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Original Article

AMY2 Gene Copy Number Variation as a Marker of Genetic Diversity in Kurds

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ABSTRACT

Genetic variation in the human genome ranges from single nucleotide polymorphisms (SNPs) to large chromosomal arrangements. Copy number variations (CNVs) are segments of DNA larger than 1kb in length with variable copies in the genome. The salivary amylase (*AMY1*) gene is a highly variable region and has been linked to dietary adaptation and body mass index (BMI), whereas the pancreatic amylase (*AMY2A* and *AMY2B)* genes are less variable and their functional implications have yet to be explored. In the European population, there are two common types of pancreatic amylase CNVs: a deletion of *AMY2A* and a duplication of a 116kb region including both *AMY2A* and *AMY2B* genes. There is limited information on *AMY2* CNVs in non-European populations. This study aims to investigate the frequency of *AMY2A*/*AMY2B* duplications in the Kurdish population using a PCR-based duplication junction assay. A total of 186 DNA samples from type 2 diabetic patients and non-diabetic controls were analysed. The assay detects a 424 bp control fragment in all individuals and an additional 323 bp fragment in duplication carrier samples, allowing the differentiation between individuals with four *AMY2* copies (duplication-negative) and those with at least six copies (duplication-positive). The results show that 23% of the Kurdish samples were duplication-positive, while 77% did not carry the duplication. The observed frequency in the Kurdish population is higher than that reported in European (12%), African (8.6%), and American (10.9%) populations and rare in East Asian (Chinese and Japanese) populations. The prevalence of duplication carrier samples in the Kurdish population reveals a notable genetic variability in pancreatic amylase genes that aligns with some global populations, possibly reflecting unique historical and dietary influences. These findings emphasise the need for further research on amylase CNVs in the Kurdish population.

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Keywords: Pancreatic amylase genes, CNVs,Gene duplication, PCR.

1 Introduction

1.1 Structural Variation and Copy Number Variation (CNV)

Genetic variation in the human genome can take several forms; it ranges from a change in a single nucleotide to large chromosomal rearrangements. Structural variations are a form of genetic variation in which a large segment of DNA is either deleted or duplicated. Upon the completion of the 1000 Genome Project, over 60,000 structural variants have been found in the human genome [1,2]. As humans are diploid, inheriting one copy of their genome from each parent, they are expected to have two copies of each gene on the homologous chromosomes. However, for some genomic regions there are more or fewer than two copies due to structural variations in the human genome; these genes with variable copies of the same gene are known as copy number variable genes [3-5]. A Copy Number Variable region (CNV) is a segment of DNA greater than 1kb in length and found in variable copy numbers within the genome [6,7]. CNVs are generally classified into two categories: the first is di-allelic copy number variants ranging from 1-4 copies per person for a duplication (or 0-2 copies for a deletion), and the second is multiallelic variants, which include the presence of a wider range of copy number alleles [5,8].

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1.2 Importance of CNVs

The effects of CNV arise due to changes in gene copy number, which in turn can change the expression of that particular gene or alter the space between a gene with their regulatory elements [5,9]. While not all the structural variants have pathological implications, numerous studies have found that CNVs have

significant effects on diseases, genetic diversity, and evolutionary processes [10-12]. Rare CNVs have been correlated with several diseases like schizophrenia, autism and Alzheimer's disease [13-16]. CNVs in Complement factor 4 (C4) have been found to be associated with Schizophrenia [17]. Other studies showed association of beta-defensin and FCGR3B CNVs with psoriasis and systemic lupus, respectively [18,19].

1.3 Methods for CNV detection

Advances in DNA sequencing technologies have facilitated the characterisation of human genomic variations, including CNVs [20]. However, genotyping multiallelic CNVs with high copy numbers remains a great challenge due to sequence complexity and similarity of these regions [20,21]. Accuracy and reproducibility are among the important aspects when measuring a copy number variable region. Several molecular methods have been developed for measuring gene copy number variations; among these are fluorescence in-situ hybridization (FISH), array-comparative genomic hybridization (Array-CGH), and PCR - based methods, such as quantitative real-time PCR, paralogue ratio test (PRT) and digital droplet PCR [6,22-24]. PCR-based methods are most commonly used because they are simpler, reliable, accurate, and high throughput. For instance, a well-designed PRT method could be used to genotype hundreds of samples for genome-wide association studies [25].

1.4 Amylase gene family and CNVs

In humans, the Alpha- amylase enzyme plays a vital role in starch digestion and is secreted by both the salivary glands and pancreas. Salivary amylase is encoded by *AMY1* genes, while the pancreatic amylase is encoded by the *AMY2* genes [26,27]. The human reference genome assembly (hg19) displays three nearly identical *AMY1* genes (*AMY1A*, *AMY1B*, and *AMY1C*) and two distinct *AMY2* genes (*AMY2A* and *AMY2B*) located on chromosome 1p21.1 [28] (Figure 1). This gene family is highly variable in copy number; for example, the salivary *AMY1* gene copies can range from to 2 to 18 [6,29].

