An old dog and new tricks:
genetic analysis of a Tudor dog recovered from the Mary Rose wreck

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Abstract

The Tudor warship the *Mary Rose* sank in the Solent waters between Portsmouth and the Isle of Wight on the 19th of July 1545, whilst engaging a French invasion fleet. The ship was rediscovered in 1971 and between 1979 and 1982 the entire contents of the ship were excavated resulting in the recovery of over 25,000 objects, including the skeleton of a small to medium sized dog referred to as the *Mary Rose Dog* (MRD). Here we report the extraction and analysis of both mitochondrial and genomic DNA from a tooth of this animal. Our results show that the MRD was a young male of a terrier type most closely related to modern Jack Russell Terriers with a light to dark brown coat colour. Interestingly, given the antiquity of the sample, the dog was heterozygotic for the SLC2A9 gene variant that leads to hyperuricosuria when found in modern homozygotic animals. These findings help shed light on a notable historical artifact from an important period in the development of modern dog breeds.

Introduction

Historical Context and redicovery

At 700 tons and provisioned with 91 guns the *Mary Rose* was one of the largest of Henry VIII's warships. Listed with a crew of 185 soldiers, 200 mariners and 30 gunners, she was a state of the art fighting machine. She sank in the Solent whilst engaging a French invasion fleet on the 19th of July 1545. The ship sank on her starboard side to a depth of 12 metres. Tidal action carried silts in suspension across the wreck site, these sediments produced anoxic conditions resulting in excellent preservation conditions. The ship was rediscovered in 1971 and between 1979 and 1982 the entire contents of the ship were excavated and the wreck raised [1]. The raised wreck included the hold of the ship and the starboard side with portions

of four decks surviving. The hull was opened to the public in 1983 and subsequently a museum containing recovered artifacts opened in 1984. A new museum built around the hull opened in early summer 2013.

Location and recovery of the sample

Between the 12th July and 14th Octber 1981, the nearly complete remains of a small dog were recovered as four separate samples (MR81S0215, MR81S0264, MR81S0328 and MR81S0444). Each sample contained animal bones together with associated sediment, so that any articulated bones remained at least closely associated, the remains were found within a 2 m radius of each other in the stern (Figure 1a). Three of the samples were found on the main deck of the ship, and the fourth (MR81S0328) directly below on the orlop deck. Two of the three samples from the main deck (MR81S0215, MR81S0264) were found within gaps created by the edges of five chests which had slid across the main deck as the ship heeled to starboard during the sinking. These chests came to rest against a partition, later identified as one of the walls of the carpenter's cabin (Figure 1b).

The third sample from the main deck (MR81S0444) was found inside the cabin, directly in line with a sliding door which was found partly open and into which the edge of one chest had penetrated. As the distribution of the bones was haphazard it was not easy to determine whether the dog drowned whilst inside or outside the cabin, although the position of the skull suggested the most likely position of the dog was just outside the cabin. The remains were assigned as Feature 69, later becoming known as the Mary Rose Dog (MRD). Here we describe genetic analysis of the both mitochondrial and genomic DNA extracted from the tooth of the MRD.

Materials and methods

DNA extraction and PCR amplification

The teeth were washed initially with Decon (5% solution), subsequently washed briefly with 1% bleach, rinsed with distilled water and dried in a clean UV laminar flow overnight. The outer layer was sandblasted with sterile sandblaster dish and pulverized with a flamed drill. The powder was collected in sterile plastic containers and stored in Lo-Bind DNA free tubes (Eppendorf) at 20 ^{0}C. Glassware was soaked overnight in 1 M HCl, rinsed with double distilled water, autoclaved at 135 ^{0}C, baked at 100 ^{0}C for 12 hours and were exposed to UV for 24 hours. Disposable plasticware was manufacturer irradiated and autoclaved, solutions were prepared fresh and exposed to UV 2-3 hours before use. DNA was extracted with an adapted spin column isolation technique [2] or the method of Binladen et. al. [3]. All DNA isolation and PCR setup were conducted in dedicated ancient DNA Laboratory facilities at the University of Portsmouth and Durham University. Strict contamination controls were exercised throughout all steps according to commonly accepted recommendations [4,5]. No contamination was detected in the negative controls of DNA extraction or PCR amplification.

