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A Study on the Prevalence of Cutaneous Manifestations of Diabetes Mellitus

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Abstract
Diabetes is one of the most common endocrine problems, affecting 8.3% of the population. Cutaneous manifestations may be the first sign of Diabetes, or may develop during the course of the disease .Individuals with type 2 diabetes is more likely to manifest cutaneous manifestations compared to type 1 patients. An observational study was conducted in a tertiary care centre among 200 patients with Diabetes and cutaneous manifestations for a period of six months .Type 2 Diabetes was common among the study population. Majority of patients were females and the common age group was 46-55 years of age. Among the cutaneous manifestations, infections constituted the majority .Fungal infections formed the major part among infections. Candidiasis and dermatophytosis were the commonest type of fungal infections .Incidence of newly detected Diabetes was common in patients with candidal balanitis. Xerosis was seen in 18 patients and most of these patients had diabetic neuropathy. The next common cutaneous manifestation was pruritus which was seen in uncontrolled diabetes and in early stages of diabetes. Cutaneous manifestations primarily due to microangiopathy were less in our study. These were manifested as diabetic ulcers in 2.5% of patient's. Awareness of dermatological manifestations helps in early detection of Diabetes in cases with pruritus and candidal balanitis and in early diagnosis of its complications like diabetic neuropathy and nephropathy

Keywords: *Prevalence, Cutaneous manifestations, Diabetes.*

Background

Diabetes is the most common endocrine disorder, affecting 8.3% of the population. Cutaneous manifestations may be the first sign of Diabetes, or may develop during the course of the disease. Individuals with type 2 diabetes is more likely to manifest cutaneous manifestations compared to type1 patients.

Introduction

Cutaneous involvement is seen in 30% of diabetic patients during the course of their disease. Although the overall prevalence of cutaneous disorders does not seem to differ between type I and type II diabetes patients, Type II patients do develop more frequent cutaneous infections, whereas type I patients develop more autoimmune-type cutaneous lesions[1]. Cutaneous manifestations usually seen in patients subsequent

to development of diabetes, but may be the first sign or may precede diabetes by many years. Diabetes exhibits a variety of multisystem complications involving the blood vessels, skin, eye, kidney, and the nervous system during the course. Abnormal carbohydrate metabolism, other altered metabolic pathways, atherosclerosis, microangiopathy, neuron degeneration, and impaired host mechanisms all play roles. Gilgor and Lazarus observed that at least 30% of patients with diabetes mellitus have some type of cutaneous involvement during the course of the disease.[2] A number of classifications on the cutaneous manifestations of Diabetes is available in literature. Sehgal and Shanker opined that no diseases of the skin are absolutely peculiar to diabetes, except three dermatoses the incidence of which is more common in diabetics than in non-diabetics.[2]

In a study cutaneous infections (45.7%) was found to be the most common skin manifestations of Diabetes ,followed by Xerosis (6.4%) and inflammatory skin disease (27.7%)[3]. Acanthosis nigricans is a commonly recognized skin manifestation of Diabetes.[4] It is a well-known fact that Diabetes alters the endothelial functions. Ultrastructure and size of human corneocytes in the upper stratum corneum layer of skin are changed in Diabetic subjects.[6]Fungal infections were the most prevalent among cutaneous infections and interdigital spaces , genital spaces genitalia and skin folds were the frequent sites of infection [7]

Materials and Methods

An observational study was conducted in the Dermatology outpatient department of a tertiary care centre in central Kerala on 200 patients who were referred from medicine department who had diabetes and cutaneous manifestations for a period of six months from January 2016 to June 2016 after getting ethical clearance from the Institutional Ethics Committee. History and clinical examination of these patients were done in detail, a dermatological examination was done and relevant investigations for the diagnosis of cutaneous manifestations and diabetic complications were done where ever necessary. Their data was entered in a proforma containing the personal details, and duration of diabetes and the details of their cutaneous manifestations. The data was entered in spss and analysed.

Results

Among the 200 study participants more than half of the patients were females (56%) and the rest were males (44%). Majority of patients included in the study had Type 2 Diabetes (98%) and only 2% of the patients were Type 1 Diabetes. Majority of the study population were of 46 to 55 year group (32%). 30.5% of the patients were of 56 to 65 years .Very few patients 2 % were of age group above 75 years. Among the cutaneous manifestations-Infections formed the major group, Candidiasis and Dermatophytosis were the commonest among the infections. Three patients(1.5%) had scleredema diabeticorum, one patient(0.5%) had diabetic bulla ,one patient with Bullous pemphigoid, seven patients(3.5%) with Lichen Planus, five patients with diabetic ulcer, Xerosis in 18(9%) patients and 20 patients(10%) had pruritus. Three patients had Kyrles disease which was seen in patients with diabetic complications mainly diabetic nephropathy.

Infections	Number	Percentage
Tinea versicolor	19	9.5%
Dermatophyte Infections	48	24%
Candidal balanitis	11	5.5%
Candidal vulvovaginitis	6	3%
pyoderma	20	10%
Chronic paronychia	7	3.5%
intertrigo	15	0.5%
Scleredema diabeticorum	3	1.5%
Photodermatitis	9	4.5%
Photolichenoid eruption	2	1%
Leg ulcers	5	2.5%
DLE	1	0.5%
Bullous pemphigoid	1	0.5%
Diabetic bulla	1	0.5%
Lichenplanus	7	3.5%
Prurigo	4	2%
Acanthosis nigricans	2	1%
Xerosis	18	9%
Psoriasis	6	3%
Eczema	25	12.5%
Pruritus	20	10%
Kyrles disease	3	1.5%
Erythema multiforme	1	0.5%
Alopecia areata	1	0.5%
Urticaria	2	1%
Miliaria	10	5%

Discussion

Majority of patients were females in the study, who had diabetes with cutaneous manifestations which was at par with other studies. Most of the patients with cutaneous manifestations were of the fifth and sixth decade which was similar to the study conducted by Rao GS & Pai GS [8].Majority (98 %) of patients were of noninsulin dependent diabetes which was similar to the study conducted by Mahajan S, Koranne RV, Sharma S K. Infections were the commonest cutaneous manifestation in our study population. Among the cutaneous infections, fungal infections were the commonest. Dermatophyte infections with extensive involvement were seen in 48 of the patients, followed by tinea versicolor in 19 patients and intertrigo in 15 patients. Another important fungal infection seen was candidiasis, among that candidal balanitis was seen in11 patients (5.5%), candidal vulvovaginitis in 6 patients (3%) and chronic candidal paronychia in 7 patients (3.5%). In this study the incidence of newly detected Diabetes was high in patients with candidal balanitis. Xerosis was seen in 18 patients and most of these patients had diabetic neuropathy. The next common cutaneous manifestation was pruritus which was seen in uncontrolled diabetes and in early stages of diabetes. Cutaneous manifestations primarily due tomicroangiopathy were less in this study which was also similar to other Indian studies [9,10]. These were manifested as diabetic leg ulcers which was seen in 2. 5% of patients and diabetic bulla in one patient (0.5%).

