression of three different novel markers in invasive breast carcinomas.

TOP2A is a key enzyme in DNA replication which catalyzes the unwinding of DNA by inducing single-stranded breaks on both DNA strands [20]. Considering the pivotal role of this enzyme in the modification of DNA topology it would appear logical to assume that TOP2A overexpression should correlate with high cell proliferation rate. Although this study revealed a strong correlation between TOP2A expression and tumor grade in several countries, including ours, the mitotic count, a well established proliferation index is incorporated into the tumor grading systems [21] no correlation was found with Ki-67 expression. This discrepancy may be the result of the small sample size of this study. Probably for the same reason, although we showed a significant association with PR-

Table 2. — *Relationships between EZH2, TOP2A and other clinicopathologic factors.*

	EZH2				TOP2A		
	+	++	+++	*p*	negative	positive	*p*
Grade							
1	1 (7.1%)	0 (0%)	0 (0%)	< 0.05	2 (16.7%)	0 (0%)	< 0.05
2	10 (71.4%)	5 (45.5%)	1 (11.1%)		5 (41.7%)	15 (42.9%)	
3	3 (21.4%)	6 (54.5%)	8 (88.9%)		5 (41.7%)	20 (57.1%)	
ER+	13 (92.9%)	7 (63.6%)	4 (44.4%)	< 0.05	11 (91.7%)	24 (68.6%)	NS
ER-	1 (7.1%)	4 (36.4%)	5 (55.6%)		1 (8.3%)	11 (31.4%)	
PR+	13 (92.9%)	5 (45.5.3%)	3 (33.3%)	< 0.05	11 (91.7%)	18 (51.4%)	< 0.05
PR-	1 (7.1%)	6 (54.5%)	6 (66.7%)		1 (8.3%)	17 (48.6%)	
Ki67+	1 (7.1%)	6 (54.5%)	7 (77.8%)	< 0.05	4 (33.3%)	18 (51.4%)	NS
Ki67-	13 (92.9%)	5 (45.5%)	2 (22.2%)		8 (66.7%)	17 (48.6%)	

NS: non significant.

negative tumors, no correlation was found with ER-negative tumors. Overall, it appears that TOP2A overexpression is related to a more aggressive tumor phenotype [22, 23]. Furthermore, in addition to its role as a proliferative and subsequently possibly prognostic marker, TOP2A has been proposed as a potential molecular target of several chemotherapy agents, including anthracyclines. Nonetheless, to date, its role as a predictive biomarker for chemotherapy remains controversial [24].

The polycomb group protein (PcG) enhancer of EZH2 is a major component for the maintenance of cell identity and cell cycle regulation [25]. Previous studies have shown that EZH2 promotes cell proliferation and tumor progression [26]. In this study we showed that the expression of EZH2 was strongly associated with increased tumor cell proliferation (as indicated by the Ki-67 expression) and higher tumor grade. These findings are indicative of the aggressive biologic behavior of EZH2 positive tumors and indirectly suggest the possible role of EZH2 overexpression in local tumor invasion and possible distant metastases. Due to lack of data no survival analysis was done, nevertheless according to previous published studies although EZH2 expression was inversely correlated with prognosis it does not appear to be an independent prognostic factor [27]. Furthermore we found that EZH2 overexpression was negatively correlated with ER and PR expression. Although previous investigators have also documented this association between EZH2 expression and hormone receptor status its role in oncogenesis remains largely unknown [28]. We should also highlight that this increasing interest regarding PcG proteins including EZH2 is also derived from the recently published studies regarding their potential role as markers for targeted therapy [7, 29].

Paxillin is a phospho-tyrosine-containing protein which is located at specific cell structures, called focal adhesions sites [30]. It is the member of a family of proteins that also contains hic-5 and leupaxin [31, 32]. Paxillin has several binding sites for other proteins with which it interacts into complexes able of transmitting signals downstream of integrins. The N-terminal half of paxillin contains several peptide sequences, such as the LD motifs, which serve as a docking site for various actin-binding and signaling proteins [33]. The C-terminal half contains the LIM domains, sequences that play an important role in the binding of paxillin to the focal adhesion sites [34].

