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IDENTIFICATION OF MSX1 GENE MUTATION IN KELANTANESE PATIENTS WITH VARIOUS TYPES OF NON-SYNDROMIC CLEFT LIP AND PALATE

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CERTIFICATE

This is to certify that the dissertation entitled

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At the end there is only my name showing on the cover of the thesis, claiming all faults of this work as mine. As for the contributors, research is not done in thin air and so there are many people I would like to thank here.

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PREFACE

Congenital cleft is the most common congenital craniofacial deformity of newborn. Cleft lip with or without cleft palate (CL/P) is among the most common of all major birth defects, occurring with an incidence of 1/500 to 1/2500 in different populations, varying with geographic location, ethnic group, and socioeconomic conditions. It is generally considered that about 70% of cases of CL/P occur as an isolated abnormality, so-called "nonsyndromic" CL/P (nsCL/P). The rest occur as part of more than 300 different multiple malformation syndromes in which CL/P is but one manifestation (Jones, 1998). The situation regarding CP is less clear. Most studies have suggested that about 80 to 85% of CP cases are nonsyndromic (nsCP) (Christensen *et al.*, 1996) (Natsume *et al.*, 2001). However, one recent study from Japan found that about 80% of cases of CP are associated with other birth defects (Nazer *et al.*, 2001), and most clinicians have come to suspect that the great majority of CP cases are, in fact, syndromic.

Orofacial clefts are of two principal types, cleft lip (CL) and cleft palate (CP). Cleft lip is a unilateral or bilateral gap in the upper lip and jaw, which form during the third through seventh week of embryonic development. Cleft palate is a gap in the hard or soft palate, which forms from the fifth through twelfth weeks of development. Thus, the embryology of CL and CP is largely distinct, a fact that has considerable significance for the nosology of these two types of clefts. Cleft lip may occur either in association with or without CP, and hence is commonly referred to as "cleft lip with or without cleft palate" (CL/P), as distinct from CP, which typically occurs without associated CL. It is unclear whether CP occurring

in association with CL results from mechanical deformation or from genes or environmental factors that affect development of both the lip and palate.

Current trend of studies in cleft are towards the genetic factor. In non-syndromic cleft deformity, a few genetic factors have been associated with the incidence of the cleft lip and/or palate. The genetic factors can either be signaling factors such as transforming growth factors (alpha or beta), retinoic acid, endothelin, etc or target genes such as Hox family, MSX genes, Pax genes etc.

Among the target genes, MSX1 gene produced the most consistent result with the incidence of non-syndromic cleft lip and/or palate. The MSX1 homeobox gene is expressed at diverse site of epithelial- mesenchymal interaction during vertebrate embryogenesis and has a critical role during craniofacial bone and tooth development. Satokata and Mass in 1994 demonstrated that mice with the MSX1 phenotype developed cleft and oligodontia.

The objective of this project is to determine the association between MSX1 and various types of non-syndromic cleft lip and/or palate in Kelantanese population. With this effort we will determine the allele frequency of the loci on MSX1 gene in the population studied.

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ABBREVIATIONS

bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
kb	Kilobase
ml	Mili liter
PCR	Polymerase Chain Reaction
V	Volt
µg	Micro gram
µl	Micro liter
CP	Cleft palate
CL	Cleft lip
CLP	Cleft lip and palate
KCl	Potassium chloride
$MgSO_4$	Magnesium sulphate
Tm	Annealing temperature
$CaCl_2$	Calcium chloride
LB	Lithium boric acid
$MgCl_2$	Magnesium chloride
nm	Nano meter
µM	Mikro mole
ddH_2O	Deionised distilled water
PAGE	Polyacrylamide gel electrophoresis

ABSTRAK

Rekahan orofasial adalah kecacatan struktur bibir dan lelangit yang melibatkan di antara 1 dalam 2000 sehingga 1 dalam 500 kelahiran. Di Kelantan ianya melibatkan 1 dalam 600 kelahiran. Gen MSX1 ialah salah satu gen sasaran dimana ia menghasilkan keputusan yang konsisten dalam insiden kecacatan bibir dan lelangit bukan sindrom. Gen ini terletak pada kromosom 4 pada lengan pendek, p16.3-p16.1. Ia mengandungi dua ekson yang mengkodkan homeodomain yang mempunyai protein dengan 297 asid amino and satu intron.