Conclusion

Diabetes is a disease which affects various systems of the body .In this study it was noticed that a high incidence of cutaneous manifestations was noticed in type 2 Diabetes. So a routine dermatological screening is recommended for longstanding cases of type 2 Diabetes.

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LETTER TO THE EDITOR

Fish skin disease and brittle bones

Joe Joseph · Joe Thomas

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20-year-old South Indian girl presented to us with generalized body pain and stooping posture, and examination revealed dry, small, and branny scales consistent with ichthyosis vulgaris (Photograph 1 and 2). Investigation showed hypocalcemia (8 mg/dl), low phosphorus (2.1 mg/dl), elevated alkaline phosphatase (1,024 IU/l low vitamin D (9 ng/ml), and elevated parathyroid hormone level (pg/ml), and X-ray (1 and 2) showed generalized osteoporosis, osteoporotic fractures, and looser zone consistent with diagnosis of osteomalacia. Ichthyosis vulgaris also called fish skin disease is an autosomal dominant inherited skin disorder in which dead skin cells accumulate in thick, dry scales on skin's surface. Osteomalacia is the softening of the bones due to impaired mineralization of bone matrix proteins secondary to inadequate amounts of available phosphorus and calcium which is also known as brittle bone disease. PubMed search revealed only one case series of congenital ichthyosis and vitamin D deficiency [1–3].

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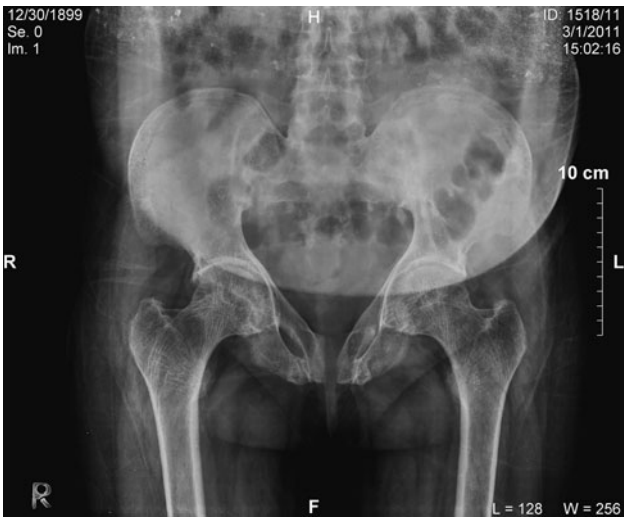

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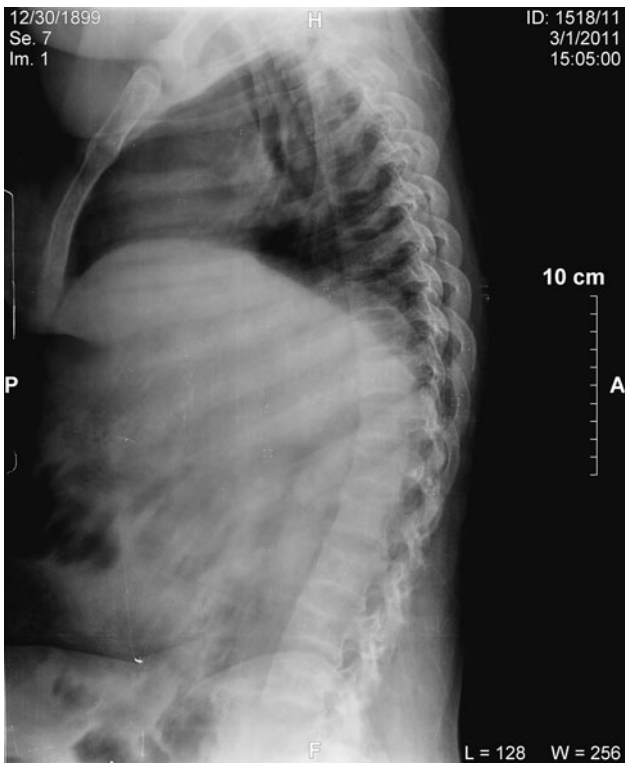

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Research Article

Variation in RASSF1A Hypermethylation in Breast Cancer Patients under Various Stages of Treatment

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Abstract

The need for new breast cancer screening tools which are accurate and sensitive is indisputably high due to limitations in the current screening procedures. Detection of breast cancer is now possible only after the mild progression of the disease. Use of molecular markers such as DNA methylation as a diagnostic technique is recommended since methylation is a frequent event in breast cancer and can be detected at an early stage. Breast cancer patients under various stages of treatment and healthy individuals were enrolled for this study. Plasma DNA was isolated from both cancer patients and healthy individuals (controls), since DNA in plasma is thought to originate from necrotic and apoptic tumor cells. The RASSF1A gene in most of the breast cancer patients were found to be methylated in accordance with the stages of their treatment while absence of methylation was observed in control samples. It was observed that the methylation of RASSF1A gene was reversed during the course of treatment. This study suggests that a non-invasive technique like plasma DNA analysis for the detection of methylation of RASSF1A gene may be used as a diagnostic and prognostic tool for breast cancer.

Keywords: RASSF1A, breast cancer, hypermethylation, molecular marker, MSPCR

Abbreviations: MSPCR – Methylation Specific Polymerase Chain Reaction

Introduction

Breast cancer is the second leading cause of cancer death among women after lung cancer. Over 1.1 million women worldwide are diagnosed with breast cancer each year and more than 410,000 of them die from this disease. Breast cancer accounts for 10% of all new cancer cases and for greater than 1.6% of mortality in women globally (Parkin *et al.,* 2002). Breast cancer was found to be the most common cancer in urban women and second most common cancer in the rural women of India (National Cancer Registry Programme, ICMR, 2006). A number of screening tests have been employed including clinical and self breast exams, mammography, genetic screening, ultrasound and magnetic resonance imaging. Mammographic screening for breast cancer uses x-rays to examine the breast for any characteristic masses or lumps (Kosters and Gotzsche, 2003). In developing countries, the extensive laboratory and clinical infrastructure required for mammographic screening and the high cost of mammography are major limiting factors in the diagnosis of breast cancer. Biopsies and tissue sampling require expert skill and tissue sample is usually removed surgically. New screening methods that could improve the detection would be highly beneficial for the timely diagnosis of breast cancer.

Cancer biomarkers are widely used for the early detection of cancers since they are measurable before overt cancer is clinically detectable. These molecular markers have high predictive accuracy, is easily measurable and reproducible, minimally invasive and acceptable to physicians (Ramirez *et al.,* 2003). Epigenetic changes, such as DNA methylation is one of the most common molecular alterations in human neoplasia (Baylin *et al.,* 1998; Bird, 1992) including breast cancer (Muller *et al.,* 2003). DNA methylation as a molecular marker has numerous favorable characteristics. It is an early event in breast carcinogenesis. Since DNA methylation is stable and detectable by PCR, aberrations can be easily analyzed from small amounts of sample. Moreover, the studies showing high concordance between the epigenetic alterations found in primary tumor specimens and in plasma (Yang *et al.*, 2004) suggest methylation analysis as a potential non-invasive screen for early cancer detection.

