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Research Article

Gene-Specific Differential Methylation in South African Ethnic Groups: A Preliminary Study

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Abstract

Global and gene-specific variation in DNA methylation has been observed between human populations. However, to our knowledge, differences in DNA methylation between South African ethnic groups have not been reported before. The present study aimed to determine if there was differential methylation in previously reported gene-specific markers between four groups. Saliva was collected from 42 healthy individuals, belonging to Black, Indian, White and Coloured ethnicities. A multiplex Methylation-Sensitive Restriction Enzyme (MSRE)-PCR assay was used to determine methylation levels of CpG sites in the USP49, DACT1, L81528 and PFN3 genes. The Coloured ethnic group displayed highest methylation levels for all selected markers, whereas the Black ethnic group showed lowest. DACT1 and L81528 markers displayed significant variations between ethnic groups (p=0.02 and p=0.03, respectively). Differences in DNA methylation levels between populations all over the world could potentially assist forensic analysts to accurately narrow down the search to ethnicity of sample donors as well as aid medical scientists in personalised medicine.

Introduction

Several studies have shown variation in genetic and epigenetic markers between human populations, which may contribute to externally visible characteristics. Studies have shown that Single Nucleotide Polymorphisms (SNPs) play substantial roles in variation of gene expression, for example between HapMap LCLs obtained from Yorubans and Caucasians [1-3]. Huang et al. [4] reported association between SNP status and ethnic groups using the latest HapMap Phase 3 release version 3, which included more sub-populations. Nearly 300 SNPs were identified, which could accurately predict the ethnicity of all HapMap populations. It has also become evident that in addition to SNPs, mRNA and miRNA expression profiles, Short Tandem Repeats (STRs) and DNA methylation may be reliably used to infer individual ancestry [5-9].

DNA methylation at cytosine residues of CpG sites is a widely characterized epigenetic modification, which plays an important role in transcriptional regulation, gene silencing and cellular differentiation that is vital for normal development [8,9]. Differential methylation patterns in human populations have been reported by several groups [10-13]. Genome wide epigenetic studies have shown that DNA methylation may contribute to phenotypic differences between human populations [11,13,14]. A recent study by Galanter et al. [15] reported differential methylation patterns in diverse Latino ethnic subgroups. About 75% of differential methylation was attributed to genetic ancestry and the remaining 25% was likely due to social or environmental factors. Zhang et al. [10] and Terry et al. [16] measured DNA methylation of peripheral blood samples between Blacks, Whites and Hispanics and found that Whites displayed highest levels of methylation, followed by Hispanics, while Blacks displayed lowest methylation levels.

Differential DNA methylation between HapMap Lymphoblastoid Cell Lines (LCLs) from Yorubans and Caucasians populations has been observed [11,13,17]. Fraser et al. [11] and Moen et al. [17] evaluated European and Yoruban populations and found over 4300 and over 36,500 differentially methylated CpG sites within and between populations, respectively. Differences in gene-specific DNA methylation were also observed; FLJ32569 and STK39 (Serine Threonine Kinase 39) genes were differentially methylated between the populations whereas there was little variation in *AP4S1* (Adaptor-Related Protein Complex 4, Sigma 1 Subunit).

DNA methylation aberrations are associated with diseases such as cancer, HIV, diabetes and several autoimmune diseases. However, the rates of incidence of such diseases differ among racial groups [8,9]. The *HLA-DPA1* locus which is correlated with chronic Hepatitis B (HBV) infection is abundant in Africans and Asians as they have higher frequency of diseases. In prostate cancer research, Enokida et al. [18] demonstrated that methylation levels of *GSTP1* (π-class Glutathione S-transferase) were significantly higher in African Americans when compared to Caucasians and Asians. Kwabi-Addo et al. [19] examined the methylation status of prostate cancer-related genes; *GSTP1, AR,*

RARβ2, SPARC, TIMP3, and *NKX2-5* in African American and Caucasian American populations and also found higher methylation levels of the genes in African Americans compared to Caucasians, which may potentially contribute to the racial differences that are observed in prostate cancer pathogenesis. These differences can be explained by different environmental exposures, varying responses to the same environmental exposures, or by intrinsic differences in the frequencies of DNA sequence or epi/genetic variants. However, some researchers state that genetic variations account for a modest amount of differences between populations while others showed that individuals of different populations are more genetically similar than individuals from a single population [7,20,21].