Tiga puluh lima pesakit kecacatan bibir dan lelangit bukan sindrom dan 35 sampel darah kawalan adalah termasuk di dalam kajian ini. Mutasi pada gen MSX1 telah berjaya dikenalpasti pada intron gen ini menggunakan pemula MSX1-CA penanda daripada Stegman *et al.* (1999). Pemula ini memperbanyakkan 9 CA ulangan dan menghasilkan 169 pasangan bes dengan menggunakan tindakbalas rantai polimerase (PCR). Apabila produk daripada tindakbalas rantai polimerase telah diperolehi, gen ini akan melalui proses elektroforesis untuk mengesan mutasi dengan memperolehi dua jalur. Ini menunjukkan CA ulangan telah memanjang atau memendek daripada 9 ulangan untuk gen mutasi.

Daripada tiga puluh lima pesakit yang diuji, lima (2%) menunjukkan variasi daripada yang lain manakala tujuh (31.4%) menunjukkan variasi pada lokus tertentu daripada sampel kawalan. Nilai P= 0.275 menunjukkan tiada perkaitan diantara mutasi pada gen MSX1 dan

kecacatan bibir dan lelangit. Akan tetapi saiz sampel adalah terhad. Saiz sampel yang lebih besar adalah diperlukan untuk kajian masa hadapan.

ABSTRACT

Orofacial cleft are congenital structural abnormalities of the lip and/or palate that affect between 1 in 2000 and 1 in 500 live births worldwide. In Kelantan, it affects 1 in 600 live births. MSX1 gene is one of the target genes which produce the most consistent result with the incidence of non-syndromic cleft lip and/or palate. This gene is located in chromosome 4 at short arm, p16.3-p16.1. It has two exons that code for a homeodomain-containing protein of 297 amino acids and one intron.

Thirty-five patients with non-syndromic cleft lips and /or palate and 35 control blood samples were included in this study. The mutation of MSX1 gene has successfully been detected in the intron of this gene using MSX1-CA. This primer amplified nine CA repeats and produced 169 base pair using polymerase chain reaction (PCR). After amplification, the product was electrophorosed to detect the mutation by getting two bands. This showed the CA repeat was expanded to more or less than nine repeats for mutated gene.

Out of thirty-five patients tested, five (2%) showed variation from the others while seven (31.4%) showed variation in particular locus from control samples. There was no association between MSX1 gene mutation and clefting (P=0.275). But the sample size is rather limited. Larger sample size is needed for future studies.

1. INTRODUCTION

1.1 Non-syndromic cleft lip and/or palate

A cleft lip is a separation of the two sides of the lip. The separation often includes the bones of the upper jaw and/or upper gum. A cleft palate is an opening in the roof of the mouth in which the two sides of the palate did not fuse, or join together, as the unborn baby was developing. Cleft lip and cleft palate can occur on one side (unilateral cleft lip and/or palate), or on both sides (bilateral cleft lip and/or palate) (Figure 1.1 and Figure 1.2). Because the lip and the palate develop separately, it is possible for the child to have a cleft lip, a cleft palate, or both cleft lip and cleft palate.

Cleft lip and cleft palate are congenital defects, or birth defects, which occur very early in pregnancy. The majority of clefts appear to be due to a combination of genetics and environmental factors (http://www.cleftline.org/aboutcl). The risks of recurrence of a cleft condition are dependent upon many factors, including the number of affected persons in the family, the closeness of affected relatives, the race and sex of all affected persons, and the severity of the clefts (http://www.cleftline.org/aboutcl).

Cleft lip and/or palate can occur in syndromic or non-syndromic form. A syndrome is a group of small abnormalities that are often seen together in children of unusual appearance, giving them a resemblance or "family air". (http://www.cleft.ie/publications/). So, cleft lip and/or palate are often associated with other abnormalities, forming part of several syndromes in a person of otherwise normal appearance. In some of the syndromes where cleft lip and/or palate are seen, the other abnormalities may be obvious and more

threatening, as in the case of some chromosomal syndromes. "Pure form", i.e. unaccompanied by any other malformation is called non-syndromic cleft lip with or without cleft palate (http://www.cleft.ie/publications/clapa_booklet/genetic.htm).

