SINGLE NUCLEOTIDE POLYMORPHISMS AND THEIR ROLE IN PREDICTING EARLOBE ATTACHMENT FOR FORENSIC IDENTIFICATION

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ABSTRACT: Forensic DNA phenotyping (FDP) has the potential to forecast externally visible characteristics from body fluid specimens, giving valuable leads in criminal investigations where conventional forms of identification are not feasible. The current study was intended to investigate the correlation of seven SNPs under consideration for the current study—rs13397666, rs2080401, rs9866054, rs263156, rs10192049, rs1342722, and rs17023457—with earlobe attachment phenotype (attached or free) in a 300-member Punjabi population sample from Pakistan. Earlobe phenotypes were classified by visual inspection, and genotypic data were examined using SNPStats with codominant, dominant, recessive, over-dominant, and log-additive models of inheritance. The strength of association was expressed as odds ratios (ORs), confidence intervals (CIs), and p-values, the fit of the model being as suggested by Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) values. Unlike other populations, for all the genetic models considered, no statistically significant correlations were obtained between any of the SNPs and earlobe attachment. The findings are indicative of a possible population-specific genetic effect or the role of other, as yet unidentified, variants. The research emphasizes studies' validation of regional phenotypic prediction models and further contributes to the world's understanding of human morphological diversity. Enhanced precision of FDP use in forensic and anthropological practice may be achieved with further study utilizing larger and more inclusive samples, genome-wide investigation, and more specific phenotypic classification.

***Keywords*:** Forensic Genetics, Single Nucleotide Polymorphism, Phenotype, Ear genetics, Genetic Markers Study design and principal results are graphically summarized in the Graphical Abstract.

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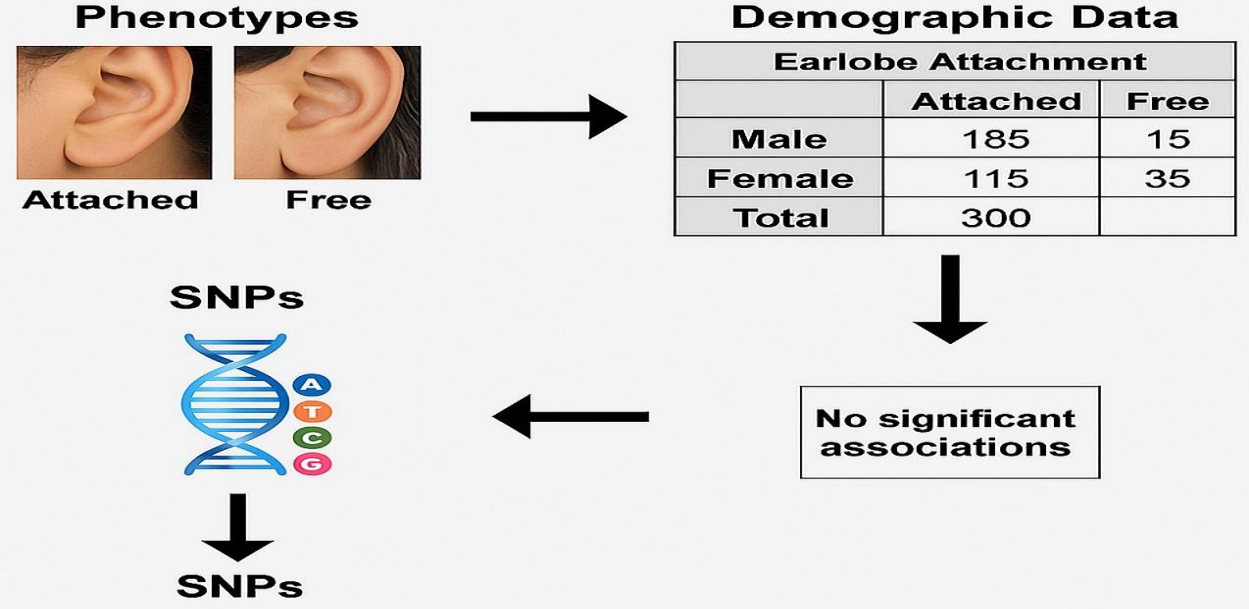

Graphical representation of the study exploring the correlation between certain single nucleotide polymorphisms (SNPs) and earlobe attachment phenotypes (attached or free) in a Punjabi Pakistani population. The study examined 300 subjects and identified no statistically significant correlations between the screened SNPs and earlobe morphology.

