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ORIGINAL PAPER

Assessing the value of CAN-gene mutations using MALDI-TOF MS

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Abstract
Purpose To identify cancer-linked genes, Sjöblom et al. and Wood et al. performed a genome-wide mutation screening in human breast and colorectal cancers. 140 CAN-genes were found in breast cancer, which in turn contained overall 334 mutations. These mutations could prove useful for diagnostic and therapeutic purposes.
Methods We used a MALDI-TOF MS 40-plex assay for testing 40 loci within 21 high-ranking breast cancer CAN-genes. To confirm mutations, we performed single-plex assays and sequencing.
Results In general, the mutation rate of the analyzed loci in our sample cohort was very low. No mutation from the 40 loci analyzed could be found in the 6 cell lines. In tissue samples, a single breast cancer tissue sample showed heterozygosity at locus c.5834G>A within the ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26).
Conclusions Sjöblom et al./Wood et al. already showed that the vast majority of CAN-genes are mutated at very low frequency. Due to the fact that we only found one mutation in our cohort, we therefore assume that at the selected loci, mutations might be low-frequency events and therefore, more rarely detectable. However, further evaluation of the CAN-gene mutations in larger cohorts should be the aim of further studies.

Keywords Breast cancer · CAN-genes · MALDI-TOF MS · Multiplex assay

Background

The decoding of the human genetic code at the beginning of the twenty-first century officially heralded the age of genomics and laid a solid foundation for the future molecular research (Collins and McKusick 2001; Venter et al. 2001). Casting a retrospective glance at the last few

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Table 1 Clinical data of patients

Patient data divided into two subgroups according to pathological tumour type. *ER positive,* Estrogen receptor positive, *PR positive,* Progesterone receptor positive

Histological type	Total no. of patients	Age (years) mean ± S.D. (range)	Stage			ER positive	PR positive
			1	2A	2B		
Invasive ductal carcinoma	14	49 ± 10.8 (28–63)	11	2	1	5	4
Medullary carcinoma	5	50 ± 7.9 (36–56)	1	4	0	0	0

years, the achievements of the human genome project accounted strongly for the development in the research of genetically determined diseases. Identifying disease-related genes and understanding their involvement in pathogenesis is one of the fundamental goals currently pursued by the scientists from different fields (The Wellcome Trust Case Control Consortium 2007; Nessling et al. 2005).

Because cancer is one of the leading causes of death worldwide and has a primarily genetic determination, cancer research now dedicates itself to the search for genes that are involved in carcinogenesis. It is already known that mutations affecting some oncogenes and tumor suppressor genes, such as TP53 and BRCA1/2, are linked with an increased cancer risk (Soussi and Lozano 2005; Walsh et al. 2006). However, to date, there are only a few genes for which the existence of such a linkage is scientifically confirmed.

For this reason, high-throughput mutation profiling using large-scale sequencing approaches has been conducted and numerous candidate genes have been identified. Sjöblom et al. and Wood et al. conducted a sequencing-based genome-wide mutation screening in human breast and colorectal cancer with the aim of identifying genes linked with these cancers (Sjoblom et al. 2006; Wood et al. 2007). By testing nearly every well-annotated gene in both cancer types and by finally using stringent statistical criteria, they identified 280 genes overall that were mutated at a significant frequency, calling them candidate cancer genes (CAN-genes). The discovery of these CAN-genes in breast cancer could prove useful for diagnostic and therapeutic applications.

To evaluate the probable applicability of these CAN-genes for clinical purposes, we used MALDI-TOF MS 40-plex assay to test 40 single-nucleotide variants on 21 high-ranking CAN-genes in 6 breast cancer cell lines and in tissues of 19 breast cancer patients and 55 healthy controls.

Methods

Cell lines and culture conditions

MDA-MB-231, MCF-7, and HS578T were grown in DMEM (high glucose with L-glutamine). BT549 and T47D were cultured in RPMI 1640 medium, and SKBR3 was grown in McCoy's 5A. All media were supplemented with 10% FCS and 1% penicillin–streptomycin. The cells were maintained in a humid incubator at 37°C with 5% CO_2.

Study cohort

The study was performed at the Laboratory for Gynecological Oncology/Department of Biomedicine, Women's Hospital Basel and approved by the Ethical Committee of the University of Umeå. Patient information can be obtained from Table 1.