The human embryo has a recognisable face at around eight weeks, when it measures 28 mm from head to rump (http://www.cleft.ie/publications/clapa_booklet/genetic.htm). At this stage the nose and lips have already been formed, probably as a result of cells migrating from the direction of the forehead and cheeks into the face. The primary palate is formed at the end of week seven, and results from growth from the inner sides of the upper jaw towards the midline, and their subsequent fusion. Fusion proceeds from front to back and is not completed until the 11 th week. The primary deformity in clefting of lip or palate occurs if this process of fusion is not completed (http://www.cleft.ie/publications/clapa_booklet/genetic.htm).

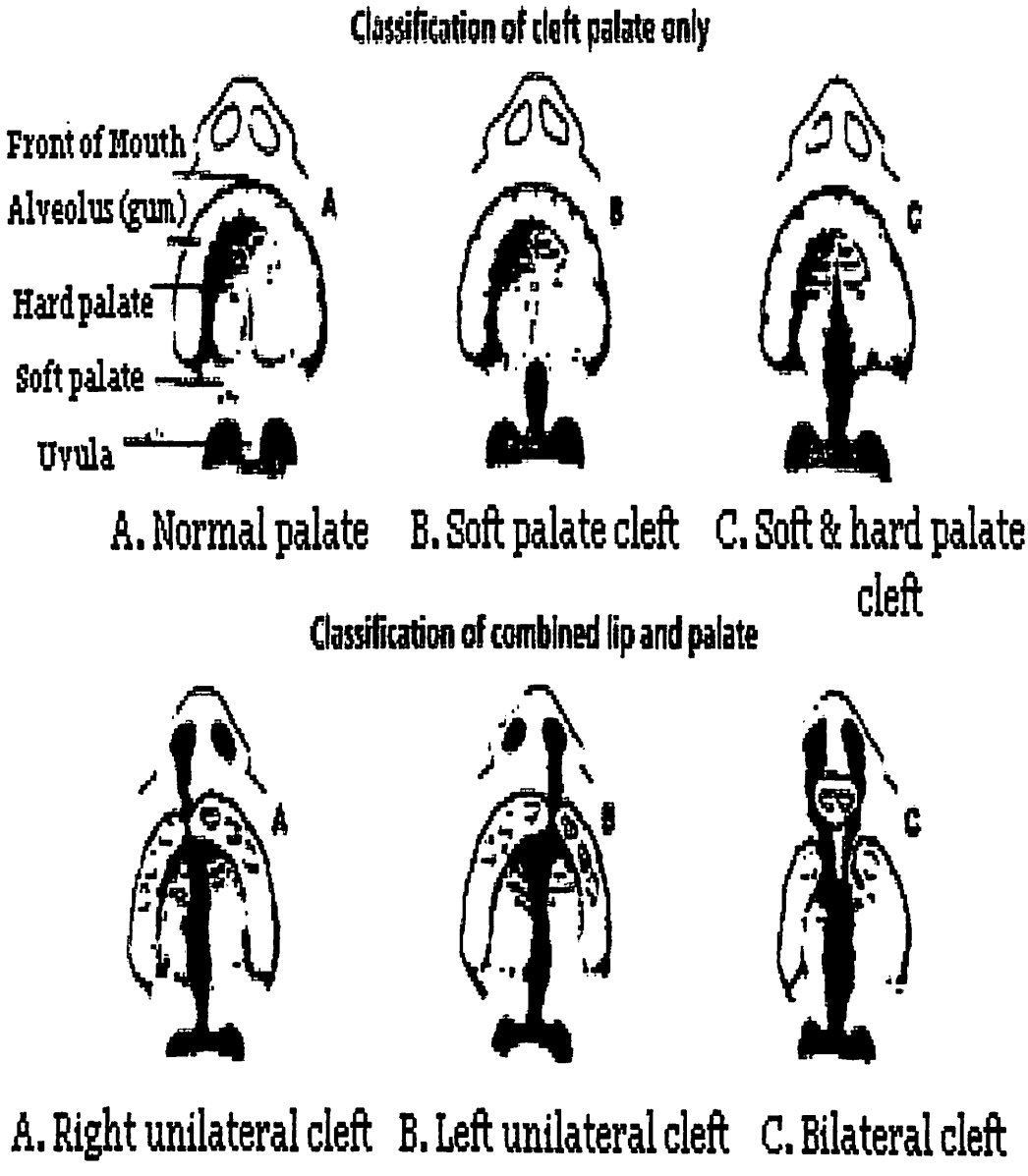

Figure 1.1 Classification of cleft lip and palate.

Figure obtained from http://www.cleft.ie/publications/clapa_booklet/intro.htm.

