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MALDI-TOF MS Genotyping of Polymorphisms Related to 1-Carbon Metabolism Using Common and Mass-Modified Terminators

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BACKGROUND: Large cohort studies may provide sufficient power to disentangle the role of polymorphisms related to 1-carbon metabolism and chronic diseases, but they require fast, accurate, high-throughput genotyping techniques. MALDI-TOF mass spectrometry has been adapted to rapid fine mapping using various approaches for allele discrimination. We developed a genotyping method based on MALDI-TOF MS and compared assay performance for formats based on standard and mass-modified terminators.

METHODS: The assay includes 20 polymorphisms of 14 genes involved in 1-carbon metabolism (*BHMT* 742G>A, *CBS* 844ins68 and 699C>T, *CTH* 1364G>T, *DHFR* del19, *NOS3* –786T>C and 894G>T, *FOLR1* 1314G>A, *MTHFD1* –105T>C and 1958G>A, *MTHFR* 677C>T and 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G and 524C>T, *SLC19A1* 80G>A, *SHMT1* 1420C>T, *TCN2* 67A>G and 776C>G, and *TYMS* 1494del6).

RESULTS: Missing calls were observed for 4.7% of the DNA samples, attributed to failed liquid sample handling. Highly accurate genotyping was obtained by mass-modified as well as standard ddNTPs, with an average error rate of ≤0.1% by analysis of sample duplicates. A semiquantitative approach enabled unambiguous identification of the *CBS* 844ins68. Cluster plots of the relative allele intensities showed allele-specific bias according to type of minisequencing terminator and revealed a potential structural variation in the *BHMT* gene.

CONCLUSIONS: MALDI-TOF MS–based genotyping using either standard or mass-modified terminators allows the accurate determination of single nucleotides as well as structural genetic variants. This was demonstrated with 20 polymorphisms involved in 1-carbon metabolism.

The methylenetetrahydrofolate reductase (*MTHFR*)[4] 677C>T polymorphism is the strongest known genetic determinant of total homocysteine (tHcy). Increased tHcy and the 677TT genotype are associated with increased risk of cardiovascular disease, colorectal neoplasias, neural tube defects (NTDs), and pregnancy complications *(1, 2)*. Several other genetic variants have been linked to 1-carbon metabolism, and their possible associations with risk of cardiovascular disease and other diseases are under investigation. These include betaine homocysteine methyltransferase (*BHMT*) 742G>A *(3)*, cystathionine β-synthase (*CBS*) 844ins68 *(4)* and 699C>T *(5)*, cystathionase (cystathionine γ-lyase) (*CTH*) 1364G>T *(6)*, dihydrofolate reductase (*DHFR*) del19 *(7, 8)*, nitric oxide synthase 3 (*NOS3*) –786T>C *(9)* and 894G>T *(10)*, folate receptor 1 (adult) (*FOLR1*) 1314G>A *(11)*, methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1 (*MTHFD1*) –105T>C (Parle-McDermott A. Personal communication, August 2006) and 1958G>A *(12)*, methylenetetrahydrofolate reductase (NADPH) (*MTHFR*) 1298A>C *(13, 14)*, 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) 2756A>G *(15, 16)*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) 66A>G *(17)* and 524C>T *(18)*, solute carrier family 19

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[4] Human genes: *BHMT*, betaine-homocysteine methyltransferase; *CBS*, cystathionine β-synthase; *CTH*, cystathionase (cystathionine γ-lyase); *DHFR*, dihydrofolate reductase; *NOS3*, nitric oxide synthase 3 (endothelial cell); *FOLR1*, folate receptor 1 (adult); *MTHFD1*, methylenetetrahydrofolate dehydrogenase ($NADP^+$ dependent) 1; *MTHFR*, 5,10-methylenetetrahydrofolate reductase (NADPH); *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; *SLC19A1*, solute carrier family 19 (folate transporter), member 1; *SHMT1*, serine hydroxymethyltransferase 1 (soluble); *TCN2*, transcobalamin II; *TYMS*, thymidylate synthetase.

(folate transporter), member 1 (*SLC19A1*) 80G>A *(19, 20)*, serine hydroxymethyltransferase 1 (soluble) (*SHMT1*) 1420C>T *(21, 22)*, transcobalamin II (*TCN2*) 67A>G *(23)* and 776C>G *(3, 23, 24)*, and thymidylate synthase (*TYMS*) 1494del6 *(25)*.

Numerous high-throughput genotyping methods have been developed during the last decade *(26)*. MALDI-TOF mass spectrometry has been proven to be a reliable and powerful tool for the determination of a limited number of genetic variants in large sample sets. Genotyping based on MALDI-TOF MS involves different approaches for allele discrimination, such as hybridization, invader, minisequencing, restriction enzyme cleavage, and ligation *(27)*. Minisequencing by primer extension has become the most popular technique for the determination of single nucleotide polymorphisms (SNPs).[5] Several formats are available, such as PinPoint, iPLEX, VSET, genoSNIP, and GOOD assays, which differ in speed, cost, and complexity of sample preparation; quality of allele separation; and level of multiplexing *(26, 27)*.

The first minisequencing approach based on MALDI-TOF MS was the PinPoint assay developed by Haff and Smirnov in 1996 *(28)*, which detects a single base extension and enables multiplexing of up to 12 SNPs *(29)*. However, the PinPoint suffers from ambiguous identification of A/T heterozygotes due to the low mass difference of 9 Da between the extension products. Use of mass-modified dideoxynucleotide triphosphates (ddNTPs) as designed by Sequenom *(30)* solves the A/T problem and also decreases the chance of mass interferences by adduct signals.