Variable methylation patterns in gene-specific CpG sites show potential for the identification of unknown individuals. Phenotypic differences between twins [22], clues to donors' ethnicity and diets have been previously estimated by CpG methylation profiling [10,14]. Since it is evident that epigenetic variation does account for differences in human populations and ethnic groups, the aim of the present study was to determine if the diverse South African ethnic groups shows differences in DNA methylation at specific CpG sites [23-25]. Southern Africa is home to populations and ethnic groups carrying significant human genomic variation. The South African population has inter- and intra-continental contributions from Bantus from Western/Central Africa, followed by the arrival of European and East-Indians [26]. Herein, the methylation profile of saliva obtained from four ethnic groups (Blacks, Whites, Indians and Coloureds) in South Africa was evaluated by Methylation-Sensitive Restriction Enzyme (MSRE) PCR, to determine if there is differential methylation of targeted CpG sites among the ethnic groups. Significant differences in DNA methylation among four ethnic groups could assist forensic analysts in future, to narrow down their search down to ethnicity for sample donors. This differential DNA methylation between ethnic groups may also be applicable in personalised medicine which may aid in tailored therapy as necessary for groups of individuals.

Materials and Methods

Sample collection

Approximately 5 mL of saliva was collected in sterile 15 mL tubes from 42 healthy volunteers (with no chronic disorders). Participants included males and females, from four ethnicities (Blacks, Indians, Whites and Coloureds). The saliva samples were stored frozen at -20°C until DNA extraction. Demographics for participants are presented in Table 1. Participants were requested to provide written disclosure of their ethnicity/race, age and gender in the form of a questionnaire and all donors provided signed informed consent. The study was conducted according to the methods specified by the Biomedical Research Ethics Committee of the University of Kwa-Zulu Natal (Westville Campus, South Africa).

Black participants can belong to any one, or be a mixture of the four major ethnic groups living in South Africa including the Nguni, Sotho, Shangaan-Tsonga and Venda groups. The Black participants were part of the Nguni languages (Zulu, Xhosa, Swati and Ndebele). All Indian participants had Indian grandparents and parents. Whites refer to individuals having only European ancestry. Coloureds of South Africa are said to be the outcome of admixture which is mating between individuals from reproductively isolated ancestral populations, therefore having mixed ancestry.

DNA extraction and quantification

DNA was extracted from 200 µL aliquots of saliva using a Quick-g DNA MiniPrep Kit (ZymoResearch) as per the manufacturer's instructions. Extracted DNA was quantified using a spectrophotometer (Nanodrop ND-2000, Thermo Fisher Scientific Inc., Waltham, MA, USA). Following quantification, all saliva samples were made up to 100 ng, and then stored frozen at -20°C until further use.

Selection of CpG sites and Primer design for PCR

For DNA methylation-based differentiation of four ethnic groups, four previously reported CpG sites in the *DACT1, USP49, L81528* and *PFN3* genes were selected as markers. CpG sites for *DACT1, USP49* and *PFN3* genes have been reported to display varying amplification in saliva [23-25] and L81528 shows no amplification in saliva as it was reported to be a semen-specific hypermethylation marker [23-25,27]. To ensure that the markers would be applicable MSRE-PCR, primers developed by Choi, et al. [23] that flanks the *Hha*I recognition sites (GCGC) of the four CpG sites were used (Table 2). Amplicon sizes were smaller than 150 bp and the forward or the reverse primers were labelled with FAM (Fluorescein), a fluorescent dye [23]. Amelogenin was used as a sex-typing marker. Primers for the amplification of an amplification control, was designed and synthesised. The artificial DNA templates of the amplification control for PCR success was obtained by PCR amplification of the 481 bp portion of the pCR® 2.1 TOPO® vector (Invitrogen, Carlsbad, CA, USA) [23,24]. The USP49, DACT1, L81528, PFN3 and Amelogenin markers were labelled with FAM, and the amplification control was labelled with NED.

