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Frequency of Met129Val allele associated with predisposition to variant Creutzfeldt – Jakob disease in the Middle ages

Research Article

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Abstract: Direct deciphering of past genes may reflect real characteristics of forebears, even of whole ancestral populations. This is obviously one of the most powerful and direct methods to follow evolutionary changes of the species. We attempted to apply ancient DNA (aDNA) technology to analyse a polymorphism at codon 129 of PRNP which probably plays a role in susceptibility to a variant Creutzfeldt – Jakob (vCJD) disease. As previously suggested, 129 Val-Val and heterozygous individuals are nearly completely protected from vCJD, in contrast to 129 Met-Met homozygous ones. We examined the frequency of the alleles encoding methionine and valine at codon 129 in DNA isolated from 100 skeletal remains of individuals who lived between 10th and 13th century. Our results confirmed significant alteration in previously studied alleles frequency between the populations of medieval Polish Lands and contemporaries. The calculated frequency of the alleles in medieval Poland (51% as compared to contemporary 65% for 129Met, and appropriately 49% vs. 35% for 129Val) implies a selection process that shaped 129 Met-Val distribution profiles in the Middle Ages. We suggest that the study of the genetic relationship between past and present-day populations could be a useful tool to follow allelic composition of particular genes (here: of the *PRNP*) over a span of time which may contribute to the understanding of evolutionary and selective mechanisms including epidemiological cases.

Keywords: *Ancient DNA (aDNA) • Prion • Prion protein gene (PRNP) • Variant Creutzfeldt – Jakob disease (vCJD)*

1. Introduction

Transmissible Spongiform Encephalopathies (TSEs) are a group of fatal neurodegenerative disorders which occur both in humans and animals. It has been suggested that certain polymorphisms of *PRNP* cause TSEs disability [1-3]. The most common of human TSE is Creutzfeldt – Jakob disese, which is associated with point mutation in the prion protein gene (*PRNP*). Polymorphism at codon 129 (Methionine/ Valine variant at codon 129) is crucial for genetic susceptibility to CJD and kuru at the genetic level [3]. Almost all vCJD patients were homozygous for methionine at codon 129 [4]. These genetic profiles strongly suggested that 129 Val–Val homozygosity and heterozygosity play a major role in resistance to vCJD, in contrast to 129 Met-Met homozygosity increasing susceptibility to he disease [5-7].

It was found that the polymorphism at codon 129 is very old and there is evidence that protective alleles exhibit marked geographic differences [8]. 129M allele frequency among contemporary Europeans accounts for

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almost 0.70. Similar distributions maintain through Middle East and North Africa, with a tendency to increase in Central/South Asia (above 0.70) and in East Asia 0.95 [8]. In contrast the frequency of 129V variant is rather low with exception of the population of Eastern Highlands of Papua New Guinea (0.55) and some Native Americans [9].

Since there is no data available on the putative event that created contemporary Polish allele frequency distribution, we decided to take advantage of ancient DNA (aDNA) methodology and searched for the genotype distribution and allele frequency in Polish medieval populations. Published data has been presented on the evaluated age of particular alleles however, the time of the allele appearance is usually calculated on the basis of contemporarily observed haplotypes [8-11]. There is no data available on Transmissible Spongiform Encephalophathies (TSEs) 35-40 generations ago, so the the comparison of the incidence is not likely. Thus, it seems reasonable to identify the alleles in DNA directly isolated from archaeological samples that were unearthed at some given location and precisely dated. These samples not only function to verify in time and space, the presence of a particular allele, but also to follow *bona fide* of past genomes, thus following presumptive evolutionary changes as represented by particular sequence frequency. Although the comparison of the past and present-day frequency data will not exhibit TSEs incidence, it can show the time scale of changes and profile modifications of allele frequencies, which in turn evidence the status of selection phenomena associated with particular units of inheritance. The study of genetic realtionships between past and modern populations seems to be useful in tracking evolutionary events.

2. Experimental procedures

aDNA was isolated from skeletal remains, originating from burial context, and collected on six Polish medieval archeological sites: Cedynia (11-12th century), Daniłowo (10-13th century), Dziekanowice (10-11th century), Gdańsk (12th century), Płock Podolszyce (11-12th century) and Stary Brześć Kujawski (11-12th century).

