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Determination of Phenotype Associated SNPs in the MC1R Gene*

ABSTRACT: Prediction of physical appearance based on genetic analysis is a very attractive prospect for forensic investigations. Recent studies have proved that there is a significant association between some genetic variants of the melanocortin 1 receptor (MC1R) gene and red hair color. The present study focuses on the potential forensic applicability of variation within this pigment-related gene. Sequencing of the complete MC1R gene was performed on a group of red-haired individuals and controls with different pigmentation. A major role in determination of red hair color is played by two MC1R variants—C451T and C478T. The optimized minisequencing assay for genotyping of the above positions and three other important red hair-related MC1R polymorphisms, C252A, G425A, and G880C was successfully applied to analyze typical forensic specimens. Determination of a homozygous or heterozygous combination can be a good predictor of both red hair color and fair skin of a subject.

KEYWORDS: forensic science, physical traits, red hair color, melanocortin 1 receptor, single nucleotide polymorphism, genetic prediction

The association between genetic variants and particular phenotypic features is the subject of extensive research in various scientific disciplines (1,2). Thanks to the sequencing of the human genome and determination of billions of single nucleotide polymorphisms (SNPs), it is now possible to study polymorphic variants in great detail (3,4). Many SNPs localized in coding or regulatory regions are expressed in phenotypes. Studies developing genotype to phenotype correlations have advanced rapidly in medicine. These studies have mainly focused on correlations between genetic variants and certain diseases or between genetic variants and individual patient drug responses (1,5). With this growing amount of data, new opportunities will also appear, which will be useful in forensic science. Genetic typing of biological traces collected at crime scenes could be a source of valuable information about a donor's physical characteristics. This type of information could be used to verify testimony from a witness relating to the description of a perpetrator. Forensic anthropological examinations enabling reconstruction of human appearance based on skeletal remains are also a potential niche for phenotype determination using genetic tests. Complementary information provided by geneticists may authenticate or add to anthropological findings. Hair color and skin type are human features that are very variable and easily recognized and therefore their prediction will be of value in forensic science. Differences in pigmentation are most striking among people of different genetic ancestries, hence variation in pigmentary genes has recently been proposed as an effective tool for genetic determination of ethnic origin (6,7).

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It is assumed that more than 120 genes are involved in the pigmentary phenotype (8). Multiple variation in hair and skin color is determined by the ratio of two melanin types. A single gene encoding the melanocortin 1 receptor (MC1R) plays a key role in black or brown eumelanin and red or yellow pheomelanin in humans and therefore has a strong influence on hair and skin color (9). The MC1R gene is located on chromosome 16 (16q24.3) and comprises a single 951 bp long exon. The gene encodes the typical G protein-coupled receptor associated with the melanocyte membrane, and its activation increases the cAMP level. Some alleles of the MC1R, described as diminished function alleles or alleles that affect the melanocyte membrane expression of the receptor, are significantly associated with the overproduction of pheomelanin, which manifests in red hair and fair skin. Variation within the MC1R gene is exceptionally high among Caucasians and has a significant impact on the pigmentary phenotype in this ethnic group (10). Red hair color has been linked to some MC1R alleles but most recent studies have shown that the same genotypes can express different hair color depending on the population studied (11). Therefore, further studies are warranted to better understand the inheritance of hair color. Broad studies addressing all forensically relevant questions are also crucial in order to open up the possibility of future application of this analysis in real cases. The aim of our study was to determine the polymorphism of the MC1R gene and examine the association of its alleles with the red hair phenotype in our population. The usefulness of the MC1R gene in forensic applications is also discussed.