Hypermethylation of a number of tumour suppressor genes have been reported to be associated with breast cancer. RASSF1A is a tumor suppressor gene located at chromosome 3p21.3 a region that frequently shows loss of heterozygosity during human cancers (Greespan and Jabionssi, 2006). Although it can be inactivated by gene deletion or point mutations, the most common contributor to loss or reduction of RASSF1A function is transcriptional silencing of the gene by inappropriate promoter methylation. De novo methylation of the RASSFIA promoter is one of the most frequent epigenetic inactivation events detected in breast cancer and leads to silencing of RASSF1A expression (Agathanggelou *et al.,* 2001). Hence, analysis of the methylation pattern of RAASF1A gene can be used as a screening technique to detect early carcinogenesis. The aim of the study is to identify the variation in the methylation pattern of RASSF1A gene in blood plasma DNA of breast cancer patients under various stages of treatment.

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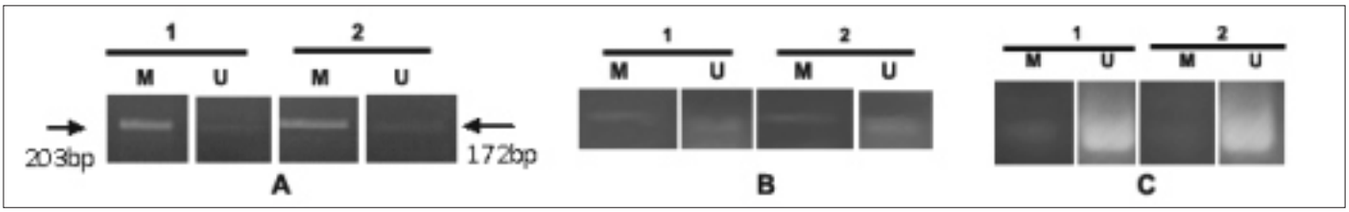

Figure 1. Stage II PCR of breast cancer positive samples. Serum DNA samples were modified by sodium bisulfite treatment and subjected to MSP analysis. M-PCR product of primers specific for methylated sequences; U- PCR product of primers specific for unmethylated sequences. (A) Samples from patients undergoing initial stages of treatment (B) Samples from patients undergoing final stages of treatment (C) Samples from recovered patients.

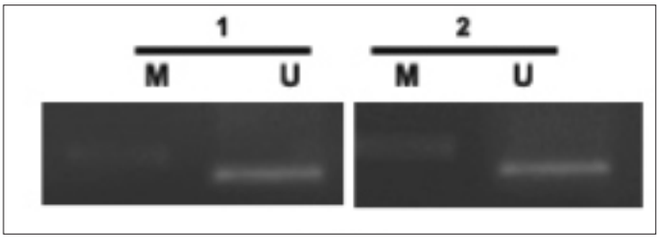

Figure 2. Stage II PCR of control samples. Faint amplification is observed with methylation specific primers while unmethylation specific primers gave good amplification.

TYPE OF SAMPLE	NO.OF SAMPLES	METHYLATED	UNMETHYLATED
CANCER	4 (Initial stage of treatment)	++	+
	3 (Final stage of treatment)	++	++
	1 (Final Stage of treatment)	+++	++
NORMAL	4 (Recovered)	+	+++
	16	-	+++
	2	+	+++

Table 1. Summary of results. +: Mild Amplification; ++ : Moderate Amplification;+++: Good Amplification ; - :Absence of Amplification

Materials and Methods

Patients

Twelve women from Southern India who were diagnosed with breast cancer were enrolled for this study. Four of these women were recovered patients; four were recently diagnosed and in the initial stages of treatment and four were in the later stages of treatment. Eighteen healthy individuals were enrolled as controls in this study. Informed consent was obtained from all the participants. Approval of research on human subjects was obtained from the Institutional Ethics Committee.

Specimen characteristics

After interviewing the participants, 3 ml blood samples were collected from them and stored at -80°C until further processing. DNA was isolated from human blood plasma using GenElute blood genomic DNA kit (Sigma-Aldrich). 200µl blood plasma was treated with Proteinase K and DNA was extracted from these samples following manufacturer's protocol.

Assay methods

Methylation specific-nested PCR

Methylation specific PCR (MSP) was used to detect the presence or absence of hypermethylation in the Rassf1A gene. Bisulfite modification is the process in which sodium bisulfite is used to determine the methylation patterns of DNA. Bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisufite treatment introduces specific changes in the DNA sequence that depends on the methylation status of individual cytosine residues The plasma DNA was modified using the EZ DNA Methylation-Gold kit™ (Zymoresearch). In MSP, methylated primers will anneal to those regions containing 5'methyl cytosine while unmethylated primers will bind to bisulphite converted regions. Three sets of primers were used for MS-nested PCR (Divine *et al.,* 2006).

Stage I PCR

PCR reaction was carried out in 20µl reaction volume, containing 1µl of the modified plasma DNA, 1.5mM $MgCl_2$, 0.2mM dNTP,10 picomoles of each outer primers, 1 unit of Taq DNA polymerase and 1X Taq buffer. Cycling conditions consisted of an initial denaturation of 10 minutes at 95ºC, followed by 40 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The amplifications were carried out in eppendorf master cycler personal with heated lid.

Stage II PCR

Two separate PCR reactions were done with two sets of internal primers for methylated and unmethylated regions of RASSF1A. Stage IIa PCR used methylation specific primers for amplifying the methylated region of the gene while stage IIb PCR used primers for amplification of the unmethylated regions of RASSF1A gene. PCR reaction was carried out in 20µl reaction volume, containing 2µl of the stage I PCR product,1.5mM $MgCl_2$,0.2mM dNTPs,10 pico moles of each primer, 1 unit of Taq DNA polymerase and 1X Taq buffer. Cycling conditions consisted of an initial denaturation of 10 minutes at 95ºC, followed by 39 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The PCR products were analysed on 2% agarose gel containing ethidium bromide.

Results

RASSF1A methylation was considered to be present if the samples

gave amplification with the methylation specific primers. The RASSF1A gene was considered unmethylated if the samples gave amplification only with the unmethylation specific primers and not with the methylation specific primers. If amplification was present in both the reactions, the samples were considered to be partially methylated. Representative examples of RASSF1A MSP are shown in figure 1 and 2.

Out of the twelve cancer samples, four were from patients who had already recovered from cancer. Four samples were from patients in the final stages of treatment and the remaining four at the initial stages of treatment. The four samples from patients undergoing initial stages of treatment showed good amplification with the methylated primers and moderate amplification with the unmethylated primers.