We recently published a PinPoint method for the simultaneous detection of 12 polymorphisms related to the 1-carbon metabolism *(31)*, which has been used in large-scale epidemiological studies including more than 20 000 samples *(3, 32, 33)*. Here we describe an improved version that includes 20 genetic variants related to 1-carbon metabolism. The method was designed to be compatible with common (standard) ddNTPs and mass-modified ddNTPs, and thus allowed comparison of both sets of minisequencing terminators for the determination of 20 polymorphisms selected on the basis of their role in 1-carbon metabolism.

Materials and Methods

DNA PURIFICATION AND MULTIPLEX PCR

DNA from 460 samples was purified as described *(31)*. We performed multiplex PCR of 20 templates in a total volume of 12.5 μL containing 7 ng DNA, 1.25 units Thermostart DNA polymerase (ABgene), 2.5 mmol/L $MgCl_2$, 1.2 μmol/L dNTPs (Amersham Bioscience), and 0.26 μmol/L of each primer. PCR primers are shown in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue1. Oligo 6.0 (Molecular Biology Insights) was used for design of primers, which were synthesized by Eurogentec. The cycling conditions (MJR Dynad thermal cycler) were 95 °C for 15 min followed by 40 cycles at 95 °C for 30 s, 62 °C for 90 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Liquid handling was performed in 384-formate using a Beckman Coulter Biomek 2000 workstation.

Excess PCR primers and dNTPs were removed by incubation of 10 μL PCR mixture with 3 μL ExoSAP-IT (USB Corp.) at 37 °C for 15 min, followed by 15 min at 80 °C.

PRIMER EXTENSION

We carried out the multiplex primer extension reactions using PCR products as template and a mixture of 20 site-specific primers (Eurogentec), compatible with the PinPoint and iPLEX assays (online Supplemental Table 1). The optimal primer concentrations were empirically determined with respect to equal signal intensity and ranged from 0.25 to 17 pmol/L. For comparison between standard ddNTPs (Amersham Bioscience) and mass-modified ddNTPs (Sequenom) by mass spectrometry, all extension reactions were carried out under identical conditions, which differed from Sequenom's iPLEX protocol. For minisequencing, the total reaction volume of 13 μL contained 3 μL PCR product, 0.63 nmol/L ddNTPs, and 2 units ThermoSequenase (Amersham Bioscience). The cycling program (MJR Dynad thermal cycler) consisted of 2 loops, where a 5-cycle loop was inside a loop of 50 cycles. After initiation at 92 °C for 30 s, the outer loop started at 92 °C for 5 s, followed by the inner loop at 57 °C for 5 s and 72 °C for 5 s. The program finished with 72 °C for 3 min. Liquid handling was performed in 384-format using a Biomek 2000.

MALDI-TOF MS AND DATA PROCESSING

Samples were desalted using cation exchange beads as described *(31)*, but incubation time could be reduced to 30 min by extensive mixing of samples and beads. MALDI-TOF MS preparation was carried out on SpectroChips (Sequenom) using a nanoliter dispensing robot spotting volumes of 10 nL. Samples were analyzed on a Reflex III (Bruker Daltonik) instrument equipped with a nitrogen laser emitting at 337 nm wavelength and 8 Hz pulse rate. The Scout 384 ion source was run in positive linear ion mode at 25 kV. To avoid detector

[5] Nonstandard abbreviations: SNP, single nucleotide polymorphism; ddNTP, dideoxynucleotide triphosphate.

saturation from matrix ions, masses of <4000 Da were suppressed by detector gating. MALDI-TOF MS analyses were run fully automated by fuzzy-logic instrument control, and the number of shots per spectrum was 50 (2 times 25).

We used software developed in-house based on the Bruker XMASS program for automated spectra processing and genotype identification. Data processing was started with background subtraction and mass calibration (*m*/*z*) by linear regression using 3 oligonucleotides (15A+2G, 23A, 32A) as internal standards. Products with relative intensities of <0.1 were set to zero. Genotypes were defined by cutoffs of wild-type to mutant allele ratios, which were >2.5 for wild-type, from 2–0.5 for heterozygous, and <0.4 for homozygous mutant variant. Peak doublets with signal ratios within the ranges 2.5–2 and 0.5–0.4 were always assigned for visual inspection to verify the mass spectra with respect to quality and potential interferences by adducts. These cutoffs were depicted as different areas in cluster plots (0, missing calls; 1, homozygotes; 2, visual inspection; 3, heterozygotes). All samples were analyzed twice on 2 chips, results were compared by data processing, and ambiguous spectra were assigned for visual inspection. If visual inspection did not clarify the source of conflicts, the genotype was classified as a missing call (failure).

ASSAY VALIDATION

The polymorphisms *CTH* 1364G>T, *DHFR* del19, *FOLR1* 1314G>A, *MTHFD1* –105T>C and 1958G>A, *MTRR* 524C>T, *NOS3* –786T>C and 894G>T, *SHMT1* 1420C>T, and *TYMS* 1494del6 were genotyped by RFLP. The remaining variants had been determined earlier *(31)* by RFLP or Taqman real-time PCR. We compared genotypes obtained by MALDI-TOF MS and reference methods and evaluated the performance of our assay in terms of failures and errors, which were determined by the relative number of missing and discrepant calls, respectively. Samples showing conflicting results between MALDI-TOF MS and the reference method were genotyped again, if the source of error was not detected by visual data inspection. Assay conditions for all polymorphisms are listed in online Supplemental Table 1.

