Haemophilia

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REVIEW ARTICLE

The 'royal disease'– haemophilia A or B? A haematological mystery is finally solved

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Summary. 'History can change blood. And blood can change the course of history'. Haemophilia is an illustration of this, as this congenital hereditary coagulation disorder, passed through the majority of royal European families at the beginning of the 20th century by Queen Victoria of England and Empress of the Indies, had indisputable political consequences, which led to one of the most defining moments of contemporary history: the Bolshevik Revolution. Today, none of Queen Victoria's living descendents carry haemophilia. Because of this, the characterization of haemophilia (deficit of either factor VIII or XI) and the identification of the causal mutation are rendered impossible. In 1991, a tomb containing the remains of Czar Nicolas II's entire family was discovered. A second tomb was discovered in 2007, allowing Russian and American scientists to fill in this gap in medical history. Following a scientific approach combining current genetic experimentation tools and the development of biological information technology, researchers were able to identify each body, allowing them to obtain precious genetic material from the young Czar Alexis, who was stricken by the disease, which revealed a causal substitution in the splice acceptor site of exon 4 in the F9 gene. This mutation that is responsible for haemophilia B had traumatized European royal families throughout the 20th century!

Keywords: Czar Nicolas II, F9 gene, haemophilia, Queen Victoria, Romanov, splice acceptor site

Introduction

Haemophilia is a prototype of recessive diseases linked to the X chromosome. This disease is secondary to a mutation of the gene coding for factor VIII (F8 gene) or factor IX (F9 gene). The genes are present on the long arm of the X chromosome. Women who carry a mutated X chromosome, thereby making them haemophilia carriers, generally do not present any FVIII or FIX deficiency themselves.

Queen Victoria of England (1837–1901) was a carrier of haemophilia, a disease that was known for a long time as the 'royal disease'. Even though the disease spared the majority of her children (only her eighth child, Leopold, was affected by the disease, and died of a brain haemorrhage at the age of 31), three of her grandchildren and seven of her great grandchildren were affected. Due to princely alliances, the disease spread through most European royal families (Fig. 1), notably in Germany, Spain and Russia. Although haemophilia may have weakened the image of the heirs to the throne in the Spanish and Russian Royal families, it is certain that the European political context at the beginning of the 20th century led to well-known consequences: The abdication of the throne in 1931 of King Alfonso XIII of Spain, and the assassination of Czar Nicolas II and his family under the orders of Lenin during the night of 16 July 1918 in the imperial villa located near Yekaterinburg. Indeed, in 1906, Alfonso XIII married Queen Victoria's favourite granddaughter, Princess Victoria Eugenie of Battenberg. Two of the couple's six children, their firstborn son, Gonzalo, and their youngest son, Alfonso, were haemophiliacs. The young age of King Alfonso XIII of Spain when he reached the throne, inexperience and

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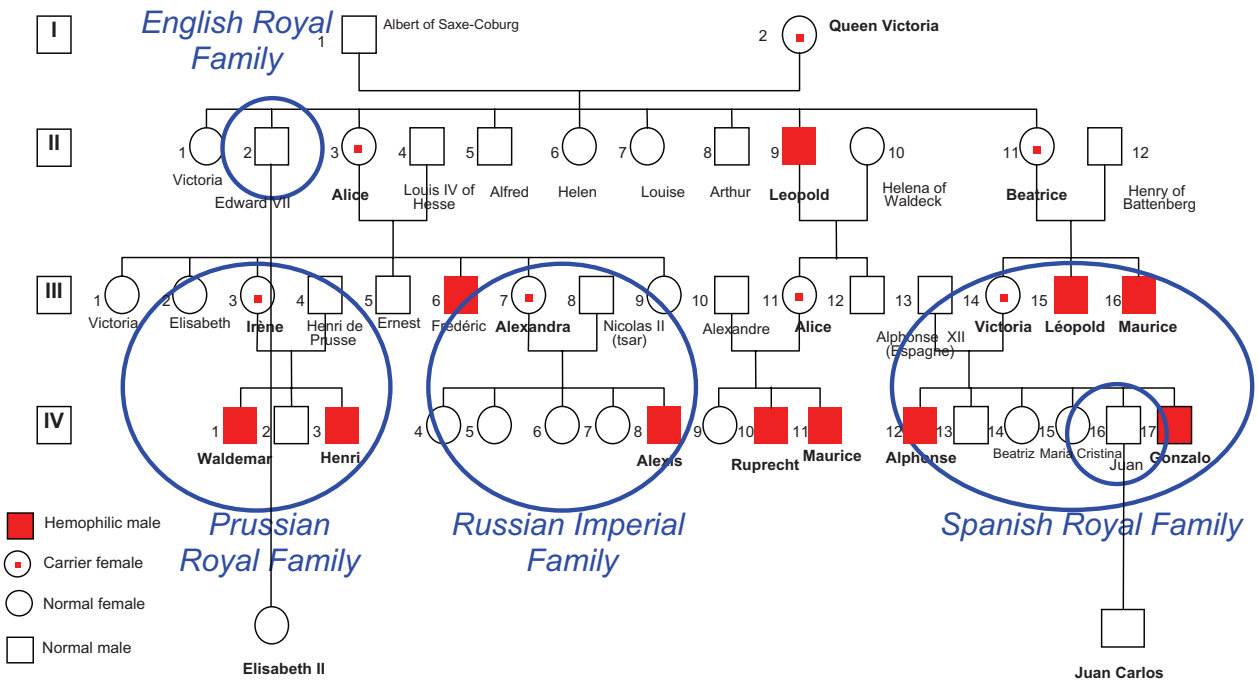

