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Expression of p16 and SATB1 in Invasive Ductal Breast Cancer – A Preliminary Study

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Abstract. *Background/Aim: An impaired cell-cycle control and genetic material organization are crucial elements of carcinogenesis. p16 is a tumor suppressor protein which decelerates promotion of the cells from G_1 to S phase, whereas special AT-rich sequence-binding protein 1 (SATB1) is a nuclear matrix protein that binds to specific regions of the DNA and ensures its proper organization and function. Increased levels of both markers are observed in various types of cancers. The aim of this study was to investigate the expression of p16 and SATB1 proteins in regard to expression of the Ki-67 antigen and available clinicopathological data (i.a. receptor status, staging and grading). Materials and Methods: The study was performed on 130 samples of archived invasive ductal breast cancers. Immunohistochemical reactions were performed on freshly prepared tissue microarrays and subsequently scanned by a histologic scanner. Reactions were evaluated separately in the cytoplasm (p16c, SATB1c) and nucleus (p16n, SATB1n, Ki-67) with use of a quantification software under researcher supervision. Results: Expression was observed for Ki-67 in 100%, p16c in 90%, p16n in 89.2%, SATB1c in 98.5% and SATB1n in 87.7% of cancer cases. Statistical analysis showed strong positive correlations: p16c vs. p16n and SATB1c vs. SATB1n ($p<0.001$ for both) and weak positive correlations: p16c vs. SATB1c and p16c vs. SATB1n ($p=0.008$, $p=0.027$; respectively). Expression of p16n was stronger in G_1 vs. G_2 ($p=0.034$) while Ki-67 expression was stronger in cases with negative progesterone receptor status ($p=0.011$). All other analyzed associations were statistically insignificant. Conclusion: A weak association between immunohistochemical expression of p16 and SATB1 indicated limited possibility of their independent usage. Further studies concerning determination of a wider panel of proteins controlling cell cycle should be considered.*

Malignant transformation of the cell is a complex process which originates from pathological gene expression. In many hypotheses, abnormalities associated with cell cycle regulation have been pointed. Transformation may be the result of the impaired function of two controlling points: p53 and retinoblastoma protein (mediated by p16). Simultaneously, structural alterations in chromatin organization can be observed. They may be considered as cell cycle dependent or independent events, resulting in gene expression dysregulation, *i.a.* by specific transcription factors involvement (1, 2).

p16 protein plays an important role in regulation of the cell cycle. It acts as a tumor suppressor by binding to cyclin-dependent kinases 4/6 and prevents interaction of cyclin D1 with retinoblastoma (Rb) protein. It ultimately inhibits the downstream activities of transcription factors, such as E2F1, and finally arrests cell proliferation. Hypermethylation, mutation, or deletion of p16 leads to downregulation of the gene which may increase the incidence of some malignancies, *i.a.* melanoma, oropharyngeal squamous cell carcinoma, cervical cancer, and esophageal cancer (3, 4). Currently, in gynecological screening tests, evaluation of p16 is performed to improve the histological diagnostic accuracy of cervical intraepithelial neoplasia grade 3. It is available as a commercial kit for combined evaluation of p16 and Ki-67 (CINtec; Ventana, Tucson, AZ, USA) (5).

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Special AT-rich sequence binding protein 1 (SATB1) is a genome organizer protein that facilitates various intracellular processes. It provides a nuclear architectural platform that binds hundreds of genes, through its interaction with specific genomic sequences. It allows parallel regulation of many genes expression enabling thereby cells to alter their function (6). SATB1 as a matrix attachment region binding protein regulates the genes by folding chromatin into loop domain (7). It was found to regulate gene expression in thymocytes and pre-B-cells but recent studies have shown that SATB1 promotes tumor growth and metastasis through chromatin gene recombination in many neoplasms such as breast, gastrointestinal tract (predominantly colorectal region), liver, laryngeal, lung, thyroid, urinary bladder, ovarian and prostate cancers, melanomas, osteosarcomas, gliomas or some leukemias and lymphomas (8-26). There is evidence that in some cancers, *e.g.* colorectal, depletion of SATB1 expression is associated with poor prognosis (12, 27) whereas in non-small cell lung cancer strong expression of SATB1 correlates with a better overall survival of patients (15). Moreover, in breast cancer it is suggested that combined evaluation of Ki67 and SATB1 as a ratio may be an independent prognostic factor of overall survival (28).