We determined the mean error rate from 368 samples, as well as after multiple analytical runs of another sample set (n = 92). Failure was attributed to the minisequencing step when unextended primers could be detected and to nanoliter dispensing when oligonucleotide signals were absent in the mass spectra. Discrepant calls obtained using MALDI-TOF MS and RFLP were inspected to assess the source of failure. To further evaluate allele discrimination, we plotted the relative signal intensities of both alleles as cluster plots. *CBS* 844ins68 was investigated separately, because the insertion required a semiquantitative approach for distinction between hetero- and homozygosity *(31)*.

STATISTICAL METHODS

We used SPSS 12.0.2 for Windows (SPSS Inc.) for linear regression of heterozygous signals and to determine significant slope differences between clusters of heterozygotes using univariate analysis. Significant differences between assay accuracy by type of terminator were tested using a *z*-test for 2 proportions (http://www.dimensionresearch.com/resources/calculators/ztest.html; Dimension Research). We used the interactive graphic package iPlots (http://www.rosuda.org/iplots) of the statistical software R (http://www.r-project.org) to compare cluster plots of the *BHMT* 742G>A polymorphism with respect to standard and mass-modified ddNTPs.

Results

MASS DETERMINATION OF TERMINATORS

Before assay validation, an exact determination of the mass differences between the ddNTPs was required. We genotyped 3 reference samples using the standard and mass-modified ddNTPs and determined the mass differences between the various terminators (online Supplemental Table 2). The difference between the standard and mass-modified ddNTPs was −27 Da for ddA, ddC, and ddG and 38 Da for ddT. Thus the mass-modified terminators generated higher mass differences for ddA/ddT (56 vs 9 Da), ddC/ddT (80 vs 16 Da), and ddG/ddT (40 vs 25 Da).

ASSAY PERFORMANCE

A typical MALDI-TOF MS spectrum after minisequencing by standard ddNTPs is shown in Fig. 1. The performance of the 20-plex assay was evaluated with respect to type of terminator using a total of 460 reference samples. The failure rate was 4.8%, and missing calls were equally assigned to sample preparation (DNA quality, liquid handling during PCR and minisequencing) and nanoliter dispensing. Missing calls were not related to type of terminator used. The average error rates (excluding the *CBS* 844ins68) were 0.11% using standard and 0.04% using mass-modified terminators (online Supplemental Table 3). The mean error rates for genotyping 5 replicates of 92 samples were 0.03% for standard and 0.08% for mass-modified ddNTPs. The differences between standard and mass-modified terminators were not significant.

In addition to the analyses of duplicates on 2 identically prepared chips, we ran 2 analysis cycles on the same chip at slightly different sampling positions and observed that the remaining spots of matrix/sample

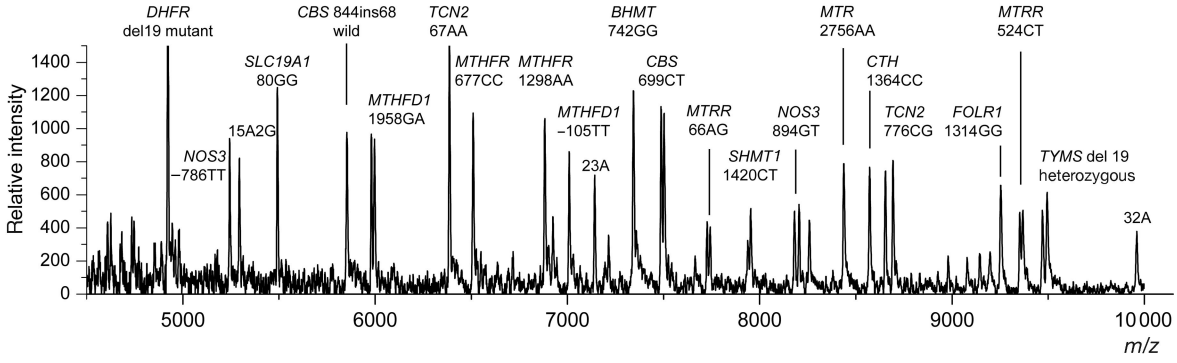

Fig. 1. MALDI-TOF MS spectrum of a 20-plex minisequencing reaction.

The extension products and 3 standards for mass calibration (labeled 15A2G, 23A, 32A) are shown. Minisequencing was performed with standard ddNTPs.

were sufficient to obtain a second set of high-quality spectra (data not shown).

SEMIQUANTIFICATION OF *CBS* 844ins68

Genotyping of the *CBS* 844ins68 polymorphism required semiquantitative measurement of the mutant to wild-type ratio, since the insertion produces a 68-bp copy of the intron 7/exon 8 boundary (http://www.uchsc.edu/cbs/cbsdata/ins68.htm). We plotted the allele ratios obtained in 460 reference samples for the mass-modified vs standard ddNTPs (Fig. 2). Heterozygotes and wild types of both formats were separated at a ratio of about 0.2; comparison demonstrated only 3 discrepant calls. Two heterozygotes could not be distinguished from background noise using mass-modified ddNTPs but were successfully distinguished by standard terminators (Fig. 2, vertical arrows). One wild-type signal was incorrectly identified as heterozygote using mass-modified ddNTPs (Fig. 2, horizontal arrow). The two homozygous mutant samples in our reference material were clearly identified at cutoffs of about 0.9 and 1.2 by standard and modified terminators, respectively. Linear regression of wild-type and heterozygous allele signal ratios obtained by the 2 methods resulted in $R^2 = 0.89$ and slope 0.90, consistent with a somewhat higher ratio of mutant (ddC) to wild-type (ddG) alleles for standards than for mass-modified ddNTPs.