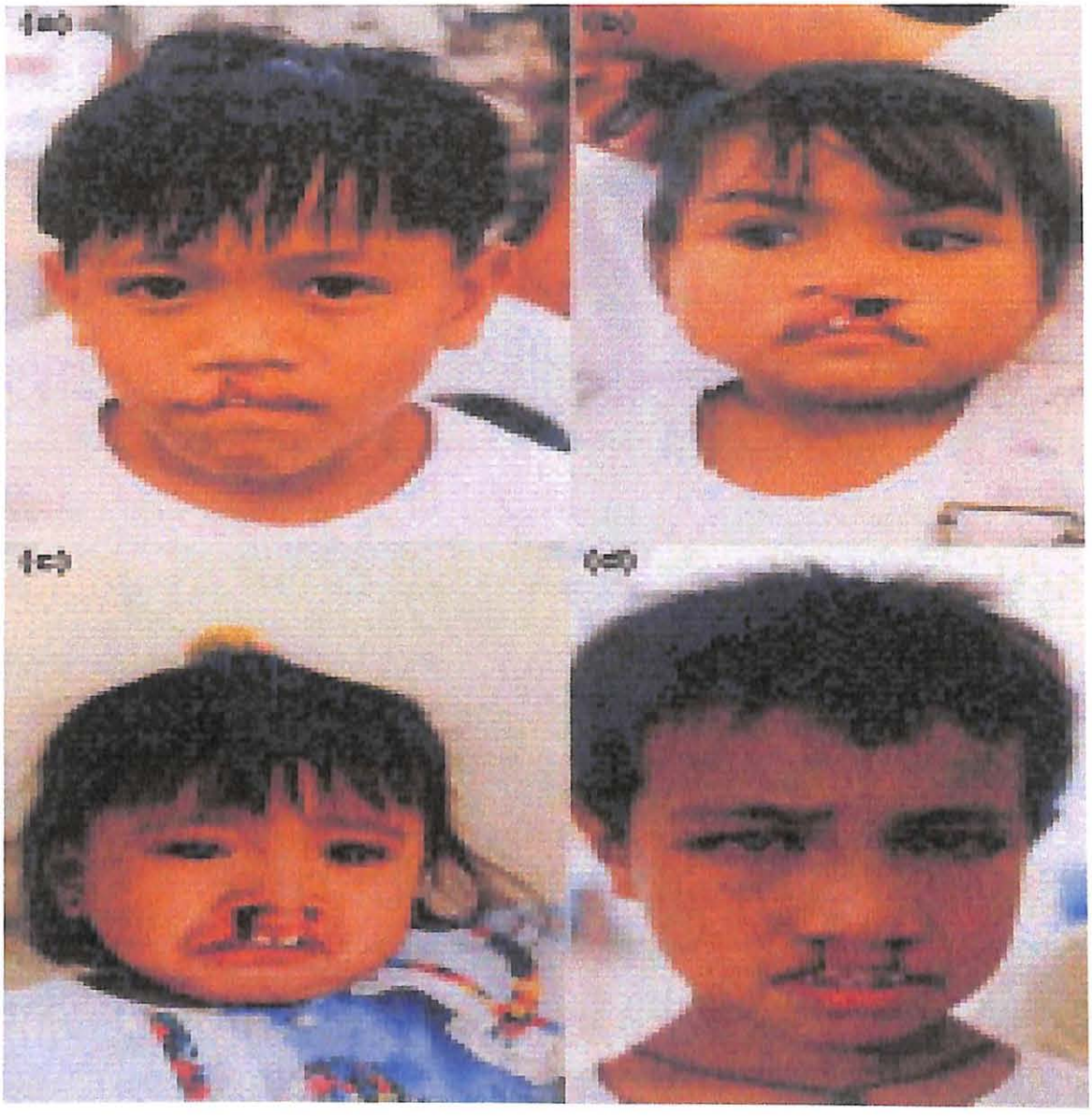

Figure 1.2 Examples of unrepaired facial clefts: (a) unilateral cleft lip only; (b) unilateral cleft lip and palate; (c)bilateral cleft lip and palate; (d) Van der Woude syndrome with a lower lip pit and bilateral cleft lip and palate.

This figure obtained from Murray JC., (2002)

1.2 MSX1 gene

Satokata and Maas (1994) found that transgenic mice rendered homozygous for a nonfunctioning Msx1 gene showed cleft palate and facial and dental abnormalities. Thus, Msx1 is a candidate gene for human cleft palate.