Materials and Methods

Samples

The donor's physical characteristics were examined by a dermatologist, who also collected buccal swabs for the purpose of genetic analysis. Data concerning phenotypic features of participants are summarized in Table 1. Samples were divided into two groups—one comprised red-haired donors and the second consisted of nonred-haired controls characterized by different pigmentation. Individuals reporting dyed hair were not included in

TABLE 1—*Characteristics of studied individuals.*

Characteristics	Total (n = 184)
Gender	
Male	76
Female	108
Hair color	
Red	40
Blond-red	36
Blond	20
Dark blond	60
Brown	13
Auburn	2
Black	13
Eye color	
Blue or gray	109
Green	33
Hazel	35
Brown or black	7

TABLE 2—*Amplification primers.*

Primer	Primer Sequence (5′-3′)	Fragment Size
MIF	TGGGCTCCCTCAACTCCACCC	268 bp
MIR	AGCAGGAGGATGACGGCCGTCT	
MIIF	AGCTCCATGCTGTCCAGCCT	193 bp
MIIR	AGCAGGACGGCCACGTGGT	
MIIIF	CTCACACTCATCGTCCTCTG	206 bp
MIIIR	TGC CCA GCA CAC TTA AAG C	

the study. The collected samples were subjected to DNA isolation, which was done by the standard organic method followed by phenol–chloroform–isoamyl alcohol extraction. DNA concentration was measured with the fluorimetric method using PicoGreen kit (Molecular Probes, Leiden, The Netherlands) and Fluoroscan Ascent FL (Labsystems, Helsinki, Finland) apparatus.

Sequence Analysis of the Complete MC1R Gene

The complete MC1R exon was amplified using primers described by Kanetsky and coworkers (12). The PCR reaction mixture consisted of 1 U Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 μM dNTP, 0.25 μM of each primer; 0.4 μL BSA (1 μg/μL), 2.0 μL 10 × concentrated PCR buffer (Fermentas, Vilnius, Lithuania) and *c*. 5–10 ng of DNA template in a total reaction volume of 20 μL. Amplification reactions were carried out in the GenAmp 9700 or GenAmp 9600 thermocycler (Perkin Elmer, Norwalk, CT) under the following PCR conditions: 95°C/1 min; (94°C/10 sec, 64°C/30 sec, 72°C/40 sec) × 32; 72°C/10 min; 4°C. PCR products were purified using Microcon 100 columns (Millipore, Billerica, MA) and subjected to cycle sequencing using the same primers as for amplification and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Products of sequencing reactions were subjected to analysis using Genetic Analyzer ABI 3100 (Applied Biosystems). Sequence analyses were done with Sequence Navigator software according to the reference consensus sequence imported from the GenBank (accession number: AF 326275).

Comparison of the allele frequencies between red-haired individuals and controls was done with Pearson's χ^2 test using statistical package for the social sciences (SPSS) software (13).

Multiplex Analysis of Forensically Valuable SNPs

Multiplex amplification of MC1R fragments encompassing significantly phenotype-correlated variable positions was optimized for the purpose of examination of forensic specimens that are often subjected to degradation. The test was developed to include polymorphic nucleotide sites: C252A (codon D84E), G425A (R142H), C451T (R151C), C478T (R160W), and G880C (D294H). Primer sequences and sizes of generated products are given in Table 2. The locations of PCR primer sites and analyzed nucleotide positions are presented in Fig. 1. The PCR mixture consisted of 5 μL of Qiagen Multiplex PCR Master Mix and 1 μL of Q solution (Qiagen, Hilden, Germany), 1 μL of premixed primers (0.125 μM each), 1–15 ng of template DNA, and distilled water up to 10 μL. Reaction conditions were as recommended by the manufacturer: preincubation step, 95°C for 15 min; denaturation, 94°C for 30 sec; annealing, 63°C for 90 sec; extension, 72°C for 90 sec, 30–34 cycles; final extension, 72°C for 10 min. Products of amplification were purified using the ExoSap kit (Amersham-Pharmacia, Freiburg, Germany) and subjected to minisequencing reactions using the SNaPshot Multiplex Kit (Applied Biosystems). The composition of the reaction mixture was as follows: 2 μL of SNaPshot mix, 1 μL of premixed extension primers and 1–2 μL of amplification products in a total volume of 10 μL. Details concerning extension primers used in the study are given in Table 3. The minisequencing products were resolved on an ABI 3100 Avant (Applied Biosystems) genetic analyzer using electrophoresis conditions as recommended by the SNaPshot manufacturer. Control DNA 007 (Applied Biosystems) was used to assess the sensitivity of the assay. The validity of the multiplex was confirmed using previously sequenced samples.

