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Genotyping *FOXA1* G559A Polymorphism in Breast Cancer Archival Samples Using the SNaPshot Technique*

Genotypowanie polimorfizmu *FOXA1* G559A w archiwalnych preparatach raka gruczołu piersiowego techniką SNaPshot

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Abstract

Background. FOXA1, a forkhead family transcription factor, is expressed in breast cancer cells and plays an essential role in the regulation of approximately 50% of estrogen receptor alpha (ERα)-dependent genes. Recent studies of expression signatures of breast cancer types have indicated its potential use as a prognostic factor in luminal type A breast cancer (BC).

Objectives. To establish the frequencies of *FOXA1* G559A polymorphisms in a Polish population of breast cancer patients diagnosed with early breast carcinoma and to test whether the SNaPshot methodology is useful for genotyping DNA extracted from formalin-fixed paraffin-embedded tissues (FFPETs).

Material and Methods. DNA was extracted from 70 FFPET blocks and *FOXA1* G559A polymorphism was successfully genotyped in 51 DNA samples using SNaPshot technology (Applied Biosystems).

Results. It is shown that the SNapShot technology based on the single-base primer extension reaction is suitable for genotyping DNA recovered from archived breast cancer tissues. The rate of successful amplifications was 94.4%. No genotypes deviated from Hardy-Weinberg equilibrium. The observed distribution of the *FOXA1* G559A genotypes for this cohort of BC patients was GG 84%, GA 14%, and AA 2%.

Conclusion. SNaPshot is a credible method for the analysis of DNA extracted from FFPETs and it can be used in large-scale *FOXA1* G559A analysis. Further study of this polymorphism and analysis of patients' case histories may provide better insight into its utility as a putative marker of breast cancer prognosis (**Adv Clin Exp Med 2009, 18, 1, 19–24**).

Key words: FOXA1, luminal breast cancer, SNaPshot.

Streszczenie

Wprowadzenie. FOXA1 to czynnik transkrypcyjny z rodziny Forkhead, ekspresjonowany w komórkach raka piersi, odgrywający kluczową rolę w regulacji około 50% genów zależnych od receptora estrogenowego alfa (ERα). Ostatnie badania niektórych typów raka gruczołu piersiowego wykazały, że FOXA1 może być potencjalnym czynnikiem prognostycznym w luminalnym raku typu A.

Cel pracy. Ustalenie częstości polimorfizmu *FOXA1* G559A w populacji polskiej pacjentek ze zdiagnozowanym wczesnym rakiem piersi oraz określenie przydatności metody SNaPshot w genotypowaniu DNA pochodzącego z utrwalonego materiału, przechowywanego w postaci bloczków parafinowych.

Materiał i metody. DNA izolowano z 70 bloczków parafinowych, a genotypowanie udało się przeprowadzić z sukcesem tylko w 51 próbkach z użyciem metody SNaPshot (Applied Biosystems).

Wyniki. Wykazano, że technika SNaPshot oparta na reakcji wydłużania starterów o jedną parę zasad pozwala na genotypowanie DNA pochodzącego z archiwalnych tkanek raka piersi. Wskaźnik udanych amplifikacji wyniósł 94,4%. Stwierdzone genotypy były ilościowo zgodne z przewidywaniami wynikającymi z prawa Hardy'ego-Weinberga. Obserwowany rozkład genotypów w badanej grupie pacjentek wynosił: GG 84%, GA 14%, AA 2%.

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Wnioski. Metoda SNaPshot okazała się niezawodną metodą analizy DNA z tkanek utrwalanych formaliną i przechowywanych w formie bloczków parafinowych i może być wykorzystana do analizy polimorfizmu *FOXA1* G559A w większej grupie pacjentów. Dalsze badania polimorfizmu, w połączeniu z analizą historii choroby, są konieczne do oceny jego przydatności jako markera prognostycznego w raku piersi (**Adv Clin Exp Med 2009, 18, 1, 19–24**).

Słowa kluczowe: FOXA1, luminalny rak piersi, SNaPshot.