All studied skeletons were uncovered in positions typical to the explored region of Poland. Also, the overall similarity of the graves' equipage and historical background of archaeological sites suggest strongly local ancestry of buried individuals.

Prior to molecular analysis, all skeletons preserved well enough were chosen for the studies. Teeth without any visible damage were considered for further procedure, and stored at low temperature without prior washing until preparation procedure. In order to avoid contemporary DNA contamination, all samples were stored and analysed according to criteria of authenticity [12].

Avoidance of samples' contamination with exogenous molecules is the crucial step during work with ancient DNA. To assure sterile conditions, all reagents were maintained at volumes suitable for a single use, and all staff involved in the procedures wore disposable clothing. Moreover, preparation, extraction of DNA, PCR and post-PCR analysis was conducted in a laboratory free of other previous DNA manipulations. Before DNA extraction, we determined biomolecules' preservation by means of collagen quantification [13].

To gain aseptic conditions, all appliances were cleaned with bleach and UV irradiated, similarly to all surfaces used. All preliminary work preparing archeological findings for DNA isolation procedure was done in a class II biosafety cabinet (Heraeus).

The surface of each undamaged tooth chosen to the analysis was mechanically removed by sterile dental drill (Dremel®). Next, tooth was exposed to highly concentrated ethanol (96%) and bleach – NaOCl [14], and UV irradiated (15-30min), then was powdered in a sterile homogenizer. First, bone powder was decalcified with sufficient amount of 0.5 M EDTA (pH 8, at room temperature for 48h with gentle mixing) and after 2 days 30 – 40 µl of 0.1 M PTB (*N*-phenacylthiazolium bromide) was added to overcome post-mortem modification [15], such as intra – and intermolecular cross-links, created during Maillard's reaction [16]. Next, 10µl (10 mg/ml) of proteinase K was added for maximum digestion of the bone proteins (overnight at 56°C). After remixing, the suspension was centrifuged (8 min, 10 000 min $^{-1}$) and the supernatant removed into the fresh sterile tubes (400 µl). Finally, each sample was processed for DNA isolation on DNA MagNa Pure Roche equipment according to the manufacturer's directions. The PCR reaction mix contained AmpliTaq Gold polymerase (Applied Biosystems) and uracil – *N*-glycosylase –UNG (Applied Biosystems), the chemical which removes deamination products of cytosine [17].

The primers used for the recognition of *PRNP* M129V gene polymorphism were: 5' – TCAGTGGAACAAGCCGAGTAA – 3' and 5' – TGTATGATGGGCCTGCTCAT – 3'.

PCR product was electrophoresed on polyacrylamid gel to confirm the presence of 129bp *PRNP* and the rest digested with *Nsp*I (recognition site: 5' – ACATG/C – 3'). 6 µl of the PCR product were incubated with 2.5 µl *Nsp*I (10 µg/ µl) for 24h, at 37°C. Digestion of obtained amplicons produced two fragments: 97bp and 32bp in length. It is considered that G/G (Val/Val) homozygote is represented by undigested 129bp fragment, heterozygote G/A (Val/Met) by two fragments (129 and 97bp)

Figure 1

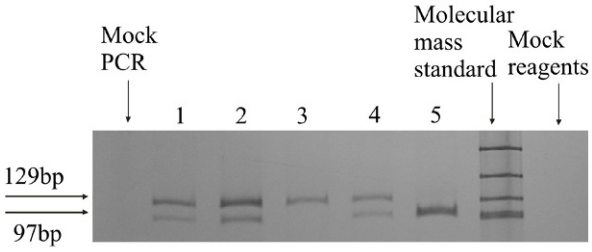
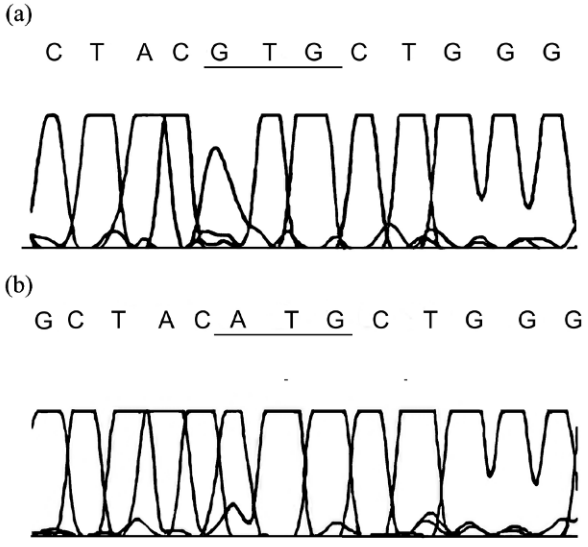