CLUSTER PLOTS

We investigated the variation of the allele signal intensity by genotyping a set of 92 samples. Each sample was analyzed 10 times. Fig. 3 shows 4 typical scatter plots of allele signals obtained with standard and mass-modified ddNTPs. Genotypes were identified by predefined cutoffs of allele signal intensities, and signal ratios are graphically presented in 4 different areas (0, missing calls; 1, homozygotes; 2, visual inspection; 3, heterozygotes). The cluster plots for *CTH* 1364G>T (Fig. 3A) showed a clear discrimination between the genotypes, and the resulting clusters enabled unambiguous genotyping. The peak doublets of the heterozygotes were balanced and could be clearly separated from the homozygotes using either standard or modified ddNTPs.

For *MTRR* 524C>T (Fig. 3B), clusters of heterozygotes were closely located in the middle of area 3, but in contrast to *CTH* 1364G>T, the linear regression showed different slopes according to assay format due to the higher signal intensity of both alleles using standard terminators. An additional cluster was observed in the area of wild types when standard ddNTPs were used. These data points could be explained by a mass interference with the homozygous mutant allele from a $(M+NH_4)^+$ adduct *(34)* for the wild-type signal caused by excess ammonium acetate. This interference was not found for mass-modified ddNTPs, since the mass difference between alleles was 80 Da, in contrast to 16 Da for standard terminators.

Similar results were obtained with *MTHFD1* –105T>C (Fig. 3C). A large cluster close to the cutoff between areas 1 and 2 again indicated a frequent mass interference using standard ddNTPs. In contrast to the signal interference for *MTRR* 524C>T, this cluster was not attributable to adducts, since the mass difference between alleles was the same (16 Da) for standard and mass-modified terminators. Visual inspection of spec-

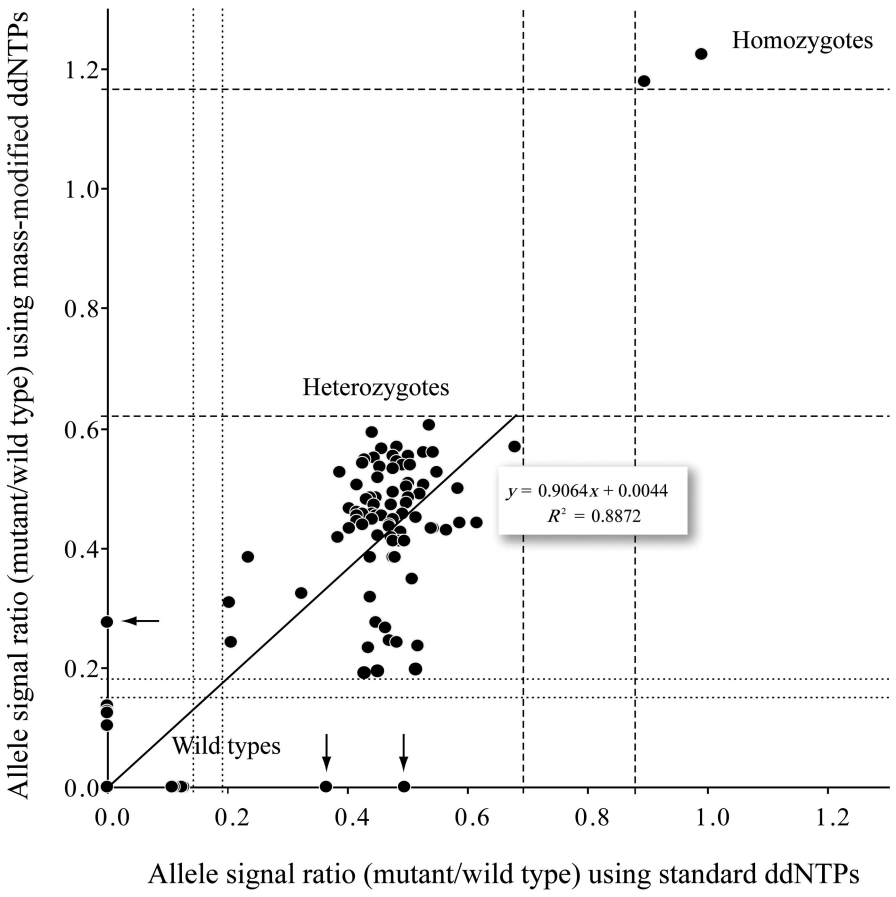

Fig. 2. Genotyping of *CBS* 844ins68 by semiquantification of allele signals.

The allele ratios of mutant to wild-type signals measured in 460 reference samples by MALDI-TOF MS were plotted for the mass-modified vs standard ddNTPs. The dashed lines indicate areas for discrimination between wild types and heterozygotes as well as heterozygotes and homozygotes. Three discrepant calls were detected for the mass-modified ddNTPs, indicated by arrows. Results of both assays were correlated by linear regression excluding the 2 homozygotes.

tra from unextended minisequencing mixtures revealed that a fragment of a larger primer in PinPoint randomly overlapped with the mutant allele signal.

The cluster plot of *DHFR* del19 (Fig. 3D) shows a large variation of both allele signals, with the widest distributions for heterozygotes observed for all investigated polymorphisms. The slopes also revealed a large asymmetry in allele ratios, which was most pronounced when using unmodified ddNTPs. Whereas heterozygotes genotyped by mass-modified ddNTPs were still located within areas 2 and 3, many heterozygotes analyzed by unmodified ddNTPs were misclassified as homozygous mutants when analyzed once. Using duplicate analysis, no wrong call was obtained for the 19-bp deletion. In addition, the allele signals of the *DHFR* del19 were among the strongest signals in the mass spectra, and the large variation of signal intensity could not be related to a low signal-to-noise ratio. Furthermore, the cluster plot showed signal interference of the mutant allele by $(M+NH_4)^+$ adducts in both assays.