Samples from three patients undergoing final stages of treatment gave good amplification with unmethylated primers and feeble amplification with methylated primers while one patient undergoing final stages of treatment gave moderate amplification with the methylated primers. All the four samples from the cured patients gave very faint amplification with the methylated primers and good amplification with the unmethylated primers indicating reduction in methylation after therapy.

All the 18 control samples showed good amplification with the unmethylated primers and very low amplification with the methylated primers. Summary of the results are given in the Table I.

Discussion

In this study the potential of hypermethylation as a molecular marker for the detection of breast cancer was analysed. Our study was focused on tumor suppressor gene RASSF1A. Reduced expression of rassf1a protein is a frequent phenomenon in many human cancers (Burbee *et al,* 2001). A number of studies on breast cancer had shown the association between RASSF1A hypermethylation and breast cancer. Since hypermethylation is a reversible phenomenon, we assumed that there should be a reversal of methylation in recovered breast cancer patients.

Our study showed that all the patients who were recently diagnosed with breast cancer were highly methylated and those undergoing treatment showed a gradation in their methylation according to the treatment stage. Although most of the control samples did not show any methylation some samples showed faint methylation indicating that they might be partially methylated. This is in accordance with the previous reports were DNA methylation has been reported in non- malignant tissues (Hoque *et al,,* 2006). Our study was conducted using DNA isolated from blood plasma. Plasma DNA has been previously used for genetic and epigenetic variation studies associated with cancer (Sozzi *et al.,* 1999; Gonzalez et al., 2000). It has been proved that plasma contains DNA from malignant cells which is modified by hypermethylation and can be used as a molecular tool for the detection of methylation (Jahr *et al.,* 2001). In this study, it was found that patients who are undergoing treatment and who had recovered from cancer showed different methylation patterns when compared to those in early stages of breast cancer treatment. It was observed that a reduction in methylation occurred after therapy. From these results it can be assumed that the RASSF1A gene of a patient who has been recently diagnosed with breast cancer might be highly methylated while that of a recovered one is very low. This difference in methylation pattern can be used as a parameter to identify whether the treatment is effective and the patient is on the way to recovery.

MSP of RASSF1A using circulating DNA is a sensitive and specific technique and it may be used as a diagnostic tool for detecting breast cancer at an early stage. Development of novel biomarkers with prognostic value will facilitate effective monitoring of the disease. Since methylation of RASSF1A seems to be linked to the stage of the disease, it may be used as a marker for confirming the treatment outcome. Other marker genes that are linked to breast cancer can also be detected using this methodology.

Conclusion

Breast cancer is a growing epidemic in many developing countries like India. Early diagnosis is crucial for the effective management of the disease. Hypermethylation of promoter region of RASSF1A gene can be detected in plasma DNA during the early stages of breast cancer. This and the variation in methylation pattern shown by this gene during the occurrence of breast cancer make it a potential candidate to become a diagnostic as well as a prognostic tool. Since this study was done with limited number of breast cancer samples, it requires validation with a larger number of samples before accepting it as a screening and prognostic method.

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The Methylation Status of Retinoic Acid Receptor Beta 2 (RARb2) Gene and Breast Cancer Risk: A Case-Control Study

Joe Joseph[1], Manju P.A[2], Jaya Vinny Eapen[3], Anu Yamuna Joseph[4]

ABSTRACT

Introduction: Breast cancer is a heterogeneous disease with very different therapeutic responses and outcomes. Aberrant hypermethylation of tumor-suppressor genes is found frequently in primary breast tumors and has been implicated in disease initiation and progression. Use of molecular markers such as DNA methylation as a diagnostic technique is recommended since methylation is a frequent event in breast cancer and can be detected at an early stage. The objective of this study was the analysis of variation in the DNA methylation patterns of RARb2 gene in the plasma DNA of breast cancer patients.

Material and Methods: DNA was isolated from the plasma of breast cancer patients and controls. The isolated DNA was modified by bisulfite treatment. The presence or absence of hypermethylation in RARb2 gene was analysed by conventional methylation specific PCR (MSP).The methylation pattern in the control and affected samples was obtained and statistical analysis was done to determine whether there is any association between hypermethylation of RARb2 gene and breast cancer in the samples under study.

Results: It was observed that 92% of the breast cancer samples showed hypermethylation whereas only 14% of the control samples showed hypermethylation in the RARb2 gene. Statistical analysis showed significant association between breast cancer and RARb2 methylation.The results showed that the methylation of *RARβ2* was associated with susceptibility to breast cancer.

Conclusion: This study confirms the detection of hypermethylation of RARb2 in breast cancer patients. The findings point to the possibility of using RARb2 methylation as a molecular marker for the diagnosis of breast cancer.

Key-words: RARb2, Breast Cancer, Hypermethylation, Molecular Marker, MSPCR

INTRODUCTION

Breast cancer is the second leading cause of cancer death among women after lung cancer. Over 1.1 million women worldwide are diagnosed with breast cancer each year and more than 410,000 of them die from this disease. Breast cancer accounts for 10% of all new cancer cases and for greater than 1.6% of mortality in women globally[1] . Breast cancer was found to be the most common cancer in urban women and second most common cancer in the rural women of India [2].A number of screening tests have been employed including clinical and self breast exams, mammography, genetic screening, ultrasound and magnetic resonance imaging. Mammographic screening for breast cancer uses x-rays to examine the breast for any characteristic masses or lumps[3]. In developing countries, the extensive laboratory and clinical infrastructure required for mammographic screening and the high cost of mammography are major limiting factors in the diagnosis of breast cancer. Biopsies and tissue sampling require expert skill and tissue sample is usually removed surgically. New screening methods that could improve the detection would be highly beneficial for the timely diagnosis of breast cancer.

Cancer biomarkers are widely used for the early detection of cancers since they are measurable before overt cancer is clinically detectable. These molecular markers have high predictive accuracy, is easily measurable and reproducible, minimally invasive and acceptable to physicians [4]. Epigenetic changes, such as DNA methylation is one of the most common molecular alterations in human neoplasia [5,6] including breast cancer[7].DNA methylation as a molecular marker has numerous favorable characteristics. It is an early event in breast carcinogenesis. Since DNA methylation is stable and detectable by PCR, aberrations can be easily analyzed from small amounts of sample. Moreover, the studies showing high concordance between the epigenetic alterations found in primary tumor specimens and in plasma [8]suggest methylation analysis as a potential non-invasive screen for early cancer detection. Hypermethylation of a number of tumour suppressor genes have been reported to be associated with breast cancer. Retinoic acid receptor beta-2 (RARb2) is known to have a critical role in the chemopreventive action of retinoids. It has been confirmed as a tumour suppressor gene whose reduced expression has been linked to many cancers[9,10,11].The role of RARb2 hypermethylation in breast cancer has been investigated in a number of studies. In 2000, Widschwendter et al[12].suggested methylation of the RAR-beta2 gene as an initial event in breast carcinogenesis. RARb2 methylation has been observed in *in situ* lesions

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from both lobular and ductal cancers [13].Aberrant promoter metylation on RARb2 has been reported by Vasilatos et al,[14] in 2009. Hypermethylation of RARb2 along with Rassf1A was reported by Yamamoto et al[15], in 2012.