In order to understand the processes leading to malignant transformation in breast cancer, the expression of p16 and SATB1 protein in regard to expression of routinely performed proliferative marker Ki-67 and available clinicopathological data (*i.a.* receptor status, staging and grading) was investigated.

Materials and Methods

Patients. The material for the study consisted of 130 archived paraffin embedded samples of invasive ductal breast cancers. The patients aged 26-81 years old (median 57 years old) were of female sex. Relevant available clinicopathological data, *i.e.* receptor status, staging and grading are presented in Table I.

Tissue microarray (TMA) construction. Hematoxylin and eosin stained (HE) 6-μm thick paraffin sections were prepared to verify histopathological diagnosis and evaluate sample usefulness for further analyses. In brief, slides were scanned utilizing histologic scanner Pannoramic MIDI (3DHistech, Budapest, Hungary). Subsequently, scans were examined by two independent pathologists and areas of interest with potentially the highest tumor cell content from non-necrotic areas were marked electronically. Afterwards, for TMA construction, from the corresponding paraffin donor blocks, triplicate tissue core punches (2 mm) for every case were obtained (TMA Grand Master; 3DHistech).

Immunohistochemistry (IHC). Immunohistochemical reactions were performed on 4-μm paraffin sections obtained from TMA blocks mounted on Superfrost Plus slides (Menzel Gläser, Braunschweig, Germany). The sections were dewaxed, re-hydrated and the epitopes were exposed using Pre-Treatment Link Rinse Station and Target Retrieval Solution (pH 6 for Ki-67; pH 9 for p16, SATB1; 97˚C, 20 min) (Dako, Glostrup, Denmark). Activity of endogenous peroxidase was blocked by 5 min exposure to Peroxidase-Blocking Reagent (Dako). The sections were then rinsed with Wash Buffer and incubated for 20 min at room temperature with the following primary antibodies against Ki-67 (MIB-1; Ready-to-use; Dako), p16 (G175-405; 1:100+linker; BP Pharmingen, CA, USA), and SATB1 (EPR3951; 1:100; GeneTex, Hsinchu, Taiwan). Secondary goat anti-mouse and anti-rabbit antibodies coupled to a dextran core, linked to horseradish peroxidase, were applied and subsequent visualization was performed using the EnVision™ FLEX+ system (Dako) according to the manufacturer's instructions. All IHC reactions were performed in an automated staining platform, Autostainer Link48 (Dako). The reactions were visualized using 3,3'-diaminobenzidine tetrachlorohydrate (DAB+ chromogen). All slides were counterstained with Mayer's hematoxylin.

Table I. *Clinicopathological features of patients with invasive ductal breast cancer.*

Feature	N	%
Grade		
G1	12	9.23
G2	77	59.23
G3	41	31.54
TNM		
pT1-2	125	96.15
pT3-4	5	3.85
pN0	77	59.23
pN1-3	53	40.77
Stage		
I-IIB	120	92.30
IIIA-IV	10	7.70
Receptor status		
ER (+)	83/130	63.85
PgR (+)	76/130	58.46
HER-2 (+)	13/70	18.58

Evaluation of IHC reaction. The slides were scanned utilizing histologic scanner Pannoramic MIDI (3DHistech). Reactions were evaluated separately in cytoplasm (p16c, SATB1c) and nucleus (p16n, SATB1n, Ki-67) with usage of Quant Center Software (3DHistech) under researcher supervision. For every case three TMA cores were quantified by the algorithm SCORE (range=0-8) and the final result was an average count.

Statistical analysis. The results were subjected to statistical analysis using the Prism 5.0 software (GraphPad, La Jolla, CA, USA) utilizing Kolmogorov-Smirnov, Spearman's rank correlation, Kruskal–Wallis, Mann–Whitney and Wilcoxon tests. Kaplan–Maier curves were performed. In all analyses, results were considered to be statistically significant for $p<0.05$.