INTRODUCTION

Forensic DNA Phenotyping (FDP) is a novel forensic science tool with great potential that provides a means of predicting a person's externally visible characteristics (EVCs) from a crime scene DNA sample (1). In contrast to comparative DNA profiling against known samples or databases, FDP is capable of giving investigation leads even without a suspect (2). This technology is especially useful where unidentified remains exist or where typical forensic evidence, such as fingerprints or dental information, is incomplete or unavailable (3). Of the EVCs, skin color, eye color, hair color, and facial morphology have been most researched (4). But newer research has added ear morphology, which entails earlobe attachment—a specific body characteristic categorized as either free or attached—to the list of FDP (5). Earlobe attachment has already been investigated as a morphogenetic trait that is caused by genetic heritage, and its phenotypic variation is potentially useful for forensic identification (6). Single Nucleotide Polymorphisms (SNPs) are small genetic differences that occur at a nucleotide position in the genome and are useful as good genetic markers for FDP (7). Several SNPs, including rs263156 (GPR126 gene), rs2080401 (SP5 gene), and rs10212419 (FOXL2 gene), have been documented for their link with ear morphology aspects like attachment and size of the earlobe (8). Although FDP from SNPs has been studied among various groups from all over the world, quite less work has been conducted in the context of South Asia, especially from Pakistan (9). As a result of the distinctive genetic profile and ethnic background of the Pakistani population, regional research is the order of the day for making the predictions more accurate and reliable in forensic science (10). The present study is interested in the Punjabi population, who constitute the largest ethnic group of Pakistan and intends to investigate the association of chosen SNP markers with earlobe attachment phenotypes. By creating a genotype-phenotype correlation, this study attempts to investigate the optimal capacity of FDP for forensic casework, particularly in native forensic databases and identification schemes. Utilizing molecular methods in DNA isolation, quantitation, and SNaPshot-based genotyping, the current study is hoped to make a noteworthy contribution to forensic genetics. The results would be used to design population-specific forensic resources and inform a broad understanding of the genetic basis for human morphological diversity, with applied value in both criminal investigations and anthropological analyses.

MATERIAL AND METHODS

Schematic overview of the study design has been shown in Fig. 1.