Confirmation of selected amplifications

The use of species-specific primers excluded the possibility of amplification of human DNA [6]. To check for the possibility of other contamination, mitochondrial analysis of the samples were independently validated at a separate ancient DNA laboratory, located in the Department of Archaeology at Durham University, UK. Mitochondrial PCR was successful and upon sequencing the DNA aligned with the sequences produced at Portsmouth

Mitochondrial PCR analysis

Mitochondrial HVI DNA sequences were amplified by two overlapping amplicons [7,8]. Resulting HVI sequences were aligned using the program of DNA alignment (www.fluxus-engineering.com). After removing primer sequences and accommodating previously published sequences [9], sequence lengths were adjusted to 205 bp. ARLEQUIN ver. 3.5.1.3 [10] was used to estimate nucleotide and haplotype diversity. The statistical significance was evaluated using 1000 permutations. Novel haplotypes used in this study were named in accordance with the nomenclature from Savolainen et. al. [11], Pang et al. [12] and Ardalan et al. [9].

STR amplification and analysis

To investigate the identity of the MRD, DNA STR (microsatellite) profiling and sex determination were employed using the Finnzymes canine 2.1 STR multiplex kit (ThermoFisher Scientific), following the manufacturer's instructions. The kit consists of a single PCR multiplex for the amplification of 18 autosomal STR markers and a single marker in the sex-linked amelogenin gene. Five separate PCR reactions were performed for the sample including appropriate negative controls in each case. The five PCR products were subsequently subjected to capillary electrophoresis on an ABI3730, with two runs per PCR product using 25 and 50 second injection times. The DNA STR data from all 10 genotyping runs for the MRD were analysed using GeneMapper v.5 (Applied Biosystems) and combined. The resulting composite profile was analysed in conjunction with a reference database for UK dogs previously used to examine individual identity [13] and breed structure [14]. The program STRUCTURE v.2.3.3 [15] was used to infer the MRD's breed through assignment to the most likely of 13 contemporary UK breeds. STRUCTURE runs used a burn-in period of 200,000 followed by 800,000 MCMC repetitions. A value of K=13 had been previously

established as the most likely number of populations, equating to the 13 named and one cross-breed group included [14]. In this analysis values of K=11-14 were employed (five runs per value) to examine assignment of the MRD to breed.

SNP genotyping by Taqman

Five common modern canine genetic disorders were selected and the corresponding single nucleotide polymorphisms (SNPs) identified in the dbSNP database (NCBI). The SNP data was used to order Taqman genotyping assays from Applied Biosystems as single pot assays. The genotyping assays were conducted on an ABI 7900 qPCR machine with all experiments repeated in triplicate on three separate occasions.

Results

Physical remains

The skull and skeleton of the MRD were complete with only a few teeth, a small number of vertebrae and some foot bones missing, no baculum (penis bone) was present. Fusion of the skull bones suggested the skeleton represents a young adult animal of good health about eighteen months to two years old at the time of death. The skull and limb bones have the average proportions of a mongrel and none of the characteristics are strongly indicative of a particular breed [16]. The mean height of the living animal, calculated by multiplying the lengths of the limb bones by the factors of Harcourt [17], was estimated to be 470mm at the shoulder. The skeleton was initially reconstructed in 2009 before a final reconstruction in 2011/12 (Figure 2a). The seated height of the final reconstructed dog from the baseboard to the top of the skull was 490mm, in good agreement with the calculated height.

Teeth were still present in the skull and were relatively small and widely spaced with the first upper left premolar and the second upper molar being absent congenitally. Two

premolar teeth was removed from both the mandibular and maxillary jaw for DNA analysis and replaced with replica teeth produced by King's College London Dental Institute, where the teeth were examined in more detail for physical characteristics. Both teeth had fully formed roots, corresponding to an adult animal. There was no evidence of tooth wear or fracture of cusps although signs of arrested noncavited caries in the bucal pit of the maxillary premolar tooth were present but with minimal evidence of calculus formation. Correlating with the fusion of the skull, the lack of wear on the teeth suggested a young adult animal. Images of one of the teeth and the replica made for display purposes are shown in figure 2b and 2c.