Results

Expression was observed for Ki-67 in 100%, p16c in 90%, p16n in 89,2%, SATB1c in 98.5% and SATB1n in 87.7% of cancer cases. Analysis of Ki-67, p16 and SATB1 expression was conducted on TMA serial sections (Figure 1A-F). Statistical analysis showed strong positive correlations: p16c *vs.* p16n and SATB1c *vs.* SATB1n (r=0.715, r=0.759;

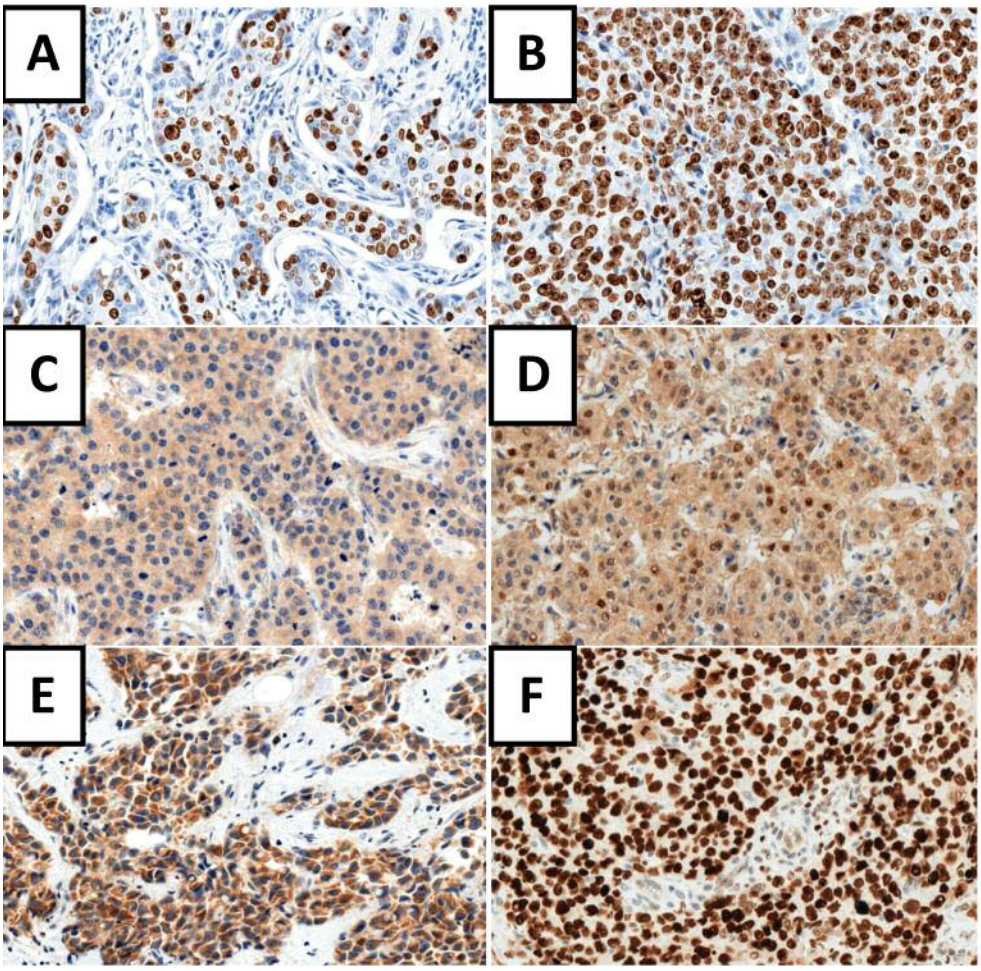

Figure 1. *Immunohistochemical expression of Ki-67 (weak, A; strong, B), p16c (cytoplasmic, C), p16n (nuclear, D), SATB1c (cytoplasmic, E), and (nuclear, F). Magnification ×200.*

respectively and $p<0.001$ for both) and weak positive correlation: p16c *vs*. SATB1c (r=0.220, $p=0.008$) and p16c *vs*. SATB1n (r=0.185, $p=0.027$) (Spearman's rank correlation test, Table II). Expression of p16n was stronger in G_1 *vs*. G_2 ($p=0.034$) and Ki-67 expression was stronger in cases with negative progesterone receptor status ($p=0.011$, Mann-Whitney test). No association between expression of studied markers and staging or TNM scale was found. Moreover, separate and combined survival analyses in regard to p16c, p16n, SATB1c and SATB1n expression disclosed statistically insignificant results (Figure 2).

