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RESEARCH ARTICLE

MULTIPLE SEQUENCE ALIGNMENT OF 538T/C IN BMP4 GENE AMONG NON SYNDROMIC CLEFT LIP AND PALATE CHILDREN

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ABSTRACT

The chromosomal region around 14q22-q23 containing bmp4 gene is extensively studied by genotyping to give evidence of linkage and one of the most functional single-nucleotide polymorphisms (SNPs) of BMP4 gene, rs17563, is currently a research focus which is a promising in NSCL/P development.

Aim: This study was done to identify Single nucleotide polymorphism that confirmed change from T to C at 538 nucleotide position (538T/C).

Method: The sequencing pattern of the base pairs at BMP4 538T/C position to find out subsequent polymorphism, resulting in an amino acid change of Val=Ala (V152A) in the polypeptide chain in cleft lip and palate patients using the most common bioinformatics analyses, Multiple Sequence Alignment (MSA) that involve comparing homologous sequences and then further alignment by Clustal Omega, a progressive method MSA.

Results: Our results showed that 71.4% of the samples represented the nucleotide as C and 28.5% showed presence of nucleotide T at SNP point mutation site.

Conclusion: Sequencing in our present study confirms the previous results of our findings of PCR restriction fragment length polymorphism on NSCLP in an Indian population that C genotype has more risk for NSCLP.

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INTRODUCTION

The identification of genetic risk factors in non syndromic cleft lip and palate has been the subject of intensive research in the last decade with the list of NSCLP candidate genes rapidly increasing. Several studies have been mainly done focusing in the search for coding mutations (Suazo, 2011). The chromosomal region around 14q22-q23 containing bmp4 gene is extensively studied by genotyping to give evidence of linkage, and one of the most functional single-nucleotide polymorphisms (SNPs) of BMP4 gene, rs17563, is currently a research focus which is a promising candidate SNP locus associated with positive risk for NSCL/P development (Hu, 2015). Our previous study performed on an Indian population of nonsyndromic cleft lip with or without palate by PCR-RFLP results has shown Single nucleotide polymorphism that confirmed change from T to C at 538 nucleotide position (538T/C).

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In this present study we did DNA sequencing to confirm this polymorphism. Sequencing means determining the order of the four chemical building blocks called bases that make up the DNA molecule. This data can highlight changes in a gene that may cause various diseases. Multiple Sequence Alignment (MSA) is essential in most bioinformatics analyses that involve comparing homologous sequences. Clustal Omega is a progressive method MSA, is highly accurate and allows alignment of almost any size to be produced (Sievers, 2011). In the present study we evaluated the sequencing pattern of the base pairs at BMP4 538T/C position to find out subsequent polymorphism, resulting in an amino acid change of Val=Ala (V152A) in the polypeptide chain in cleft lip and palate patients using this technique.

MATERIALS AND METHODS

Samples and settings

A Subsample was obtained from a hospital based study done on hundred cleft lip with or without palate children from a Craniofacial Project research centre, Karnataka. Six months to

eighteen years old children with no family history of clefts or any neural crust cell disorder were taken as the study sample. Written informed consent was obtained from all participants.

Procedure

Genomic DNA was extracted from EDTA peripheral blood leukocytes by HiMedia blood DNA extraction kit. The standardization of PCR condition for BMP4F/R primer was done in a programmable gradient thermocycler (Corbett). The primer sequences used were BMP4 F-5'-CCTAACTGTGCCTAG-3' and BMP4 R-5'-CATAACCT CATAAATGTTTATACGG-3'.

the PCR products of the study population were later processed for sequencing using Sanger method, which is based on the selective incorporation of chain terminating dideoxynucleotides by DNA polymerase enzyme, and a blast was performed to compare the nucleotide sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. The sequence was further analyzed using Multiple Sequence Alignment (MSA) in Clustal Omega.