IDENTIFICATION OF 2 CLUSTERS FOR *BHMT* 742GA

Fig. 4 shows that the cluster plots of the *BHMT* 742G>A differed from results of other polymorphisms. Using standard ddNTPs, homozygous mutant genotypes again showed interference from $(M+NH_4)^+$ adducts and a fragment from a larger primer. In contrast to all other polymorphisms investigated here, the *BHMT* 742GA heterozygotes cluster was split into 2 subgroups in both assay formats. The allele ratios were calculated for all heterozygotes and

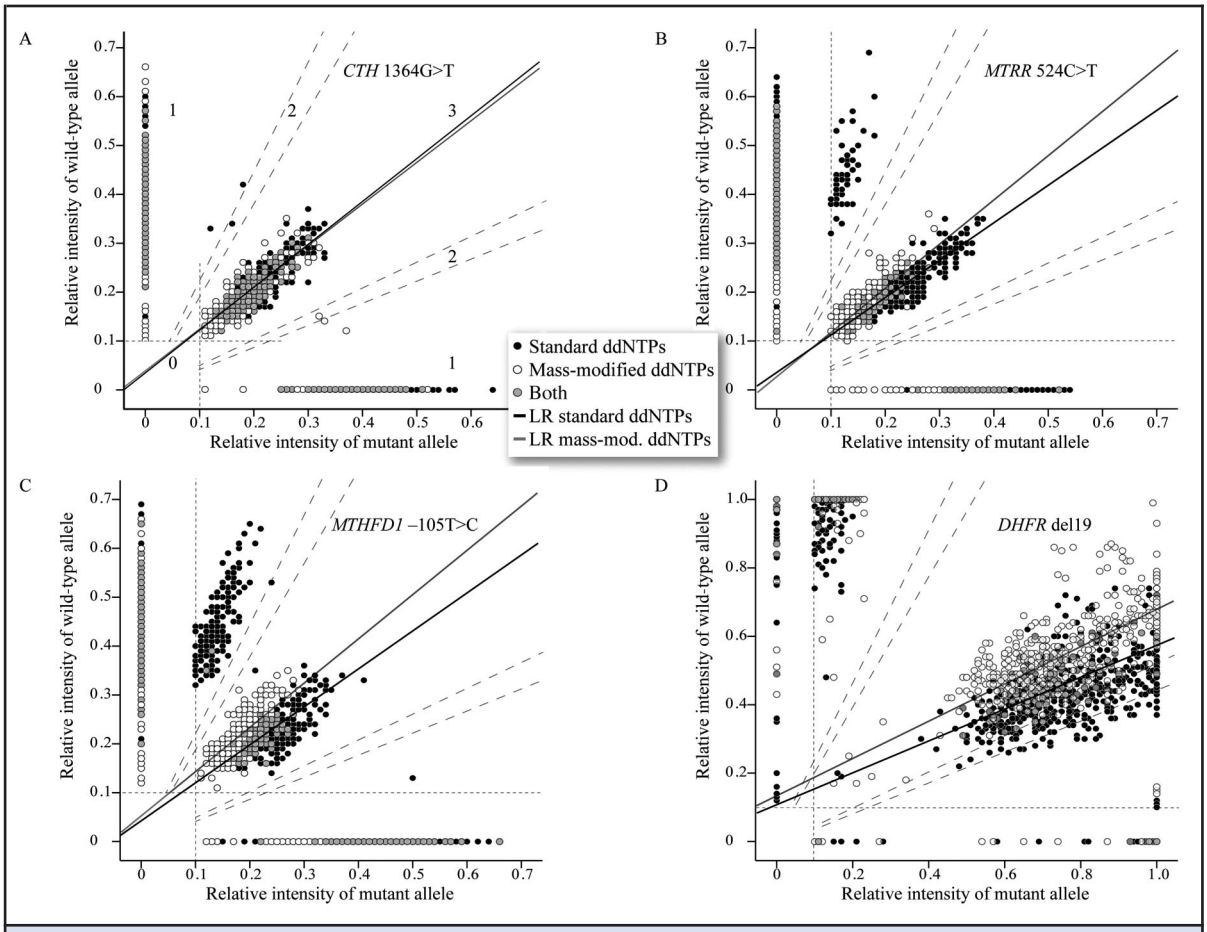

Fig. 3. Cluster plots of relative allele signal intensities for 4 polymorphisms.

Signals with relative intensities below a cutoff of 0.1 could not be discriminated from background noise and were set to 0. The cluster plot of *CTH* 1364G>T illustrates decision areas (0–3) for genotype identification (0, missing calls; 1, homozygous; 2, visual inspection requested; 3, heterozygous). Clusters of heterozygotes according to type of terminators were fitted by linear regression analysis (solid lines). mass-mod., mass-modified.

plotted for mass-modified vs unmodified terminators (Fig. 4C). Different labeling of both subgroups demonstrated that the discrimination was not related to assay format.

To evaluate if these results were caused by primer design, we redesigned the PCR primers and minisequencing probe. All 92 samples were genotyped again by duplex minisequencing using standard ddNTPs, including the original and the modified site-specific probes. The obtained cluster plots showed the same subgroups for heterozygotes as observed in the 20-plex assays (data not shown).

ALLELE SIGNAL RATIOS OF HETEROZYGOTES ACCORDING TO TYPE OF TERMINATOR

Allele-specific signals were evaluated in more detail with respect to bias related to type of minisequencing terminator. The mean allele signal ratios of heterozygotes are shown in Fig. 5 for both modified and standard ddNTPs and were grouped according to single nucleotide changes. *CBS* 844ins68 was excluded due to the theoretical allele ratio of 2:1 for heterozygotes.