Breast cancer (BC) is one of the most common malignancies affecting women worldwide. It accounts for 23% of all cancers, with an estimated one million new cases each year [1]. Despite advances in the early detection and understanding of breast cancer biology, about 30% of patients with early-stage BC experience recurrent disease. The clinically used prognostic and predictive factors include age, tumor size, lymph node status, histological tumor type, grade, mitotic rate, and hormonal receptor status [2]. However, these factors are unable to predict and explain events of progression of the disease. Therefore the search for new molecular based prognostic and predictive factors is necessary. At the same time, establishing laboratory tools which will be suitable and reliable for molecular testing is also needed.

Some studies have recently identified FOXA1, a member of the forkhead family of transcription factors and an important regulator of estrogen receptor (ER), as a potential predictive factor in luminal type A breast cancer [3–5]. FOXA1 is known to influence the expressions of approximately 50% of ERα estrogen-regulated genes by opening the chromatin and enhancing the binding of ER-α to its target genes [6, 7]. One of its known important downstream targets is the gene *GATA3*, which encodes a protein participating in breast tissue morphogenesis as well as tumorigenesis [8]. Furthermore, as part of the ER network, FOXA1 may have an effect on differentiation and control of the cell cycle, i.e. by regulating the expressions of the cell cycle inhibitor p27kip1 and the cell adhesion molecule E-cadherin [9]. Therefore, due to its function, some studies hypothesized that FOXA1 may prevent metastatic progression of luminal type A breast cancer [4, 5].

Currently, the investigation of the impact of *FOXA1* single-nucleotide polymorphisms (SNPs) on making treatment decisions or in BC prognosis seems to be underestimated. Since SNPs have revolutionized human molecular genetics by providing a powerful panel of genetic markers distributed across the entire genome and are responsible for over 90% of all sequence polymorphisms in the human genome [10], their investigation is of great significance. The study of SNPs has received even greater attention since its application in large-scale genetic association studies [11, 12]. Therefore, study of *FOXA1* G559A polymorphisms may provide essential input into the clinical management of patients with luminal type A breast cancer.

Formalin-fixed paraffin-embedded tissues (FFPETs) have been described as a valuable source of biological specimens because of their wide availability in pathology archives together with the accessibility of clinical data. However, limitations of PCR-amplification have been reported when using DNA recovered from FFPETs due to its poor quality caused by cross-links and fragmentation or base modifications [13–16]. This makes the use of FFPETs in molecular testing challenging. Therefore the development and validation of a suitable genotyping technology before use in a large-scale analysis is required. The aim of this pilot study was to test the applicability of DNA recovered from archival tissues for genotyping FOXA1 polymorphisms by the SNaPshot technology as well as to test the distribution of these polymorphisms among Polish breast cancer patients and compare it with the genotype frequencies in a general European population.

Material and Methods

FFPET samples from 70 patients diagnosed with early breast cancer and surgically treated between 1987–2000 at the Wroclaw Medical University Hospital were collected from the Archive of the Wroclaw Medical University, Department of Histopathology. The study was approved by the Institutional Bioethics Board. No clinical information about the treatment type and histopathological characteristics of the tumor was available.

DNA Extraction

Tissue processing and genomic DNA isolation from the 70 paraffin-embedded breast cancer tissues were performed as previously described [17].

Initial PCR Amplification

A 227-bp fragment containing the *FOXA1* G559A (rs714465) polymorphic site in exon 2 was amplified by PCR using specific primers (Table 1).

Table 1. Primer sequences for initial PCR amplification and SNaPshot reaction

Tabela 1. Sekwencje starterów użyte do reakcji PCR i reakcji SNaPshot

Name (Nazwa)	Primer sequence (Sekwencja starterów)
FoxA1 Forward	5`- ATGACTACGAGCGGCAACAT -3`
FoxA1 Reverse	5`- CCCAGGCCATTCATGGAG -3`
SNaPshot Forward	5`- GACTGACT GCCTGAGTCCCGGCGCAGTA -3`
SNaPshot Reverse	5`- AGCCCCCCGGCATGCCGG -3`

Each reaction was performed in a total volume of 20 µl containing 20 ng genomic DNA, 1.5 mM $MgCl_2$, 0.3 µM of each primer, and 0.3 mM each of d-NTP and 1U Taq polymerase (DynaZyme, Finnzymes, Finland). An initial denaturating step was performed at 95°C for 3 min; the PCR cycling conditions were denaturation at 94°C for 30 s, annealing at 56°C for 20 s, extension 72°C for 30 s, and after 35 cycles a final elongation step at 72°C for 5 min.