Fig. 1. Queen Victoria, grandmother of Europe.

concerns about the disease's effects on his heirs must surely have played their part in his abdication of the throne and the later rise of Spanish dictator General Francisco Franco. In the Imperial House of Russia, Alexei Nikolaevich, Tsarevich of Russia, was the youngest child and the only son of Emperor Nicholas II of Russia and Princess Alix, granddaughter of Queen Victoria who adopted the Russian Orthodox Church name Alexandra Feodorovna following her marriage. Within a few months of his birth, his parents realized that Alexis had haemophilia. To help their suffering child, the Czar and Czarina turned to the monk Rasputin, a spiritualist who claimed that he could help Alexis. This brought Rasputin intimately close to the imperial family, where he had a huge influence over Alexandra and indirectly was able to influence affairs of state. In a context of First World War, the growing unpopularity of Rasputin helped to discredit the Czarist government, leading to the fall of the Romanov dynasty.

Though there is no doubt in terms of the diagnosis of haemophilia among the royal family, based on clinical and hereditary manifestations, the type of haemophilia (A or B) and its molecular origin had never been identified, as there are no known living descendents of Queen Victoria who carry the disease. Indeed, as Beatriz (IV–14) and Maria Cristina (IV–15) had an *a priori* risk of 50%, many of their living female descendants may be possible carriers of the disease. However, the discovery of the two tombs and the identification of the presumed remains of Czar Nicolas II's family have enabled researchers to solve this mystery.

Identification of the remains of Czar Nicolas II and his family

On 11 July 1991, the supposed Romanov tomb, located in the forest near Yekaterinburg, was opened by a group of scientific experts, accompanied by members of the police and the Parquet. They discovered close to 850 bones and remains, in various stages of preservation, corresponding to nine skeletons. This allowed researchers to identify them as the remains of the Romanov family and their closest subjects. In fact, it was possible to identify each cadaver, based on size, approximate age of the victims at the time of their death, dental records and DNA analysis. For the latter, two different types of tests were performed, taking into account the presence of two types of DNA in human cells: nuclear DNA in the cell nucleus and mitochondrial DNA (mtDNA) in the cell mitochondria. Gill *et al.* [1] first investigated the nuclear DNA markers. These allowed them to determine that the remains belonged

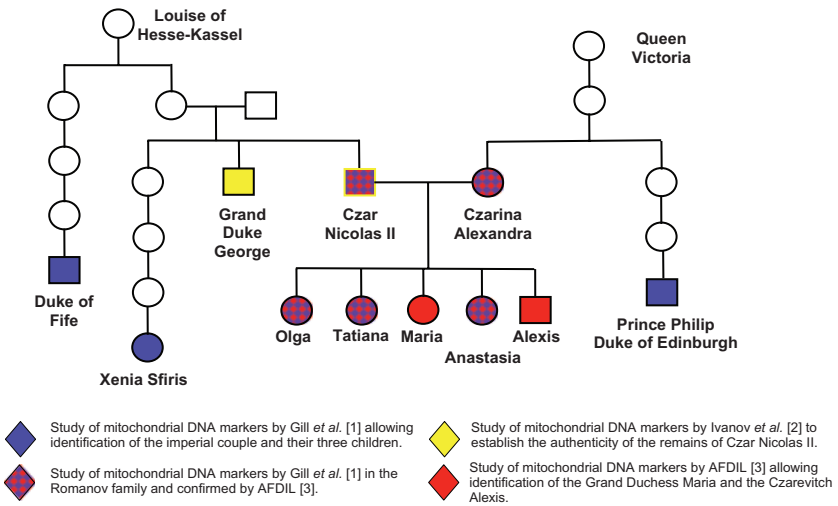

Fig. 2. Mitochondrial DNA study in the Romanov family.

to four men and five women, among which there was a family of two parents and their three daughters.