HhaI restriction reaction

Multiplex PCR was developed to amplify *Hha*I recognition sites (GCGC) of the markers of *USP49, L81528, DACT1* and *PFN3* genes. Amelogenin is devoid of a restriction site. Approximately 100 ng of DNA from each participant was digested with *Hha*I in a 10 µL reaction containing 1 µL of Cut Smart Buffer (New England Biolabs, Ipswitch, MA, USA) and 0.2 U of the *Hha*I restriction enzyme (New England Biolabs, Ipswitch, MA, USA). To ensure complete digestion, the unmethylated artificial DNA template (481 bp portion of the pCR®2.1 TOPO® vector) was restricted. Additionally, each sample was run with an unrestricted control reaction. Herein the enzyme was not added therefore the reaction simply contained 100 ng of DNA and 1 µL of Cut Smart Buffer (New England Biolabs, Ipswitch, MA, USA). All digestions were incubated at 37°C for 30 minutes with subsequent heat inactivation at 65°C for 20 minutes on a BIORAD T100™ Thermal Cycler [23,24].

Multiplex MSRE-PCR

Multiplex PCR was carried out in a 96-well plate, in 20 µL reaction volumes that contained 10 µL of enzyme-digested DNA and undigested DNA controls, 2.0 U of AmpliTaq Gold DNA Polymerase, 1 µL of Gold STAR 10 × Buffer (Promega, Madison, WI, USA) and the stated primer concentrations (Table 2). PCR was conducted under the following Hot Start conditions: 95°C for 11 minutes, 28 cycles of 94°C for 20 seconds, 59°C for 60 seconds, and 72°C for 30 seconds and final extension at 60°C for 60 minutes [23] on a BIORAD T100™ Thermal Cycler.

Table 1: Demographics for South African participants.

Ethnic group	Gender	Number of Participants (n)	Age Range (years)
Black	Male	11	Below 30 n=6
			30-50 n=5
	Female	10	Below 30 n=7
			30-50 n=3
Indian	Male	9	30-50 n=8
			Above 50 n=1
	Female	10	Below 30 n=10
White	Male	12	Below 30 n=5
			30-50 n=6
			Above 50 n=1
	Female	8	Below 30 n=5
			30-50 n=3
Coloured	Male	10	30-50 n=10
	Female	10	Below 30 n=4
			30-50 n=5
			Above 50 n=1

Analysis of PCR products

To prepare samples for analysis, a mix containing 1 µL of amplification products, 20 µL of Hi-Di formamide and 0.2 µL of GeneScan™-500 LIZ® internal lane size standard was denatured at 95°C for 5 minutes followed immediately by 3 minutes on ice [23,24]. The amplification products were run on an ABI 310 genetic analyser (Applied Biosystems) according to manufacturer's instructions.

The electropherograms were analysed using GeneMapper ID Software 5 (Applied Biosystems). The threshold for a positive peak was set to 100 relative fluorescent units (rfu).

The methylation status is shown as peak height ratios of each of four markers to Amelogenin for each sample in MSRE-PCR. For men, the peak height of Amelogenin was calculated by the sum of two peak heights [24].

Statistical analyses

To determine whether the candidate CpG sites showed a significant difference in DNA methylation profiles of saliva between the four ethnic groups, ANOVA (Analysis of Variance) was carried out using SAS Software Version 9.2 (SAS Institute Inc., Cary, NC, USA). Effect of age and gender on methylation status was also analysed. Comparison of methylation status of each marker, for each ethnic group, was performed by calculating the peak height ratios/ marker. Differences were statistically significant when p values were less than 0.05. To test for normality of data, Shapiro-Wilk, Kolmorogorov-Smirnov Cramer-von Mises and Anderson-Darling tests were carried out. All tests confirmed that the data followed a normal distribution.