Genotyping

FOXA1 G559A polymorphism typing was performed using the SNaPshot method (Applied Biosystems). The SNaPshot primers were designed to bind to a complementary sequence one base adjacent to the polymorphism (Table 1). Five microliters of PCR product was incubated with 1U shrimp alkaline phosphatase (SAP) (Fermentas) and 2U Exonuclease I (ExoI) (Fermentas) at 37°C for 40 min followed by 80°C for 15 min. The SNaPshot reaction was performed using a SNaPshot Multiplex Kit (Applied Biosystems) in 5 µl volume using 1 µl of digested PCR product, 0.1 µM of extending primer, 1.5 µl of ready reaction mix, and 2.5 µl of deionized H_2O. The reaction mixture was run under the following conditions: denaturation at 96°C for 10 s, annealing at 57°C for 5 s, and extension at 60°C for 30 s for 25 cycles. After the SNaPshot reaction, 1U of SAP was added to digest unincorporated ddNTPs and the mixture was incubated at 37°C for 60 min. Prior to analysis in an ABI PRISM® 310 genetic analyzer (Applied Biosystems), 1 µl of clean SNaPshot product was mixed with 9 µl of Hi-Di formamide and the GeneScan-120 LIZ standard (Applied Biosystems). The samples were denaturated at 95°C for 5 min and placed on ice before loading to the genetic analyzer. The results were analyzed using GeneMapper-ID v3.2 software (Applied Biosystems).

Results

DNA was obtained from all 70 archival tissue blocks. However, agarose gel electrophoresis showed a high content of degraded DNA in 16 of them (22.8%). These samples underwent fixation in 1987–1990. Due to the poor quality of the nucleic acid, the genotyping analyses were performed only in 54 of the samples. Successful genotyping was obtained for 94.4% of the samples. The other 5.6% did not amplify, probably due to high DNA degradation; these were obtained exclusively from FFPETs prepared before 1990.

The SNapShot reaction discriminated between carriers of the wild-type, heterozygous, and variant *FOXA1* G559A genotypes. The detected alleles are seen on the electrophoregram as peaks (Fig. 1). Patients were assigned as having the wild-type homozygote when there was a single peak (blue) at the 30-bp position. The heterozygous genotype was described by the occurrence of peaks for both the wild-type and variant alleles, and a variant genotype by the presence of a single peak (green) at the 32-bp position.

All genotypes were in accord with Hardy-Weinberg equilibrium (HWE) and the genotype frequencies were GG 84%, GA 14%, and AA 2%. When compared with the frequencies available from HapMap-CEU (www.hapmap.org) obtained from residents of Utah of Northern and Western European ancestry, large discrepancies in the genotype frequencies were observed (Fig. 2), the HapMap-CEU frequencies being GG 13%, GA 48%, and AA 38%. The samples were analyzed in duplicate and the genotyping results were matched for each of them.

Discussion

Breast cancer is characterized by high rates of incidence and mortality among women worldwide [1]. In the era of personalized medicine there is a need to identify prognostic and predictive biomarkers for early detection and better management of the disease, but also for determining the most suitable analysis tools to provide efficient and reliable molecular diagnostic methods. In this study the suitability of the SNapShot technology was tested to analyze *FOXA1* G559A polymorphisms in formalin-fixed paraffin-embedded breast cancer

