Clinical Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Development and validation of molecular methodologies to assess *PALB2* expression in sporadic breast cancer

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ARTICLE INFO

Article history:
Received 26 April 2015
Received in revised form 11 October 2015
Accepted 14 October 2015
Available online xxxx

Keywords:
PALB2
DNA methylation
Pyrosequencing
RT-qPCR
mRNA expression
Breast cancer

ABSTRACT

Objectives: Recent reports have included *PALB2* (Partner and localizer of *BRCA2*) in the growing list of hereditary cancer genes. *PALB2* mutations confer a moderate breast cancer risk in heterozygotes and Fanconi anemia in biallelic mutation carriers. PALB2 protein co-localizes with BRCA2 and BRCA1 in nuclear structures and enables error-free homologous recombination repair of double-stranded DNA breaks. This important contribution could be severely diminished if affected by epigenetic mechanisms such as promoter CpG island methylation. The aim of our study was to develop molecular methodologies in order to assess accurately *PALB2* expression in breast cancer tissues.

Design and methods: DNA and RNA were extracted from 91 sporadic fresh-frozen breast tissues with known histopathological data. DNA was subjected to sodium bisulfite conversion reaction and the CpG island of the *PALB2* promoter was analyzed by pyrosequencing. RNA was converted to cDNA and analyzed by a newly developed and validated RT-qPCR assay based on a hydrolysis probe (TaqMan) in the Light Cycler.

Results: *PALB2* promoter was not methylated in any of the samples tested. 87 out of 91 (95.6%) primary tumors were positive for *PALB2* expression, as checked at the mRNA level. When levels of *PALB2* mRNA were compared to histopathological data (tumor size, grade, lymph node involvement, metastasis, hormone receptors and *HER2* overexpression), no significant statistical correlation was found.

Conclusions: DNA methylation is an unlike mechanism for *PALB2* transcriptional regulation. *PALB2* mRNA expression does not seem be a promising prognostic biomarker for sporadic breast cancer.

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1. Introduction

Breast cancer is one of the most commonly diagnosed cancers worldwide [1]. *BRCA1* and *BRCA2* were the first two breast cancer susceptibility genes that were discovered and are considered to be highly penetrant [2–4]. Recent reports have also included *PALB2* in the growing list of hereditary cancer genes [5,6]. *PALB2* mutations confer a moderate breast cancer risk in heterozygotes [7–11] and Fanconi anemia in biallelic mutation carriers (another name for this gene: *FANCN*) [12].

PALB2 gene is located in a 38.2 kb area on chromosome 16 (16p12.1). It consists of 13 exons transcribing a mRNA which encodes a protein of 1186 amino-acids. PALB2 protein co-localizes with BRCA2 (*PALB2*: *P*artner *A*nd *L*ocalizer of *BRCA2*) but also with BRCA1 in nuclear supercomplexes and enables error-free DNA repair of double-stranded breaks via the homologous recombination (HR) mechanism [13–15]. C-terminal end of PALB2 protein is anchoring the N-terminal end of BRCA2 protein [16] and N-terminal end of PALB2 interacts with BRCA1 protein via coiled coil regions [15]. Previous studies have shown that without the PALB2 protein, the BRCA2 protein is unable to enter the nucleus, thus the BRCA1-PALB2-BRCA2 complex -which is very central to the terminal HR mechanism- is not functional [5,13,17]. The inactivation of the HR pathway is associated with an increased risk of cancer. Deleterious mutations in *PALB2* gene are responsible for its dysfunction especially in hereditary tumors. However, in sporadic tumors *PALB2* may also be inactivated by other pathways besides mutations [18].

