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RT-PCR ANALYSIS OF TOPBP1 GENE EXPRESSION IN HEREDITARY BREAST CANCER

Abstract: Hereditary predisposition to breast cancer determined in large part by loss of function mutations in one of two genes *BRCA1* and *BRCA2*. Besides BRCA1 and BRCA2 other genes are also likely to be involved in hereditary predisposition to breast cancer. TopBP1 protein is involved in DNA replication, DNA damage checkpoint response and transcriptional regulation. Expression of *TopBP1* gene at the mRNA level was analyzed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in 94 samples of hereditary breast cancer. Analysis of *TopBP*1 mRNA level showed that expression of *TopBP1* is significantly downregulated in poorly differentiated breast cancer (grade III according Bloom-Richardson system ($P<0.05$).

Key words: TopBP1, gene expression, RT-PCR, hereditary breast cancer

1. INTRODUCTION

Hereditary breast and ovarian cancer syndrome is an inherited cancer susceptibility syndrome. The hallmarks of this syndrome are multiple family members with breast or/and ovarian cancer, the presence of both breast and ovarian cancer in a single individual, and early age of breast cancer onset (LU *et al.* 2009).

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Breast and ovarian cancers are among the most common malignancies of women in Western countries. About 5 – 10% of the cases are considered familial, and 40 – 50% of them can currently be explained by mutations in two main susceptibility genes, *BRCA1* and *BRCA2*. Of the remaining cases no more than 5% are caused by defects in other studied genes, such as *TP53*, *PTEN*, *ATM* and *CHK2* (KARPPINEN *et al.* 2006; EASTON 1999). TopBP1 (topoisomerase IIβ binding protein 1) displays structural as well as functional similarities with BRCA1, and both proteins have been suggested to function partly in the same cellular processes (KARPPINEN *et al.* 2006). Based on its biological significance KARPPINEN *et al.* (2006) suggested that TopBP1 is a plausible susceptibility gene for hereditary breast and/or ovarian cancer. Aberrant expression of *TopBP1* may be involved in the deregulation of processes controlled by this protein and have pathological consequences. The aim of this study was to investigate the expression of *TopBP1* at the mRNA level in hereditary breast cancer.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples of 94 hereditary breast cancers were obtained from patients (age range 28 – 67 years) undergoing surgery for breast neoplasms in Polish Mather's Memorial Hospital, Poland. The inclusion criteria were: (1) at least one first-degree relative with breast cancer, regardless of age, or (2) breast cancer diagnosed below 40 years of age. None of the patient received neoadjuvant endocrine therapy, chemotherapy and radiotherapy. The pathological evaluation report was obtained for each patient (Table 1). Immediately after resection, samples of breast cancer tissue were fixed in 10% neutral buffered formalin and embedded in paraffin blocks following standard histological protocols. For our studies tissue sections were cut from the blocks with a microtome blade, extra paraffin was removed, and tissue sections was placed in a 1.5-ml microcentrifuge tube.

Table 1. Characteristics of patients and tumor samples

Characteristics	Number of patients
Age at diagnosis	
range	28 - 67
mean ± SD	55.3 ± 8.5
Type of cancer	
ductal carcinoma	79
lobular carcinoma	13
tubular carcinoma	2
Tumor grade according to Bloom-Richardson system	
I	9
II	64
III	21
Lymph node metastasis	
No	52
Yes	42
Menopausal status	
premenopausal	55
postmenopausal	39
ER status	
Negative	46
Positive	48
PR status	
Negative	42
Positive	52