MATERIAL AND METHODS

Sample Collection

In the present case-control study, fifty women from Kerala, India who were diagnosed with breast cancer were enrolled as cases and fifty healthy females were enrolled as controls. Informed consent was obtained from all the participants. Approval of research on human subjects was obtained from the Institutional Ethics Committee. After interviewing the participants, 3 ml blood samples were collected from them and stored at -80°C until further processing.

Genomic DNA Extraction

DNA was isolated from human blood plasma using GenElute blood genomic DNA kit.(Sigma-Aldrich). 200µl blood plasma was treated with Proteinase K and DNA was extracted from these samples following manufacturer's protocol.

Methylation specific-nested PCR

Methylation specific PCR (MSP) was used to detect the presence or absence of hypermethylation in the RARb2 gene. Bisulfite modification is the process in which sodium bisulfite is used to determine the methylation patterns of DNA. Bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. Thus, bisufite treatment introduces specific changes in the DNA sequence that depends on the methylation status of individual cytosine residues The plasma DNA was modified using the EZ DNA Methylation-Gold kit™ (Zymoresearch). In MSP, methylated primers will anneal to those regions containing 5'methyl cytosine while unmethylated primers will bind to bisulphite converted regions. Three sets of primers were used for MS-nested PCR[16].

Stage I PCR

PCR reaction was carried out in 20µl reaction volume, containing 1µl of the modified plasma DNA, 1.5mM $MgCl_2$, 0.2mM dNTP's,10 picomoles of each outer primers, 1 unit of Taq DNA polymerase and 1X Taq buffer.Cycling conditions consisted of an initial denaturation of 10 minutes at 95ºC, followed by 40 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The amplifications were carried out in eppendorf master cycler personal with heated lid.

Stage II PCR

Two separate PCR reactions were done with two sets of internal primers for methylated and unmethylated regions of RARb2. Stage IIa PCR used methylation specific primers for amplifying the methylated region of the gene while stage IIb PCR used primers for amplification of the unmethylated regions of RARb2 gene. PCR reaction was carried out in 20µl reaction volume, containing 2µl of the stage I PCR product,1.5mM $MgCl_2$,0.2mM dNTPs,10 pico moles of each primer, 1 unit of Taq DNA polymerase and 1X Taq buffer. Cycling conditions consisted of an initial denaturation of 10 minutes at 95ºC, followed by 39 cycles of 94ºC for 30 seconds, 60˚C for 30 seconds and 72ºC for 30 seconds. A final elongation of 72ºC was given for 10 minutes. The PCR products were analysed on 2% agarose gel containing ethidium bromide.

STATISTICAL ANALYSIS

The significance of asscociation of RARb2 hypermethylation between breast cancer and normal samples was determined using the chi-squaretest. Odds ratios (OR) and 95% confidence intervals (95% CI) were measured using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Statistical significance was assumed at the $p < 0.05$ level.

RESULTS

The mean age of the patients was 52.48 ± 12.05 years (range 31–70 years). 9 (18%) patients were aged below 40 years.

RARb2 methylation was considered to be present if the samples gave amplification with the methylation specific primers. The RARb2 gene was considered unmethylated if the samples gave amplification only with the unmethylation specific primers and not with the methylation specific primers.

When the fifty cancer samples were analysed by PCR, 46 samples gave amplification with methylation specific primers and very mild amplification with umnethylation specific primers. Four cancer samples gave amplification only with unmethylation specific primers indicating the absence of hypermethylation in these samples. Out of the 50 control samples, only 7 samples showed amplification with both methylation specific primers and unmethylation specific primers while 43 samples showed amplification only with unmethylation specific primers.

Out of the fifty cancer samples, hypermethylation was observed in 46 samples (92%) while it was not observed in 4 samples (8%). Among the control samples, only 7 samples (14%) showed hypermethylation while 43 samples (86%) did not show hypermethylation (χ^2 =61.06, p<0.001). The results show that the methylation of *RARβ2* was associated with susceptibility to breast cancer (OR =70.64 95% CI 19.31 – 258.4 (Table 1).

RARb2 hyper methylation	Without Breast cancer		With breast Cancer		Odds (95% CI)
	Count	Percent	Count	Percent	
Absent	43	86.0	4	8.0	1
Present	7	14.0	46	92.0	70.64 (19.31 – 258.4)
$\chi2$ =61.06, p<0.001					

Table-1: Association of RARb2 hyper methylation with breast cancer

DISCUSSION

In this study the potential of hypermethylation as a molecular marker for the detection of breast cancer was analysed. Our study was focused on tumor suppressor gene RARb2. Reduced expression of RARb2 protein is a frequent phenomenon in many human cancers. A number of studies on breast cancer had shown the association between RARb2 hypermethylation and breast cancer.

Our study showed that all the patients who were recently diagnosed with breast cancer were highly methylated. Although most of the control samples did not show any methylation some samples showed faint methylation indicating that they might be partially methylated. This is in accordance with the previous reports where DNA methylation has been reported in non- malignant tissues [17]. Our study was conducted using DNA isolated from blood plasma. Plasma DNA has been previously used for genetic and epigenetic variation studies associated with cancer [18,19]. It has been proved that plasma contains DNA from malignant cells which is modified by hypermethylation and can be used as a molecular tool for the detection of methylation[20]. In this study, it was found that patients with breast cancer showed different methylation patterns when compared to the control population.

A number of studies have suggested that the methylation profiles of cancers may be ethnicity specific [21,22]. It has been proposed that that differences in exposure patterns among racial/ethnic subgroups might lead to differences in cancer susceptibility, irrespectively of any intrinsic genetic differences between groups. Also, breast cancer prognosis was found to be considerably influenced by the ethnicity[23]. These findings advocate the necessity for the development of population specific molecular markers for the diagnosis and prognostic prediction of breast cancer. MSP of RARb2 using circulating DNA is a sensitive and specific technique and it may be used as a diagnostic tool for detecting breast cancer at an early stage. Development of novel biomarkers with diagnostic value will facilitate effective monitoring of the disease.

CONCLUSION

Breast cancer is a growing epidemic in many developing countries like India. Early diagnosis is crucial for the effective management of the disease. Hypermethylation of promoter region of RARb2 gene can be detected in plasma DNA during the early stages of breast cancer. This makes it a potential candidate to become a diagnostic tool. Since this study was done with limited number of breast cancer samples, it requires validation with a larger number of samples before accepting it as a screening method.