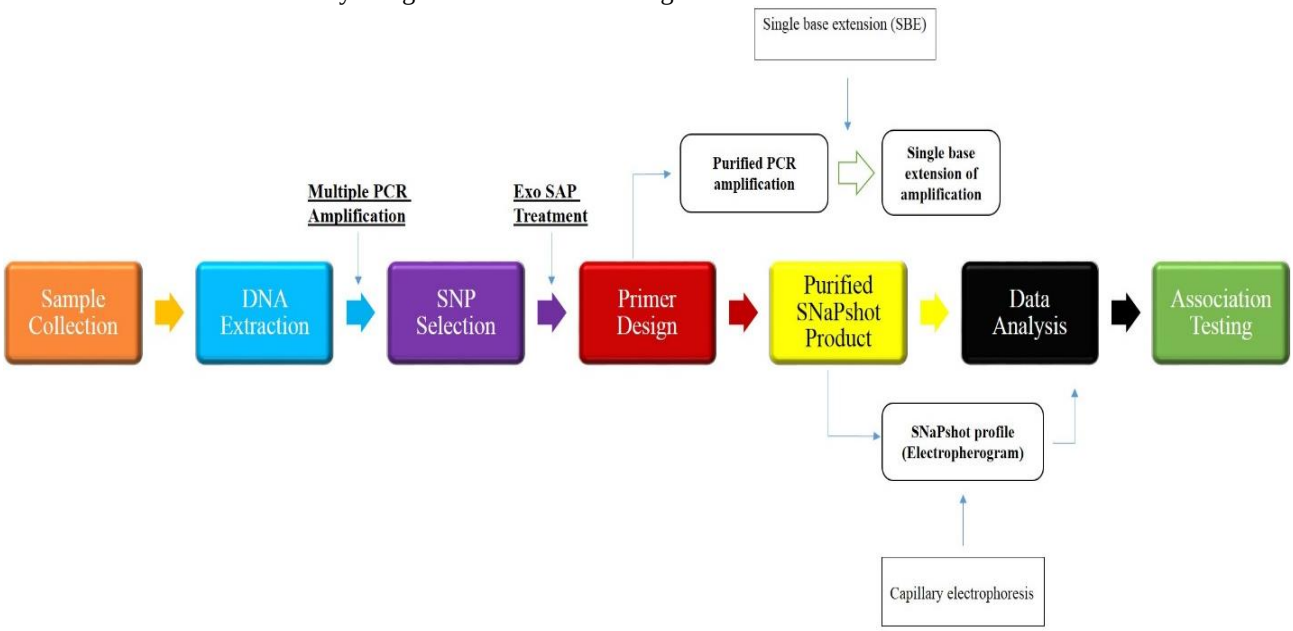

Fig. 1: A Schematic Overview of the study design

Study Population and Design: The cross-sectional study aimed to evaluate the correlation between chosen Single Nucleotide Polymorphisms (SNPs) and the earlobe attachment phenotypes in the Punjabi population of Pakistan. 300 unrelated, healthy individuals (150 males and 150 females) were recruited by purposive sampling from five prominent districts of Punjab: Lahore, Faisalabad, Rawalpindi, Multan, and Gujranwala.

Inclusion and Exclusion Criteria: Subjects chosen for this research were healthy adults between 18 and 50 years old with no known ear deformities. For each subject, photographs and blood samples were taken. People were excluded if they had ear deformities, ear piercings, known diseases of the ear, or were not within the age bracket of 18 to 50 years.

Ethical Considerations: The research was approved ethically by the Ethical Review Committee of the University of Lahore (UOL). Written informed consent was sought from all participants before blood sampling and photography.

Phenotype Classification: Earlobe attachment was phenotyped by direct visual examination and standardized digital photographs. The participants were categorized as: Free earlobes and Attached earlobes. The photographs were obtained with a Nikon D5600 DSLR camera ((Nikon D5600; Effective Pixels (Megapixels) 24.2 million and Sensor Size: 23.5 mm. x 15.6 mm) under standardized illumination with the head in Frankfort horizontal plane.

Genomic DNA Extraction: Genomic DNA was isolated from human blood samples in a modified salting-out procedure followed by phenol-chloroform extraction. In short, blood samples were pipetted into 15 mL centrifuge tubes and three volumes of Cell Lysis Buffer-I (1 M Tris-Cl, 0.5 M EDTA, distilled water) were added. The samples were kept on ice for 30 minutes and then centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant was removed, and the pellet was re-suspended in 10 mL of lysis buffer and re-centrifuged with the same parameters for 25 minutes. The supernatant was removed, and the pellet was re-suspended in 4.75 mL of STE buffer. Then 25 µL of 10% SDS was added dropwise while mixing, followed by 10 µL of Proteinase K (20 mg/mL). The solution was incubated overnight at 55°C to allow protein digestion. For phase separation, 5 mL of cold phenol: chloroform: isoamyl alcohol (25:24:1) was added, and the samples were shaken gently for 10 minutes and centrifuged at 2500 rpm for 30 minutes at 4°C. The aqueous upper phase was pipetted to new tubes using a cut-tip pipette to avoid DNA shearing. DNA was precipitated after adding 500 µL of 10 M ammonium acetate and 5 mL cold isopropanol (or 10 mL absolute ethanol). White DNA strands could be seen on gentle mixing, and samples were left overnight at −20°C (or 15 minutes at −70°C). DNA was centrifuged at 2500 rpm for 60 minutes at 4°C, washed in 5 mL of 70% ethanol, and then re-centrifuged at 2500 rpm for 40 minutes at 15°C. The thus-obtained DNA pellet was left to air-dry at room temperature and re-suspended in low TE buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0).