In recent years, several studies highlighted the importance of CNVs within the amylase gene family. The *AMY1* gene copy number has been linked to dietary starch intake; populations with high starch-diet tend to carry higher *AMY1* gene copy numbers compared to those with low starch-diet [30]. Additionally, low copies of *AMY1* gene were linked with a predisposition with obesity [31-33]. However, this association has not been consistently replicated in subsequent studies using more accurate genotyping methods and larger sample sizes [24,34,35]. The pancreatic amylase genes on the other hand are less extensively variable; humans can have 2 to 12 copies in total, with the majority having four copies (2 copies each of *AMY2A* and *AMY2B* per diploid genome) [6,36]. Studies have shown that in European population samples there are generally two types of CNVs observed at pancreatic amylase genes; the first is a deletion of *AMY2A* gene and the second is a duplication of a 116 kb region including both *AMY2A* and *AMY2B* genes (Figure 1). This duplication created a new junction sequence that can be identified by the PCR method [6]. A carrier of this duplication will at least have a haplotype containing two copies of *AMY2A* and *AMY2B* genes, and this haplotype is common in the European and African populations but rare in East Asians (Chinese and Japanese) [6]. Moreover, family segregation analysis using PRT and fibre- FISH methods has identified higher order expansion (duplications) of *AMY2A*/*AMY2B* in African population samples; some haplotypes may carry up to 5 copies of each *AMY2A* and *AMY2B* genes [36]. Despite the extensive research on amylase CNVs, particularly the implications of *AMY1* for dietary starch intake and obesity, and the structural characterisation of *AMY2* genes, studies on pancreatic amylase CNVs within non-European populations are rare, especially in Kurdish populations which have a unique genetic background and dietary history.

To date, no studies have investigated CNVs specifically in the Kurdish population, which is known to have a distinctive genetic background [37]. By examining the CNVs of pancreatic amylase genes (particularly *AMY2A* and *AMY2B* duplications) this research provides a valuable chance to explore genetic diversity in this group. It can also offer valuable insights into the extent of amylase gene variation and contribute to a better understanding of CNVs within the Kurdish population. Accordingly, this study aims to characterise pancreatic amylase CNVs, focusing on *AMY2A*/*AMY2B* duplications, using a PCR-based duplication junction assay.

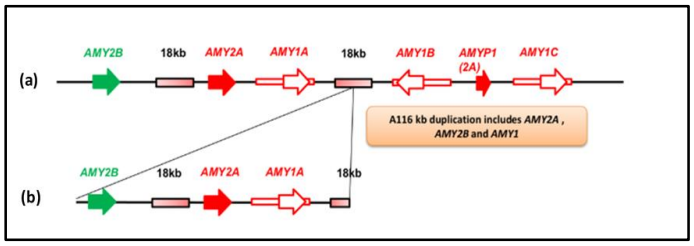

Figure 1: A schematic representation of the amylase gene family on chromosome 1p21.1. The upper panel (a) shows amylase gene family on human reference assembly (hg19), including three copies of the AMY1 genes (AMY1A, AMY1B, and AMY1C) and two copies of the AMY2 genes (AMY2A and AMY2B). The AMYP1 represents a pseudogene copy of AMY2A. The lower panel (b) shows a 116 kb duplication encompassing the AMY2B, AMY2A, and a copy of AMY1 genes, which creates a unique sequence junction that can be detected by PCR [23].

2 Materials and Methods

2.1 DNA Samples

In this study, 186 unrelated samples (51% males and 49% females) were used to characterise the pancreatic amylase CNV. These samples consisted of previously extracted genomic DNA from blood of Type 2 diabetic patients (T2D) and non-diabetic controls [38]. The inclusion of T2D was not intended to explore the role of pancreatic amylase gene CNVs in T2D specifically, but rather to allow for a broader investigation of genetic diversity in the Kurdish population. The purity and concentration of the

DNA samples were measured by the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), with concentration ranging from 8.5-346 ng/µl, and a purity (at A260nm/280nm) range of 1.79-2.1.