1.2.1. Mapping

Ivens *et al.*, (1990) found that the gene maps to 4p16.1, slightly proximal to the Huntington Disease (HD) locus. This region shows homology of synteny with part of mouse chromosome 5 where the murine HOX7 gene is located (Robert *et al.*, 1989). Figure 1.3 showed an idiogram of MSX1 gene.

1.2.2. MSX1 gene function

Targeted inactivation of Msx1 in transgenic mice leads to an arrest of tooth development at the bud stage. These mice exhibit also other developmental defects, e.g. cleft palate (Satokata *et al.*, 1994). If also the MSX2 gene is rendered inactive ("double knockout"), the tooth development is arrested even earlier, at the initiation stage.

There is also evidence of involvement of human MSX1 in tooth development. A single point mutation in the homeodomain of MSX1 was found to segregate with oligodontia (lack) of all permanent second premolars and third molars (Vastardis *et al.*, 1996).

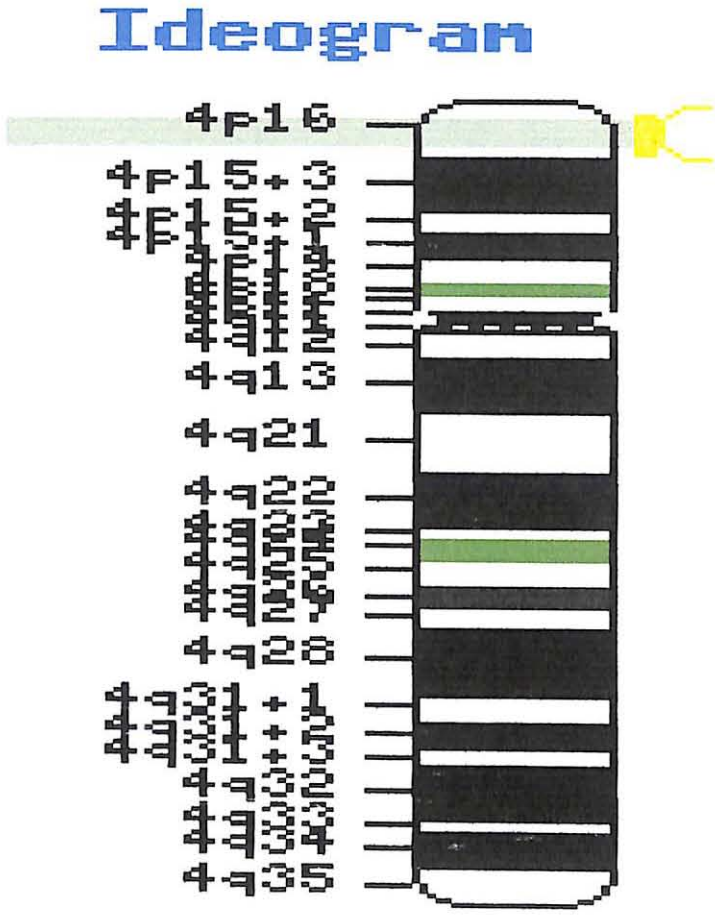

Figure 1.3: Ideogram of MSX1 gene

This figure was obtained from http://www.ncbi.nlm.nih.gov/mapview/maps.cgi

Most affected members also lacked some other teeth (first permanent and molars, the lack of which is very uncommon, or first incisors). However, the affected members of the family were all reported to have complete deciduous dentition, a state that would be in accordance with the lack of defects in heterozygous mice. Functional analysis later showed that the point mutation causes structural perturbation and reduced thermostability of the MSX1 protein, thus suggesting that the phenotype is caused by smaller amount of the functional protein (haploinsufficiency, Hu *et al.*, 1998). The selective effect on only certain human teeth of the single amino acid change in a protein that appears to be present in all teeth can best be interpreted in terms of an existing threshold in some point of the development that must be overcome to produce a complete tooth.

MSX1 has also been implicated with oral clefting. The homozygous null mutant mice exhibit cleft palate (Satokata *et al.*, 1994). A polymorphism in MSX1 was associated with clefting (Lidral *et al.*, 1998), and recently several alterations in MSX1 were found in a wide study of oral cleft patients (Jezewski *et al.*, 2003). Different types of clefts were also found in some patients in one of the families with a MSX1 exon 1 nonsense mutation (van den Boogaard *et al.*, 2000). However, oral clefts were not seen in patients with heterozygous MSX1 deletions (Nieminen *et al.*, 2003).