Quantification: Quantification was achieved using a NanoDrop spectrophotometer by measuring absorbance values at 260 nm and 280 nm. The purity of the DNA was determined by the A260/A280 ratio, and values between 1.7 and 2.0 were considered acceptable. A ratio of >2.0 was an indication of phenol contamination and a ratio of <1.7 was an indication of protein contamination. The concentration of the DNA was determined using the equation: A260 × 50 µg/mL. The NanoDrop method needed only 1–2 µL of sample and gave quick and precise quantitation by UV-Vis absorbance (Desjardins et al., 2010). Representative DNA concentrations of chosen samples were given in the supplementary data.

Choice of SNP Markers: Seven SNPs previously linked with earlobe shape were chosen from the literature that are; rs263156, rs2080401, rs10212419, rs17023457, rs13397666, rs10192049, and rs1342722. They were intronic, intergenic, and regulatory variants.

Primer Design and PCR Amplification: Genotyping was conducted with multiplex SNaPshot assays. Briefly, genomic DNA was isolated by the in-house standard protocol of phenol-chloroform isoamyl alcohol followed by qualitative and quantitative analysis. Six genes were chosen by a survey of the literature, rs263156, rs10212419, and rs2080401 were intronic SNPs in gene GPR126, MRPS22, and SP5. While rs1702345, rs13397666, and 1092049is regulatory variant. Primer pairs flanking each of the chosen SNPs were designed using the Primer 3 online tool. By using Primer 3 with default settings, single-base extension primers and primer pairs were designed using a melting temperature of 60 °C and GC content of 30%. Melting temperature and amplicon length were calculated using the UCSC genome browser. Autodimer determined hairpin and primer dimer formation in multiplex PCR primers for their performance evaluation. Forward and reverse single-base extension primers were generated for the multiplex system. PolyT-tails on SBE primers' 5' ends offer full capillary electrophoresis separation of multiplexes' SBE products. Gradient PCR was utilized to optimize all primers. For performing multiplex PCR in 10µl final reaction volume 1 ng of DNA, 1x Qiagen PCR Multiplex Mix (Hilden, Germany), and primer concentrations were utilized. Then, thermal cycling was carried out using the Veriti 96 well thermocycler (Applied Biosystems).

Multiplex PCR and SNP Genotyping: Four chosen SNPs were co-amplified at optimized conditions through multiplex PCR. Each 10 µL reaction contained 5 µL of Qiagen multiplex mix, 3 µL of primer premix (0.2 µM each), 1 µL of genomic DNA (10 ng), and 1 µL of PCR-grade water. Thermal cycling conditions were optimized for maximal yield and specificity. PCR products were cleaned by adding 1 µL ExoSAP (ExoproStart™, Illustra™) and incubating for 60 minutes at 37°C followed by inactivation of the enzymes at 75°C for 15 minutes to remove any residual primers and dNTPs. **2.9**

SNaPshot Multiplex Single Base Extension (SBE) Assay

For the SNaPshot assay, 1 µL of SNaPshot™ Ready Reaction Mix (Thermo Fisher), 1 µL of SBE primer premix (0.4 µM), 1 µL of purified PCR product, and 2 µL of water were used in a 5 µL final volume. The SBE thermal profile included 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 30 seconds, run on a Veriti 96-well thermocycler.