Breed type

Mitochondrial analysis

As previous morphological analysis of the skeletal remains suggested that the MRD may have been related to either modern Whippets or Manchester terrier breeds we supplemented an already existing database of 169 sequences from different breeds of modern dogs [9] with ten modern British Terrier and nine British Whippet samples and compared the HVSI region of the mitochondrial DNA. Phylogenetic analyses on the mtDNA results were performed using the programs MEGA 5.05 [18] and Network 4.610 [19]. A median-joining network was constructed and to reduce the complexity of the phylogenetic network due to mutation rate heterogeneity in the control region, seven polymorphic sites were low-weighted in the input file for the subsequent analyses. In this way, sites are allowed to appear more than once in the network and so reduce the reticulations observed. The results of this analysis are shown in figure 3. The network of mtDNA haplotypes consists of three large star-like clusters for haplogroups A, B and C connected to smaller terminal star-like clusters for haplogroups D, E and F with the MRD belonging to cluster B. However in agreement with previous canine

mtDNA [20,21] studies our analysis shows that distribution of mtDNA haplogroups is, at best, only loosely connected with breed. Indeed in the network historically unrelated breeds, such as poodle and whippet, cluster together. Further, in parts of the network some haplotypes, for example Whippets 7, 8 and 10 share the founder haplotype of cluster B with a variety of breeds from different geographical regions, suggesting that extensive gene flow has occurred among different dog breeds in the past [22].

Short tandem repeat (STR) analysis

As mitochondrial DNA was not able to provide a clear answer concerning breed type of the MRD we next looked at genomic STR markers. The ten genotyping runs allowed a composite DNA profile to be generated that consisted of twelve STR markers and the amelogenin marker. These thirteen markers were all below 250 bp in size with the amelogenin marker conclusively identifying the MRD as a male dog, the remaining six larger markers failed to amplify. The STRUCTURE analysis consistently assigned the MRD to the Jack Russell Terrier breed (Fig. 4a and 4b) with lower assignment to English Springer Spaniel. The lack of DNA profiling markers observed above 250 bp is typical of historic samples indicating fragmentation of the DNA, the reduced length of intact DNA molecules preventing the PCR amplification of the longer markers [23].

Although the DNA profile results for the MRD categorically identified the remains as male; the inference of breed requires far more cautious interpretation. Since the MRD was alive in 1545 dogs in the UK have been subjected to increasing levels of selective breeding and the introduction of studbooks by the Kennel Club in the 1870s has enabled consistent breeding within pedigree. The genetic structure of contemporary breeds is therefore very different to that of the 1540s when many breeds did not exist, including the Jack Russell Terrier that only dates back to the mid-19th century. The breed assignment of the MRD to the Jack Russell is

therefore interpreted as identifying him as a 'Terrier-type', an inference that is consistent with the use of terrier dogs on board ships as rat-catchers. Even such limited interpretation must be taken with caution, especially since certain critics consider the term "breed" problematic in relation to animals alive prior to modern breed practices [22]. The beginning of the process of selection for particular classes of dog (ratters, hounds, giant dogs etc.) emerges during the prehistoric archaeological record. For example, three differently sized dog types have been recorded at the 8,000-y-old Svaerdborg site in Denmark [24]. However modern breeding practices based on strict aesthetic requirements and closed bloodlines, only emerged 150 years ago, and claims for the antiquity (and long-term continuity) of modern breeds are based upon little or no historical or empirical evidence. Furthermore, recent historical records clearly demonstrate that most modern breeds experienced significant population fluctuations within the past 100 years, probably as a consequence of the socio-political upheavals of the past century. Therefore the vast majority of modern breeds emerged from what was a relatively homogeneous gene pool formed as a result of millennia of human migration and the subsequent merging of multiple, previously independently evolving dog lineages. This history, along with the closed gene pools and small effective population sizes associated with recent breed formation, also explains the difficulty to resolve relationships between ancient dogs by genetic methods.

Coat colour

A number of genes were postulated as early as the 1950s that could explain the inheritance of dog colour and pattern in dogs [25,26]. Recent developments in molecular genetics have pointed numerous mutations with complex associations that are responsible for particular colour dog characteristics [27,28]. To gain an insight on the MRD phenotype, we examined single nucleotide polymorphisms (SNPs) that affect the expression of three major proteins

responsible for major colour patterns. The MRD lacks a mutation at the E locus (306R) of the melanocortin MR1C gene that is responsible for the expression of eumelanin [29]. Moreover, it exhibits a heterozygous character (Figure 5) for the agouti signal peptide protein (ASIP) with a probable phenotype of light brown (fawn) colour [28].