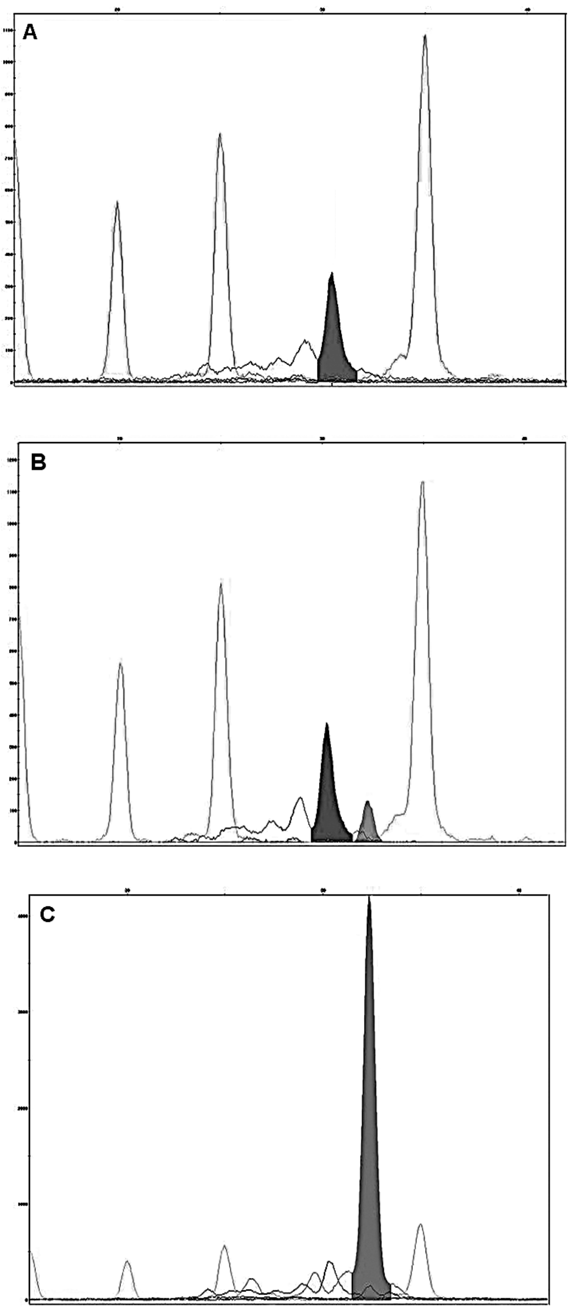

Fig. 1. Examples of electrophorograms for *FOXA1* G559A polymorphisms determined by SNaPshot genotyping. Allele discrimination is based on primer size and color as a result of fluorescence-labeled ddNTPs (terminators) incorporated in the extension reaction. A – blue peak, homozygote wild-type (GG); B – blue and green peaks, heterozygote (GA); C – green peak, mutant homozygote (AA)

Ryc. 1. Elektroforogram prezentujący genotypowanie polimorfizmów *FOXA1* G559A metodą SNaPshot. Allele określano na podstawie wielkości starterów i kolorów znakowanych fluorescencyjnie ddNTPs (terminatorów) włączanych podczas reakcji wydłużania staretów. A – niebieski pik, homozygota typu dzikiego (GG); B – niebieski i zielony pik, heterozygota (GA); C – zielony pik, homozygota zmutowana (AA)

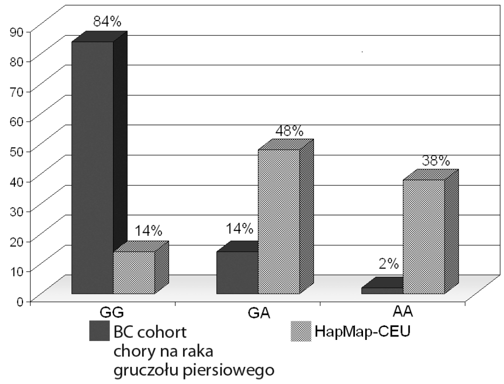

Fig. 2. Distribution of the *FOXA1* G559A polymorphisms in the breast cancer cohort compared with the frequencies available in the HapMap-CEU database