DNA extraction

For DNA extraction from cell lines and paraffin-embedded tissue samples, the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) was used according to the manufacturer's protocol. Before extraction, cells were washed with 1× PBS. For patient samples, paraffin-embedded tissue sections were pretreated as proposed in the kit manual. The DNA was finally eluted in 100 µl elution buffer and stored at −20°C until further use. DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Biolab, Mulgrave, VIC, Australia).

Assay design for detection of CAN-gene mutations

Wood et al. defined 140 genes containing more than 334 mutations as CAN-genes for breast cancer using cancer mutation prevalence (CaMP) score, which is calculated based on the likelihood that the amount of mutations in any gene is higher than those expected from a background mutation rate. Of the 140 CAN-genes, we selected 21 genes containing 40 loci (Table 2). For the design of the capture and the extension primers, the DNA sequences containing the CAN-gene mutations were entered in the software MassArray Assay Design version.3.1 (Sequenom, San Diego, CA, USA). Information about sequences and mass of capture and extension primers can be obtained from Supplementary 1 (Table 1a).

Genotyping using MALDI-TOF MS

For SNP genotyping, the iPLEX Gold assay (Sequenom, San Diego, CA, USA) was used. The assay consists of 3

Table 2 First CAN-gene assay

Gene	CCDS accession	CaMP score	Nucleotide (genomic)	Nucleotide (cDNA)	Amino acid (protein)
THBS3	NM_007112	3.5	g.chr1:151978713A>G	c.2863A>G	p.R955G
SP110	NM_004509	3.81	g.chr2:230907125T>C	c.23T>C	p.M8T
TLN1	NM_006289	2.98	g.chr9:35690282C>T (homozygous)	c.6566C>T	p.A2189V
ZNF646	NM_014699	3.06	g.chr16:30999156A>T	c.4010A>T	p.N1337I
TRIOBP	NM_001039141	3.54	g.chr22:36443733A>T	c.670A>T	p.R224W
ZNF569	NM_152484	3.28	g.chr19:42609051C>G	c.85C>G	p.Q29E
XDH	NM_000379	3.76	g.chr2:31500578C>G (homozygous)	c.2371C>G	p.R791G
VEPH1	NM_024621	5.34	g.chr3:158461684_158461683delAA	c.2443_2444 delAA	Fs
XDH	NM_000379	3.76	g.chr2:31501422C>T	c.2287C>T	p.L763F
ZFYVE26	NM_015346	3.06	g.chr14:67302874G>A	c.5834G>A	p.R1945Q
ZNF569	NM_152484	3.28	g.chr19:42597140A>G	c.260A>G	p.E87G
TP53	NM_000546	55.19	g.chr17:7519167A>G (homozygous)	c.488A>G	p.Y163C
ZFP64	NM_199427	3.39	g.chr20:50134614G>C	c.1827G>C	p.K609 N
TP53	NM_000546	55.19	g.chr17:7520091C>G	c.321C>G	p.Y107X
TP53	NM_000546	55.19	g.chr17:7518335A>T (homozygous)	IVS5-2A>T	Sp
ZFYVE26	NM_015346	3.06	g.chr14:67321561C>A	c.3491C>A	p.A1164E
TMEM123	NM_052932	4.7	g.chr11:101777989A>C	c.259A>C	p.N87H
TRIOBP	NM_001039141	3.54	g.chr22:36436328T>A	c.515T>A	p.V172E
VEPH1	NM_024621	5.34	g.chr3:158581776G>T	c.998G>T	p.S333I
TP53	NM_000546	55.19	g.chr17:7519095G>C	IVS4 + 1G>C	Sp
TIMELESS	NM_003920	2.96	g.chr12:55101032C>G	c.3022C>G	p.Q1008E
TP53	NM_000546	55.19	g.chr17:7517747C>T (homozygous)	c.916C>T	p.R306X
TP53	NM_000546	55.19	g.chr17:7517831C>T	c.832C>T	p.P278S
TLN1	NM_006289	2.98	g.chr9:35693800C>T	c.6329C>T	p.A2110 V
TG	NM_003235	5.84	g.chr8:134030233C>G (homozygous)	c.5264C>G	p.P1755R
TP53	NM_000546	55.19	g.chr17:7518937C>T	c.637C>T	p.R213X
TMPRSS6	NM_153609	3.28	g.chr22:35810313G>A	c.668G>A	p.R223H
TECTA	NM_005422	4.56	g.chr11:120504208T>A (homozygous)	c.2312T>A	p.I771N
TACC2	NM_206862	2.65	g.chr10:123834397C>G	c.2392C>G	p.L798V
TECTA	NM_005422	4.56	g.chr11:120494285G>A	c.851G>A	p.R284H
TG	NM_003235	5.84	g.chr8:133994534C>G	c.4220C>G	p.S1407X
TG	NM_003235	5.84	g.chr8:133968052A>G (homozygous)	c.1253A>G	p.D418G
TDRD6	NM_001010870	2.69	g.chr6:46764550C>G	c.726C>G	p.F242L
TMEM123	NM_052932	4.7	g.chr11:101777516T>C	c.509T>C	p.M170T
TG	NM_003235	5.84	g.chr8:134103511G>T	c.6970G>T	p.A2324S
TCF1	NM_000545	3.48	g.chr12:119900103G>A	c.1721G>A	p.S574N
SLC6A3	NM_001044	3.66	g.chr5:1456172A>C	c.1632A>C	p.R544S
TECTA	NM_005422	4.56	g.chr11:120505627delA	c.2438delA	Fs
TDRD6	NM_001010870	2.69	g.chr6:46769240G>C	c.5416G>C	p.E1806Q
SULF2	NM_018837	3.84	g.chr20:45728625T>C	c.1591T>C	p.Y531H