2.2. Total RNA extraction and cDNA synthesis

Xylene deparaffinization: Sections were deparaffinized by two rinses in xylene for 10 min at room temperature with shaking, followed by centrifugations at

room temperature for 5 min at 12,000g. After deparaffinization, we introduced a rehydratation step (rinsing in 100% ethanol, 85%, 70% ethanol, all prepared with DEPC treated dH_2O, for 5 min). The tissue was collected by centrifugation at 12,000g for 5 min. After the final wash, alcohol was aspirated and the tissue pellets were resuspended in 500 µl of digestion buffer (10 mM NaCl, 500 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1% SDS) and 1 mg/ml proteinase K was added. Sections were incubated at 45°C overnight. Prior to RNA purification, in same samples we inactivated proteinase K at 97°C for 10 min. The digested samples were extracted using TRI Reagent (Sigma Aldrich, USA) according to manufacturer's protocol. RNA was eluted in 20 µl RNase-free water, quantified by spectrophotometry at 260 nm and stored at -20°C. RNA with a 260/280 nm ratio in range 1.8 – 2.0 was considered high quality. First-strand cDNA was synthesized from each RNA pool using PCR Kit ver. 3.0 (Takara Bio Inc. Japan) according to the manufacturer's instructions. Briefly, 1 µg RNA was combined with 2.5 pmol of oligo dT-adapter primer, 4 µl of 25 mM $MgCl_2$, 2 µl 10 x RNA PCR buffer, 2 µl of 10 mM dNTP mixture, 20 units of RNase inhibitor, 5 units of AMV Reverse Transcriptase XL, and RNase-free water to total volume of 20 µl. The reaction took place at 42°C for 30 min, followed by 95°C for 5 min and 5°C for 5 min in a GeneAmp PCR System 9700 (Perkin-Elmer Co, USA). cDNA was stored at -20°C.

2.3. RT-PCR

One microliters of cDNA sample was used as a substrate for PCR reaction in a 20 µl volume with 1 µM of forward and reverse primers, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.0 µl 10 x PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) and 1 units Taq polymerase (Takara Bio Inc., Japan). Specific oligonucleotide primers for *TopBP1* were as follows F: 5'GCTTCATCGCTCCTACCTTG3', R: 5'TTCCACCCACTAAATGCTCC3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with primer sequences F: 5'GAAGGTGAAGGTCGGACTC3', R: 5'GAAGATGGTGATGGGATTTC 3' was used as an endogenous control for the PCR amplifications. PCR reaction was carried out as follows: initial denaturation of 4 min at 94°C) followed by 40 cycles of 1 min at 94°C, 30 s at 53°C for *TopBP1* and

60°C for *GAPDH* and 30 s at 72°C, final extension step of 10 min at 72°C. Negative control and already amplified cDNA were included in all the PCR amplifications. The size of amplified fragments was 210 and 225 bp for *TopBP1* and *GAPDH*, respectively. After amplification 10 µl of PCR products were combined with 2 µl gel loading buffer and the mixture was separated on a 8% polyacrylamide gel. The gel was silver stained. For qualitative and quantitative analysis of silver nitrate stained gels video densitometr (Biotec-Fischer, Germany) with the software program Gel-Pro® Analyzer 3.0 (Media Cybemetics, USA) was used. All RT-PCR reactions were repeated 2 times for each samples. The integrated optical density (IOD) of the bands in a digitalized picture was measured. *TopBP1* gene expression was determined as the ratio of *TopBP1* to *GAPDH*.

2.4. Statistical analysis

All data are presented as mean ± SEM. Because results obtained from RT-PCR were not normally distributed (Kolmogorov-Smirnov test), therefore nonparametric Mann-Whitney U-test (for two categories) and the Kruskal-Wallis test with post hoc multiple comparisons (for three categories) were used. Statistical significance was designated at $P<0.05$.

