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SHORT COMMUNICATION

# *TIMP3* Promoter Methylation Represents an Epigenetic Marker of BRCA1ness Breast Cancer Tumours

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**Abstract**
Tumours presenting BRCAness profile behave more aggressively and are more invasive as a consequence of their complex genetic and epigenetic alterations, caused by impaired fidelity of the DNA repair processes. Methylation of promoter CpG islands represents an alternative mechanism to inactivate DNA repair and tumour suppressor genes. In our study, we analyzed the frequency of methylation changes of 24 tumour suppressor genes and explored their association with BRCAness profile. BRCA1ness profile and aberrant methylation were studied in 233 fresh frozen breast tumour tissues by Multiplex Ligation-dependent Probe Amplification (MLPA) and Methylation Specific (MS)-MLPA methods, respectively. Our analyses revealed that 12.4% of the breast cancer (BC) patients had tumours with a BRCA1ness profile. *TIMP3* showed significantly higher ($p = 5.8x10^{-5}$) methylation frequency in tumours with BRCA1ness, while methylation of *APC*, *GSTP1* and *RASSF1* promoters was negatively associated with BRCA1ness (p = 0.0017, p = 0.007 and p = 0.046, respectively). *TIMP3* methylation was also associated with triple negative (TN) BC. Furthermore, TN tumours showing BRCA1ness showed stronger association with *TIMP3* methylation ($p = 0.0008$) in comparison to TN tumours without BRCA1ness ($p = 0.009$). In conclusion, we confirmed that *TIMP3* methylation is a marker for TN tumours and furthermore we showed for the first time that *TIMP3* promoter methylation is an epigenetic marker of BRCA1ness tumours.

**Keywords** *TIMP3* · BRCA1ness · MS-MLPA · Breast cancer

## Introduction

The BRCAness profile is defined as a phenotypic trait that some sporadic tumours share with BRCA hereditary tumours. Recently, it has been described as a condition in which a homologous recombination repair (HRR) defect exists in a tumour in absence of a *BRCA1/2* germline mutation [1]. Tumours harbouring BRCAness profile generally behave more aggressively: they are dividing rapidly and are poorly differentiated [2]. The aggressive behaviour is one of their main hallmarks as a consequence of complex genetic and epigenetic alterations caused by impaired fidelity of DNA repair processes. In addition to DNA mutations and loss of heterozygosity (LOH), methylation of promoter CpG islands represents an alternative mechanism to inactivate DNA repair and tumour suppressor genes, transcription factors and cell cycle regulators [3]. Up to date, a few studies have investigated the aberrant DNA methylation profile in tumours with BRCAness phenotype. Therefore, the aim of our study was to characterize the frequency of methylation changes of selected panel of tumour suppressor genes and to explore their association with BRCAness profile.

## Materials and Methods

We analysed 233 fresh frozen tumours from unselected patients with invasive breast cancer (BC) obtained from the Clinical Hospital Acibadem Sistina. Informed written consent was obtained from each patient and the study was conducted according to the Declaration of Helsinki and was approved by the Ethical Committee of the Macedonian Academy of Sciences and Arts. Macroscopic tissue dissection was performed by a pathologist