A balanced signal ratio (>0.95, <1.05) was observed for 8 heterozygous variants, 4 by standard and 4 by mass-modified terminators. A mean ratio different from 1.0 was significant for most variants, with the exception of *FOLR1* 1314G>A using mass-modified ddNTPs.

The allele signal ratios of C/T were <1.0 for 5 of 6 SNPs when measured by standard ddNTPs and >1.0 for 5 SNPs using modified ddNTPs. Allele ratios of G/T extensions were >1.0 for standard ddNTPs and > and <1.0 for the mass-modified ddNTPs.

Heterozygotes for AG represented the largest group in our assay, and for 6 of 8 variants, ratios

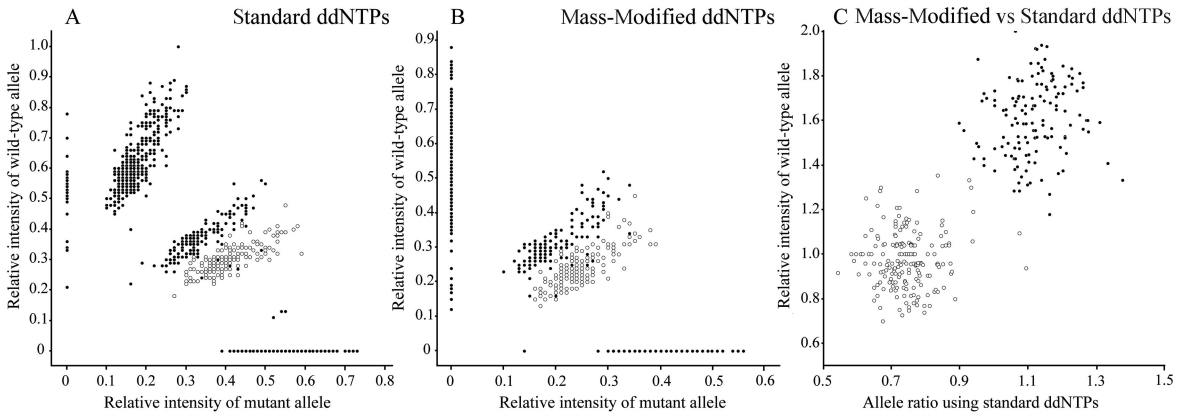

Fig. 4. Cluster plots of relative allele signal intensities for *BHMT* 742G>A.

The genotypes are plotted in 2 separated scatter plots using standard (A) and mass-modified (B) ddNTPs. Plotting the allele signal ratios of heterozygotes obtained by mass-modified vs standard terminators (C) allowed simultaneous selection and labeling of samples in all 3 plots, using the iPlots package for R.

were <1.0 using standard ddNTPs, whereas for 5 variants, allele ratios were >1.0 using mass-modified terminators.

Independent of assay formats, variants for AC showed ratios >1.0, whereas *TCN2* 67CG ratios were <1.0.

We also evaluated if the slopes of the regression lines fitted to clusters of heterozygotes were related to the type of incorporated ddNTP (Fig. 6). Significantly different slopes between assay formats were observed for heterozygotes of *BHMT* 742G>A, *CBS* 699C>T, *MTRR* 524C>T, *NOS3* 894G>T, *DHFR* del19, *MTHFD1* –105T>C, *MTR* 2756A>G, *MTRR* 66A>G, *NOS3* –786T>C, and *TCN2* 776C>G. Among these heterozygotes, mass-modified ddNTPs caused higher slopes than standard ddNTPs for transitions of C>T and A>G, with the exception of *MTR* 2756A>G. Significantly lower slopes by unmodified terminators were obtained for G>T and C>G transversions, where the latter was consistent with results for *CBS* 844ins68.

Discussion

PRINCIPAL FINDINGS

This multiplex genotyping method based on MALDI-TOF MS enables the determination of 17 SNPs, 2 deletions, and 1 insertion related to the 1-carbon metabolism. The assay includes semiquantification of *CBS* 844ins68, is compatible to standard and mass-modified terminators, and enables highly accurate genotyping. Cluster plots of the relative allele intensities gave insights into genotyping performance for both single nucleotide and structural polymorphisms, assessed allele-specific bias according to type of minisequencing terminator, and detected potential structural variation in the *BHMT* gene.

ASSAY PERFORMANCE

Our method is characterized by low failure and error rates. The mean failure rate of about 5% was independent of assay format and is comparable to the success rates of other MALDI assays *(30, 35)*. Because failed liquid handling during sample preparation and MALDI preparation contributed equally to all missing calls, improved liquid-dispensing techniques would increase the efficiency of our method.

Average error rates of both assay formats were comparable and very low (≤0.1%) when analyzing samples in duplicate. Hence, duplicate analyses represent a simple approach when highest genotyping accuracy is required. Alternative methods using flexible cutoffs for each polymorphism—by bootstrapping, for example—may be more efficient for evaluation of genotypes but were not supported by our software.

