Expression of MIR125A is diminished in breast tumors, however the reason for the hsa-miR-125a decrease in the cancer is not known. HER2 is encoded by ERBB2, a target for hsa-miR-125a which interacts with the 3'UTR of ERBB2 mRNA. The present study reveals that a polymorphism (rs12976445) within the pri-miR-125a sequence correlates with the amount of mature hsa-miR-125a in breast tumor samples. miRNA, RNA and DNA were extracted from breast cancer samples obtained from 26 patients. Following immunohistological evaluation of the samples, the ERBB2, PGR and ESR1 mRNA profiles were also analyzed using real-time PCR. Genomic DNA was sequenced using MIR125A flanking primers. PCR products were analyzed using a BaeGI restriction enzyme specific to the rs12976445 variant. The rs12976445 variant (C/T and C/C) correlated with a lower level of hsa-miR-125a in comparison with the T/T variant. The expression of HER2 mRNA was increased in tumors with the rs12976445 variant (C/T and C/C) compared with T/T. We conclude that rs12976445 may be a potential prognostic marker of HER2 expression in breast cancer. Its predictive value on the efficacy of trastuzumab treatment in patients with HER2-positive breast cancer warrants further study.

Introduction

The MIR125A gene is located on chromosome 19 in a cluster with MIR99B and MIR7E. Mature hsa-miR-125a interacts with a conserved 8-nt binding element, CUCAGGGA, located within the proximal 3'-UTR of ERBB2 and is able to bifunctionally mediate ERBB2 transcript decay and translational inhibition (1). Besides ERBB2, HuR, Rock-1, KLF13 and ARID3B mRNA levels are also diminished by hsa-miR-125a (2,3). Decreased levels of hsa-miR-125a have been detected in breast cancer and gastric cancer (4,5). Furthermore, increased levels of miR-125 in breast cancer cells inhibit cell growth via the suppression of cell proliferation and by promotion of apoptosis (6). In the nucleus, pri-miR-125a is transformed into mature hsa-miR-125a by the Drosha system (7). This process is obstructed by estrogen receptor (ER) α (8). Germline mutations in BRCA1/2, ATM, PTEN and CHEK2 are common in familial breast cancer, but they explain only one-quarter of the familial risk (9). Thus, it is likely that there are a number of unidentified genes which contain loci encoding miRNAs that confer susceptibility to breast cancer. An SNP (rs12975333) has been observed in the hsa-miR-125a miRNA precursor sequence, which blocks the pri- to pre-miR-125a processing step (10).

In our study, we hypothesized that three other known genetic variants of pri-miR-125a (rs10404453, rs12976445 and rs143525573) should correlate with levels of mature hsa-miR-125a in breast cancer cells. Consequently, ERBB2 mRNA levels would be increased in breast tumors with these genetic variants, suggesting that genetic variants that influence hsa-miR-125a expression have potential as genetic markers of breast cancer. In particular, these variants may have predictive value in designing treatment with drugs against HER2.

Materials and methods

Patients and samples. Tissue samples were obtained from 26 Polish patients undergoing surgery for breast cancer in the Department of Surgery, Chair of Oncology of Poznan University of Medical Sciences (PUMS; see Table I for patient and tumor characteristics). No preoperative radiotherapy or chemotherapy was used. The study protocol was approved by the bioethics board of PUMS. Tumor tissue and blood samples for comprehensive experiments were collected after obtaining written informed consent from all participants.

Immediately after surgery, the tissue samples were stored in liquid nitrogen. Formalin-fixed paraffin-embedded (FFPE)
tissue samples of breast tumors were collected separately. Subsequently, the patients' cases were classified according to the TNM classification of tumors (7th edition). Prior to RNA extraction, corresponding hematoxylin and eosin (HE) stained tumor tissue sections were made and the percentage of cancer cells in the sections was evaluated using a light microscope (Olympus BX41, Olympus, Tokyo, Japan). In the present study, the average percentage of tumor cells per section was 76%.

**Blood sampling and measurements.** Blood samples were obtained by puncture of the antecubital vein, in the Department of Surgery, Chair of Oncology at PUMS.

**RNA and DNA extraction.** miRNA and mRNA were extracted from frozen tissue using the mirVana™ miRNA Isolation kit (Life Technologies, Carlsbad, CA, USA). Total RNA and DNA from the paraffin-embedded tissues was extracted using the RecoverAll™ Total Nucleic Acid Isolation kit (Life Technologies).

DNA from frozen tissue and blood was extracted using GenElute™ Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Luis, MO, USA). The quantity of obtained nucleic acid was assessed using a BioPhotometer™ (Eppendorf, Hamburg, Germany).