Results and Discussion

Population Data

The complete population sample consisted of 76 red-haired individuals and 108 nonred-haired controls. Individuals included in the redheaded group had different shades of red color but for simplicity were classified into two subgroups—pure red and blond red (strawberry blond). The most frequent phenotype characteristics for nonred-haired individuals were dark blond hair and blue eyes. Data concerning pigmentary traits are summarized in Table 1 and were selected to adequately reflect the phenotype distribution characteristic for the studied part of Europe. All but two participants were unrelated males and females from Poland, mainly from the southern region. One of two studied redheaded sibs was included in further population calculations. The obtained results show that MC1R variation in the studied population sample is high and is similar to the level of variation characteristic for other

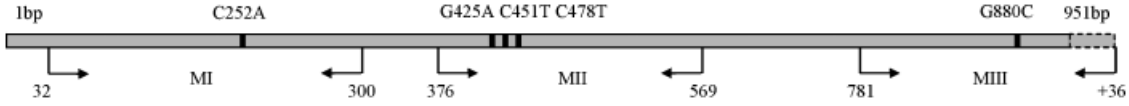

FIG. 1—*Diagram presenting location of the primers and five tested polymorphic single nucleotide polymorphism positions included in the optimized minisequencing assay. MI, MII, MIII, fragments of the melanocortin 1 receptor gene encompassing tested sites (compare also with Table 2).*

TABLE 3—*Extension primers.*

Amino Acid	DNA	Neutral* 5′ → 3′	Target 5′ → 3′	Concentration
D84E	C252A	–	C TGC CTG GCC TTG TCG GA	0.2 μM
R151C	C451T	T CTG ACA A	TCT CCA TCT TCT ACG CAC TG	0.2 μM
R142H	G425A	AA AGT CTG ACA A	GCC ATC GCC GTG GAC C	0.2 μM
R160W	C478T	AA AGT CTG ACA A	ACA GCA TCG TGA CCC TGC CG	0.2 μM
D294H	G880C	A CGT CGT GAA AGT CTG ACA A	CTG CAA TGC CAT CAT C	0.1 μM

*Neutral sequence according to (22): 5′-AAC TGA CTA AAC TAG GTG CCA CGT CGT GAA AGT CTG ACA A-3′.

populations of northern European origin (12,14,15). Our survey ascertained 21 variable sites: 16 transitions (9 C/T and 7 A/G), four transversions, and one insertion A (Table 4). The study revealed only two alleles at each of the examined polymorphic sites. Sixteen of the variable sites result in nonsynonymous amino acid changes. A relatively high number of variants causing amino acid alterations have usually been observed for this particular gene in European populations. Differences in pigmentation between various ethnic groups have been explained by selective pressure affecting pigment-related genes and this hypothesis was proved in the case of MC1R (10,16).