ALLELE DISCRIMINATION BY STANDARD AND MASS-MODIFIED TERMINATORS

The major benefit of the chosen mass-modified ddNTPs is the discrimination of A and T allele signals, but this could not be proven here because the SNPs investigated did not include any A/T polymorphic site. These transversions occur at the lowest frequency (approximately 7%) of all SNPs *(36)* and represent <3% of SNPs found in coding regions of 24 genes related to 1-carbon metabolism *(37)*. An expected advantage of

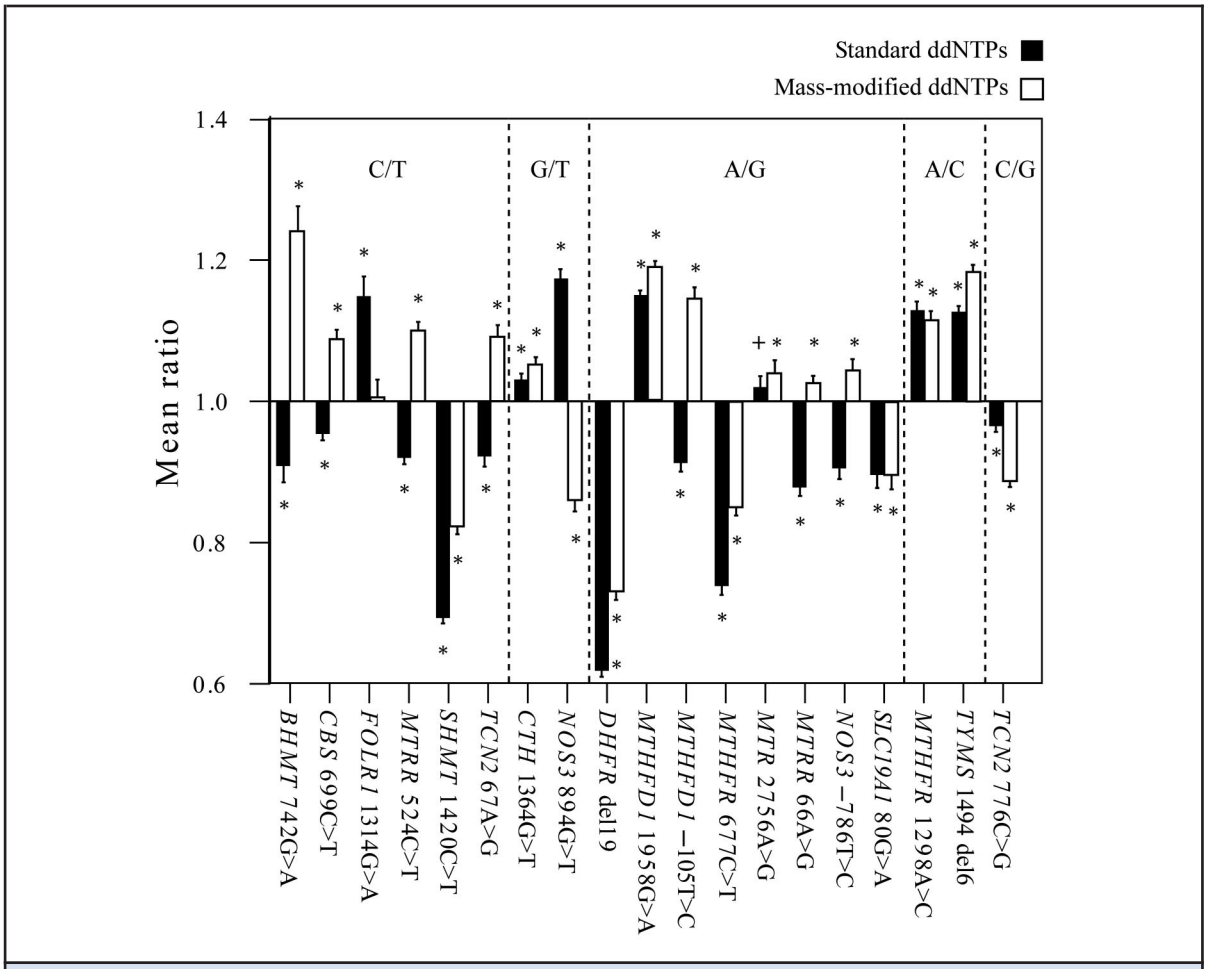

Fig. 5. Mean allele ratios in heterozygotes.

The mean allele ratios (95% CIs) obtained from the 10 replicates of 92 samples were plotted for all clusters of heterozygotes according to type of terminators. Genetic variants were grouped by type of transversion/transition, which were complementary when downstream primers were used in minisequencing. Significant differences of mean allele ratios from 1.0 are indicated ($+P < 0.05$; $*P \leq 0.001$).

mass-modified ddNTPs with respect to our assay was the elimination of mass interferences by ammonium adducts. However, these adducts, as well as random fragments of primers occurring after inadequate primer synthesis or numerous thaw-freeze cycles, could be perfectly separated from the heterozygous genotypes by standard ddNTPs using uniform, fixed cutoff values. In addition, sodium adducts were efficiently suppressed in all samples by desalting and by data processing at the appropriate discrimination level.

SEMIQUANTIFICATION OF *CBS* 844ins68

We have previously described a semiquantitative approach for genotyping *CBS* 844ins68 in a 12-plex PinPoint assay *(31)*. Owing to the simultaneous presence of the wild-type sequence and the insertion sequence on the same chromosome, heterozygotes and homozygotes can be distinguished only by analyzing the relative amount of both alleles. The results confirmed that the cutoffs defined earlier for genotype discrimination were still valid for the PinPoint assay with the multiplexing level increased to 20 polymorphisms. Also, the mass-modified ddNTPs enabled unambiguous identification of *CBS* 844ins68 genotypes and had good between-assay agreement. The mass-modified ddNTPs tended to provide less efficient discrimination between wild types and heterozygotes because of a lower average allele ratio. Separation of heterozygotes from homozygous mutants seemed to be adequate for standard and modified ddNTPs, although an accurate determina-