RESULTS

Our sequencing was analysed through Clustal Omega and all the samples compared with BMP4 rs17563 SNP database,

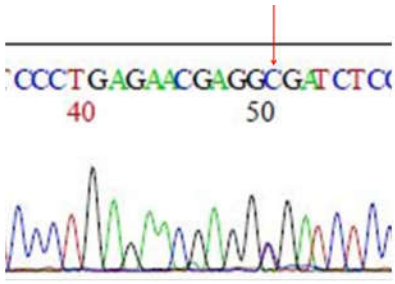

Figure 1. T allele converting to C allele was shown in the result of heterozygote for BMP4 538T/C (arrow) by direct sequencing

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P                 ----------------------------------GCGCTTCCTTTGGCCCGGGTGTAACT
N                 TCCTAACTGTGCCTAGAACATCTGGRGRACRTSC--------CAGGGACCAGTGMAAACT
M                 TCCTAACTGTGCCTAGAACATCTGGAGAACATCCCAGGGTGTTTGTTACCAGTGAAAACT
J                 TCCTAACTGTGCCTAGAACATCTGGAGAACATCCCAGKGACC--------AGTGAAAACT
D                 TCCTAACTGTGCCTAGAACATCTGGAGAACATCCCAGGGTACCRG---TGAAGTGCAACT
B                 TCCTAACTGTGCCTAGAACATCTGGWGAACATCCCAGGGA----C---M-AGTGMAAACT
A                 -CCTAACTGTGCCTAGAACATCTGGAGAACATCCCAGGTGWCMAG---T-KAAGTGAACT
BMP4_rs17563      ------------------------------------------------------------

P                 CTGCTTTTCGTTTCCTCTTTAGCCTCAGCAGCATCCCTGAGAACGAGGCGATATCCTCTG
N                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGTGATCTCCTCTG
M                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGTGATCTCCTCTG
J                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGCGATCTCCTCTG
D                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGCGATCTCCTCTG
B                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGCGATCTCCTCTG
A                 CTGCTTTTCGTTTCCTCTTTAACCTCAGCAGCATCCCTGAGAACGAGGCGATCTCCTCTG
BMP4_rs17563      ----------------------CTCAGCAGCATCCCTGAGAACGAGGCGATCTCCTCTG
                                        ************************** *** *******

P                 GAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATTGGCAAAGGGGCTTCC
N                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGKGCCCTGATTGGGAAASGGGC----
M                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATGTRKCGTACCAAGGGG
J                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATTGGGAAA--GGGGCT-
D                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATTGSGAMM--GKSRCT-
B                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATTGGGAAM--GGGGCT-
A                 CAGAGCTTCGGCTCTTCCGGGAGCAGGTGGACCAGGGCCCTGATTGGGAAM--GGGTACG
BMP4_rs17563      CAGAGCTTCGGCTC--------------------------------------------------
                  **************

P                 ACC-----GTA-TGACATTTATGAGGTTATGAT
N                 TTACCACCGTATAAACATTTATGAGGTTATGA-
M                 CTTCCACCGTATAAACATTTATGAGGTTATAAA
J                 --TCCACCGTATAAACATTTATGAGGTTATGA-
D                 --TCCACCGTATAAACATTTATGAGGTTATAA-
B                 --TCCACSGTATAAACATTTATGAGGTTAT---
A                 CTTCCACCGTATAAACATTTATGAGGTTATGA-
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Figure 2. Clustal Omega (1.2.1) multiple sequence alignment showing SNP point mutation on 538th position as either C/T (highlighted in red)

The BMP4 primers generated PCR amplicons of 197 bp. The PCR product (197 bp) was digested with HphI. HphI digestion produced a fragment in the A147 allele that lacks the HphI site, while the digestion detected 110 bp and 87 bp fragments. HphI restriction fragment length polymorphism was done to analyse the single-nucleotide polymorphism (SNP) of rs17563 and difference in the frequency of SNP rs17563 in cases compared with controls to estimate the relative risk of genotypes of BMP4T538C on the occurrence of NSCLP. A subsample from showed SNP point mutation as either C/T highlighted region marked in red (Figure 1). 71.4% of the study population represented the nucleotide as C and 28.5% showed presence of nucleotide T at SNP point mutation site. 14.28% of the total sample size showed other point mutations too at other regions other than the SNP point mutation for rs17563. Figure 2 represents the multiple sequence alignment pattern of the BMP4 gene fragment.

DISCUSSION

Our study was performed using polymerization chain reaction-restriction fragment length polymorphism on 200 samples. Results showed a significant association between C allele and CL ± P compared with T allele. When haplotypes and cleft were investigated, increased risk of NSCLP was found for homozygous CC and heterozygous TC genotype. We wanted to confirm the allele sequencing, so, we carried out DNA sequencing on a subsample from both cases and controls. The term DNA sequencing refers to methods for determining the order of the nucleotides bases in a molecule of DNA and has developed substantially over the years into a more cost-effective and accurate technique for scientific advancement in medical diagnostics, forensics, systematics, and genomics. The knowledge of DNA sequences of genes is indispensible for basic research studies. In our present study Sanger method of DNA sequencing was done to determine SNP mutations at the 538th position of BMP4 gene (chromosome (14q22-q23) in NSCL/P patients. Sanger dideoxy chain termination sequencing was preferred in our study as it is currently one of the simple, most established and popular sequencing methods which is used widely in smaller scale projects for obtaining long contiguous DNA sequence reads (Sanger et al., 1977).