Paxillin regulates various biological events such as cell migration and proliferation. Despite the extensive research to date, the precise function of paxillin remains elusive [35]. Although many reports implicate paxillin as a positive regulator of motility some investigators have published opposite results suggesting that paxillin could in fact inhibit cell motility [36, 37]. In the same manner the role of paxillin in breast cancer remains controversial. A study by Vadlamudi et al in human breast cancer cells demonstrated an increase in paxillin expression with HER2/HER3 pathway and grade 3 breast cancer tumors [38]. Similarly in a recent report by Hicks *et al*. paxillin expression correlated well with HER2 amplification, but failed to show any association with tumor grade [39]. On the other hand Madan *et al*., although also failing to show any correlation between paxillin and tumor grade, found no association between paxillin and HER2 expression [40]. Interesting, however, the latter researchers demonstrated that high paxillin expression was associated with lymph node negative status and thus less aggressive forms of breast cancer. In agreement with the previous studies we failed to show any association between paxillin and estrogen/progesterone expression as well as between paxillin and HER2 expression or tumor grade and stage. We assume that the reasons for these discrepancies could be the relatively small number of patients that were enrolled in these studies and the analysis of a heterogeneous group of breast neoplasms.

In conclusion, we demonstrated a significant association between EZH2 and TOP2A expression with unfavorable prognostic markers such as higher tumor grade. These data indirectly support the possible prognostic role of these two markers. On the other hand this study failed to show any correlation between paxillin and other clinicopathologic factors. Further studies are needed to confirm our results and help us better understand the biologic role and possible clinical implications of these markers.

References

[1] Nelson H.D., Tyne K., Naik A., Bougatsos C., Chan B.K., Humphrey L.: "U.S. Preventive Services Task Force. Screening for breast cancer: an update for the U.S. Preventive Services Task Force". *Ann. Intern. Med*., 2009, *151*, 727.

[2] Nappi O., Carrillo G.: "Prognostic and predictive factors of breast carcinoma: Beyond hormonal receptors and HER2". *Eur. J. Cancer*, 2008, (suppl. 6), 1.