Mitochondrial DNA is inherited exclusively from the mother, as one ovule contains up to 1000 mitochondrial samples, and because, at fertilization, there are no sperm mitochondria found in the fertilized egg. The mtDNA study thus allows researchers to establish parental lineages between female ascendants and descendants. The Empress had one sister, Victoria de Battenberg, one of whose daughters, Princess Alice of Greece, had three of her own daughters and one son – none other than Prince Phillip, Duke of Edinburgh, and the husband of Queen Elizabeth II of England. The Czarina's grand-nephew was asked and agreed to provide a blood sample. Results showed that his mitochondrial genotype was identical to that of the Czarina and her three children, confirming the lineage (Fig. 2).

Results from the skeleton of Nicolas II were controversial. Indeed, researchers were able to identify the skeleton using two different DNA samples, one obtained from Countess Xenia Cheremetev Sfiris, the great granddaughter of Princess Irina, niece of Nicolas and the other from James Bannerman Carnegie, the third Duke of Fife, a descendent, on his maternal side, of Louise of Hesse-Kassel, Nicolas II's maternal grandmother. The Duke of Fife and the Countess' mtDNA corresponded very well to the presumed DNA of Nicolas II, though not totally (98.5%), given a slight difference at position 16 169. Indeed, a heteroplasmy (C/T) was identified, while the collaterals presented only a T at this position, which was difficult to interpret. To confirm the authenticity of this difference, Czar Nicolas II's youngest brother, the Grand Duke Georges Alexandrovitch, who died of tuberculosis in 1899, was exhumed during the night in July 1994. The same heteroplasmy was found in his mtDNA sequence, allowing researchers to reach a conclusion, with 1.3×10^8 reliability, that the remains were indeed those of the Romanov family [2].

The discovery of a second tomb in 2007

However, two bodies were still unaccounted for, namely that of Alexis, aged 13 at the time of the tragedy, and his sister Maria, aged 19. According to the memoires of political commissary Jacob Yourovski, who was charged with the assassination, their bodies had been burned and buried not far from the original location. A second tomb was discovered in August 2007, allowing for the recovery of teeth, skull fragments and the heavily damaged pelvic bones and tubular bones of two people. Archaeologists very quickly confirmed that these were the remains of a young boy aged between 10 and 13 years, and of a young woman between 18 and 23 years at the time of death.

The results of mtDNA, nucleic DNA and Y chromosome markers taken from minimal amounts of bone fragments carried out by a large research group under the direction of Professor Evgeny Rogaev in independent laboratories specialized in the study of old DNA (Russian Academy of Sciences, University of Massachusetts, Armed Forces DNA Identification Laboratory, Gregor Mendel Institute in Austria), allowing Edouard Rossel, governor of the Sverdlovsk region, to proclaim, on 30 April 2008, 'The United States' largest genetic laboratory has confirmed the identity of the remains recovered

in August 2007; 'they are indeed the bodies of the children of Alexandra and Czar Nicolas II, more precisely the Grand Duchess Maria and the Czarevitch Alexis […] We have now recovered the entire family' [3,4].

Discovery of a mutation at the splice acceptor site of exon 4 in the F9 gene

Genomic DNA extraction from bone fragments recovered from the two tombs also allowed researchers to study the genes responsible for haemophilia, and to identify which of both forms affected Queen Victoria's male descendents. Czarina Alexandra's DNA was first analysed by researchers at the University of Massachusetts, School of Medicine, and by PhD E. Rogaev, head of the Russian Academy of Medical Sciences' molecular genetics laboratory [5].

Several scientific problems arose during the investigation, due to significant contamination, likely of microbial origin, and the presence of heavily damaged DNA, which forced researchers to work on extremely small sequences (a mean of 117 pb), and to apply new sequencing technologies (Illumina GA sequencing). The gene study required PCR amplification of 180 overlapping fragments to cover the 26 exons coding for the F8 gene and of 32 amplicons for the eight exons coding for the F9 gene. It should be noted that this technical approach does not make it possible to detect the intron 22 and intron 1 inversions in the F8 gene identified at almost half of the severe HA cases; that would have to leave the haematologic enigma unsolved.

Fortunately, DNA analysis for the Czarina revealed an A>G intronic mutation located 3 bp upstream of exon 4 (intron–exon boundary IVS3-3A>G) of the gene coding for the coagulation FIX at the heterozygotous stage, due to a mixture of normal and mutated sequences found among female carriers (Fig. 3).

This mutation is responsible for a splice anomaly, which refers to the process through which RNAs transcribed from genomic DNA may undergo coupling and ligation steps, which lead to the suppression of introns in the messenger RNA, while preserving the exons which will be translated at the protein level. Any mutation that alters the intronic sequences very close to the exon renders splicing impossible or modified.