Abbreviations: CISH, Chromogenic in situ hybridization; Cq, Quantification cycle; CV, Coefficient of variation; FFPE, formalin-fixed paraffin embedded tissue in blocks; HR, homologous recombination; IHC, Immunohistochemistry; LOD, limit of detection; MLPA, Multiplex Ligation-dependent Probe Amplification; (MS-) HRMA, (Methylation Sensitive-) High Resolution Melting curve Analysis; PARP, Poly (ADP Ribose) Polymerase; PCR, Polymerase Chain Reaction; PTT, Protein Truncation Test; qMSP, quantitative Methylation specific PCR; RT-qPCR, Reverse Transcriptase-Quantitative Polymerase Chain Reaction; SB, sodium bisulfite.

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http://dx.doi.org/10.1016/j.clinbiochem.2015.10.009
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Please cite this article as: N. Poumpouridou, et al., Development and validation of molecular methodologies to assess *PALB2* expression in sporadic breast cancer, Clin Biochem (2015), http://dx.doi.org/10.1016/j.clinbiochem.2015.10.009

One such mechanism is DNA methylation, which is an epigenetic process that is present in mammalian cells and is an important regulator of gene transcription. It has been shown that DNA hypermethylation is silencing the expression of tumor suppressor genes and is a common phenomenon in human cancers [19–21] and especially in breast and ovarian tumors [22–24]. Potapova et al. have reported that *PALB2* methylation occurs at a frequency of 8% in sporadic breast and ovarian cancer cell lines and patient cases [18] while another more recent study debated this finding in a set of ovarian cancer tumors [25]. Whether a breast tumor sample is *PALB2* hypermethylated or not could be important, since it might have implications on the selection of patient therapy (e.g. with novel PARP inhibitors) as it has been shown for the tumor hypermethylation of other HR genes [26].

Therefore, we decided to investigate the methylation status of *PALB2* gene in DNA from sporadic breast cancer tissues by a novel, thorough, pyrosequencing method. In order to corroborate the findings, we also measured *PALB2* mRNA expression in our tumor samples by developing and validating a reverse-transcriptase quantitative Polymerase Chain Reaction method (RT-qPCR) based on a hydrolysis (TaqMan probe) in the Lightcycler platform. Finally, we investigated whether there was any correlation between differential expression of *PALB2* mRNA and tumor histopathological parameters for its use as a prognostic biomarker in sporadic breast cancer.

2. Materials and methods

2.1. Patients

Ninety-one tissues were collected from the Pathologic Anatomy Laboratory of Evgenidio Hospital from consecutive female breast cancer patients residing mostly in the Athens Metropolitan area during the period 2007–2011. Main criteria were the availability of the material, the presence of >70% tumor cells in the frozen section and the informed consent of the patients (family history was not used as a criterion for inclusion in the study). The study was approved by both bioethics and scientific committees of the Evgenidio Hospital. Most of the specimens originated from lumpectomy surgery and the mean size was 2.0 cm (range: 1.0–5.5 cm). A small part of the resected specimens was immediately stored in RNAlater (Life Technologies Ambion, USA) for 1–2 days at 4 °C and then transferred to −80 °C until DNA and total RNA extraction for molecular analysis. The larger part of the resected specimens was formalin-fixed paraffin embedded (FFPE) and was used for histopathological examinations. The majority of the tumors (~80%) were ductal infiltrating carcinomas (the rest were lobular mostly, papillary and mucinous) and were classified according to the Bloom-Richardson grading system as grade I (3 samples), grade II (59 samples) and grade III (21 samples). Grades I and II were grouped together as the low grade group because of the small number of grade I tumors. As normal controls, four samples were selected among mammoplasty and benign breast hyperplasia samples. The presence or absence of estrogen and progesterone hormone receptors was investigated with routine immunohistochemistry (IHC) methods and positivity was defined as a score > 1 in IHC. HER2 protein overexpression was examined with IHC and in case the score was 2 in the 0–3 scale, it was further examined with chromogenic in situ hybridization (CISH) at the DNA level; therefore, we were able to dichotomize all samples as being either negative or positive. Lymph node involvement was o examined in most of the samples and the presence of any recurrences or metastasis was also recorded for those patients with sufficient follow-up data. The characteristics of the 91 tissues and patients with sporadic breast cancer are summarized in the first column of Table 1.