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Original Article

Carrier Frequency of Connexin26 W24X Mutation in the Population of Kerala, India

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ABSTRACT:

Purpose: The aim is to study the carrier rate of connexin 26 (Cx26) W24X gene mutation among the general population of Kerala, India. **Subjects and Methods:** This study included 248 normal-hearing individuals from the general population of Kerala, India. Polymerase chain reaction-restriction fragment length polymorphism was performed to detect the presence of Cx26 W24X mutation. **Results:** Of the 248 individuals, 7 cases were heterozygous and 241 cases were homozygous normal. The results showed a carrier frequency of 2.82% for the Cx26 W24X mutation in the Kerala population. **Conclusion:** Among the selected Kerala population sample, the Cx26 gene mutation showed a high carrier rate. Early detection of nonsyndromic hearing impairment has been proven to be very helpful in speech therapy and language development. Given the high carrier rate of Cx26 W24X mutation in the Kerala population, we propose the genetic screening of the suspected newborns for W24X mutation in the Cx26 gene.

Keywords: Connexin26, Kerala population, sensorineural deafness, W24X mutation

Introduction

Congenital deafness is a major malady affecting one in 1000 newborns across different populations. Roughly, half of the severe childhood deafness is attributed to genetic causes. Connexin 26 (GJB2) was the first gene reported to be responsible for autosomal recessive nonsyndromic deafness.[1] GJB2 codes for Cx26 protein which is involved in the formation of gap junctions. It has been suggested that these gap junctions are involved in the local circulation of potassium ions between the fluids of the inner ear.[2-4] Defects in connexins may therefore reduce the efficiency of potassium ion circulation and consequently lead to impaired hearing sensitivity.

Mutations in Cx26 gene are the most common cause of hearing loss in different populations. It is estimated that GJB2 mutations are responsible for 50% of autosomal recessive nonsyndromic hearing loss in the Mediterranean population.[5-7] In the United States, GJB2 mutations account for 21% of congenital hearing loss whereas in Asian nations these are found to be responsible for 20% of cases of severe to profound hearing loss.[8-11] It has been observed that the prevalence of specific mutations is population specific. The most commonly found mutations are 35delG which predominate among Caucasian and Mediterranean,[7,12] 235delC and V37I in Japanese,[13] 167delT in Ashkenazi Jews[14] and W24X in the Indian population.[15] The predominance of different mutations in different populations suggests an ethnic predilection for GJB2 mutations.

Genetic studies in the Indian deaf population have shown that mutations in GJB2 account for the majority of genetic deafness cases. A high carrier rate was reported for the W24X mutation in normal-hearing control samples from southern and Western India.[15,16] W24X is caused by a G to A transition at nucleotide position 71. This creates an Amber (TAG) stop codon at codon 24, replacing Tryptophan. A stop codon at this position results in the production of a truncated protein. Estimating the allele frequency of a mutation in the general population will help to decide whether to adopt widespread genetic screening

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for that mutation. In this study, we tried to estimate the carrier rate of Cx26 W24X mutation in the normal-hearing population of Kerala, India.

Subjects and Methods

Sample size and data collection

This was an observational study approved by the Institutional Ethics Committee, Mar Athanasius College. A total of 248 unrelated healthy individuals with normal hearing from the South Indian state of Kerala were enrolled for this study. Written informed consent and a brief medical history was obtained from all the participants. 3ml blood sample from each participant was collected into ethylenediaminetetraacetic acid coated tubes.

Genotyping

Genomic DNA was isolated from blood samples by the salting out method.[17] Cx26 W24X mutation was analyzed by the Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers used for the PCR reaction were designed using the software PERL PRIMER. Gene sequence from Gene bank was used as reference gene (GeneBankACC.No.NG_008358.1).

The primers were 5'CAGAGTAGAAGATGGATTGGG-3' and 5'GGGAAGTAGTGATCGTAGCAC -3'. The partial exon2 of the Cx26 gene was amplified using these primers. PCR reaction was carried out in 20µl reaction volume, containing 50ng of template DNA, 1.5mM $MgCl_2$, 0.2mM dNTPs (*Fermentas, USA*), 10 picomoles of each primer, 1U of Taq polymerase (Sigma-Aldrich, India) and 1X Taq buffer. The cycling conditions consisted of an initial denaturation of 2 min at 95°C, followed by 35 cycles of 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s. A final extension was given at 72°C for 10 min. The amplified PCR products were visualized by agarose gel electrophoresis.

PCR-RFLP analysis was done for the detection of mutation. For this purpose, the PCR products were digested with the AluI restriction enzyme. Restriction digestion was done at 37°C for 1hour using 5 units of AluI enzyme (Thermoscientific, USA). The digested products were checked by agarose gel electrophoresis.

Results

A total of 248 DNA samples isolated from healthy individuals with normal hearing from different parts of Kerala was analyzed. A 214 bp fragment containing the W24X region was amplified by PCR. The PCR products were used as template for RFLP analysis and the size of the products were confirmed by compairing with 100bp ladder. The presence of the W24X mutation results in the generation of an AluI restriction site at this region (CTGG to CTAG). It was observed that of the 248 samples, 241 samples were undigested by the AluI enzyme indicating the absence of G to A nucleotide change at the 71st position of the Cx26 gene. This indicated that these samples were normal with respect to the W24X site. Samples from seven individuals when digested with Alu1enzyme gave three bands corresponding to 214bp, 131bp, and 83bp size. This indicated that these seven individuals were carriers of the G to A mutation at the 71st position of the Cx26 gene. The results of this analysis showed that 7 out of 248 individuals were carriers of the W24X mutation in the Cx26 gene. This indicated a carrier rate of 2.82% for the W24X mutation in the Kerala population [Table 1].

Discussion

In this study, we screened for the presence of the mutation in the general population of Kerala. From the data, a carrier rate of 2.82% could be estimated for the W24X mutation in the Kerala population. The results indicate a deviation from the previously indicated data which showed a 6.45% carrier rate in the Kerala Population.[16] The higher carrier rate observed in the previous study may be due to sample size bias. The results confirm that there is indeed a high carrier rate for the W24X mutation in the Cx26 gene in the Kerala population.

Genetic studies in the Indian deaf population have been going on for some time now. So far, the most prominent gene in these studies is GJB2 accounting for the majority of genetic deafness cases. In 2003, Ramshankar *et al*.[15] reported GJB2 mutation as the major genetic cause of profound sensorineural deafness in patients from Southern and Western India. About 17.67% of the subjects showed mutation in this gene. Although three different mutations were reported, one particular mutation, W24X accounted for 94% of the GJB2 mutations and 16.44% of all the deafness cases analyzed. A carrier frequency of 2.4% was observed for W24X mutation in the normal-hearing control samples. A parallel study on cases predominantly from South India and Delhi conducted by Maheshwari *et al*.[18] in the same year again reported W24X as the major cause (8.8%) of genetic deafness. They also reported two compound heterozygotes with 35delG/W24X and R143W/W24X.