Genotype–Phenotype Associations

Penetrance of Red Hair

The proportion of individuals with a particular genotype that is expressed in the red hair phenotype (the penetrance of red hair) is given in Table 5. The complete sequence data determined for the MC1R gene proved that variants R151C and R160W are significantly associated with red hair color in our region ($p \leq 0.001$). A recent study on functional implications of the MC1R variants suggested that these changes are responsible for reduced expression of the receptor on the melanocyte membrane (17). All six homozygotes of R151C and all nine homozygotes of R160W, as well as 24 of 25 heterozygous variants R151C and R160W had red hair. The study indicated that 97.5% of individuals who possessed the above genotypes had red hair. Based on the limited sample size in this study, there is a strong correlation between the presence of the above genotypes and the presence of red hair. Moreover, our study showed that the variants R151C and R160W appeared in redheaded individuals in combination with some other polymorphisms and finally 65 of 76 redheaded individuals had at least one of these alterations. The above mutations together with D84E, R142H, and D294H have been considered in the literature as strongly associated with the red hair phenotype (15,18). The obtained data also confirmed the significant association of these variants with red hair, but their role in determination of this phenotype is significantly smaller due to their lower frequency in the studied population. Examination of the above five alleles should be considered as valuable for the purpose of phenotype prediction. Comparative analysis of our data with variation characteristic for other populations with European origins showed that the variation patterns are very similar, though minor shifts in the role of particular polymorphisms in determination of red hair color were noticeable. For example, a higher frequency of allele R142H was noted among redheaded individuals in Poland, U.K., and U.S. than, e.g., in Australia, so its contribution among the cohort of redheads in Europe and U.S. can be considered as more significant. The opposite situation occurred in the case of the D84E allele (12,14,15). Still, the role of R151C and R160W has been most significant in the currently studied populations. As many as 69 of the analyzed red-haired individuals carried more than one allele variant. Six individuals (five of them strawberry blond) were simple heterozygotes. This result is consistent with previous suggestions that only 10–20% of redheads have a combination of one consensus and one variant allele (15,18). A simple heterozygote, even in a case where a mutation strongly decreases the performance of the receptor, apparently has no predictive value. In our study, simple heterozygous individuals with one red hair-related allele, e.g. R151C/consensus or R160W/consensus, represented a whole range of scalp hair colors from blond red to black. One individual with the strawberry blond phenotypic feature had two consensus alleles. This phenomenon has been sporadically observed in other studies and can be explained by different mechanisms. It can be caused by variation in regulatory regions of the MC1R that could, e.g., diminish allelic expression. It can also occur due to variation in other genes involved in pigmentation processes, like the proopiomelanocortin gene, the precursor of α-MSH or agouti signaling protein (19,20). Determination of two consensus alleles of the MC1R gene in a sample has no exclusive value for red hair color prediction.

TABLE 4—*Melanocortin 1 receptor (MC1R) allele frequencies in 75 unrelated red-haired individuals and a group of 108 controls.*

Nucleotide Change	Amino Acid Change	Red Hair, 150 Alleles (%)	Controls, 216 Alleles (%)
Consensus		5.3	51.9
86.1A	N29insA	2.7	0
G178T	V60L	2.7	8.3
C199T	R67W	0.7	0
C252A	D84E	0.7	0
G274A	V92M	5.3	9.7
C325T	R109W	0	0.5
G425A	R142H	6	0.5
C444T	*Y148Y*	*0*	*0.5*
C451T	R151C	33.3	4.6
C456A	Y152OCH	1.3	0
T464C	I155T	2.7	0.9
C478T	R160W	34.7	9.3
G488A	R163Q	1.3	4.2
C561T	*A187A*	*0*	*0.5*
G652A	A218T	0	0.5
G699A	*Q233Q*	*0*	*0.5*
C766T	P256S	0.7	0
A832G	K278E	0	0.5
G880C	D294H	2.7	0
A942G	*T314T*	*6.7*	*9.7*
C948T	*S316S*	*0*	*0.5*

Synonymous changes are printed in italics.