The table shows the genes analyzed in the MALDI-TOF MS 40-plex assay, the associated CCDS accession number, the cancer mutation prevalence score (CaMP score), the nucleotide and genomic position, and the affected amino acid. To optimize the signal-to-noise ratio, the 40 CAN-gene positions have been divided into four mass groups indicated by the parting lines

major steps: 1. Capture PCR (amplification of the amplicon containing the locus of interest); 2. Shrimp alkaline phosphatase (SAP) treatment (removal of unincorporated dNTPs); and 3. iPLEX reaction (Primer extension).

1. The Capture PCR was carried out in a 10 μl-PCR volume containing 1 μl DNA (10 ng/μl), 1.625 mM $MgCl_2$, 500 μM dNTP mix, 0.5U Hotstart Taq DNA polymerase (Quiagen), and primer mix (containing all

40 amplification primer pairs). PCR amplification was performed using a Mastercycler gradient (Eppendorf, Germany) under the following conditions: Preincubation at 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 3 min.

2. For the removal of unincorporated dNTPs, SAP treatment using shrimp alkaline phosphatase (Sequenom) was performed at 37°C for 40 min and 85°C for 5 min, followed by a final cooling to 4°C.
3. For the iPLEX reaction, a PCR cocktail mix was made using iPLEX buffer (10×), 0.4 µl iPLEX termination mix, 0.08 µl iPLEX enzyme, and the primer mix (consisting of all 40 extension primers). As there is an inverse relationship between peak intensity and analyte mass that influences the signal-to-noise ratio, a classification of different mass groups is required when performing high-plex assays. To ensure an optimal signal-to-noise ratio, we divided our assay into 4 different mass groups (7, 9.66, 10.33, and 14 µM). The PCR was carried out in a Mastercycler gradient (Eppendorf, Germany) using a 200-short-cycle program consisting of two cycling loops. The first loop of five cycles is located within a second loop of 40 cycles. The PCR starts with a first denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 94°C for 5 s, primer annealing at 52°C for 5 s, and extension at 80°C for 5 s. Within these 40 cycles, the primer annealing and extension step is repeated 5 times resulting in a total of 200 cycles. After the 200 cycles, a final extension is done at 72°C for 3 min, and the product is cooled down to 4°C.

To optimize the mass spectrometric analysis, the iPLEX reaction products were desalted using clean resin and then dispensed on a 384-element SpectroCHIP bioarray by a Nanodispenser (Sequenom). For measuring the assay reproducibility, each sample was run in duplicate and found mutation(s) were reconfirmed with single-plex assay and sequencing. For the processing and analysis of the iPLEX SpectroCHIP, the MassARRAY Compact system and the MassARRAY Workstation software version 4.0 (Sequenom) were used.