In 2009, Padma *et al*.[19] reported 10.9% deafness cases from in and around Hyderabad in South India to be harboring GJB2 mutations of which 18.2% were carriers. Although seven different variations were identified in the GJB2 gene, W24X was the predominant one accounting for 87% of the mutant alleles. In the same study, screening for GJB6 mutations yielded negative results. In another study, a muation frequency of 36% was reported in the GJB2 gene in patients with profound sensorineural deafness in Kerala, South India.[16] Two different mutations were reported in these samples, namely W24X and R32L. As reported earlier, here too

Table 1: Genotype and Allele frequency of connexin 26 W24X mutation in the Kerala population

Genotype frequency (%)		Allele frequency	
GG	241/248 (97.18)	G	0.986
GA	7/248 (2.82)	A	0.014
AA	0/248 (0)		

W24X was the predominant mutation with 32.5% frequency among the deaf samples. A carrier frequency of 6.45% was observed among the normal hearing control samples which was significantly higher than the previously reported 2.4%.[15] A study involving patients from Western and Southern India reported mutations in GJB2 as the cause of hearing impairment with 18.8% homozygous and 21.5% heterozygous for GJB2 mutations.[20] Here too, W24X was the most common mutation followed by W77X. In another study, screening of W24X mutations in hearing-impaired samples showed that 26% were positive for this mutation.[21]

The prevalence of W24X mutaion among the deaf population prompted many authors to suggest a founder effect for this mutation in the Indian population.[15,16] It is evident that this generalization is not possible for the Indian population as a whole as it has been reported that 35delG also contributes significantly to genetic deafness in certain regions of India. All the studies which report W24X as the major GJB2 mutation were done on samples taken mostly from Southern and Western India. On the other hand, the reports which show 35delG as the predominant GJB2 mutation were based on studies conducted in the North Indian population. These data suggest a clustering of these mutations in a region-specific manner. Such clustering is not surprising in a country like India which is an amalgamation of diverse ethnic groups which still preserve their ethnic identity to a great extent.

There is a strong recommendation for the early detection of nonsyndromic hearing impairment, as there is a high incidence of recessive hearing impairment. Early detection has been proven to be very helpful in speech therapy and language development. These early detection programs also help for enrolling the patients for various treatments like cochlear implantation. Given the high carrier rate of W24X mutation in the Kerala population, we propose the genetic screening of the suspected newborns for W24X mutation in the Cx26 gene.

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Conflicts of interest

There are no conflicts of interest.

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VDR FOK-I POLYMORPHISM IN THE POPULATION OF KERALA, INDIA

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ABSTRACT

BACKGROUND

Vitamin D receptor (VDR), a member of the steroid hormone receptor family is involved in a variety of biological processes such as bone metabolism, modulation of immune response, regulation of cell proliferation and differentiation. Polymorphisms in VDR gene has been linked to a number of diseases like osteoarthritis, cancer, diabetes, etc. Since ethnic variations has been reported in the allele frequency of VDR polymorphisms, population specific data has to be generated before conducting a valid genetic association study.

The aim of this study was to identify the distribution of VDR Fok-I polymorphism in the healthy individuals of Kerala, South India.

MATERIALS AND METHODS

This study was conducted on 152 unrelated individuals of Kerala. Detection of VDR Fok-I polymorphism was done by PCR-RFLP. The allele and genotype frequencies were calculated from the genotype data. Genotype frequencies of different populations were compared with that of ours using Chi square test.

RESULTS

The genotype distribution of VDR Fok-I in this population followed Hardy-Weinberg equilibrium. Significant variations were observed on comparing our genotype distribution with that of data from previous studies in different populations confirming ethnic variation in genotype frequency. Variations between different population groups within India were also observed.

CONCLUSION

This study confirms ethnic variations in the VDR Fok-I genotype distribution indicating the need for generating population specific data for different ethnic groups. Establishing such specific databases is essential for valid genetic association studies.

KEYWORDS

VDR, Polymorphism, Kerala Population, PCR-RFLP.

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BACKGROUND

Vitamin D, also known as Calciferol, is a fat soluble vitamin. Calcitriol, the biologically-active form of vitamin D, regulates the concentration of calcium and phosphate in the bloodstream. It also affects neuromuscular function and inflammation, cell proliferation, differentiation and apoptosis.[1] Vitamin D receptor (VDR) is a member of the steroid hormone receptor family that binds the active form of Vitamin D (1,2,5-dihydroxyvitamin D3) and interacts with the target cell nuclei to produce variety of biological effects. The binding of vitamin D to VDR is essential for the maintenance of calcium and phosphorous levels in the blood and the maintenance of mineral density. VDR is also known to be involved in cell proliferation and differentiation. A role for vitamin D in a number of diseases like, diabetes, cancer, cardiovascular diseases, etc. has been reported. The biological activity of Vitamin D is exerted through VDR-mediated control of target genes.[2]

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Vitamin D receptor (VDR) is expressed throughout the body on a wide variety tissues and cells such as heart, kidney, immune cells etc. Hence, it has been associated with several renal, cardiovascular and inflammatory diseases. Several DNA sequence variations known as polymorphisms have been reported in VDR gene. These polymorphisms can have biological effects. Polymorphisms in the VDR gene is known to be associated with a number of diseases like osteoarthritis, diabetes, cancer, rickets, immunological diseases, etc. The effect of VDR polymorphisms on disease susceptibility has been widely investigated.[3]

VDR protein is encoded by the VDR gene located on human chromosome 12 q12-q22 region.[4] It consists of 11 exons that span approximately 75 kb. The non-coding 5' end of the VDR gene includes exons 1A, 1B and 1C. Eight exons encode the structural portion of the VDR gene which is 427 amino acids long with a molecular weight of 48289 daltons.[5]

Most of the polymorphisms in the VDR gene has been found to be in the 5' promoter and 3' UTR regions. The three adjacent RFLPs BsmI, ApaI and TaqI at the 3' end and the thymine/cytosine polymorphism at the first potential start site of VDR gene are the most frequently studied. The polymorphisms in the 3' UTR region are most probably associated with mRNA stability[6] while the Fok-I polymorphism has been shown to affect the activity of the protein. The short 424 aa VDR protein variant corresponding to the C allele (F) was found to be 1.7-fold more active than the long 427 aa variant corresponding to the T allele (f).[7]

Genetic and epidemiological studies in the VDR gene will provide insight into the association between disease and VDR alleles. Since ethnic variation in allele frequency is widely documented,[3,8] population specific data of VDR polymorphism has to be generated before conducting a valid genetic association study. In this study, we determined the frequency of VDR Fok-I polymorphism in a sample of Indian population. The allele frequencies were compared with those of other populations. We also verified whether the allelic distribution followed Hardy-Weinberg equilibrium.