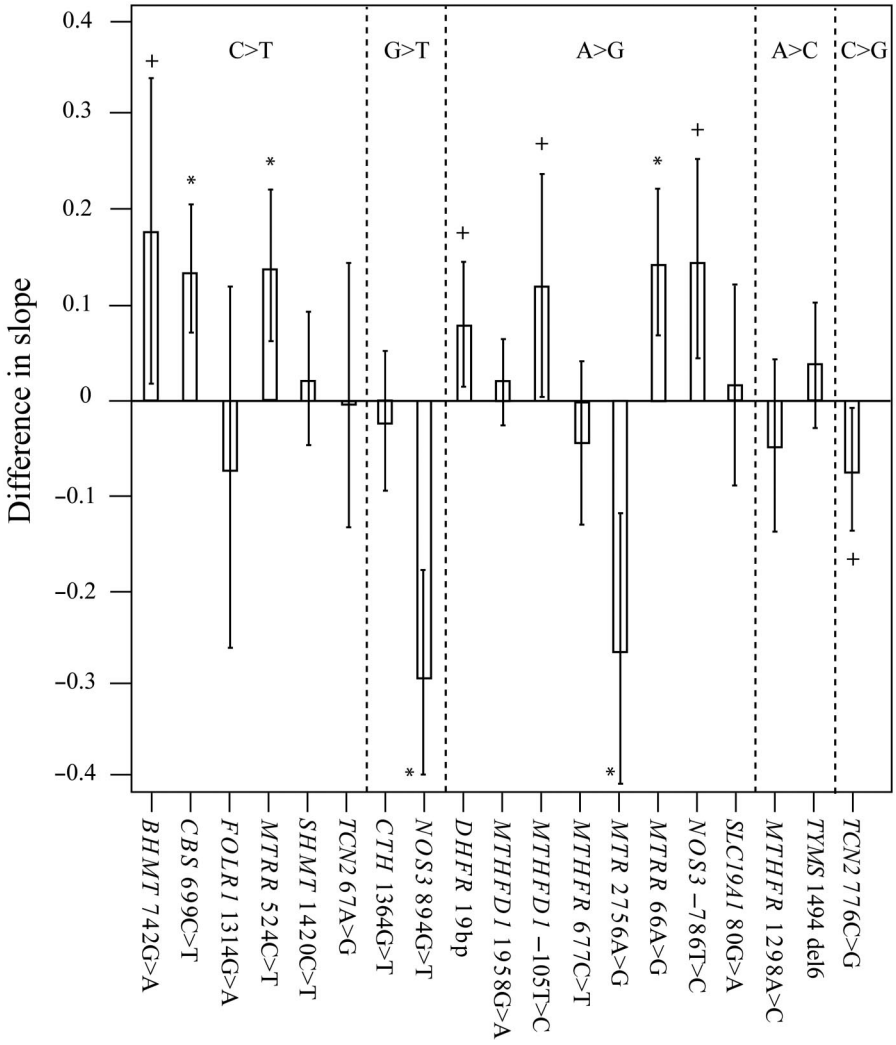

Fig. 6. Difference in slopes of regression lines (95% CIs) fitted to clusters of heterozygous samples genotyped with standard and mass-modified ddNTPs.

The slopes were calculated for all clusters of heterozygotes obtained from the 10 replicates of 92 samples, and the differences between types of terminators were plotted for each polymorphism. Genetic variants were grouped by type of transversion/transition, which were complementary when downstream primers were used in minisequencing. Significant differences in slope are indicated ($+P < 0.05$; $*P \leq 0.001$).

tion of the cutoff was hindered by the small number of homozygous mutants in the reference material.

ALLELE-SPECIFIC BIAS

The accuracy of genotyping depends on the variability of the allele signals. Cluster plots demonstrated that fixed cutoffs of allele ratios were well suited for genotype discrimination of various polymorphisms, but could fail if pronounced allele-specific bias occurred. Although mass interferences essentially did not affect the error rates of our assay, markedly unbalanced or variable signal ratios of heterozygotes could impede genotyping, as observed for the *DHFR* del19. To achieve high accuracy not affected by allele-specific bias, it is paramount to optimize data processing by using cluster analysis algorithms calculating flexible cutoffs for each polymorphism or by comparing duplicate analyses.

Allele-specific bias can be caused by unbalanced PCR, unbalanced minisequencing, or interference in MALDI MS spectra. The bias resulting from these fac-

tors became visible in the clusters of heterozygote samples. In the case of *DHFR* del19, the high variability in allele signal intensities may be related to low extension efficiency of the minisequencing primer, which was the shortest primer used in our multiplexing assay. We could also observe allele-specific bias in heterozygotes according to the type of transitions/transversions as well as terminator species. These differences may be caused either by different incorporation rates of ddNTPs by ThermoSequenase enzyme or variation in ionization/fragmentation probabilities of the extension products in MALDI-TOF MS.

POTENTIAL STRUCTURAL VARIATION IN *BHMT* GENE

The separate clusters observed for the *BHMT* 742GA genotype demonstrated 2 different doses for 1 allele and may indicate an underlying structural variation within the *BHMT* gene. Copy number variations (CNVs) are frequent DNA changes in the human genome *(38)* and can be detected by quantitative allele determination using MALDI-TOF MS *(39)*. So far, no CNV has been reported for the *BHMT* gene (http://www.sanger.ac.uk/humgen/cnv/), and further investigations are required to identify and confirm this structural variation.

In summary, we have developed a genotyping assay based on MALDI-TOF MS for the simultaneous detection of 20 genetic variants involved in 1-carbon metabolism. The assay was adapted to both standard ddNTPs and mass-modified terminators and enabled genotyping at very low error rates for the selected genetic polymorphisms related to 1-carbon metabolism.

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