Table II. *Spearman correlation test results.*

	Ki-67	p16n	p16c	SATB1n	SATB1c
Ki-67		NS	NS	NS	NS
p16n			r=0.715 $p<0.001$	NS	NS
p16c				r=0.185 $p=0.027$	r=0.220 $p=0.008$
SATB1n					r=0.759 $p<0.001$
SATB1c					

Discussion

The p16 tumor suppressor gene (*CDKN2A*) located on chromosome 9p21 is a member of the INK4 class of cell cycle inhibitors. The p16 protein binds to cyclin-dependent kinases 4/6 what prevents formation of active complex with cyclin D1. This blocks phosphorylation of Rb protein hindering dissociation of E2F transcription factor and finally stopping transition from G1 to S phase (29, 30). Expression of p16 protein is increased in aging cells inducing apoptosis

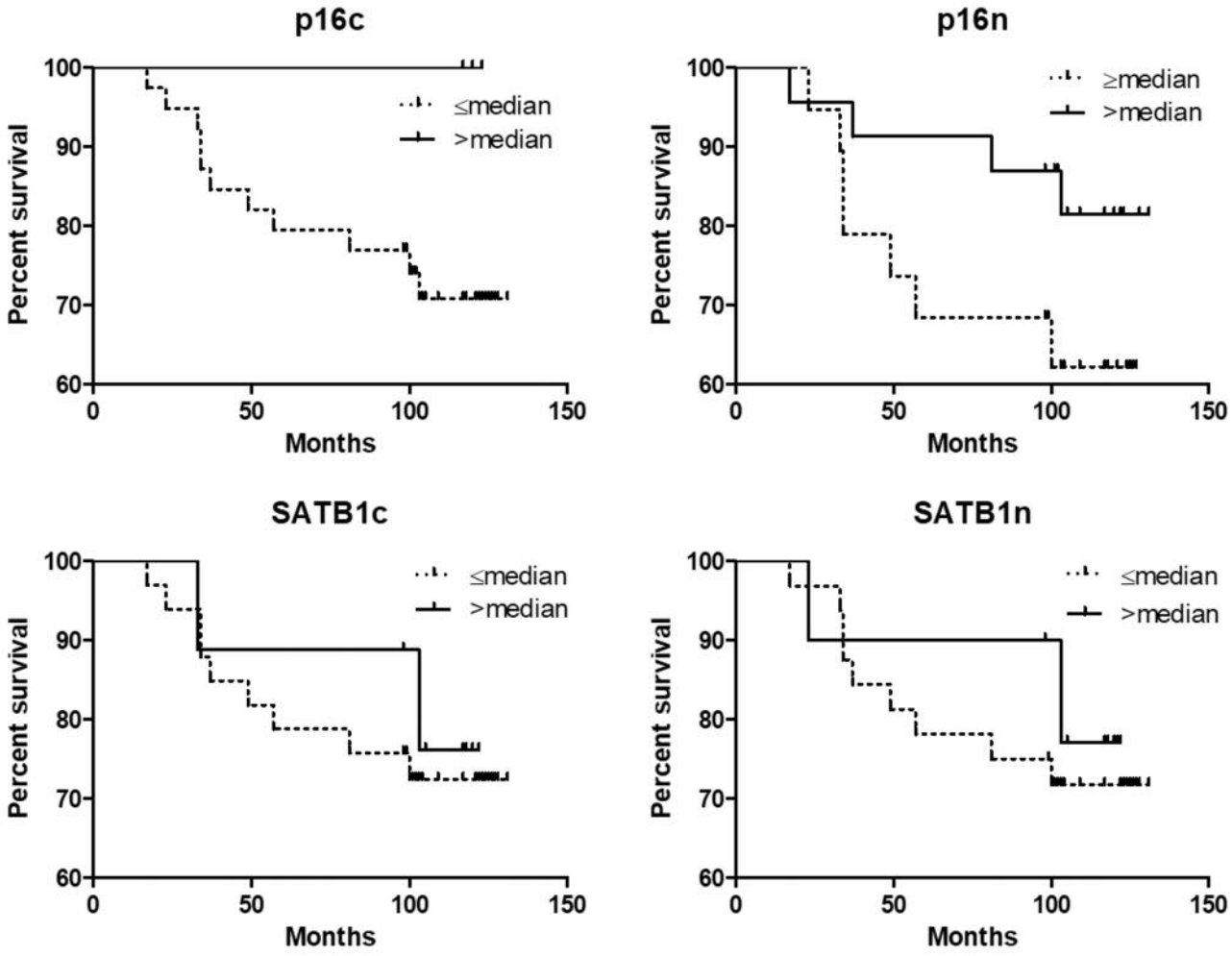

Figure 2. *Kaplan–Meier curves for studied markers – p16 and SATB1.*

process. Similar mechanism was disclosed in metaplastic and atrophic cells (30, 31). There is evidence that in non–HPV-related malignant neoplasms, *i.e.* breast, pancreas, colon tumors as well head and neck carcinomas are related to smoking, p16 function is lost by gene deletions, mutations, or epigenetic silencing. This results in negative IHC findings in the abovementioned cancers (3, 29-31). On the other hand, IHC expression of p16 is routinely performed in gynecological diagnostics, mostly in cervical dysplasia screening, combined with Ki-67 evaluation using commercial kits, *e.g.* CINtec (4). The disruption of the p16-cyclin D1-CDK4/6-Rb pathway is pointed in many human cancers, however its significance in breast carcinogenesis is still controversial. In the present study correlation between p16c and p16n was strong (r=0.715, $p<0.001$). However, no association with other studied markers, *i.e.* Ki-67 and SATB1 was found. Expression of p16n was stronger in G_1 *vs.* G_2 ($p=0.034$). It indirectly stays in line with observation by Peurala *et al.* who postulated that p16 expression correlates with a better prognosis and may function independently (without CDK4) in human breast cancer (32). However, there is also evidence that p16 promotes growth and mobility potential of breast cancer by activation IL-6/JAK2/STAT3 pathway (33). We performed also separate survival analysis for p16c and p16n expression as well in combination with Ki-67 expression. Results were statistically insignificant. There are some speculations about potential of 9p21 locus as a therapeutic target and prognostic marker in breast cancer, *i.a.* due to hypermethylation of the p16 gene in malignancies (34). Currently, studies are ongoing.

SATB1 (special AT-rich sequence-binding protein-1) located on chromosome 3p24 is a global chromatin organizer and transcription factor, mostly responsible for higher-order chromatin architecture and gene regulation (35,