3. RESULTS

The expression of *TopBP1* in hereditary breast cancer was estimated at the mRNA level by semiquantitative RT-PCR analysis with *GAPDH* applied as a reference gene. Representative electropherogram of *TopBP1* mRNA expression in hereditary breast cancer with *GAPDH* mRNA as standard is presented in Fig. 1. Expression of *TopBP1* gene at the mRNA level was observed in 81 of 94 (86.2%) hereditary breast cancer samples. The comparison of transcript level of *TopBP1* gene with clinicopathological parameters of tumors is shown in Table 2, where ratio of integrated optical density *TopBP1* to *GAPDH* representing the mean level of mRNA, are used for statistical analysis. Analysis of *TopBP1* mRNA level showed significantly lower expression of *TopBP1* in the poorly differentiated hereditary breast cancer (grade III according to Bloom-Richardson scale) in comparison with

moderately and well-differentiated cancer (P<0.05). No statistically significant differences occured between *TopBP1* mRNA level in grade I and II tumors (Fig. 2).

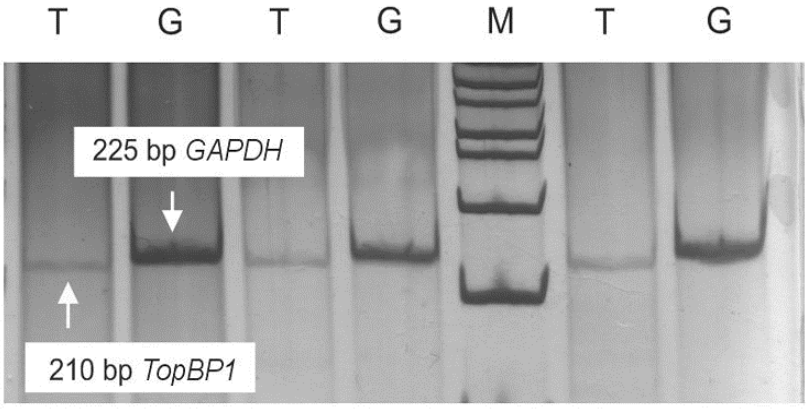

Fig. 1. Representative electropherogram of *TopBP1* mRNA expression in hereditary breast cancers with *GAPDH* mRNA as standard (M molecular weight markers). Amplification products were separated on the 8% polyacrylamide gel. T – *TopBP1*; G – *GAPDH*; M – molecular weight markers.

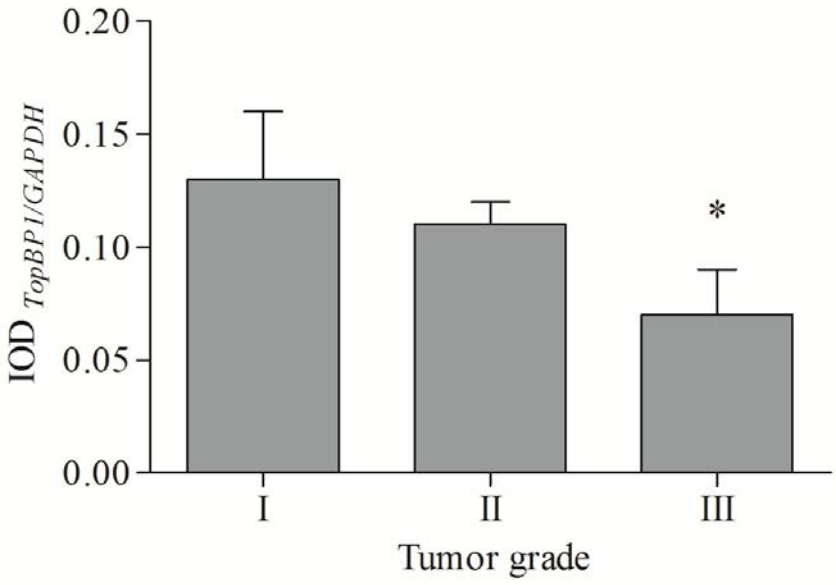

Fig. 2. Expression of *TopBP1* mRNA in hereditary breast cancers in relation to tumor grade. Asterisk indicates significant differences at P<0.05.

There were no significant associations between level of TopBP1 mRNA expression and other clinicopathological parameters, such as estrogen and progesterone receptor status, appearance of metastasis in the axillary lymph nodes and type of cancer.