Epistasis and Phenotype

Genetic background can obviously influence the ultimate effect of MC1R action. A recent study indirectly showed that the effect

TABLE 5—*Proportion of red hair in a genotyped population sample. Cells indicate red hair/whole number of variant carriers.*

	N29shift	V60L	R67W	D84E	V92M	R142H	R151C	Y152OCH	I155T	R160W	R163Q	P256S	D294H	Cons
N29shift	0/0													
V60L	0/0	0/0												
R67W	0/0	0/0	0/0											
D84E	0/0	0/0	0/0	0/0										
V92M	0/0	***1/2***	0/0	0/0	0/1									
R142H	***1/1***	0/0	0/0	0/0	0/0	***1/1***								
R151C	0/0	***2/3***	***1/1***	0/0	***2/2***	***4/4***	***6/6***							
Y152OCH	0/0	0/0	0/0	0/0	0/0	***1/1***	0/0	0/0						
I155T	***1/1***	0/0	0/0	0/0	0/0	0/0	***2/2***	0/0	0/0					
R160W	***1/1***	***1/3***	0/0	***1/1***	***3/5***	0/0	***24/25***	***1/1***	***1/1***	***9/9***				
R163Q	0/0	0/0	0/0	0/0	0/1	0/0	***1/2***	0/0	0/0	***1/2***	0/0			
P256S	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	***1/1***	0/0	0/0		
D294H	0/0	0/0	0/0	0/0	0/0	***1/1***	***1/1***	0/0	0/0	0/0	0/0	0/0	***1/1***	
Cons	***1/1***	0/14	0/0	0/0	***2/17***	0/1	***1/8***	0/0	0/2	***2/16***	0/6	0/0	0/0	***1/35***

All 76 redheaded individuals are included. Variants with noted red hair phenotype are printed in bold and italics.

of MC1R variants can be masked by other genes. The study revealed individuals homozygous for well-known red hair-associated variants who obviously did not have red hair (11). It may be that this phenomenon is observed in some populations, especially with darker pigmentation. Definitely, this problem is of great relevance to phenotype prediction based on genetic tests and thus needs further, more detailed studies. The influence of other genes on phenotypic features was also visible in our work. We noted a few cases exemplifying differences between phenotypic features of individuals with the same MC1R genotype. As mentioned above, 24 of 25 heterozygous variants R151C and R160W revealed red hair, but among them were 14 pure redheads and 10 blond-red haired. One individual with this genotype had dark-blond hair and—which is also very rarely observed in the case of these mutations—brown eyes. In a family study, two sibs revealed the same MC1R genotype (homozygotes R160W), but an explicitly different phenotype. While the older brother had dark-red hair color, the younger one was typically carrot-red haired. We can speculate that differences in shade of hair color can originate from heterozygosity/compound heterozygosity of genotyped samples and can also be associated with total melanin content characteristic of an individual, so can be dependent on the performance of other pigmentary genes. The obtained results and literature data indicate that it is not possible to predict the shade of red color on the grounds of MC1R analysis.

Pleiotropic Effect of the MC1R

The influence of MC1R variants is not limited to hair color. It had been reported by many authors (and was also supported by our study) that most redheads reveal pale skin often associated with freckling. Therefore, determination of MC1R variants strongly associated with red hair determination, such as R151C or R160W, can be considered as a strong indication that the individual has red or strawberry-blond hair and most probably fair skin color, possibly associated with freckles. As discussed above heterozygous individuals with one red hair-related and a second consensus allele are often not redheaded. Interestingly, the action of mutation diminishing receptor function can still affect physical features of the individual. We noted examples of males with the above genotypes who did not have red scalp hair but revealed red hair beards. Similar observations were also presented in the work of Flanagan et al. (21). One definitely cannot speculate about the eye color of an individual based on MC1R analysis. Although some studies have demonstrated associations of MC1R variants with blue iris color, others have not supported this observation (6,22). Our results also did not reveal a correlation between any eye colors and the MC1R variant. Literature suggestions concerning the V60L codon change and its association with fair, blond, or light brown hair color (23) also seem to be very uncertain. Our work showed weak correlation of V60L ($p \leq 0.005$) with dark-blond hair, but determination of this alteration obviously cannot be considered as predictive. We are aware of the fact that some correlations may be biased by population substructure.