SBE Product Purification: The extended products were purified by adding 1 µL of Shrimp Alkaline Phosphatase (SAP, Applied Biosystems) into the 5 µL reaction and then incubated at 37°C for 70 minutes and then deactivated the enzyme by incubating it at 72°C for 20 minutes. The reaction was then heated at 100°C for 2 minutes and then cooled again for 2 minutes.

Capillary Electrophoresis and Data Analysis: After purification, 10 µL of Hi-Di formamide and 0.4 µL of GeneScan-120 LIZ size standard were added to each reaction. Samples were run on a 3130xl Genetic Analyzer (Applied Biosystems) with the POP-7 polymer, 36-cm capillary, and E5 dye set, with injection voltage at 2.5 kV for 10 seconds and a run time of 500 seconds at 60°C. Allele calling and genotype analysis were done using GeneMapper™ ID software v3.1.

Association Testing and Statistical Analysis: The association between earlobe morphology and the chosen SNPs was examined by the web application SNPStats (Solé et al., 2006). This application facilitates detailed statistical modeling for association studies based on SNPs in a three-step process. First, data were manually inputted or uploaded as a tab-delimited file, in which genotype data were denoted with slashes (e.g., "T/T", "C/C"). In step 2, SNPStats typed variables as quantitative, categorical, or SNP to enable users to specify covariates and response types (binary or continuous) for analysis. SNP types could be rearranged for comparisons, and covariates for inclusion in regression analyses. In the third step, the user chose particular statistical outputs like odds ratios, p-values, or genotype distributions.

RESULTS

Population Data: The research in this study was conducted on 300 unrelated healthy individuals belonging to the Punjabi population of Pakistan to analyze the relationship between chosen SNPs and earlobe attachment phenotype. The data were gender-balanced with 150 males and 150 females. Phenotypic distribution showed that 185 males had attached earlobes while 15 had free earlobes. Among the females, 115 exhibited attached and 35 had free earlobes. The distribution reflected a higher prevalence of the attached earlobe phenotype in the population under study (Table 1).

Table 1: Demographic Characteristics of Study Participants

Variable	Category	Frequency (n = 300)	Percentage (%)
Gender	Male	150	50
	Female	150	50
Age Group	18–30	130	43.3
	31–40	100	33.3
	41–50	70	23.4
Earlobe Type	Free	165	55
	Attached	135	45

Observed Ear Phenotypes: The two most prominent phenotypes seen were attached and free earlobes. Each subject was classified by direct visual examination and standard photographic analysis. Phenotypic variation was employed as the foundation for applying observed characteristics to genotypes.

SNP Association Analysis using SNPStats: Association between seven SNPs—rs13397666, rs2080401, rs9866054, rs263156, rs10192049, rs1342722, and rs17023457—and earlobe attachment phenotype was evaluated with the aid of SNPStats software. It worked in five inheritance models: codominant, dominant, recessive, over-dominant, and log-additive. The choice of model was based on AIC and BIC values, with lower values being a sign of the best-fit model. Association strength was assessed by Odds Ratios (ORs) with 95% Confidence Intervals (CIs), and statistical significance was declared based on a p-value cutoff of < 0.05.

Relationship of rs13397666 with Earlobe Attachment: The rs13397666 SNP showed no statistically significant association with the earlobe attachment phenotype in any of the genetic models. All three codominant, dominant, and recessive models gave OR values close to 1.00, and p-values greater than the significance level. The over-dominant and log-additive models also did not show significant association, implying that this SNP is not involved in earlobe attachment variation in the population that was under study (Table 2).

Link between rs2080401 and Earlobe Attachment: SNP rs2080401 analysis revealed no significant connection with the earlobe phenotype under any model of inheritance. While individuals with A/C and C/C genotypes were rather more frequent among attached earlobe subjects, the differences in distribution failed to lead to statistically significant results. All OR values were near 1.00, and the p-values did not provide for any significant genetic effect of this variant (Table 3).