2.2. DNA extraction

After a brief snap-freeze of the frozen tissues in liquid N_2, DNA was extracted with the use of the NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. DNA concentration was determined by the Quant-iT dsDNA Broad range Assay kit in the Qubit 1.0 Fluorometer (Life Technologies Invitrogen, USA).

2.3. DNA conversion and pyrosequencing methylation analysis

Tumor DNA and appropriate negative and positive controls were modified after treatment with sodium bisulfite (SB) that converts all unmethylated cytosine to uracil while leaving methylated cytosines untouched [19] by using the EZ-DNA Methylation Gold Kit (Zymo, USA) according to manufacturer's instructions. Converted DNAs were stored up to 3 months at −20 °C. The fully-methylated positive control was a SB-converted male fetal DNA sample treated by *SssI* methylase in the presence of S-adenosyl methionine (both from NEB, USA) while the negative unmethylated control was the same converted sample but *SssI* untreated. Serial mixtures of the aforementioned positive and negative controls were constructed in order to validate the *PALB2* pyrosequencing assay (range of the constructed controls: 2.5–80% positive). Specific primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Germany): PALB2 F1 (forward biotinylated): 5′- TTAGGTGGTTTATTGGGAT-3′, PALB2 R1 (reverse): 5′- ACCAATTAAA TCCACCATT-3′, PALB2 S1 (for sequencing, with one additional base underlined compared to R1): 5′- ACCAATTAAATCCACCATTC-3′ (Eurofins MWG Operon, UK). Our *PALB2* pyrosequencing assay covers a region between −174 and −288 nucleotides (relative to the ATG start of the coding gene area) and interrogates 12 CpGs in the CpG island existing between the promoter and exon 1 of the gene (identified by CpG Island Searcher software: http://cpgislands.usc.edu/). Fig. 1 shows the location of this area but also the *PALB2* genomic areas covered by the other two preexisting assays from Potapova et al.[18] and Mikeska et al.[25]. The primers were designed in order to amplify the SB-converted DNA regardless of the level of methylation as they do not hybridize on CpG dinucleotides. Polymerase Chain Reaction (PCR) was performed by using both forward and reverse primers in a 200 nM final concentration, 50 ng of the SB-modified genomic DNA as the template, 200 μM dNTPs, 0.15 μl HotStar Polymerase (Qiagen, Germany) and 3 μl of its corresponding Coral Load 10X Buffer in a 30 μl total volume. The cycling conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30s, 53 °C for 30s, 72 °C for 30s and a final extension at 72 °C for 10 min. The quality and quantity of the 114 bp PCR products were confirmed by electrophoresis of a 10 μl aliquot in a 2% agarose gel. In the rest of every product, 50 μl PyroMark Binding Buffer (Qiagen, Germany), 2 μl Streptavidin-Sepharose High Performance Beads (Qiagen, Germany) and 18 μl ddH_2O were added and mixed well at room temperature for about 10 min. Then, 1.5 μl of the S1 primer (100 μM final concentration) and 43.5 μl PyroMark Annealing Buffer (Qiagen, Germany) were added to the above mixtures, followed by washing steps and finally, the pyrosequencing analysis. Additionally, in order to check the quality of our tumor samples and to assist in the selection of normal samples, a LINE-1 pyrosequencing assay was undertaken as previously described [27].

2.4. Total RNA isolation

After passing the liquid N_2 snap frozen tissues through special filter columns (shredders) in order to homogenize them and to reduce viscosity, total RNA was extracted by the NucleoSpin RNA kit (Macherey-Nagel, Germany). DNA was removed by an in-column recombinant DNase treatment. Total RNA was eluted in RNase-free water and stored at −80 °C until further use. RNA concentration was determined by the Quant-iT RNA Assay kit in the Qubit 1.0 Fluorometer (Life Technologies Invitrogen, USA) that employs a dye specific for RNA and not for DNA. All RNAs were of adequate quantity.

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