SNaPshot Test for Determination of Forensically Relevant Polymorphisms

The proportion of individuals with variants R151C and R160W that express the red hair phenotype has been reported as significantly high in many populations. Approximately 85% of redheads examined in our work were affected by one of these mutations. It therefore seems that determination of these MC1R variants could be considered as a valuable tool for forensic purposes. We chose three additional codon positions that had been considered as very important in determination of red hair color in different population samples—R142H, D294H, and D84E and developed a SNaPshot-based assay providing multiplex selective analysis of these five variable sites. As short amplicons are crucial for amplification of problematic forensic specimens, which are frequently subjected to degradation, we optimized a triplex amplification of the MC1R segments, encompassing the above-mentioned nucleotide positions. Primers were designed to generate fragments of length below 300 bp, which is widely considered as applicable to analysis of degraded forensic samples. The amplification was optimized with the Qiagen Multiplex PCR kit. The extension primers were designed according to rules described by Sanchez et al. (24). An example of results for the optimized multiplex from a sample revealing the forensically relevant heterozygote R151C/R160W is given in Fig. 2. Performed experiments showed that positive results can be obtained in the range of 50 pg to 15 ng of template DNA, but its excess can result in additional unspecific PCR products. The sensitivity of the assay was assessed by analysis of good quality DNA template—007 control, included in the SGM Plus kit (Applied Biosystems). PCR was performed using standard conditions and 34 cycles. Excellent minisequencing results were obtained for samples with 100 pg of PCR template DNA. In order to confirm correct performance of the developed protocol, we analyzed several previously sequenced samples and verified that the genotyping done with the developed protocol yielded results that