Furthermore, analysis of the tyrosinase related protein gene demonstrated the presence of b^s and b^c loci that code for brown eumelanin. Based on these observations a MRD with brown characteristics ranging from light to dark brown is likely, these results are tabulated in Table 1. It has recently suggested that humans actively selected for colour novelty and thus allowing for the proliferation of new mutations in coat colour genes [30,31,32]. However it remains unclear if the MRD coat colour was the result of such a process or independent of human selection.

Table 1: Genes and loci involved in the pigmentation of the Mary Rose Dog

Gene	Locus	Mutations	Phenotype	Colour prediction
Melanocortin Receptor Gene (MC1R)	MC1R_Exon	Wild type	E	Eumelanin (black, brown, blue) can be produced
Tyrosinase related protein (TYRP)	TYRP_Exon 2	S41C	bs	Brown eumelanin can be produced
	TYRP_Exon 5	Q331ter	bc	

Agouti Signal Peptide Protein (ASIP)	CFA24	A82S, R83H/Wild type	a^y/a^w	Fawn-sable (cream to yellow to red with dark tips)

Recessive disease

The MRD preceded the period of intensive breed selection by several hundred years. As the genetic material from the MRD was well preserved we decided to look for a panel of Single Nucleotide Polymorphisms that lead to disease phenotypes and have become fixed in some modern breeds. We screened for five mutations known to be associated with disease phenotypes (DNM1, SLC2A9, M-PFK, SOD1 and PRCD) by qPCR SNP assays (ABI), only the disease causing SNP in SLC2A9 gave a positive result. This SNP lies in the SLC2A9 transporter gene and is associated with the autosomal recessive disorder Hyperuriosuria in dogs [33]. To confirm the presence of the SNP in the SLC2A9 gene the PCR was repeated using different primers and the product sequenced where the presence of the SNP was again confirmed. Hyperuricosuria in dogs was first determined in 1916 where it was initially recognised as a uric acid defect in the Dalmatian dogs predisposing them to formation of stones in their bladders and kidneys. However such defects have been since detected in 127 breeds with variable frequency [34,35]. Bannach et al. [33] have previously suggested that the mutation must be quite old and predate breed formation. Our data support this view since the specimen under study lived centuries before intensive breeding in UK.

Discussion and Conclusions

In conclusion our results demonstrate the power of genetic analysis for the factual reconstruction of museum specimens that would be otherwise impossible. The Mary Rose Dog was found to exhibit male genotype in contrast to skeletal examination. Furthermore, STR analysis suggested that the dog belonged within the "terrier types", in agreement with historical records of dogs of this type on board ships to kill rodents. In addition, SNP analysis suggested that the animal was in the color range of brown to liver while it was also a carrier of hyperurioria, a genetic diseases that affects modern dogs. Finally, the production of a tooth replica demonstrates that it is possible to recover molecular information from exhibited specimens combining minimal sampling and restoration techniques.

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Figure legends

Figure 1. Location of the remains of the *Mary Rose Dog*. (a) Photograph of the location of the recovery site (b) Schematic showing the location of the carpenters cabin in the intact ship.

Figure 2. Reconstructed MRD skeleton, CT scanned tooth and it's replica. (a) Final reconstructed skeleton. (b) Computerised Tomography scan of one of the teeth removed for DNA extraction (c) A replica tooth prepared for replacement into the skeleton.

Figure 3. Genetic relationships between the mtDNA CR sequences. MJ network showing the relationships between the haplotypes in dog clades and the representation of the MRD

haplotypes in B cluster. Yellow circles indicate the presence of haplotypes and the size of the circles is correspondent to the number of within that haplotype. The MRD is shown in green while the nine Whippet dogs are labelled in blue and ten Terrier Dogs are labelled in red.

Figure 4. Breed assignment results from STRUCTURE and example STR profile. (a) Each vertical bar represents an individual dog, colours indicate the proportion of times it is assigned to different breeds. Dogs are grouped into thirteen UK breeds and one cross-breed group (2nd row, centre) (adapted from Mellanby et al. 2012). MRD is predominantly assigned to the Jack Russell terrier (73%), with secondary assignment to the English Springer spaniel (18%). (b) Example DNA profile result for MRD showing the 13 STR markers and one sex marker distributed across the four rows (dye colours). The heterozygote amelogenin marker identifies the MRD as male (black oval), the dashed line around 250 base pairs indicates the maximum fragment size that could be amplified from the DNA sample, with six larger STR markers failing.