Association of rs9866054 with Earlobe Attachment: The findings for rs9866054 also did not indicate any statistically significant association with the earlobe phenotype. Genotype frequencies (G/G, A/G, A/A) revealed very little variation between groups. All models—codominant, dominant, recessive, over-dominant, and log-additive—provided non-significant p-values and ORs near 1.00. This suggests that rs9866054 would not be likely to be involved in the determination of earlobe morphology in this population (Table 4).

Table 2:rs13397666 association with response STATUS

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	56 (40%)	67 (42.1%)	1.00	NA	596	1698.7
	A/G	66 (47.1%)	69 (43.4%)	NA (NA-NA)			
	G/G	18 (12.9%)	23 (14.5%)	NA (NA-NA)			
Dominant	A/A	56 (40%)	67 (42.1%)	1.00	NA	596	1698.7
	A/G-G/G	84 (60%)	92 (57.9%)	NA (NA-NA)			
Recessive	A/A-A/G	122 (87.1%)	136 (85.5%)	1.00	NA	596	1698.7
	G/G	18 (12.9%)	23 (14.5%)	NA (NA-NA)			
Over-dominant	A/A-G/G	74 (52.9%)	90 (56.6%)	1.00	NA	596	1698.7
	A/G	66 (47.1%)	69 (43.4%)	NA (NA-NA)			
Log-additive	---	---	---	NA (NA-NA)	NA	596	1698.7

(n=299, adjusted by Sample. ID+ Sex)

Table 3: rs2080401 association with response STATUS (n=299, adjusted by Sample.ID+Sex)

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	49 (35%)	74 (46.5%)	1.00	NA	596	1698.7
	A/C	69 (49.3%)	62 (39%)	NA (NA-NA)			
	C/C	22 (15.7%)	23 (14.5%)	NA (NA-NA)			
Dominant	A/A	49 (35%)	74 (46.5%)	1.00	NA	596	1698.7
	A/C-C/C	91 (65%)	85 (53.5%)	NA (NA-NA)			
Recessive	A/A-A/C	118 (84.3%)	136 (85.5%)	1.00	NA	596	1698.7
	C/C	22 (15.7%)	23 (14.5%)	NA (NA-NA)			
Over-Dominant	A/A-C/C	71 (50.7%)	97 (61%)	1.00	NA	596	1698.7
	A/C	69 (49.3%)	62 (39%)	NA (NA-NA)			
Log-Additive	---	---	---	NA (NA-NA)	NA	596	1698.7

Table 4: rs9866054 association with response STATUS

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	94 (67.1%)	121 (76.1%)	1.00	NA	596	1698.7
	A/G	31 (22.1%)	22 (13.8%)	NA			
	A/A	15 (10.7%)	16 (10.1%)	NA			
Dominant	G/G	94 (67.1%)	121 (76.1%)	1.00	NA	596	1698.7
	A/G-A/A	46 (32.9%)	38 (23.9%)	NA			
Recessive	G/G-A/G	125 (89.3%)	143 (89.9%)	1.00	NA	596	1698.7
	A/A	15 (10.7%)	16 (10.1%)	NA			
Over-Dominant	G/G-A/A	109 (77.9%)	137 (86.2%)	1.00	NA	596	1698.7
	A/G	31 (22.1%)	22 (13.8%)	NA			
Log-Additive	---	---	---	NA	NA	596	1698.7

(n=299, adjusted by Sample.ID+Sex)

Relationship of rs263156 with Earlobe Attachment: SNP rs263156, within the GPR126 gene and earlier implicated in literature to be affecting ear morphology, could not demonstrate any association with earlobe attachment in this study. Genotype distributions were equally split across phenotypic groups, and all five models of inheritance yielded non-significant values.

ORs hovered at or near 1.00, corroborating the non-association in the Punjabi population examined (Table 5).

Connection of rs10192049 with Earlobe Attachment: The rs10192049 SNP also did not exhibit any statistically significant connection. Genotypic and phenotypic frequencies were almost the same in both groups. All of the genetic models lacked significant variation in the likelihood of free or attached earlobes. ORs and p-values corroborated the lack of any significant association between this SNP and earlobe morphology (Table 6).