36). Aberrant expression of SATB1 has been shown to promote growth and metastasis of various neoplasms (8-26). The expression of SATB1 simultaneously increases with the progression of cancers and it dynamically reprograms the expression of genes that are involved in carcinogenesis (36). It determines specific epigenetic modifications at target gene loci, directly up-regulating metastasis-associated genes while down-regulating tumor-suppressor genes. As SATB1 reprogrammes chromatin organization and the transcription profiles to promote growth and metastasis in breast cancer, it is presumed that mutation of *SATB1* gene might affect not only cell cycle progression but also apoptosis pathway in breast cancer (37). In this study, the association between SATB1 protein expression with routinely performed marker of proliferation – Ki-67 antigen as well one of very important controller of cell cycle – p16 was investigated. Similarly to p16, SATB1 expression was analyzed in cytoplasm and nucleus separately. Except of the strong correlation between SATB1c and SATB1n no statistically significant associations were found. This stays in line with results of our previous study (38) as well as of the study by Laurinavicius *et al*. (39). They analyzed a set of 10 IHC markers – ER, PR, HER2, Ki-67, AR, BCL2, HIF-1α, SATB1, p53, and p16 in invasive ductal breast cancer. They observed insignificant associations between SATB1 and p16 in regard to other tested markers, except HIF-1α. They speculated that SATB1 and HIF-1α may be important markers of estrogen–positive cancers, whereas their biological and clinical significance remains to be elucidated (39). In our previous study we analyzed expression of SATB1 and Ki-67 in regard to receptor status. We found moderate correlation between mentioned markers (r=0.392, p=0.032) only in estrogen–negative tumors (38). Nevertheless, in the present study no associations with receptor status and the studied markers were disclosed. Moreover, Laurinavicius *et al*. suggested that in breast cancer combined evaluation of Ki67/SATB1 ratio may be an independent prognostic factor of overall survival (28). We also performed separate survival analysis for SATB1c and SATB1n expression as well in combination with Ki-67 expression. Results were statistically insignificant.

Conclusion

Both studied proteins are present in diagnostic pathology. Their use in screening, differential diagnostics and prognostic value in breast cancer is still unknown. A weak association between immunohistochemical expression of p16 and SATB1 indicates limited possibility of their independent usage. In regard to received results and literature data, further studies concerning the determination of a wider panel of proteins controlling cell cycle should be considered, probably in combination.

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