Table 2. Comparison of the transcript levels of *TopBP1* gene with clinicopathological parameters of the tumors

Clinicopathological features (N)	Semiquantitative RT-PCR ($IOD_{TopBP1/GAPDH}$)	P
Type of cancer		
ductal carcinoma (79)	0.14 ± 0.01	
lobular carcinoma (13)	0.12 ± 0.02	0.45
Tumor grade		
I (9)	0.13 ± 0.03	
II (64)	0.11 ± 0.01	
III (21)	0.07 ± 0.02	0.60
Lymph node status		
No (52)	0.13 ± 0.01	
Yes (42)	0.11 ± 0.01	0.67
ER status		
Negative (46)	0.12 ± 0.01	
Positive (48)	0.10 ± 0.01	0.64
PR status		
Negative (42)	0.11 ± 0.01	
Positive (52)	0.13 ± 0.01	0.20

4. DISCUSSION

TopBP1 protein plays a key role in various aspects of DNA metabolism. It is required for the initiation of DNA replication, the maintenance of DNA replication forks when replication is stalled and for DNA damage signaling and checkpoints

(JEON *et al*. 2007; KIM *et al*., 2005; KUMAGAI *et al*. 2006; LIU *et al* 2006; MORISHIMA *et al*. 2007; SCHMIDT *et al* 2008; YAMMANE *et al.* 2003). TopBP1 is also involved in the process of mitosis in somatic cells and during meiotic recombination in germ cells (PERERA *et al.* 2004; REINI *et al.* 2004). Most of TopBP1 does not colocalize at site of ongoing DNA replication in irradiated cells but is relocalized to stalled replication forks upon DNA damage. The involvement of TopBP1 in DNA replication is supported by the demonstration that incubation of an antibody against the sixth BRCT motif of TopBP1 inhibits replicative DNA synthesis in a in vitro HeLa cell nucleus replication assay (KIM *et al.* 2004; MAKINEMI *et al*. 2001). TopBP1 can interact with human polymerase ε, checkpoint protein Rad9, Miz-1, E2F1, human papilliomavirus type 16 (HPV16) transcription/replication factor E2 (BONER *et al*. 2002; DONALDSON *et al.* 2007; DELACROIX *et al.* 2007; HEROLD *et al.* 2002; LIU *et al.* 2003, 2004, 2006; MAKINIEMI *et al.* 2001) . TopBP1 also participates in ATR activation in ATRIP-dependent manner (BURROWS, ELLEDGE 2008; CIMPRICH, CORTEZ 2008; KUMAGAI *et al.* 2006; LEE *et al.* 2007). In addition to control DNA replication, TopBP1 is also required for cell survival. Inhibition of TopBP1 expression induces apoptosis. TopBP1 is involved in several important aspects of regulation cell growth (LIU *et al.* 2003; YAMANE *et al.* 2002). The aim of this study was investigate the transcript level of *TopBP1* gene. Analysis of *TopBP1* expression shown that tumor progression is accompanied by a decrease of *TopBP1* mRNA level. Expression of TopBP1 is regulated by Rb/E2F1 and is induced when cells enter into S phase (LIU *et al.* 2004, 2009; YOSHIDA, INOUE 2004). Therefore, disruption of Rb/E2F1 pathway can lead to overexpression of TopBP1 protein in breast cancers. In the other hand, TopBP1 regulates activity of E2F1 and overexpression of TopBP1 suppressed E2F1 transcriptional activity (LIU *et al.* 2003, 2004). Thus, decreased transcriptional activity of E2F1 by TopBP1 can repress the expression of E2F1 target genes, including *TopBP1*. In the other hand, downregulation of *TopBP1* mRNA expression may be caused by aberration expression of E2F1. Many studies have found that expression of E2F1 protein in breast cancer tended to decreased as the grade

increase (HO *et al.* 2001; KWON *et al.* 2009). However, regulation of *TopBP*1 expression will need to be studied.

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