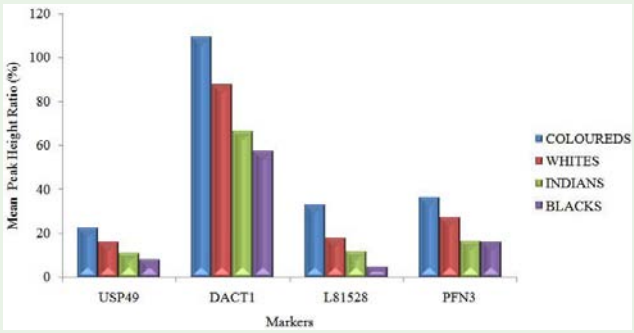

Figure 1: Methylation status of the USP49, DACT1, L81528 and PFN3 markers in saliva DNA of four ethnic groups in South Africa. Methylation status is indicated as (mean) peak height ratios of each of four markers to Amelogenin for each sample in MSRE-PCR. For men, peak height of Amelogenin was calculated by the sum of two peak heights. Blacks, n=9; Indians, n=10; Whites, n=15; Coloureds, n=8. All four CpG sites display highest methylation in the Coloured ethnic group and lowest methylation in the Black ethnic group.

Results

Individual DNA methylation profiling using MSRE-PCR

MSRE-PCR was conducted on 100 ng of saliva DNA from 42 individuals; comprising of 9 Blacks, 10 Indians, 15 Whites and 8 Coloureds from South Africa.

Calculation and comparison of methylation levels of selected CpG markers between four ethnic groups

The peak height ratios for each individual in each ethnic group are listed in Supplementary Tables 1- 4. Peak height ratios revealed a distinct variation of methylation levels for all four sites between the four ethnic groups. The DACT1 marker displayed the highest methylation level across all ethnicities. The Coloured ethnic group displayed highest methylation levels for all CpG sites, whereas lowest methylation was found in the Black ethnic group. A graphical representation of the mean peak height ratio for each marker in each ethnic group is shown in figure 1.

Statistical analyses of CpG markers

The USP49 marker showed slight variation between the four ethnic groups (p=0.05) (Supplementary Table 5). Significant differences in methylation levels were observed between the Coloured (highest methylation) and Black (lowest methylation) ethnic groups (Figure 2).

Table 2: Multiplex PCR primers for amplification of markers [23].

Marker	Forward primer sequences (5'→3')	Reverse primer sequences (5'→3')	Conc (µM)	Amplicon size (bp)
USP49	GTAGCAGGTGTTGCCCAGGTT	FAM-CCCTCCCTACCTCACGCAGA	1.0	107
DACT1	FAM-CACTCCTCCCCTGCTGTCTA	GATAAACTGGCTTGACCA	0.70	118
L81528	FAM-CTTCTGGGGCGACTACCTG	AGTCAGCCTCATCCACACTGA	0.40	128
PFN3	CCTGGCAGCCTCTAGACTCA	FAM-GGGCCAAATAAACTGTGACC	0.20	137
Amelogenin	CCCCTTTGAAGTGGTACCAGAG	FAM-GCATGCCTAATATTTTCAGGGAATAA	0.25	81; 84
Amplification Control	CTGTTCTTCTAGTGTAGCCGTAGTT	NED-CAACCCGGTAAGACACGACT	0.15	131

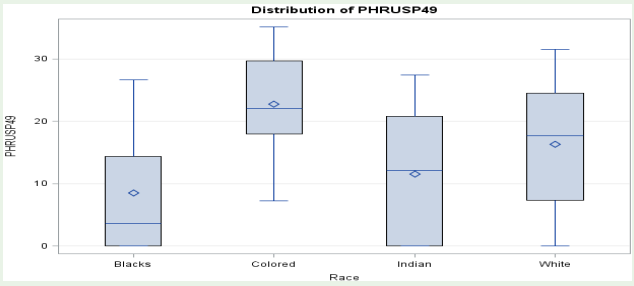

Figure 2: Box-Whisper plot depicting the effect of ethnicity on methylation status of the USP49 tDMR across four ethnic groups from South Africa.