2.2 *AMY2A*/*AMY2B* Duplication Assay

The *AMY2*A/*AMY2B* duplication assay is a PCR- based method developed to identify the duplication in the human pancreatic amylase genes that encompasses both *AMY2*A and *AMY2B* genes [6]. The assay includes three primers carefully designed to differentiate samples carrying the duplication from those without the duplication. Primers *AMY2B*F: 5′-TCC TCC AAG ACA TTA TTT TTG C-3′ and *AMY2B*R: 5′-TCA ATT AGG AAA TGA AGA TAT TGT TGA-3′ produce a 424 bp product in all samples, regardless of the duplication status, and serve as a control to confirm successful amplification. The primer *AMY2B*D: 5′-TGC CAT AGA CAA AAT CTG TTG G-3′ pairs with *AMY2B*R to amplify a 323 bp product from the duplication junction. A copy number inference of total pancreatic amylase genes could be deducted from this assay as follow: any sample negative for the duplication will have 4 copies (2 copies of *AMY2*A and 2 copies of *AMY2B*) similar to the reference genome, while samples positive for the duplication will have at least 6 copies (3 copies of *AMY2A* and 3 copies of *AMY2B*).

2.3 PCR reaction mix and conditions

The PCR reactions were conducted by adding all the three primers in a single tube for each sample. The PCR mixture consisted of 10 µl of PCR master mix (Add T*aq* Master; Addbio, Korea), 1µl of DNA sample (10ng/µl), 1µl of each primer (10µM), and then sterilized deionized distilled water was used to reach the final volume of 20µl. The PCR steps were one cycle of initial denaturation at 95°C for 3 minutes, followed by 36 cycles of denaturation at 95°C for 30s, annealing at 48°C for 30s and extension at 65°C for 1min, and a final extension at 72 °C for 10 minutes. The PCR products were stained with safe stain (Addbio, Korea) and resolved on 2% (w/v) agarose gel electrophoresis run at 120 volts for 1 hour, and finally visualised with a UV transilluminator.

3 Results and discussion

In this study, 186 DNA samples were genotyped to characterise the pancreatic amylase gene CNV in the Kurdish population. The duplication assay was carefully designed for this purpose, and it successfully genotype samples with pancreatic amylase CNV. The PCR assay produced different banding patterns on the agarose gel for both duplication-positive and duplication-negative samples. In all the samples it amplified a 424 bp product regardless of the presence of the duplication or not, which can be used as a control for a successful PCR reaction. In addition, the assay amplified an extra 323 bp product in samples harbouring a duplication of the pancreatic amylase genes (*AMY2A* and *AMY2B*). This clear banding pattern allowed reliable differentiation between duplication-positive and duplication-negative samples; indicating copy number variability among the studied samples (Figure 2).

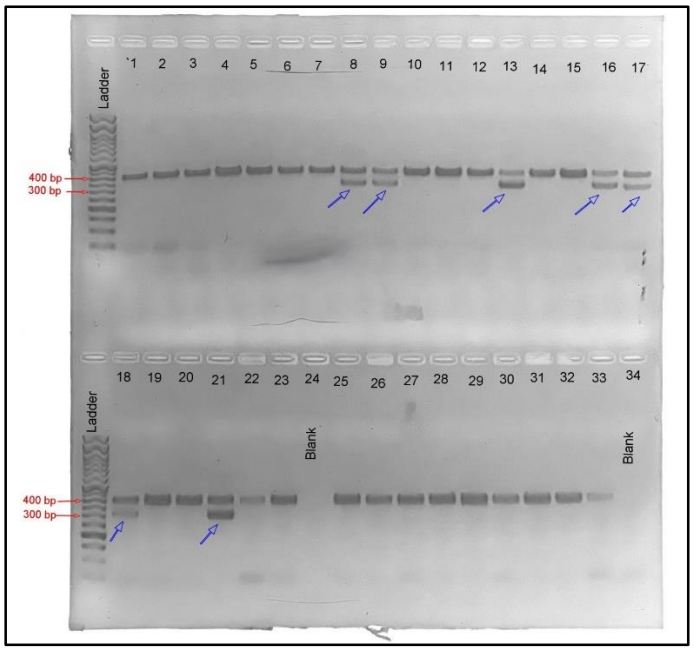

Figure 2: Agarose gel electrophoresis of *AMY2A*/*AMY2B* duplication junction assay, showing samples positive for the duplication. The first lane in both panels represents a 50 bp Ladder, followed by PCR products of each sample. A product size of 424 bp is a control band produced in all samples, indicating a successful PCR amplification. An extra band of 323 bp indicates that the sample carries the *AMY2A*/*AMY2B* duplication junction (blue arrows). The products were resolved on 2% agarose gels run at 120 volts for 1 hour.

Among 186 samples, 144 (77%) showed a negative duplication assay, indicating that these samples have no variable pancreatic amylase genes (Figure 3). This means that they have in total four copies of the pancreatic amylase genes (2 copies each of *AMY2A* and *AMY2B*), and these copies are distributed equally on each haplotype, similar to the reference genome assembly (*AMY2B-AMY2A*).