Figure 5: **Single nucleotide polymorphisms in genes responsible for color in the Mary Rose Dog.** The loci examined were, the Melanocortin Receptor gene (MC1R), Tyrosinase Related Protein gene (TYRP_A & TYRP_B) and the Agouti Signal Peptide (ASIP) gene. The Mary Rose Dog exhibits a homozygous wild type character at the MC1R for 306R, a serine to cysteine change (S41C) at exon 2 in TYRP gene, a premature stop codon in exon 5 (Q331t) in TYRP gene and a heterozygotic character with two amino acid changes, A82S and R83H, in comparison to wild type in the ASIP gene.
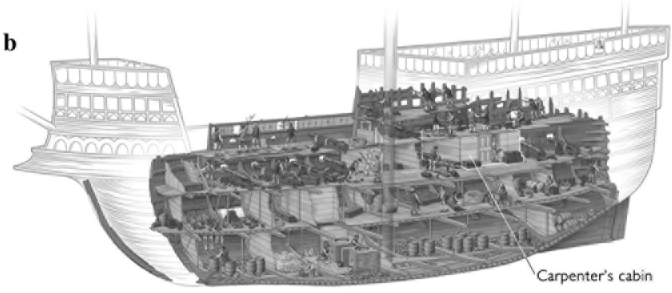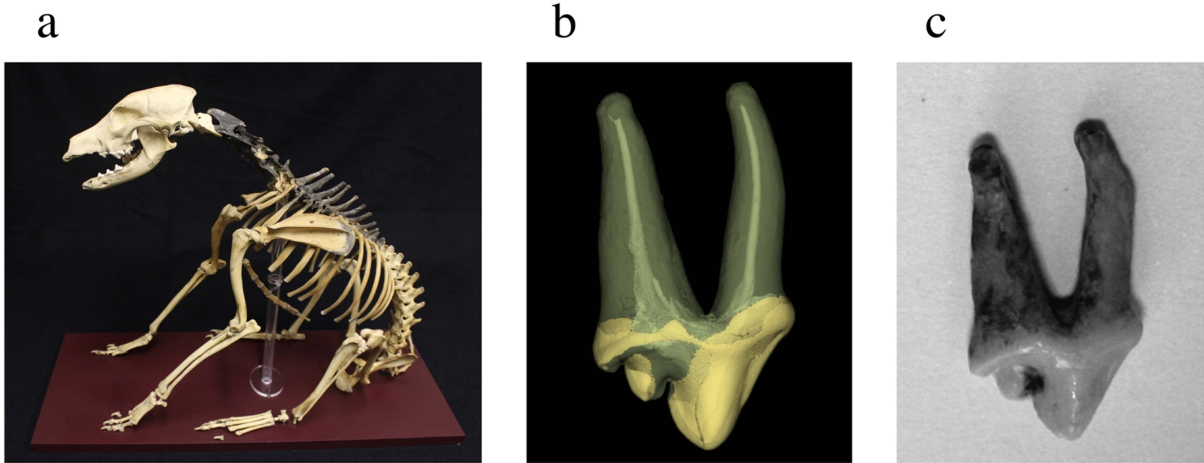
Figure 1