[3] Tang P., Skinner K.A., Hicks D.G.: "Molecular classification of breast carcinomas by immunohistochemical analysis: are we ready?". *Diagn. Mol. Pathol*., 2009, *18*, 125.
[4] O'Connor J.K., Hazard L.J., Avent J.M., Lee R.J., Fischbach J., Gaffney D.K.: "Topoisomerase II alpha expression correlates with diminished disease-free survival in invasive breast cancer". *Int. J. Radiat. Oncol. Biol. Phys*., 2006, *65*, 1411.
[5] Raaphorst F.M., Meijer C.J., Fieret E., Blokzijl T., Mommers E., Buerger H. *et al*.: "Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene". *Neoplasia*, 2003, *5*, 481.
[6] Zhu L., Li Y.F., Chen W.G., He J.R., Peng C.H., Zhu Z.G., Li H.W.: "HER2 and topoisomerase IIalpha: possible predictors of response to neoadjuvant chemotherapy for breast cancer patients". *Chin. Med. J. (Engl.)*, 2008, *121*, 1965.
[7] Kirmizis A., Bartley S.M., Farnham P.J.: "Identification of the polycomb group protein SU(Z)12 as a potential molecular target for human cancer therapy". *Mol. Cancer Ther*., 2003, *2*, 113.
[8] Schaller M.D.: "Paxillin: a focal adhesion-associated adaptor protein". *Oncogene*, 2001, *20*, 6459.
[9] Bantis A., Gianopoulos A., Gonidi M., Liossi A., Aggelonidou E., Petrakakou E. *et al*.: "Expression of p120, Ki-67 and PCNA as proliferation biomarkers in imprint smears of prostate carcinoma and their prognostic value". *Cytopathology*, 2004, *15*, 25.
[10] Greene D.R., Taylor S.R., Wheeler T.M., Scardino P.T.: "DNA ploidy by image analysis of individuals foci of prostate cancer: a preliminary report". *Cancer Res*., 1991, *51*, 4084.
[11] Koss L.G., Melamed M.R., Koss L.G., Melamed M.R. (eds.): "Recognizing and classifying cells. Koss/diagnostic cytology". 5th edition, Philadelphia, Lippincott Williams and Wilkins, 2006, 119.
[12] Werner M., Chott A., Fabiano A., Battifora H.: "Effect of formalin fixation and processing on immunohistochemistry". *Am. J. Surg*., 2000, *24*, 1016.
[13] Creager A.J., Geisinger K.R., Perrier N.D., Shen P., Shaw J.A., Young P.R. *et al*.: "Intraoperative imprint cytologic evaluation of sentinel lymph nodes for lobular carcinoma of the breast". *Ann. Surg*., 2004, *239*, 61.
[14] Aihara T., Munakata S., Morino H., Takatsuka Y.: "Touch imprint cytology and immunohistochemistry for assessment of sentinel lymph nodes in patients with breast cancer". *Eur. J. Surg. Oncol*., 2003, *29*, 845.
[15] Aihara T., Munakata S., Morino H., Takatsuka Y.: "Comparison of frozen sections and touch imprint cytology for evaluation of sentinel lymph nodes metastasis in breast cancer". *Ann. Surg. Oncol*., 2004, *11*, 747.
[16] Flens M.J., van der Valk P., Tadema T.M., Huysmans A.C., Risse E.K., van Tol G.A., Meijer C.J.: "The contribution of immunohistochemistry in diagnostic cytology. Comparisons and evaluation with immunohistochemistry". *Cancer*, 1990, *65*, 2704.
[17] "World Health Organization Classification of Tumors: Pathology and Genetics of Tumors of the Breast and Female Genital Organs". Tavassoli F., Devilee P. (eds.), Lyon, IARC Press, 2003, 10.
[18] Page D.L., Anderson T.J.: "Diagnosis histopathology of the breast". Edinburgh, Churchill Livingstone, 1987, 303.
[19] "Cytology Subgroup of the National Coordinating Committee for Breast Cancer Screening Pathology. Guidelines for cytology procedures and reporting on fine needle aspirates of the breast". *Cytopathology*, 1994, *5*, 316.
[20] Nielsen K.V., Ejlertsen B., Møller S., Jørgensen J.T., Knoop A., Knudsen H., Mouridsen H.T.: "The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCG trial 89D". *Acta Oncol*., 2008, *47*, 725.
[21] Genestie C., Zafrani B., Asselain B., Fourquet A., Rozan S., Validire P. *et al*.: "Comparison of the prognostic value of Scarff-Bloom-Richardson and Nottingham histological grades in a series of 825 cases of breast cancer: major importance of the mitotic count as a component of both grading systems". *Anticancer Res*., 1998, *18*, 571.