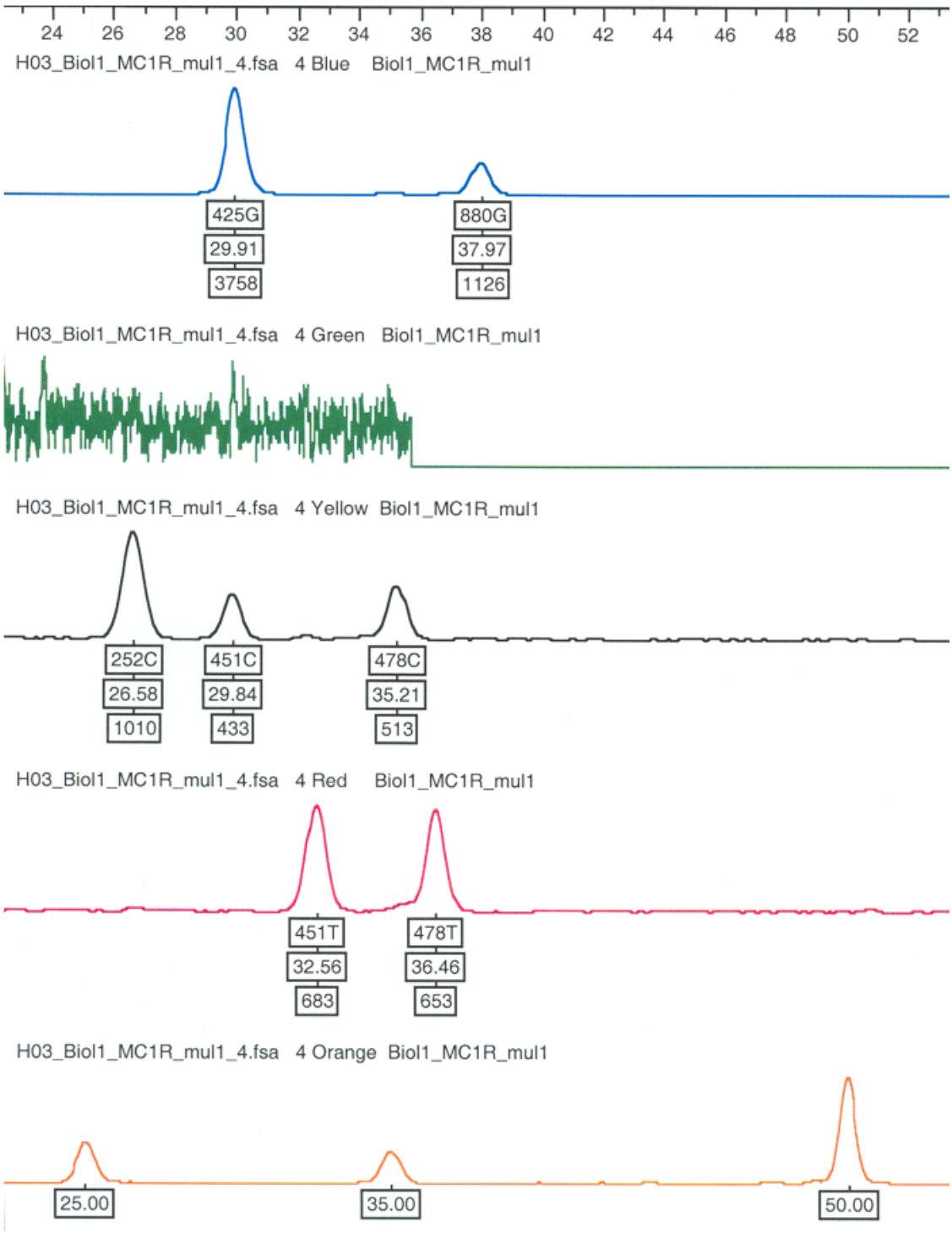

FIG. 2—*Example of results of the SNaPshot test. The first four panels refer to the sample heterozygote R151C/R160W. The lowest panel presents three fragments of the GeneScan-120LIZ size standard. The profile of the sample is 252C, 451C/T, 425G, 478C/T, 880G. The individual had carrot-red hair color. Each peak is accompanied by information concerning the name of the single nucleotide polymorphism and the detected variant, e.g., 425G, real size of the extension product and peak height.*

were completely concordant with the sequence data. Furthermore, typical casework samples were subjected to analysis in order to evaluate the effectiveness of the assay in the case of heavily degraded specimens. Positive results were obtained for several blood and semen stains and five bone samples. The method was tested under standard PCR conditions and 34 amplification cycles. The genotyper macro prepared for genotyping of the MC1R positions is available upon request.

Conclusions

The study performed on the population of 76 redheaded individuals and 108 nonred-haired controls revealed 16 nonsynonymous and five additional synonymous changes in the MC1R gene. The relatively high population frequency of variants R151C and R160W and their very high association with red hair color makes analysis of these particular positions very interesting from the

point of view of phenotype prediction. Three important variants affecting MC1R performance—D84E, R142H, and D294H, were less frequent in the studied population, but, due to their significant impact on hair color, their analysis still seems to be valuable. The optimized test based on multiplex amplification and selective genotyping enabled effective analysis of these five positions in typical forensic specimens. Determination of one of the above-mentioned MC1R variants in the homozygous state or a heterozygous combination can be considered as a strong indicator that the sample donor has red or strawberry-blond hair and fair skin color. Our study also showed that determination of two consensus alleles in a sample does not exclude the possibility that the donor has red hair but the probability for this is very low. Another issue is that potential inconsistency between genetic indication and witness testimony concerning the hair color of a perpetrator does not need to be related to test weakness or a mistake by the witness. Irrespective of the obtained results, the possibility that the perpetrator had artificial or dyed hair always has to be taken into account in the investigation. We can conclude that analysis of particular sites within the MC1R gene can, in special circumstances, be useful in forensic science for phenotype prediction. Further research encompassing other populations, especially ones carrying different pigment characteristics, and also analysis of other pigmentary genes, would be useful in developing more accurate genetic predictions of pigmentary status—which could be applied in the forensic field.

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