Odelberg *et al.*, (2000) presented evidence that terminally differentiated murine myotubes can be induced to dedifferentiate. Ectopic expression of MSX1 in C2C12 myotubes reduced the nuclear muscle proteins MyoD myogenin (MyoG; Mrf4 and p21 to undetectable levels in 20 to 50% of the myotubes. Approximately 9% of the myotubes cleaved to produce either smaller multinucleated myotubes or proliferating, mononucleated cells. Clonal populations of the myotube-derived mononucleated cells could be induced to

redifferentiate into cells expressing chondrogenic, adipogenic, myogenic, and osteogenic markers. These results suggested that terminally differentiated mammalian myotubes can dedifferentiate when stimulated with the appropriate signals and that MSX1 can contribute to the dedifferentiation process.

Blin-Wakkach *et al.*, (2001) demonstrated the presence of an endogenous MSX1 antisense RNA in mice, rats, and humans. In situ analysis revealed that this RNA is expressed only in differentiated dental and bone cells with an inverse correlation with MSX1 protein. These in vivo data and overexpression of MSX1 sense and antisense RNA in an odontoblastic cell line showed that the balance between the levels of the two MSX1 RNAs is related to the expression of MSX1 protein. To analyze the impact of this balance in the MSX-DLX homeoprotein pathway, Blin-Wakkach *et al.*, (2001) analyzed the effect of MSX1, MSX2, and DLX5 overexpression on proteins involved in skeletal differentiation. They showed that the MSX1 antisense RNAs is involved in crosstalk between the MSX-DLX pathways because its expression was abolished by DLX5. MSX1 was shown to downregulate a master gene of skeletal cell differentiation, Cbfa1. All these data were interpreted as strongly suggesting that the ratio between MSX1 sense and antisense RNAs is an important factor in the control of skeletal terminal differentiation. The initiation site for MSX1 antisense RNA transcription was located by primer extension in both mouse and human in an identical region, including a consensus TATA box, suggesting evolutionary conservation of the antisense RNA-mediated regulation of MSX1 gene expression.

By investigating MSX1 function in repression of myogenic gene expression, Lee *et al.*, (2004) identified a physical interaction between MSX1 and H1B, and found that MSX1 and H1B bind to a key regulatory element of MYOD, a central regulator of skeletal muscle

differentiation, where they induce repressed chromatin. Moreover, MSX1 and H1B cooperated to inhibit muscle differentiation in cell culture and in Xenopus animal caps. Lee *et al.*, (2004) concluded that their findings defined a theretofore unknown function for linker histones in gene-specific transcriptional regulation.

1.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an *in vitro* technique that is commonly used for amplification of specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequence. PCR rapidly amplifies a single DNA molecule into many billions of molecules. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

The starting material for PCR, the target sequence, is a gene or segment of DNA. Complementary strands of double-stranded molecule of DNA are separated or denatured by heating. Two small pieces of synthetic DNA each complimenting a specific sequence at opposite ends of the target sequence and lie on opposite strands of DNA serve as primers. Each primer binds or anneal to its complimentary sequence when the reaction mixture is cooled to a certain temperature. Polymerase starts at each primer and copy the sequence of that strand. Within a short time, exact replicates of the target sequence are produced. The cycle of denaturation annealing and ultimately synthesis of DNA product is repeated. In subsequent cycle, double-stranded molecule of both the original DNA and the copies are separated; primers binds again to complementary sequence and the polymerase replicates them. At the end of many cycles, the pool is greatly enriched in the small pieces of DNA

that have the target sequences, and this amplified genetic information is then available for further analysis. The product of PCR is a segment of double stranded DNA which length is defined by the distance between the two primers. Sets are available to perform PCR that contain the DNA polymerase, deoxyribonucleoside phosphates (dNTPs) and 10X buffers. There are various thermostable polymerase enzymes available commercially for use in PCR the most common of which are *Taq* DNA polymerase, obtained from *Thermus aquaticus* and *Pfu* DNA polymerase, obtained from *Pyrococcus furiosus*. The buffer solutions contain potassium chloride (KCl) and magnesium chloride for *Taq* DNA polymerase and KCl and magnesium sulphate ($MgSO_4$) for *Pfu* DNA polymerase. Divalent cations are very important for the proper function of DNA polymerase enzymes. Figure 1.4 shows the diagram depicting the PCR process.