**PCR, sequencing and restriction analysis.** DNA specimens were amplified using standard PCR protocols. The PCR primers corresponding to pri-pre-miR-125a used for MIR125A sequencing were: 5'-TTTTGTTCTTCTGCTCTG-3' and 5'-TGAGGGAGGTATGAGGAGT-3'. The PCR products were purified with the Gel-out purification kit (A&A Biotechnology, Gdynia, Poland) and sequenced at the DNA Sequencing and Oligonucleotide Synthesis Laboratory of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences (Warsaw, Poland). The sequencing results were analyzed using BioEdit Sequence Alignment Editor. In addition to sequencing, the SNP (rs12976445) was sequenced. A single nucleotide change from T to C (variant rs12976445) was identified in the sequence of pri-miR-125 in 25% of the samples. Three other variants were not present in these samples.

**Real-time PCR.** To evaluate the MIR125A expression level, TaqMan microRNA Assays (Life Technologies) for real-time RT-PCR were used. Similar assays were also used for hsa-miR-206, hsa-miR-125b, hsa-miR-17 and hsa-miR-27b; U6 RNA was used as a reference gene. All samples were reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit and specific starters from the TaqMan microRNA Assay. TaqMan Universal PCR Master Mix and specific primers from the TaqMan microRNA Assays were used to quantify the samples in a Roche (Indianapolis, IN, USA) LC 480 cycler. The relative amounts of all miRNAs were calculated using standard curves and compared as ratios using the U6 reference gene.

**ERBB2, ESR1 and PGR mRNA levels** were analyzed by reverse transcription (Life Technologies) and TaqMan real-time PCR (Roche). **HMBS and POL2** were used as reference genes.

**Statistical analysis.** Statistical analysis was conducted using Instat software. P≤0.05 was considered to indicate statistically significant differences. A t-test was used to compare the differences in mean expression levels between the groups of samples from the real-time RT-PCR experiment. The mean of the log₂ of the ratio between target gene expression levels and reference gene expression levels was also calculated.

**Results**

**Frequency of the rs12976445 variant.** A total of 26 surgically removed breast tumors were analyzed with the aim of identifying genetic polymorphisms in the gene encoding hsa-miR-125a. Of the samples, 21 were congealed in liquid nitrogen and 5 were paraffin-embedded archival samples. The group of 26 samples was analyzed routinely using histopathological methods. The patients were classified by the TNM system and the samples were also grouped into ER, PR and HER positive and negative cases. The results are presented in Table I. DNA extracted from the samples was amplified using primers spanning pri-, pre- and mature hsa-miR-125a. There are four known SNPs in the amplified fragment of MIR125A: rs12976445, rs10404443, rs12975333 and rs143525573 (11). Subsequently all amplicons from the tumor samples were sequenced. A single nucleotide change from T to C (variant rs12976445) was identified in the sequence of pri-miR-125 in the breast cancer patients (15 nucleotides downstream from the start of pri-miR-125, 54 nucleotides upstream from the start of pre-miR-125, 68 nucleotides upstream of the miR-125a-5p).

The three other variants were not present in these samples.
To confirm the results of the MIR125A sequencing and to establish the frequency of the variant, the DNA extracted from the congealed tumors and paraffin-embedded tissues was amplified using the same pair of primers as for the sequencing. Subsequently the products were digested using the restriction enzyme BaeGI (GKGCM^C). The restriction analysis revealed that 8 out of 26 patients were T/T homozygotes (30.8%), 15 (57.7%) were T/C heterozygotes and 3 were C/C homozygotes (11.5%; Fig. 1). For 14 tumors, corresponding blood samples were available and the same genetic variant was investigated using restriction analysis with BaeGI (Fig. 1B). The results revealed exactly the same distribution of variants in the blood as in the corresponding tumors.

**MiRNA level.** The expression of MIR125A was analyzed in the tumors using real-time PCR (Fig. 2). The hsa-miR-125a level was decreased by 85% (P=0.024) in the samples with the variants CC and CT, compared with the TT variant. When the T homozygous variant, heterozygotes and the C homozygotes were compared, a gradual decrease of hsa-miR-125a levels was observed. In the present samples, no correlation was observed between the expression of hsa-miR-125b, hsa-miR-27, hsa-miR-17-5 or hsa-miR-206 and the rs12976445 variant.

mRNA of the ERBB2 gene was increased by 2.4-fold (P=0.044) in the tumor samples with the variant C/C and C/T MIR125A variants, compared with the T/T variant. ESR1 and PGR mRNA levels did not correlate with the rs12976445 variant.

**Discussion**

The present study shows that in breast cancers the rs12976445 variant (in the T/C and C/C types as opposed to the T/T type)
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