[22] Miyoshi Y., Kurosumi M., Kurebayashi J., Matsuura N., Takahashi M., Tokunaga E. *et al*.: "Topoisomerase IIalpha-positive and BRCA1-negative phenotype: association with favorable response to epirubicin-based regimens for human breast cancers" Collaborative Study Group of Scientific Research of the Japanese Breast Cancer Society. *Cancer Lett*., 2008, 8, *264*, 44.
[23] Fritz P., Cabrera C.M., Dippon J., Gerteis A., Simon W., Aulitzky W.E., van der Kuip: "H.c-erbB2 and topoisomerase IIalpha protein expression independently predict poor survival in primary human breast cancer: a retrospective study". *Breast. Cancer Res*., 2005, *7*, R374.
[24] Pritchard K.I.: "Are HER2 and TOP2A useful as prognostic or predictive biomarkers for anthracycline-based adjuvant chemotherapy for breast cancer?". *J. Clin. Oncol*., 2009, *27*, 3875.
[25] Jacobs J.J., and van Lohuizen M.: "Polycomb repression: from cellular memory to cellular proliferation and cancer". *Biochim. Biophys. Acta*, 2002, *1602*, 151.
[26] Raaphorst F.M., Meijer C.J., Fieret E., Blokzijl T., Mommers E., Buerger H., Packeisen J., Sewalt R.A., Otte A.P., van Diest P.J.: "Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene". *Neoplasia*, 2003, *5*, 481.
[27] Collett K., Eide G.E., Arnes J., Stefansson I.M., Eide J., Braaten A. *et al*.: "Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer". *Clin. Cancer Res*., 2006, *12*, 1168.
[28] Pietersen A.M., Horlings H.M., Hauptmann M., Langerød A., Ajouaou A., Cornelissen-Steijger P. *et al*.: "EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer". *Breast. Cancer Res*., 2008, *10*, R109.
[29] Tang X., Milyavsky M., Shats I., Erez N., Goldfinger N., Rotter V.: "Activated p53 suppresses the histone methyltransferase EZH2 gene". *Oncogene*, 2004, *23*, 5759.
[30] Turner C.E., Glenney J.R. Jr, Burridge K.: "Paxillin: A new vinculin-binding protein present in focal adhesions". *J. Cell. Biol*., 1990, *111*, 1059.
[31] Ishino K., Kaneyama, Shibanuma M., Nose K.: "Specific decrease in the level of Hic-5, a focal adhesion protein, during immortalization of mouse embryonic fibroblasts, and its association with focal adhesion kinase". *J. Cell. Biochem*., 2000, *76*, 411.
[32] Lipsky B.P., Beals C.R., Staunton D.E.: "Leupaxin is a novel LIM domain protein that forms a complex with PYK2". *J. Biol. Chem*., 1998, *273*, 11709.
[33] Brown M.C., Curtis M.S., Turner C.E., Paxillin L.D.: "Motifs may define a new family of protein recognition domains". *Nat. Struct. Biol*., 1998, *5*, 677.
[34] Brown M.C., Perrotta J.A., Turner C.E.: "Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding". *J. Cell. Biol*., 1996, *135*, 1109.
[35] Schaller M.D.: "FAK and paxillin: regulators of N-cadherin adhesion and inhibitors of cell migration?". *J. Cell. Biol*., 2004, *166*, 157.
[36] Hagel M., George E.L., Kim A., Tamimi R., Opitz S.L., Turner C.E. *et al*.: "The adaptor protein paxillin is essential fornormal development in the mouse and is a critical transducer of fibronectin signaling". *Mol. Cell. Biol*., 2002, *22*, 901.
[37] Yano H., Uchida H., Iwasaki T., Mukai M., Akedo H., Nakamura K. *et al*.: "Paxillin a and crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation". *Proc. Natl. Acad. Sci*., USA, 2000, *97*, 9076.
[38] Vadlamudi R., Adam L., Tseng B., Costa L., Kumar R.: "Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells". *Cancer Res*., 1999, *59*, 2843.
[39] Hicks D., Yoder B., Short S., Tso E., Choueiri T., Budd G. *et al*.: "The expression of the focal adhesion protein paxillin in breast cancer correlates with HER2 amplification and may help predict a better response to chemotherapy". *Mod. Pathol*., 2005, (suppl. 1), 36A.
[40] Madan R., Smolkin M.B., Cocker R., Fayyad R., Oktay M.H.: "Focal adhesion proteins as markers of malignant transformation and prognostic indicators in breast carcinoma". *Hum. Pathol*., 2006, *37*, 9.

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