Figure 2



and homozygote A/A (Met/Met) by 97bp fragment (Figure 1). Primary structure of codon 129 *PRNP* was confirmed by sequencing of PCR product (Figure 2). Overall data obtained were compared to results of contemporary DNA samples described by Bratosiewicz [18] and Grzeszczak [19]. To achieve reliable data, each result was confirmed in at least two separate analyses of different samples from the same individual. The presence of contaminating, contemporary molecules in historic samples was verified by using primers producing long enough (977bp) fragment of *AMLX* gene (aDNA is unlikely to be isolated in fragments longer than ~250bp) at each step of isolation procedure, and authentication of historic sample was followed *via* identification of its mtDNA haplogroup and comparison to all members of staff involved along whole procedure, starting from uncovering the remains (data not shown).

3. Results

We analysed *PRNP* codon 129 variants in DNA isolated from teeth samples collected on six medieval Polish archeological sites. Only DNA samples found negative for contamination were taken for further identification. We approached DNA isolation from 147 historic samples, succeeding in 100 cases. Out of them, 22 were G/G homozygote at codon 129 (Val/Val), 24 were A/A homozygote (Met/Met) and 54 were G/A heterozygote (Val/Met). The frequencies do not deviate from the Hardy-Weinberg equilibrium. The frequency of the allele among medieval inhabitants differs significantly from the distribution within contemporary gene pool (Table 1). According to Bratosiewicz [18] and Grzeszczak [19] the distribution of genotypes in modern population accounts for 0.65:0.35 Met:Val, in contrast to results obtained for historic population – 0.51:0.49 Met:Val. For the comparison of the medieval and modern data collected by Bratosiewicz [18] and Grzeszczak [19], the Pearson Chi-square test has been used. Our results indicate that the frequency of the allele in medieval Poland differs significantly from the frequency characterising contemporary Polish population (P = 0,000163, df = 2).

4. Discussion

Variant of Creutzfeldt – Jakob disease is one of the lethal transmissible spongiform encephalophaties triggered by prions. vCJD was described as acquired disease which affected relatively young people, similar to bovine spongiform encephalopathy (BSE) [20,21]. Although vCJD is acquired, it has been suggested that susceptibility to the disease is associated with Met – Val variant at codon 129 of *PRNP*. It is considered, that genotypes 129V and M129V influence on resistance phenotype, in contrast to 129M.

It was found that the polymorphism at codon 129 is very old one and the origin of the 129 Met-Val variant is estimated for 200±100ky under a constant population

Table 1

Site	N	A/A (Met)	A/G (Met/Val)	G/G (Val)
Cedynia	16	4	9	3
Dziekanowice	35	6	19	10
Stary Brześć Kujawski	33	11	18	4
Other sites	16	3	8	5
All sites	100	24 (24%)	54 (54%)	22 (22%)
Contemporary Poland (Bratosiewicz et al.2001)	109	49 (45%)	43 (39%)	17 (16%)
Contemporary Poland (Grzeszczak and Juźwiak 2005)	915	383 (41,86%)	419 (45,79%)	113 (12,35%)