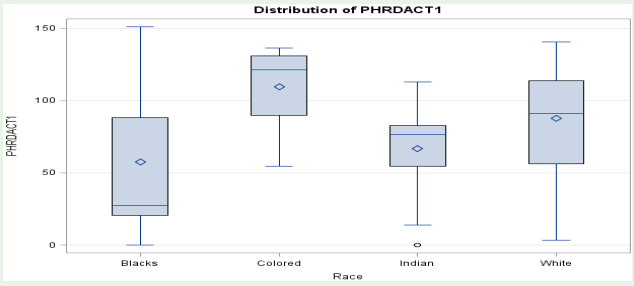

Figure 3: Box-Whisper plot depicting the effect of ethnicity on methylation status of the DACT1 marker across four ethnic groups. The largest differences between the Black and Coloured ethnicities were observed for the marker from South Africa.

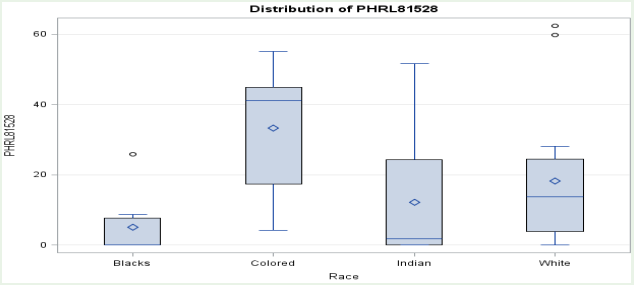

Figure 4: Box-Whisper plot depicting the effect of ethnicity on methylation status of the L81528 marker across four ethnic groups from South Africa. The largest differences between the Black and Coloured populations were observed for the marker.

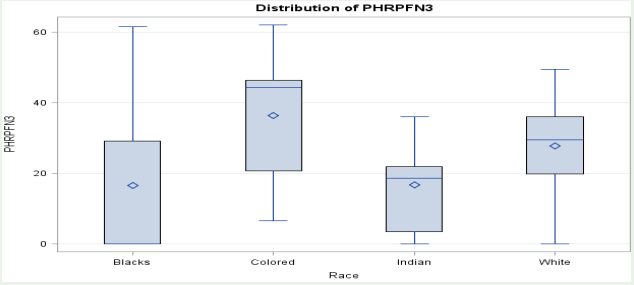

Figure 5: Box-Whisper plot depicting the effect of ethnicity on methylation status of the PFN3 marker across four ethnic groups from South Africa.

The DACT1 marker showed significant methylation variation between the Coloured and Black ethnicities (p=0.02) (Supplementary Table 6). The Coloured group displayed the highest methylation level and the Black group displayed minimum methylation levels (Figure 3). A consistent level of methylation of the DACT1 marker in subjects <30 years and between 30-50 years of age was observed.

The methylation status of the L81528 marker showed significant variation between Coloured and Black ethnicities (p=0.03) (Supplementary Table 7). Similar to USP49 and DACT1, the Coloured group displayed the highest methylation level and the Black group displayed minimum methylation levels (Figure 4). Methylation levels for this marker across all age groups did not significantly differ.

The PFN3 marker showed slight variation between the four ethnic groups (p=0.05) (Supplementary Table 8). The Coloured group displayed the highest methylation level and the Black groups displayed minimum methylation levels (Figure 5). A consistent level of methylation of the PFN3 marker in subjects <30 years and between 30-50 years of age was observed. Interaction of race and age, as well as gender, did not have an effect for all tested CpG sites (data not shown).

Discussion

Predominantly for medical research purposes, studies have proven that variations in human populations exist at the genetic and epigenetic level [20,28-30]. South Africa; being a multi-ethnic country, comprises various races, religions and traditions. While variation in outward appearances is apparent, little is understood of the extent of these variances at the epigenetic level. Thus, the present study was undertaken to explore this epigenetic variation in the context of differential DNA methylation. The aim was to determine if the methylation profile of previously reported CpG sites in four genes in saliva differs between four ethnic groups in South Africa, namely; Blacks, Whites, Indians and Coloureds. The analysis of these CpG-based biomarkers may be of relevance in surveying phenotypic and ethnic traits for possible forensic and medical applications.