On the other hand, 42 (23%) samples were positive for the duplication assay, indicating variable copy numbers of pancreatic amylase genes (Figure 3). In duplication-positive samples, the inferred copy number increases to at least six copies of pancreatic amylase genes (three copies each of *AMY2A* and *AMY2B*) because of the duplication event. This structure includes a haplotype carrying two copies of the pancreatic amylase genes (one copy of each *AMY2A* and *AMY2B*), and another haplotype with four copies of the pancreatic amylase genes, with two copies of each gene arranged in the order (*AMY2B-AMY2A-AMY2B-AMY2A*). Although homozygous or higher-order duplications may be present in some individuals, this assay cannot identify samples carrying the same duplication junction multiple times, even the more extensive and complicated duplications of the pancreatic amylase genes observed in African populations are nearly always of this type, and would be detected as positive by

the assay [36]. Additionally, this assay was designed to detect the *AMY2A*/*AMY2B* duplication, but it is possible that other, independent duplications exist that would appear as negative in the assay [6,29].

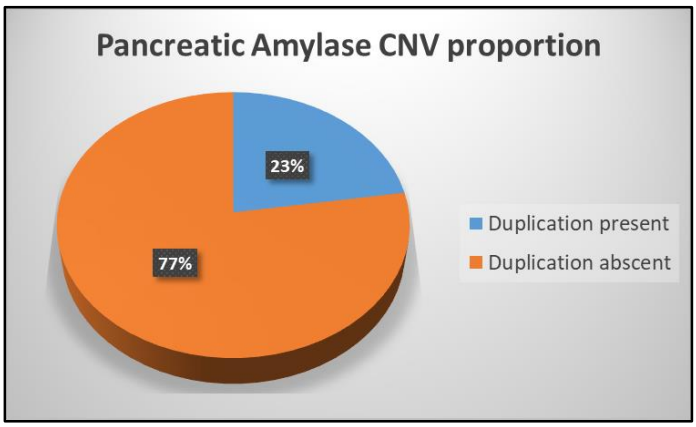

Figure 3: This chart shows the proportion of pancreatic amylase CNV in 186 genotyped samples for *AMY2A*/*AMY2B* duplication. 23% of the samples (42 out of 186 samples) were positive for the duplication, indication a variable copy number of the pancreatic amylase, due to the presence of the duplication junction. The remaining 77% (144 out of 186 samples) were negative for the duplication assay, and have four copies of *AMY2* genes, consistent with the reference genome, indicating non-variable pancreatic amylase genes.

The findings from this study highlight the variability of the pancreatic amylase genes within the Kurdish population, which need to be further explored in terms of copy number variations. The 23% duplication- positive in this study suggests that the frequency of duplications of *AMY2A*/*AMY2B* in the Kurdish population aligns more closely to the European, African, and American populations, where similar duplication frequencies are seen, and less common in East Asians (Chinese and Japanese) from samples of the International Hap Map project (Table1) [6,36]. This higher frequency in the Kurdish population might be attributed to a dietary history and practice, as populations with high starch diet are generally seen to have higher amylase copy numbers [30]. Therefore, the high frequency in the Kurdish population might indicate an adaptive evolutionary response to environmental factors, suggestion similar selective pressure may have influenced gene CNVs in this population [29]. Although no studies have found a direct association between pancreatic amylase CNV and a disease susceptibility, the findings highlight the importance of studying CNV in different populations to better understand the evolutionary implications of gene CNV [25].

Although the PCR based duplication assay used in the current study reliably distinguishes between duplication- positive and duplication- negative samples, it cannot distinguish higher order duplication more than six copies. This limitation may result in underestimation of the full range of *AMY2A*/*AMY2B* copy number variation [36]. Future studies involving larger sample size using high resolution paralogue ratio test (PRT) and sequencing are necessary to characterise the full range of amylase genes variations in the Kurdish population.

Table 1: Frequencies of pancreatic (*AMY2A*/*AMY2B*) duplication across different populations around the world, as reported by Carpenter et al. (2015) [6], compared to the Kurdish population.

Population	***AMY2A*/*AMY2B* duplication**
European	12 %
East Asian	0 %
African	8.6 %
American	10.9 %
Kurds	23 %

Conclusions and future works

This study provides the first preliminary analysis of pancreatic amylase CNVs in the Kurdish population. The prevalence of duplication carrier samples reveals a notable genetic variability that aligns with some global populations but diverge from others, possibly reflecting unique historical and dietary influences. Future works should expand these findings by using larger sample size, and high-resolution sequencing, such as long read sequencing, would help in accurately characterise the higher order duplications in the Kurdish population. Additionally, to better understand the potential adaptive role of these variations it is recommended to explore the metabolic effect of *AMY2* CNVs and compare it with neighbouring populations which have different dietary practice and history. This may provide a better understanding on how CNVs at the amylase gene family contribute to the genetic diversity and adaption.

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Conflict of interests

The author declares no conflict of interest.

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None

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