model [8]. In order to join the scientific discussion on the explanation of present-day frequency of the *PRNP*, we started to search for the polymorphism at codon 129 in the past populations, and a medieval one as first. Analysis of ancient DNA in medieval inhabitants of Poland showed increased frequency of 129V variant, while decreased of 129M allele when compared to moderns. We found the frequency of Met-Val heterozygous genotype in medieval population almost twice as high as distribution of 129M or 129V homozygous variants. In contrast, 129M homozygous and heterozygous individuals are represented in contemporary population by similar allele frequency. Comparison of heterozygous genotype distribution indicates its considerably higher presence in medieval genomes than in moderns. The results obtained for the individuals inhabiting medieval Poland suggest that some selective processes took place within the last millennium. Migration as a phenomenon influencing the frequency of alleles should not be considered since the differences between explored archaeological sites were high enough to reject the possibility. Poland appears to be a very suitable region for such comparisons as the population has always been uniform with very little ethnic minorites and no major migration or imigrant influx. The Polish language functions as an evidence to this fact; it is deprived of any major variability throuhout the entire country. The relative continuity between the medieval and contemporary populations may contribute to understanding of evolutionary and selection mechanism pressured favoring more resistant variants and thus resulting in selective advantage of heterozygote M129V.

The high frequency of heterozygous 129 genotype within historical populations implies balancing selection at the locus, similar mechanism which was proposed for contemporary worldwide population by Mead. On the basis of the worldwide high frequency of heterozygote M129V in moderns [22,23] an occurrence of a strong balancing selection pressure in favor resistant variants has been suggested. According to Mead, the high level of heterozygous genotype presence in moderns is probably associated with evolutionary selective pressure leading to gene diversity [22]. His hypothesis assumes that currently observed high frequency of heterozygous genotype resistant to prion diseases was formed as an effect of cannibalistic practices over the evolution time of modern humans. Balancing selection as a strong factor pressing on *PRNP* allele diversity was suggested for the first time after genotyping of Fore living women from Eastern Highlands of Papua New Guinea who were only M129V heterozygous and survived until elderhood [24]. The disease (kuru) spread as an effect of cannibalistic feasts usually causing death of young homozygote individuals.

This remains in accordance with our results since we observed even higher frequency of protecting genotypes. Probably cannibalism as a strong selecting agent has to be rejected in relation to Polish medieval population since there is no evidence concerning such practices among these people, although, in principle, it could have occured in earlier times. Anthropological and archaeological analysis of the skeletal remains completely excluded the cannibalistic practice among Polish medieval populations. All studied skeletons were very well preserved with no visible marks of pre-and post-mortem mutilation on the bones, typical for sacrificial cannibalism practiced among the Aztecs [25]. Moreover, any medieval skeletal fragments did not reveal typical cut marks for some Neanderthals and all the braincases and long bones were naturally closed [26].

Besides, other factors responsible for the high frequency of heterozygous genotype should be taken into account. Studies on ethiopathology of TSE diseases indicated that prions can be collectively associated with soil particles after decomposition of diseased carcasses, burial of infected material or even urination by infected individuals and therefore persist as infectious even longer than unbound [27-31]. Prion particles adhere to soil minerals like silicates (montmorillonite) and keep infectivity for years [27,32], increasing the capability of oral transmission of TSE diseases. There is a strong evidence indicating development of neurological encephalopathies among ruminants after food ingestion contaminated with the soil [32]. Consumption of TSE infected food including dirty vegetables, plants and meat of infected animals could also be a likely reason of morbidity incidence among generations preceding studied medieval Polish population. Because oral transmission of soil originated prions is likely, we suggest that the observed majority of resistant heterozygous individuals living at that time seem to be a plausible effect of the selection.

5. Conclusions

The results obtained for M129V genotypes in medieval DNA differ remarkably from the data collected during the examination of modern population. Each of the agents – poor living conditions, poverty, widespread starvation among humans run-up to Middle – Ages as well as high possibility of oral transmission of infected meat or vegetables might contribute to rising the genotype determining resistance to prion infection. It is not clear which of the agents, if not all of them, stimulated selection since there is no valid data conferring it. We are, however, convinced that ancient DNA research enables following directly, the changes of particular allele fre-

quencies, which we noticed clearly analyzing medieval remains. The acquisition of such data will surely contribute to reconstruction of evolutionary changes concerning past populations, reflected by the frequency of traits/sequences transmitted between generations.

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