Connection of rs1342722 with Earlobe Attachment: Lastly, rs1342722 analysis revealed no indication of a strong association with earlobe attachment in any model. Even though the frequency of the G/G genotype was slightly more common in individuals with free earlobes, differences were not statistically significant. OR values remained with no effect, and all models did not reach significance (Table 7).

Table 5: rs263156 association with response STATUS

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	C/C	52 (37.1%)	55 (34.6%)	1.00	NA	596	1698.7
	A/C	64 (45.7%)	73 (45.9%)	NA			
	A/A	24 (17.1%)	31 (19.5%)	NA			
Dominant	C/C	52 (37.1%)	55 (34.6%)	1.00	NA	596	1698.7
	A/C-A/A	88 (62.9%)	104 (65.4%)	NA			
Recessive	C/C-A/C	116 (82.9%)	128 (80.5%)	1.00	NA	596	1698.7
	A/A	24 (17.1%)	31 (19.5%)	NA			
Over-Dominant	C/C-A/A	76 (54.3%)	86 (54.1%)	1.00	NA	596	1698.7
	A/C	64 (45.7%)	73 (45.9%)	NA			
Log-additive	---	---	---	NA	NA	596	1698.7

(n=299, adjusted by Sample.ID+Sex)

Table 6: rs10192049 association with response STATUS

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	61 (43.6%)	72 (45.3%)	1.00	1	598	1704.4
	A/G	61 (43.6%)	66 (41.5%)	1.00 (0.00-NA)			
	G/G	18 (12.9%)	21 (13.2%)	NA			
Dominant	A/A	61 (43.6%)	72 (45.3%)	1.00	1	598	1704.4
	A/G-G/G	79 (56.4%)	87 (54.7%)	1.00 (0.00-NA)			
Recessive	A/A-A/G	122 (87.1%)	138 (86.8%)	1.00	NA	596	1698.7
	G/G	18 (12.9%)	21 (13.2%)	NA			
Over-	A/A-G/G	79 (56.4%)	93 (58.5%)	1.00	1	598	1704.4
Dominant	A/G	61 (43.6%)	66 (41.5%)	1.00 (0.00-NA)			
Log-additive	---	---	---	1.00 (0.00-NA)	1	598	1704.4

(n=299, adjusted by Sample.ID+Sex)

Table 7: rs1342722 association with response STATUS

Model	Genotype	STATUS=Ca	STATUS=Co	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	86 (61.4%)	99 (62.3%)	1.00	1	598	1704.4
	A/G	43 (30.7%)	54 (34%)	1.00 (0.00-NA)			
	G/G	11 (7.9%)	6 (3.8%)	NA			
Dominant	A/A	86 (61.4%)	99 (62.3%)	1.00	NA	596	1698.7
	A/G-G/G	54 (38.6%)	60 (37.7%)	NA			
Recessive	A/A-A/G	129 (92.1%)	153 (96.2%)	1.00	1	598	1704.4
	G/G	11 (7.9%)	6 (3.8%)	1.00 (0.00-NA)			
Over-Dominant	A/A-G/G	97 (69.3%)	105 (66%)	1.00	1	598	1704.4
	A/G	43 (30.7%)	54 (34%)	1.00 (0.00-NA)			
Log-additive	---	---	---	1.00 (0.00-NA)	1	598	1704.4

(n=299, adjusted by Sample.ID+Sex)

Summary of SNP Analysis: In none of the seven SNPs that were analyzed was there a statistically significant association with earlobe attachment phenotype among the Punjabi population sampled. Minor differences in the frequencies of genotypes were noted, but these did not portend to predictive models of genetics. The results indicate that earlobe attachment might be determined by other genetic loci that were not present in this panel or polygenic and environmental interactions outside the scope of this project.