Sequencing analysis

The PCR was carried out in 25 µl total volume containing 17.4 µl H_2O, 2.5 µl 10× PCR buffer (Quiagen), 0.5 µl dNTP mix (25 mM) (Quiagen), 1.25 µl of each primer, 0.1 µl Hotstart Taq (Quiagen), and 2 µl DNA. The PCR was performed under the following conditions: initial denaturation at 94°C for 15 min, followed by 45 cycles at 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 3 min. Removal of primers and dNTPs was done using Exo1/SAP treatment. To 25 µl of the PCR, 0.3 µl Exo1, 0.6 µl Exo1 buffer (10×) (both Fermentas), 3 µl SAP (Sequenom), and 2.1 µl H_2O were added and incubated under the following conditions: 37°C for 40 min and 85°C for 5 min, followed by a final cooling to 4°C. Sequencing was done by Microsynth (Balgach, Switzerland). Information on primers used for sequence analysis is listed in Supplementary 1 (Table 1b).

Results

Quality of the multiplex assay

In the previous studies, the sensitivity and specificity of the method itself has been proven by us as well as by other research groups (Garritsen et al. 2009; Thomas et al. 2007; Xiu-Cheng Fan et al. 2008). Therefore, we only analyzed several assay quality parameters including call rate and call probability. For every analyzed locus, these parameters are automatically calculated by the software MassArray Typer (Sequenom, Inc.). Assay quality was also assessed by visual analysis of MALDI-TOF spectrograms. The peak pattern was quite homogenous, with some slight variations in peak intensity (Fig. 1a).

Mutational analysis of breast cancer cell lines and patient samples

Using the MALDI-TOF MS-based 40-plex assay, we analyzed the mutational status of 40 variants on 21 CAN-genes in 6 human breast cancer cell lines and tissues of 19 breast cancer patients and 55 healthy controls; no CAN-gene mutations have been found at the analyzed loci in any of the cell lines. Regarding the tissue samples, only a single breast cancer tissue sample showed heterozygosity at locus c.5834G>A within the ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26). This heterozygosity could also be confirmed using a single-plex assay and sequencing (Fig. 1b, c).

Discussion

Using MALDI-TOF MS 40-plex assay, we evaluated 21 CAN-genes and 40 loci in 6 breast cancer cell lines and in tissue samples of 19 breast cancer patients and 55 healthy controls. The frequency of the mutations in our sample cohort was very low. No mutation was found at the analyzed loci within the 6 cell lines. Only one breast cancer patient was found to be heterozygous at one locus within