Carpenter's cabin

Figure 2

a

b

c

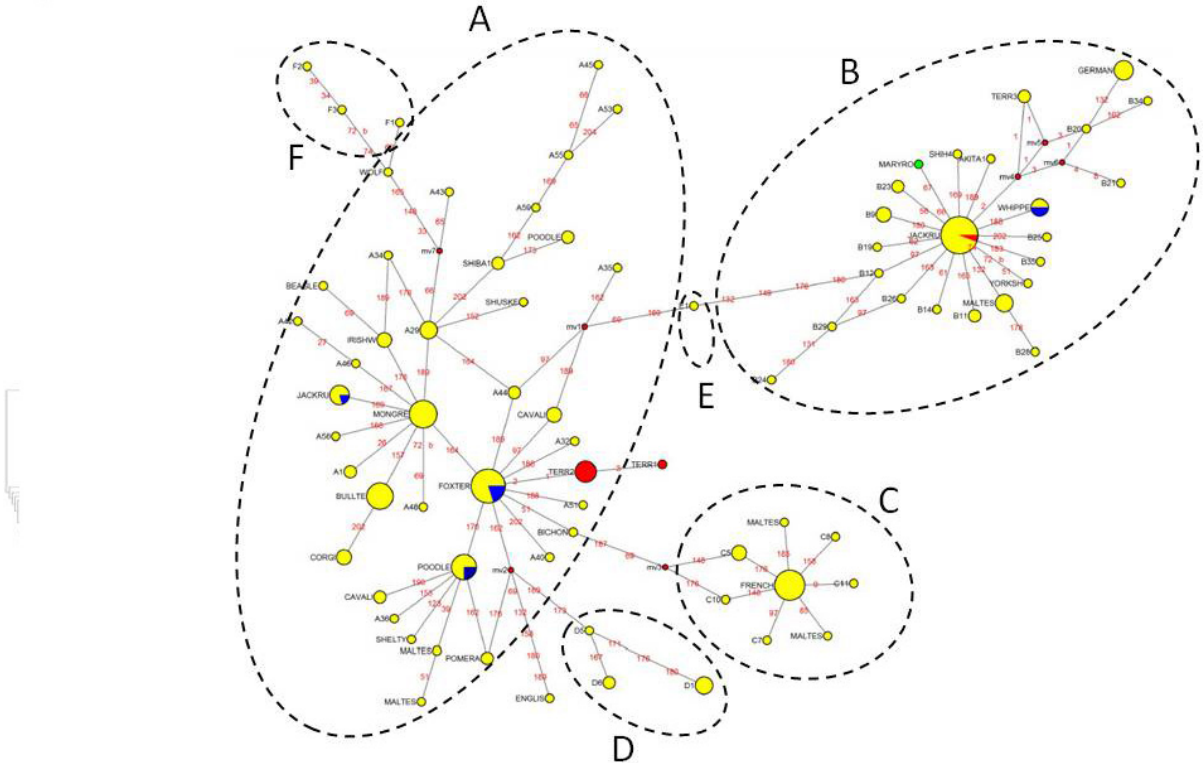Figure 3
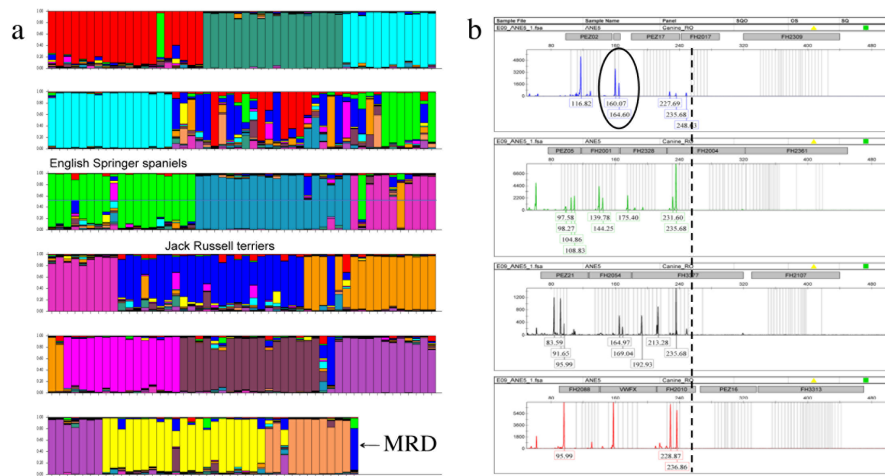
Figure 4

Figure 5

MC1R

CGC AGC CAG GAG CTC CGA AAG ACT CTC AAG AGG TAG TGC TAT GTT

R   S   Q   E   L   R   K   T   L   Q   E   V   V   L   C

306R

TYRP1_A

AAT TGG TGT GTG TGC CCA GAC CTG TCC CCA GTG TCT CTG GGC CTG

G   G   V   C   C   P   D   L   S   P   V   S   G   T   D

S41R

TYRP1 B

ATG GTG CAA CGT CTT CCT GAA CCA TAG GAT GTC GCT CAG TGC TTG GAA

M   V   Q   R   L   P   E   P   D   V   A   Q   C   L   E

STOP
Q331t

ASIP

AAA AAG GCT TCG ATG AAG AAC GTG TCT CAT CCC CGG CCC CCG
                                A   G

K   K   A   S   M   K   N   V   S   H   P   R   P   P

A82S R83H