Supported by indisputable bioinformatic and experimental arguments, the new splicing site, created by the mutation, generates a site 5500 times stronger than the normal site, which massively produces (99.98) abnormal messenger RNA, compatible with a severe form of type B haemophilia.

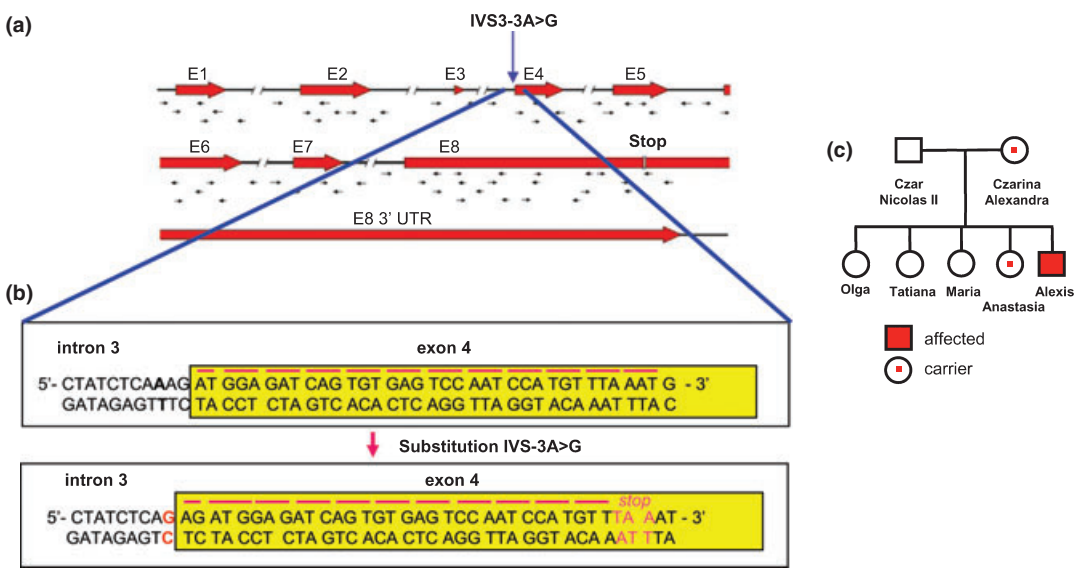

Fig. 3. Presence of a mutation at the splice acceptor site of exon 4. (a) Schematic representation of the eight exons of the F9 gene, as well as the localization of primers having allowed the amplification of exons coding for the gene. (b) Detail of the intron/exon junction of exon 4. In position 3 as compared to the beginning of the exon 4 sequence on the 5′–3′ fragment, in the DNA of Czarevitch Alexis, the A nucleotide normally present has been replaced by a G. This substitution creates a new cryptic splice acceptor site which produces an aberrant frameshift transcript that results in a stop 11 codons downstream. (c) Among the Imperial couple's four daughters, the IVS-3A > G genetic anomaly is found at the heterozygote state only in Anastasia, confirming that she was a haemophilia carrier.

Given that this mutation has been reported in at least three cases of severe HB patients with a residual FIX coagulant activity inferior to 1% [6,7], and as this mutation is not found among the 928 X chromosomes studied in a normal population, there is no doubt about its deleterious character and its responsibility for the presence of haemophilia among Queen Victoria's descendents. This mutation has also been found in Czarevitch's DNA, at the hemizygote status (as he had only one X chromosome), as well as in the DNA of his youngest sister, the Grand Duchess Anastasia, at the heterozygote status.

Conclusion

A simple base mutation among the three billion bases which make up the human genome can change the course of history. This was the case of Romanovs and haemophilia. Even if this disease did not directly cause the 1917 Communist Revolution, it certainly played a role in the Revolution's success. A simple substitution in one of the two splicing sites at exon 4 from the F9 gene was responsible for the severe form of type B haemophilia in the young Czarevitch Alexis. He inherited this disease from his mother, grandmother and great grandmother, the Queen Victoria. If, based on current knowledge, genetic counselling had been available while the members of the imperial family were still alive, a geneticist would have been able to reassure the couple's three oldest daughters, Grand Duchesses Olga, Tatiana and Maria, that they were not carriers of the disease. On the other hand, the youngest daughter, Anastasia, would have had 50% chance of giving birth to a haemophiliac boy and 50% a haemophilic girl.

The discovery of these remains puts an end not only to one of the oldest mysteries in history regarding the fate of the Romanov children (during the 20th century, many women claimed to have been the Grand Duchess Anastasia, and to have escaped the massacre), but also to the scientific curiosity regarding the genetic identification of haemophilia in this family.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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