Saliva was selected to screen the diverse South African ethnic groups as advantages lie in ease of access and handling, non-invasive collection techniques, as well as no cost involvement [31,32]. Thus, saliva samples were collected from 42 healthy individuals (with no history of any chronic disorder). As volunteers were from different age groups, the effect of age on methylation status of the selected markers was also investigated. To facilitate the differentiation of the four ethnic groups using the saliva DNA, a multiplex PCR assay was used to detect the methylation status of markers. Four markers from the *USP49, DACT1, L81528* and *PFN3* genes were selected. These markers have been previously reported to display differential methylation between human body fluids including saliva, blood, semen and vaginal fluid [23-25]. However, for the present study the aim was not to differentiate between human body fluids, but rather to analyse the methylation patterns of the CpG sites in a single body fluid to determine if they differ between individuals of different ethnicities.

Methylation profiling of saliva indicated a distinct variation in the methylation status of the four markers; especially between the Coloured and Black ethnic groups. The methylation levels of

all markers for all study subjects were graphically represented as peak height ratios. A trend in methylation levels is apparent; the Coloured ethnic group displayed highest methylation levels for all four markers, followed by Whites, Indians and Blacks. The Coloured ethnic group of South Africa is said to be the outcome of admixture, which is mating between individuals from reproductively isolated ancestral populations; in this case, the admixture of various ethnic groups namely Khoi-San, Xhosa, and descendants from European, South East Asian, Indian and Middle East immigrants [33]. Notably, the Black ethnic group shows the lowest methylation levels for all four markers. Similar results were observed by Zhang et al. [10] and Terry et al. [16] who compared DNA methylation levels of blood DNA obtained from Hispanics, Whites and Blacks and found lowest levels of methylation in the Black ethnicities. In this regard, there have been reports of similar methylation patterns between saliva and blood [27,34-36].

In the present study, the DACT1 marker displayed the highest peak height ratio, followed by PFN3, L81528 and USP49 which displayed the lowest average/mean peak height ratio (between the four markers). The DACT1 marker was also found to display highest methylation followed by USP49 and PFN3 in saliva obtained from 34 Korean males and females in the study by Choi and colleagues [23]. An and colleagues [24] found highest peak height ratio for the DACT1 marker followed by PFN3, USP49 and PRMT2 markers; and Lee et al. [25] found approximately 97% methylation for the DACT1 marker.

ANOVA analysis showed that methylation levels for the USP49 and PFN3 markers did not differ significantly across all races (p=0.05). However, the DACT1 (p=0.02) and L81528 (p=0.03) markers displayed significant variation in methylation levels across all four ethnic groups. Since the L81528 CpG site is a semen-specific hypermethylation marker [23] either complete unmethylation or hypomethylation should be observed in saliva. Consistently low, yet varying methylation levels was observed across all races and ages. A significant variation in methylation levels were observed across all four races (p=0.03), and analogous to USP49 and DACT1, largest differences in methylation levels between the Coloured and Black ethnicities were observed.

There was no effect of age and gender on the methylation profile of saliva for the ethnic groups; however a larger sample size would shed more light on the effect of age on methylation levels of markers. Promising results were found in this study for two markers, DACT1 and L81528 as CpG sites of both these markers displayed significant variations between the Coloured and Black ethnic groups. The analysis of DNA extracted from saliva has been proven to be informative in medical as well as forensic research [31,37-39]. However, detecting methylation status of the markers in other body fluids, such as blood, semen, vaginal fluid and menstrual blood obtained from the four ethnic groups will further introduce more accuracy and precision in the analysis.

The analysis of differential DNA methylation holds much promise as it provides a cost-effective, reproducible, robust, sensitive and specific alternative to techniques based on protein and RNA-based markers [40,41]. Using methylation-based markers requires low amounts of template DNA [23,27,42] are not influenced by fluctuating temperatures, varying methods of collection and handling, transport and storage. Multiplexing enables rapid analysis of large amounts of samples within a short time period, and in this study as it facilitated rapid amplification of the four CpG sites in a single reaction [24,27,39].