Ryc. 2. Rozkład polimorfizmów *FOXA1* G559A w badanej grupie pacjentów z rakiem gruczołu piersiowego w porównaniu z częstościami dostępnymi w bazie danych HapMap-CEU

tissues. SNapShot is a method based on a single-base primer extension reaction [18], and so far it seems that the present authors are the first to report its assessment in the genotyping of DNA recovered from breast cancer archival tissues. Although it has been previously shown that FFPET samples are a valuable source for SNP genotyping using technologies such as MALDI-TOF mass spectrometry and multiplex PCR with minisequencing [16, 17, 19], the amplification of DNA fragments longer than 200–300 bp might be problematic. The usefulness of FFPETs in PCR-based methods is limited, especially if the tissues were processed using unbuffered formalin, which was routinely practiced before 1990. Fixation time was shown to be a critical factor affecting the sensitivity of PCR amplification [20]. Due to the high impact of the fixation conditions on DNA quality and quantity, the use of amplicon sizes shorter than 180 bp is recommended [21]. Therefore, technologies such as the GeneChip Human Mapping Assay (Affymetrix) and the Infinium Genotyping Assay (Illumina), which require a relatively large amount of intact DNA, will be not as practical for typing archival DNA [16]. However, even these methods could be utilized in archival samples, especially when considering that archival tissues together with collectable clinical information are a very informative source for genetic analysis [22].

In this study a 227-bp product was amplified and 94.4% successful genotype identifications could be obtained. Due to the inclusion of DNA extracted from archival tissues prepared before 1990, an ~5.6% dropout rate in genotyping was noted. This is mostly because of poor DNA quality caused by nucleic acid degradation occurring during fixation performed in an unbuffered fixation bath with an acidic pH. However, other factors, such as autolysis of the sample prior to fixation, tissue type, and the time and conditions of tissue storage, may also contribute to DNA modifications [23]. The high percentage of successful amplifications obtained in the present study is comparable with results published for other genotyping methods, i.e. an 86–100% success rate for multiplex PCR with minisequencing with trends to higher successful genotyping rates after exclusion of poor DNA material [16]. In the present study, 16 samples (22.8%) were excluded from a total of 70 because they showed too high DNA degradation on agarose gel. This helped focusing on higher quality DNA, which ensured a higher rate of successful genotyping with only a 5.6% dropout rate.

The high percentage of successful genotyping in 51 samples shows that the FFPET preparations and extraction methods were compatible with the SNapShot methodology. SNapShot is a sensitive reaction which enables the amplification and detection of very minute DNA and is ideal for carrying out SNP analysis and thus makes it useful in analyzing FFPETs.

The distribution of *FOXA1* genotypes within the cohort of breast cancer patients of the present study was different from that published by HapMap for a general population. This discrepancy can be the result of the difference in origin of our patients and the subjects analyzed by HapMap, although the latter group was of North and Western European ancestry and such great differences in genotype frequency should not have been observed. However, this can also be explained by population bias, since our study consisted only of patients with breast cancer. As there were no healthy controls, the frequencies obtained for the breast cancer cohort could not be compared and it was not possible to establish the genotype frequencies of the *FOXA1* G559A polymorphisms for the general Polish population.

Furthermore, the present group of patients was relatively small; therefore all the genotype frequencies will be subsequently confirmed in a greater number of breast cancer patients. Also, the accessibility to archival tissues was limited and the present authors were unable to obtain any clinical information, including treatment type and histopathological parameters, necessary to perform a determination of the clinical value of *FOXA1* G559A polymorphisms.

Most studies on FOXA1 in the literature focus only on the application of its expression in the prognosis and prediction of breast cancer. So far, FOXA1 expression has been associated with ERα-positive status and favorable prognosis in patients with luminal type A breast cancer and, recently, also with a good prognosis in breast cancer patients treated by adjuvant anthracycline-based chemotherapy [24–26]. On the other hand, not much is known about the functionality and importance of *FOXA1* G559A and other known *FOXA1* polymorphisms. This indicates the necessity of investigating FOXA1 polymorphisms with regard to their potential contribution to either personalized therapy or prognosis for luminal type A breast cancer.

In summary, the SNapShot methodology is a useful and trustworthy tool for genotyping FFPETs. This technology could be used with a greater number of breast cancer patients to evaluate the potential prognostic role of *FOXA1* G559A polymorphism on a larger scale.

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