This method is based on the key principle of using dideoxy nucleotide triphosphates (ddNTP) as DNA chain terminators. The chain termination method requires a single-stranded DNA template, DNA primer, DNA polymerase, radioactively or fluorescently labelled nucleotides, and modified nucleotides that terminate DNA strand elongation. In our study, the DNA sample was divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP, dTTP) and the DNA polymerase. To each reaction was added only one of the four dideoxynucleotide (ddATP, ddGTP, ddCTP, ddTTP) which are the chain terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length. The newly synthesized and labelled DNA fragments were heat denatured, and separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of the four individual lanes (lanes A, T, G,C), the DNA bands were then visualized by UV light, and the DNA sequence could be directly read off the gel image (Barh, 2006).

In the study, after sequencing, alignment of the sequences was done using Multiple Sequence Alignment (MSA) and was preferred over other methods like Pair wise Sequence Alignment as the latter reads only two biological sequences at a time, whereas MSA can read three or more sequences. From the output, homology can be inferred and the evolutionary relationships between the sequences can be studied in MSA. An MSA can be observed as a representation that offers a unified picture of sequence similarity by averaging out matched residues that perhaps cannot be reliably matched over the entire lengths of the sequences. This is because of evolution, mutations, insertions, and deletions of sequence fragments. So the sequence alignments inconsistencies can well arise under divergent evolution. Given these difficulties, building a reliable MSA for a query set of sequences is an overwhelming task. In this unit it has been made strong that the increased attention to multiple sequence alignment methodology has ensued in recent developments regarding most of its facets (Notredame, 1996). Unlike other sequence alignment tools and homology search tools, MSA rely extensively on approximate string matching techniques but do not focus on providing accurate residue-level alignments of the returned sequences. Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more sequences. Hidden Markov models are probabilistic models that can assign likelihoods to all possible combinations of gaps, matches, and mismatches to determine the most likely MSA or set of possible MSAs. In a number of typical multiple alignments (Notredame, 1996) CLUSTAL OMEGA was found to be much faster than other algorithms such as SAGA algorithms, so was preferred in the present study. The results of our sequencing pattern are similar to the one shown in a Brazilian population where the T allele is replaced by C allele.

The results of our study showed that SNP of rs17563 was a high risk factor for NSCL/P among carriers of the C allele, which was in accordance with that of a case-control study performed among Chinese children in 2008 by Lin, where the authors analysed a similar increased risk of NSCL/P among carriers of the C allele (Lin, 2008). L Jianyan, in his hospital-based case-control study in Chile identified the interactions between 538 (T-C) polymorphic site of BMP4 gene and the exposures in pregnancy with NSCLP showed homozygosity for polymorphism (CC) resulted in significantly raised risk compared to TT genotypes However the authors did not find an increased risk for heterozygous TC genotype compared with the TT genotypes. Similar findings of Bings' case-control study on Chinese population evaluated BMP4T583C polymorphism resulting in change of amino acid VI52A out of 184 patient genotypes suffering with NSCLP. Also, 205 controls were detected using PCR-RFLP strategy where the 538C allele carriers were associated with significantly increased risk of NSCL/P as compared to no carriers. Our results are in contrast to a study by Araujo et al in a Brazilian population found different results of the 538C allele having a protective effect on NSCLP (Lin, 2008). However, NSCLP is a multifactorial disease where multiple etiologic factors, both genetic and environmental, could either act solely or in combination with different levels of cell to cell interactions or during different signalling pathways. So it is very hard to declare a single variant of a particular gene to be responsible for NSCLP-based on gene association studies and several other restriction segments of different genes are also studied individually or in combination with environmental factors thus there is a need for further research in this area in different population to be elucidated.

Conclusion

Sequencing in our present study confirms the previous results of our findings of PCR restriction fragment length polymorphism on NSCLP in an Indian population that C genotype has more risk for NSCLP. However NSCLP is a multi-factorial disease and further studies are required to better understand the aetiology behind NSCLP.

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