The multiplex MSRE-PCR used in the present study for differentiation of ethnic groups based on methylation levels of CpG sites was designed to use a standard capillary electrophoresis platform; which is compatible with STR-typing methods. The method may easily be incorporated into applications of forensics and medicine; it exudes high sensitivity, specificity, and reliability. The only factor to be wary of is efficient restriction of the DNA template. Hence, unmethylated DNA was used as a control. The method was selected due to the numerous advantages described in Frumkin et al. [27].

Developments in novel technologies for analysis of DNA methylation enable easy compatibility with current STR-typing methods which makes the method more attractive [25]. Analysis of DNA methylation status in methylation-based markers, especially DACT1 and L81528, may be potentially implemented in differentiation of ethnic groups in real forensic casework. In this regard, the DNA methylation status of DNA from a body fluid (not only saliva) found at a crime scene will help to narrow down the search to ethnicity of individuals that were present. There is also potential to apply gene-specific differential DNA methylation for personalised medicine. The DACT (Dishevelled-associated antagonist of β-catenin) family of proteins are said to play a vital role in tumorigenesis. Deng et al. [43] found that 28.3% of 459 patients with gastric cancer displayed DACT1 promoter methylation and this was associated with poor survival rates of patients. Another recent study by Guo et al. [44] found that high methylation status of transcription start sites of DACT1 and DACT2 led to reduced expression and thus, tumour progression in oesophageal squamous cell carcinoma. The authors suggested that the proteins may serve as prognostic methylation biomarkers for individuals with oesophageal squamous cell carcinoma [44]. However, to confidently apply the markers for forensic and medicinal use, and to substantiate the present findings much work lies ahead. Future work will include identifying associations of disease and aberrant DNA methylation in more genes/markers in individuals of different ethnic groups over a larger geographic location, which will pave the way for tailored therapy. Further quantification of the methylation status of the markers, as well as testing in a larger sample size is also necessary. Not only can the method benefit the present ethnic groups studied, but once larger scale studies are performed, we believe that the applications may have widespread benefit.

The present research focuses on a controversial aspect of dividing a population into specific ethnic groups or classes. The notion of human classification is commonly dismissed as it is thought of as a form of discrimination, an infringement of privacy and basic rights. However, the method of direct phenotyping described here, which is the inference of solely externally visible traits and appearance directly from biological samples [9,45,46] does not cross the line to inference of sensitive aspects which range from susceptibility to diseases and disorders, and propensity toward homosexuality [9,45]. If the information gained from use of biological samples is used purely for investigative purposes in forensics and medicine and will add value to the research in question, DNA methylation-based phenotyping may be a fine tool to assist in these applications.

Conclusion and Future Work

The present study has demonstrated that the analysis of differential DNA methylation holds much potential in forensics and medicine, and once implemented, will likely be an indispensable tool to infer ethnicity of sample donors and aid in personalised medicine. Two CpG sites in the *DACT1* and *L81528* genes showed potential to differentiate between Coloured and Black ethnic groups of South Africa. As this study was based solely on DNA from saliva, future work will involve analysis on other body fluids to determine if the same methylation status/level is observed. While studies have reported genetic variation between ethnic groups in South Africa [33,47], these were based on STR markers. Thus, to our knowledge differential DNA methylation between South African ethnic groups has not been previously studied. However, the application of the present work is not limited to ethnic groups in a single location. As this is a preliminary study, more research is required for medical and forensic science applications. Future work in this regard would also involve testing the potential population-specific markers on a larger sample size, over a larger geographic area. The results are encouraging and pave the way for application of differential DNA methylation for inference of ethnicity in forensic applications, and tailored therapy in medicinal use.

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Ethical Approval

All procedures performed in the present study involving human participants were in accordance with the ethical standards of the Biomedical Research Ethics Committee of University of Kwa-Zulu Natal (Westville Campus, South Africa); Reference Number BE221/14.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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