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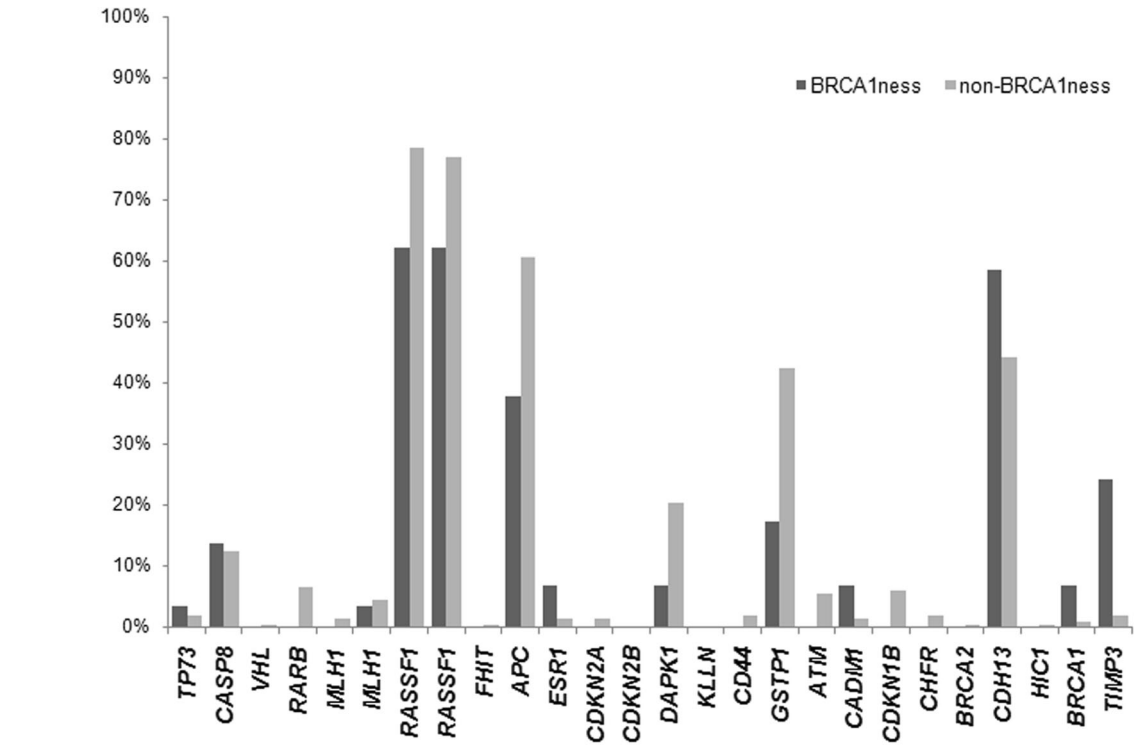
to ensure high tumour content (>80% of tumour cells in a sample). The frozen tissues were pulverized with liquid nitrogen and DNA was isolated using standard phenol-chloroform isolation procedure. BRCA1ness classification was done by Multiplex Ligation-dependent Probe Amplification (MLPA) method using P376 BRCA1ness probemix (MRC-Holland, Amsterdam, the Netherlands), as described in the literature [4] and data analysis was performed using Coffalyser.NET software (MRC-Holland) [5]. The relative copy number ratios from Coffalyser.NET for all target-specific probes were used in the prediction analysis for microarrays (PAM) in R statistical software. Samples with BRCA1ness scores ≥0.5 were classified as BRCA1ness. Aberrant methylation of promoter regions of 24 different tumour suppressor genes was assessed by Methylation Specific (MS)-MLPA method [6] using ME001-C1 Tumour suppressor-1 probemix containing HhaI-sensitive probes targeting *TIMP3, APC, CDKN2A, MLH1, ATM, RARB, CDKN2B, HIC1, CHFR, BRCA1, CASP8, CDKN1B, PTEN, BRCA2, CD44, RASSF1, DAPK1, VHL, ESR1, TP73, FHIT, IGSF4, CDH13*and *GSTP1* gene promoters. The data analysis was performed with Coffalyser.NET software. We considered promoter methylated if the methylation dosage ratio was ≥0.10, which corresponds to 10% of methylated DNA. Statistics were performed using SPSS ver19 software and chi-square or Fisher's exact tests were applied to compare the proportions of methylation of the studied genes between the BRCA1ness and non-BRCA1ness groups.

## Results

Our analyses revealed that 29 out of 233 or 12.4% of the BC patients had tumours with a BRCA1ness profile and 53.6% (15/28, data was not available for one patient) BRCA1ness tumours had triple negative (TN) receptor status. Average number of methylated genes per patient was 3.1 ± 1.6 *SD* for the BRCA1ness group and 3.8 ± 2.0 *SD* for the non-BRCA1ness group. Twelve genes (*VHL, RARB, FHIT, CDKN2A, CDKN2B, KLLN, CD44, ATM, CDKN1B, CHFR, BRCA2* and *HIC1*) did not show promoter methylation in the BRCA1ness group, while only two genes (*CDKN2B* and *KLLN*) were found to be unmethylated among the non-BRCA1ness group. Four of the analyzed genes showed higher methylation frequency in BRCA1ness tumours compared to non-BRCA1ness ones: *ESR1* (6.9% in BRCA1ness tumours vs 1.5% non-BRCA1ness tumours), *CADM1* (6.9% vs 1.5%), *BRCA1* (6.9% vs 1%) and *TIMP3* (24.1% vs 2%) (Fig. 1). Among them, only*TIMP3* showed significantly higher ($p = 5.8 \times 10^{-5}$) methylation frequency in tumours with BRCA1ness profile. Moreover, TN tumours with