DISCUSSION

The current study aimed to explore the genetic linkage among seven selected single nucleotide polymorphisms (SNPs) and earlobe attachment phenotype in Punjabi inhabitants of Pakistan by using a series of various genetic models. In contrast to forecast and observations within some of the earlier reports, none of the SNPs in question—rs13397666, rs2080401, rs9866054, rs263156, rs10192049, rs1342722, and rs17023457—were found to be statistically significantly correlated with earlobe attachment in the examined participants. These results differ from research such as that of Adhikari et al. (2015), where SNPs like rs263156 and rs2080401 were found to be of great association with various ear morphology traits, like earlobe attachment, in multi-ethnic individuals. Other research conducted in European and East Asian populations also supported a great genetic contribution of some of these variants to external ear morphology. However, the lack of association in this study reinforces the possibility of population-specific genetic effects or differences in allele frequency that could result in opposing outcomes between ethnic groups. There are several possible reasons why the present study and previous observations differ. One possible reason is the sample size. Although 300 were covered, detection capacity for associations—especially among low minor allele frequency or low effect size SNPs—may have been on the margin. In addition, the population was ethnically homogeneous, consisting only of people from the Punjabi province of Pakistan, whereas numerous of the cited studies covered different or admixed populations. The homogeneity of the sample may reduce variation, and the associations could be less apparent since they could be hidden relative to those in a genetically heterogeneous population. A second possible reason that no association was detected is that earlobe attachment is caused by a collection of genetic variants not included in the SNPs selected for this study, or by regulatory elements in linkage disequilibrium with other variants. Environmental circumstances, developmental stages, or epigenetic alterations can also play roles in the formation of this trait and were not included in this research. Secondarily, one should also point out that the dichotomization of earlobe attachment as simply two phenotypes—attached and free—may be overly simplistic in capturing the complex variation of ear morphology. Intermediate grades or gradual variety are perhaps more appropriately investigated with quantitative trait methods rather than binary typing. Additionally, although a number of various genetic models were tested using SNPStats, multiple testing was not controlled for in the analysis, which could impact borderline association interpretation, if any. That all models used odds ratios with broad confidence intervals and non-significant p-values only serves to further support the conclusion that these SNPs, in this specific cohort, play little part in controlling earlobe phenotype. Despite the unfavorable outcomes, this study meaningfully adds to the literature by providing population-specific genetic data on an underrepresented South Asian group, filling a key gap in forensic genetics literature. It emphasizes the importance of reproducing genotype-phenotype association studies in representative populations to affirm the universality or specificity of previously noted genetic markers. This lack of association does not negate the value of forensic DNA phenotyping but rather suggests that the best approach is a more comprehensive one using genome-wide analyses or applying polygenic risk scores inclusive of many small-effect variants. Future studies should expand the sample size and include participants from different ethnic populations throughout Pakistan to see if different genetic backgrounds influence the association pattern. Genome-wide association studies (GWAS) or whole-genome sequencing could also uncover new variants influencing ear morphology that had not been researched before. Additionally, gene-environment interaction and developmental factor investigation may help elucidate the complexity of earlobe attachment biology. Locally adapted studies such as this one will be instrumental in improving the accuracy and applicability of trait prediction models as the forensic DNA phenotyping field continues to develop.

Conclusion: Overall, the results displayed no major correlations between earlobe attachment and chosen SNPs in the Punjabi Pakistani population to support possible ethnic-specific genetic variation or the character of polygenic traits. Although the findings are contrary to other populations' work, they do bring forward the importance of localized studies in generating precise and representative forensic DNA phenotyping tools. Further research with larger cohorts, more genetic markers, and newer genomics tools will be needed to explore further the genetic basis of earlobe shape as well as enhance trait prediction in mixed populations.

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Supplementary Data

DNA Concentration of Selected Samples

Sample ID	DNA Concentration (ng/µL)
SD078	83.6
SD254	84.6
SD068	94.6
SD069	85.0
SD150	85.2
SD178	86.6
SD197	89.2
SD032	89.8
SD132	91.0
SD021	91.8
SD221	93.2
SD089	96.0