MATERIALS AND METHODS

Sample Size and Data Collection

This was an observational study approved by the Institutional Ethics Committee. sample size used for convenience. Written informed consent was obtained from all the participants. 3 mL blood sample from each participant was collected into EDTA coated tubes.

Genotyping

Genomic DNA was isolated from blood samples by salting out method.[9]

VDR Fok-I polymorphism was analysed by PCR-RFLP method. The primers used for the PCR reaction were forward - 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and Reverse - 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'.[10] PCR reaction was carried out in 20 µL reaction volume, containing 50ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 10 picomoles of each primer, 1U of Taq polymerase (Sigma-Aldrich, India) and 1X Taq buffer. The cycling conditions consisted of an initial denaturation of 2 minutes at 95°C, followed by 35 cycles of 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 45 seconds. A final extension was given at 72°C for 10 minutes. The PCR products were confirmed by agarose gel electrophoresis.

For PCR-RFLP analysis, the PCR product was digested with Fok-I restriction enzyme. Restriction digestion was done at 37˚C for 5 minutes using 5 units of Fok-I fast digest enzyme (Fermentas, USA). The digested products were checked by agarose gel electrophoresis. Three different patterns were obtained: the FF genotype without the Fok-I restriction site showed only a single uncut band of 265 bp size, ff genotype generated two bands of 196 and 69 bp and heterozygous Ff showed three bands of size 265, 196 and 69 bp.

Statistical Analysis

The genotyping data was used to estimate the allele frequencies and genotype frequencies. To verify whether the VDR Fok-I genotype distribution was in Hardy-Weinberg Equilibrium, the data was analysed using the chi-square test and the Hardy-Weinberg equilibrium calculator.[11] Genotype frequencies of different populations were compared with that of ours using chi square test.[12]

RESULTS

VDR Fok-I allele frequencies and genotype distributions in the Kerala population are shown in Table 1. Of the total 152 samples analysed, 84 were homozygous FF, 56 were heterozygous Ff and 12 were homozygous ff. The allele frequency of F was 73.86% and that of was 26.31%. The data obtained was analysed to verify if it was in Hardy-Weinberg equilibrium. The observed genotype frequency was used to calculate the expected frequency. Chi-square analysis was done to compare the observed and expected frequencies. A chi-square value of 0.38 with a p-value of 0.8269 at 0.05 significance level was obtained. This confirmed that the difference in the observed and expected genotype values were due to chance alone and hence the VDR Fok-I genotype distribution in the Kerala population is in Hardy-Weinberg equilibrium.

The frequency distribution of VDR Fok-I genotype of our population was compared with those found in previous studies (Table 2). Considerable variations were observed in the genotype distribution between ours and other populations. A goodness of fit test revealed that significant variations exist in the VDR Fok-I genotype distribution of Japanese, French, Canadian, European and Chinese Han populations when compared with our population. As expected, the genotype distribution of South Indian population obtained from a previous study was not significantly different from that of ours, but there was significant difference in the VDR Fok-I genotypes between North Indian population and our population.

	Genotypes n (%)			Allele Frequencies n (%)	
N	FF	Ff	Ff	F	f
152	84 (55.26)	56 (36.84)	12 (7.89)	224 (73.86)	80 (26.31)

Table 1. Genotype Distribution and Allele Frequencies of VDR Fok-I Polymorphism in the Healthy Population of Kerala, India

Country/ Ethnicity	No.	Genotype (n)			p-value	Reference
		FF	Ff	Ff		
Kerala, India	152	84	56	12	**ref***	**Present Study**
North Indian	346	152	170	24	0.0385 (S) †	13
South Indian	80	43	29	8	0.8624 (NS) ‡	14
North Indian	160	80	79	1	0.0014 (S) †	15
Japanese	249	92	127	30	0.0015 (S) †	16
Chinese Han	176	55	78	43	0.0000028 (S) †	17
European	2154	804	1036	314	0.00004 (S) †	18
French Canadian	1381	517	647	217	0.00005 (S) †	19

Table 2. VDR Fok-I Genotype Distributions of Various Populations in Comparison with Kerala Population

* Reference population, † Significant; ‡ Not significant ($p>0.05$)

DISCUSSION

Large numbers of biological process are modulated by the vitamin D endocrine system including bone metabolism, modulation of the immune response, and regulation of cell proliferation, calcium absorption from the gut, and differentiation. Any defect in the VDR gene could modulate the metabolism of calcium thereby increasing the risk of developing different diseases mainly osteoporosis and calcium stones. Variation in the vitamin D receptor sequence have been linked to several other diseases like diabetes, cancer, asthma, SLE, cardiovascular diseases, tuberculosis, etc. Genetic and epidemiological studies in VDR gene facilitate the study of association between disease conditions and molecular sequences. Since genotypic frequency of each

population is different, population specific data of VDR polymorphism has to be generated before conducting a valid genetic association study.

Variations in the vitamin D receptor sequence have been linked to several diseases. The most frequently studied polymorphisms in VDR gene are BsmI, ApaI, and TaqI, at the 3' end of the VDR gene and thymine/cytosine polymorphism (Fok-I) located at the first potential start site. Polymorphisms in 3' UTR region are probably non-functional, and are in linkage disequilibrium (LD) with one or more truly functional polymorphisms elsewhere in the VDR gene. However, Fok-I polymorphism is not in LD with any of the other polymorphisms and hence is considered as an independent marker in itself especially in diseases related to calcium metabolism.

In this study, polymorphism present in the exon 2 of VDR gene was detected using the enzyme Fok-I by PCR-RFLP analysis. VDR Fok-I genotype frequency was obtained for the control population. In case-control studies, the control population must be in Hardy-Weinberg Equilibrium. Otherwise that will be a faulty base data. In this study, the genotype frequency analysis was done and it was found to be in Hardy-Weinberg Equilibrium confirming that this can be definitely used as a background data for large population studies in future.

The allele frequency differences between ethnic groups most likely results from evolutionary process and population genetic behaviour. The findings of this study confirm ethnic variations in the VDR Fok-I genotypes. These variations are relevant in that they form the basis for the correlation between genotype and incidence of different diseases in such groups. In a previous study, variation in VDR Fok-I genotype distribution between Indian and world population was reported.[12] Our study reports variation between different population groups within India. The VDR Fok-I genotypes of the population of Kerala which is a South Indian state were similar to that of an earlier study conducted in South Indian population which included samples from Tamil Nadu, another South Indian state.[13] But our results were significantly different from the results of two different studies conducted in North Indian population.[14,15] India being a multi-ethnic population, these results suggest that separate genotype data should be generated for each (ethnic) group before establishing epidemiological databases for valid genetic association studies.

CONCLUSION

The functional effect of the VDR polymorphisms might be same since the physiological role of VDR remains unchanged in different ethnic populations. However, these polymorphisms may be helpful in predicting the incidence of disease/phenotype between such groups. Association studies can indicate which VDR genotype is most likely associated with the disease causing genes, and hence can serve as diagnostic markers.

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