| | TP73 | CASP8 | VHL | RARB | MLH1 | MLH1 | RASSF1 | RASSF1 | FHIT | APC | ESR1 | CDKN2A | CDKN2B | DAPK1 | KLLN | CD44 | GSTP1 | ATM | CADM1 | CDKN1B | CHFR | BRCA2 | CDH13 | HIC1 | BRCA1 | TIMP3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BRCA1ness (%) | 3.4 | 13.8 | 0 | 0 | 0 | 3.4 | 62.1 | 62.1 | 0 | 37.9 | 6.9 | 0 | 0 | 6.9 | 0 | 0 | 17.2 | 0 | 6.9 | 0 | 0 | 0 | 58.6 | 0 | 6.9 | 24.1 |
| non-BRCA1ness (%) | 2 | 12.4 | 0.5 | 6.5 | 1.5 | 4.5 | 78.6 | 77.1 | 0 | 60.7 | 1.5 | 1.5 | 0 | 20.4 | 0 | 2 | 42.3 | 5.5 | 1.5 | 6 | 2 | 0.5 | 44.3 | 0.5 | 1 | 2 |

**Fig. 1** Methylation frequency of 24 tumour suppressor genes in BRCA1ness and non-BRCA1ness tumours

BRCA1ness profile showed stronger association with *TIMP3* methylation ($p = 0.0008$) in comparison to TN tumours without BRCA1ness profile ($p = 0.009$). Furthermore, we observed that aberrant methylation of *APC*, *GSTP1* and *RASSF1* promoters was negatively associated with BRCA1ness profile ($p = 0.0017$, $p = 0.007$ and $p = 0.046$, respectively).

## Discussion

Changes in the genes' promoter methylation during tumour progression have been described in several breast tumour subtypes, but only a small number of studies have investigated promoter methylation alterations in tumours with BRCA1ness phenotype. Our study revealed that four (*ESR1*, *CADM1*, *BRCA1* and *TIMP3*) of the 24 genes have higher methylation frequency among the BRCA1ness group. However, only *TIMP3* showed significant association with tumours presenting BRCA1ness profile. Tissue inhibitors of metalloproteinase (TIMPs) are natural inhibitors of matrix metalloproteinases (MMPs) and the balance between them is important for maintaining the integrity of the extracellular matrix. They are involved in diverse biological processes such as cell growth, tumour progression, apoptosis, invasion, metastasis and angiogenesis [7]. In breast cancer cells, *TIMP3* repression has been linked to promoter DNA methylation, which has been reported in approximately 20–27% of the breast carcinomas [8, 9]. In our study *TIMP3* promoter methylation was observed in 5% of the studied breast tumours (with and without BRCA1ness profile), contrary to the higher frequencies reported in the previous studies. Low levels of TIMP3 protein expression have been correlated with an aggressive breast tumour phenotype (high tumour grading and lymph node metastasis) and poor disease-free survival [8–10]. Those features are typically related to TN and basal-like breast tumours, as well as tumours harbouring BRCA1ness phenotype. Additionally, *TIMP3* methylation was observed more frequently in TN BCs and was found to be associated with estrogen negative breast tumours [11, 12]. In our study, we found that TN tumours with BRCA1ness profile are more strongly associated with *TIMP3* methylation. Thus, we confirmed that *TIMP3* methylation is a marker for tumours that are hormone receptor and HER2 negative and furthermore for tumours that are presenting BRCA1ness profile. Therefore, we propose that silencing of *TIMP3* expression by promoter methylation and consequently incensement of the tumour cell proteolytic activity might be in part responsible for their aggressive biological behaviour.

Regarding the promoter methylation of other genes, we found that aberrant methylation of *APC*, *GSTP1* and *RASSF1* promoters was negatively associated with BRCA1ness tumour profile. Methylation of these genes was also proposed as a marker for estrogen and HER2 negative tumours by two independent studies [13, 14]. Recently, Murria Estal et al. selected promoter methylation status of *APC*, *ATM* and *RASSF1* as independent parameters strongly linked with BRCA1ness profile [15]. They have found that *APC* promoter methylation was associated with BRCA1ness profile, while *ATM* and *RASSF1* metylation with non-BRCA1ness tumours.

In conclusion, our study showed for the first time that methylation of *TIMP3* promoter is an epigenetic feature of BRCA1ness BC tumours. However, additional functional studies are needed to elucidate its role in the development of tumours with BRCA1ness features.

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**Author Contribution** All co-authors contributed to the conception and design, analysis and interpretation of data. IMK and DPK drafted the article and DPK is a guarantor for the article.

All co-authors have seen and agree with the content and conclusions in the submitted version of the article, confirm that the work has not been published/submitted elsewhere and agree with submission manuscript.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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