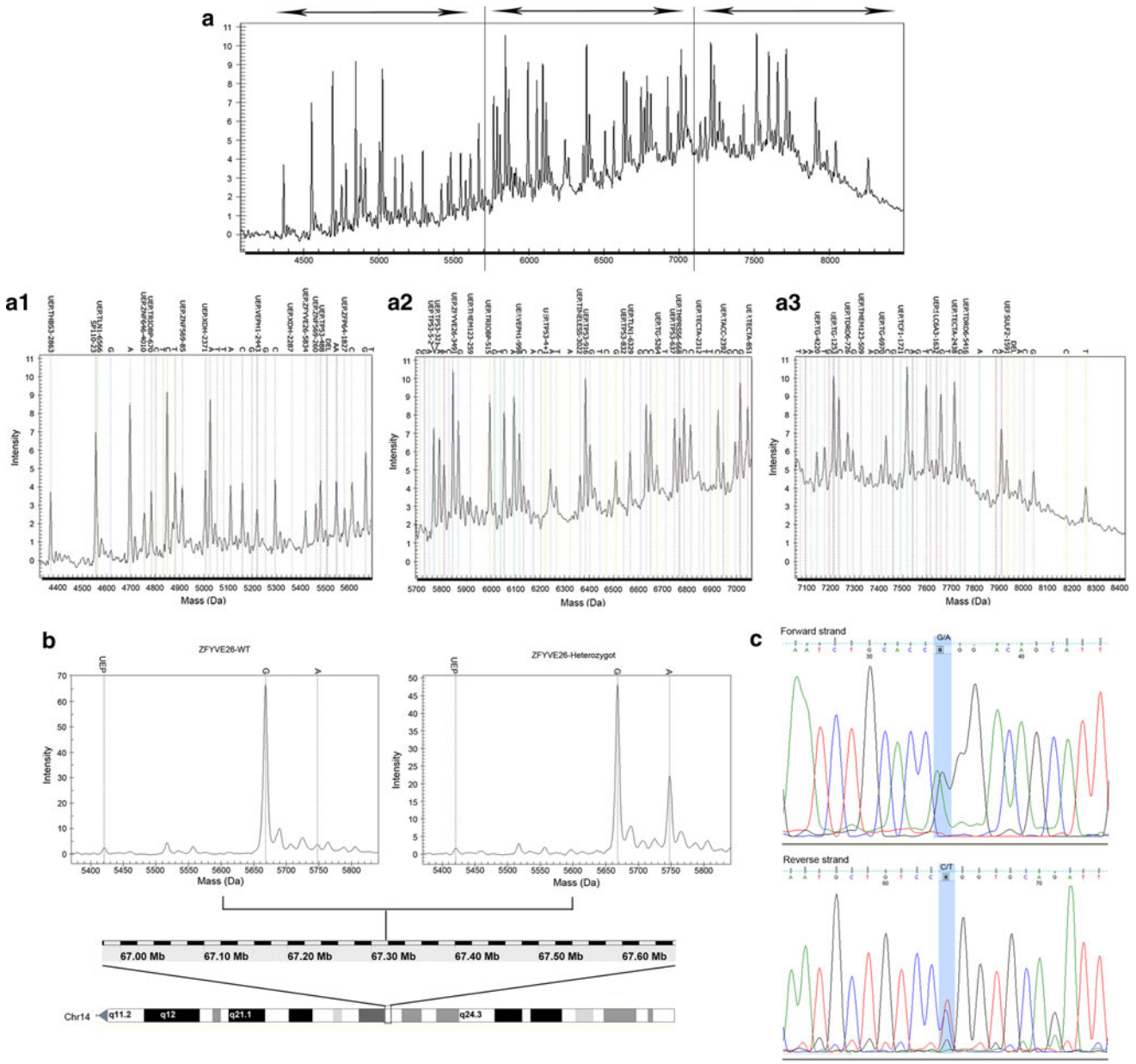

Fig. 1 Spectrograms of one 40-plex assay and a single-plex assay/sequencing confirming heterozygosity in ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26). **a** Spectrogram of one 40-plex assay with detailed view in the augmentations a1, a2, and a3. The *dashed lines* represent the unextended primers (UEP, followed by gene name and nucleotide position of the allele) and the respective alleles. **b** Single-plex assays for two patients; one homozygote for G and the other heterozygote for G/A at locus g.chr14:67302874 within the ZFYVE gene. **c** Sequencing results for confirmation of heterozygosity at locus g.chr14:67302874 within the ZFYVE gene

the ZFYVE26 gene, which was also confirmed by single-plex assay and by sequencing. Defects in ZFYVE26 are known to be the cause of spastic paraplegia autosomal recessive type 15 (Goizet et al. 2009). However, ZFYVE seems to play a role in cytokinesis as well. Sagona et al. showed that depletion of the ZFYVE26 protein in HeLa cells led to the arrest of cells in cytokinesis (Sagona et al. 2010). A direct association of the gene with breast cancer has not been reported.

To get an idea whether others had already described cancer-related mutations at the same positions that we analyzed in this study, we investigated the COSMIC (Catalogue of Somatic Mutations in Cancer) database, Sanger (http://www.sanger.ac.uk/genetics/CGP/cosmic/), which contains information about previously reported somatic mutations in cancer. Interestingly, it seems that most of the CAN-gene loci analyzed in this study have only been analyzed by Sjöblom et al until now, and most of

them have not been directly implicated in breast cancer so far. Only few of the positions, which were included in our study, have previously been associated with breast cancer, and mutations at those loci have been found by other groups. Alsner et al. analyzed the heterogeneity in TP53 mutations in 315 breast cancer patients. Using tumour material from those patients, they identified 74 TP53 mutations. At position c.488, which is located in the exon 5 of the TP53 gene and was also analyzed in our study, they found a base exchange from A>G (Alsner et al. 2000).

To conclude, it is widely accepted that the landscapes of cancer are quite complex (Vogelstein and Kinzler 1993). Sjöblom et al. and Wood et al. showed that just a humble amount of genes are mutated at higher frequencies, while most of the genes are mutated at a relatively low frequency in cancer. Although our study included high-frequency as well as low-frequency genes/loci, one have to consider as well the mutational inter- and intra-variations within/between cancer types, which makes it more difficult to select the real driver mutations out of a background of passenger mutations. To really get a conclusion about the probable value of these CAN-genes for a diagnostic/therapeutic purpose, evaluation of a higher number of CAN-genes/CAN-gene loci in a larger cohort should be the aim of further studies.

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

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