 https://doi.org/10.34836/pk.2022.318.1

Identification of monozygotic twins in the era of next-generation sequencing

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Abstract

Monozygotic twins pose a challenge to forensic science due to the lack of standardized tools to differentiate them within a pair, and thus making it difficult or impossible for a court to determine which of the twins is the perpetrator of the crime. The advent of next-generation sequencing technologies, whole-genome sequencing, and analyses of the human epigenome, has opened the way to the identification of monozygotic twins for forensic purposes. The percentage of monozygotic twins that can be differentiated and the level of reliability of results remain an issue open for investigation. Identification of monozygotic twins through whole-genome sequencing raises doubts in the courts. New technologies raised a number of questions regarding the parameters: the power of differentiation, the level of reliability of the result, validation method and legal issues. New twin identification technologies require adapting to the requirements for forensic methods: verification, validation and standardization, as well as a uniform approach to the interpretation of results, so that they can be widely used in forensics and recognized by courts as reliable evidence.

Key words: monozygotic twins, next-generation sequencin, epigenome analysis, genetic identification of twins

Introduction

The studies conducted at the beginning of the 21st century found that monozygotic twins do not necessarily have identical genomes and may have mutations in their DNA that occurred after the division of the zygote and/or the formation of two independent embryos (Van Dongen et al., 2012). At that time, the extent of genome diversity within pairs of monozygotic twins remained poorly researched, but it was expected that the development of whole genome sequencing (WGS) technology should bring a better understanding of the differences between twins, especially in the context of their susceptibility to diseases, both infectious and non-infectious (Van Dongen et al., 2012). These assumptions were recently confirmed. Whole-genome sequencing was used to detect mutations that occurred at the zygote stage in monozygotic twins (Jonsson et al., 2021). However, genetic variation is not the only source of information about differences in human genomes. Almost 50 years ago, it was postulated that gene expression and the resultant phenotype could be regulated by epigenetic mechanisms (Holliday & Pugh, 1975; Riggs, 1975). In the second decade of the 21st century, significant knowledge about methylation of the human genome was collected, which confirmed the assumptions regarding the influence of cytosine methylation in DNA (also referred to as genetic or genomic imprinting) on gene expression and cell differentiation. It has been

recognized that epigenetic imprinting is transmitted to offspring (Jones, 2012), but there are also doubts about the extent of this phenomenon in humans compared to other vertebrates (Horsthemke, 2018). Advanced studies of the location of methylated cytosines, defined as the fifth base (Lister & Ecker, 2009), conducted due to their impact on gene functioning and the heredity of imprinting, became possible thanks to the invention of DNA bisulfite conversion technology (Frommer et al., 1992) and the development of next-generation sequencing (NGS) (Lister & Ecker, 2009). Bisulfite conversion allows for the conversion of all unmethylated cytosines to uracil (Hayatsu, 2008). This allows to determine which cytosines were methylated in unmodified DNA, because only these cytosines will not be converted to uracil. Next-generation sequencing methods enable not only the analysis of entire genomes or a large representation of their fragments, but also the detection of cytosine methylation in DNA subjected to an appropriate procedure, e.g. bisulfite conversion. The examination of the methylation status of cytosines in the genome allowed for the differentiation of identical DNA sequences based on this status and even for the assessment of the level of methylation. A new level of DNA differentiation was achieved, apart from the DNA sequence (Lister & Ecker, 2009). The DNA methylation status undergoes dynamic changes at the stage of foetal development and cell differentiation. Changes in the pattern and level of cytosine methylation in the genome occur continuously throughout life (Dor & Cedar, 2018). The process of cytosine demethylation is also intensively studied and its mechanisms are well described (Seethy et al., 2021). Because methylation affects gene expression, it also affects phenotype (Kukla-Bartoszek et al., 2019). The level of DNA methylation changes with age (Horvath & Raj, 2018; Ryan et al., 2020), depends on nutrition (Cavalli & Heard, 2019) and the history of infectious diseases (Fitzgerald et al., 2021). In cancer diseases, methylation levels and patterns are perturbed throughout the genome (Klutstein et al., 2016). Epigenome research has also become an area of interest in forensic science, as both patterns and levels of methylation can be used to determine phenotypic features and age (Kukla-Bartoszek et al., 2019; Pośpiech et al., 2020; Spólnicka et al., 2018; Zbieć-Piekarska et al., 2015). People with identical sequences of DNA regions may differ in the pattern of cytosine. The aim of this study is to present the current state of knowledge and prospects for the use of next-generation sequencing (NGS) technology in the study of genomes and epigenomes in monozygotic twins for forensic purposes.

Twin genome testing

Identification of monozygotic twins for investigative and trial purposes is a problem for prosecuting authorities and the system of justice. Determining paternity is also difficult in the case of monozygotic twins. All over the world, cases are reported in which it is impossible to determine which of a pair of twins committed a given act, left biological traces or was the father. Short tandem repeats (STR) analysis, which is the gold standard for identifying individuals, is typically ineffective in monozygotic twins (Turrina et al., 2021; Vidaki et al., 2017). There was a case in which one of a pair of twins had a mutation (insertion) in the STR regions that allowed it to be distinguished from the other twin in the pair (L. F. Wang et al., 2015). However, such an event is very unlikely and therefore extremely rare. The extremely low, often impossible, probability of distinguishing twins using STR profile tests (Z. Wang et al., 2015), in the absence of other evidence (Tvedebrink & Morling, 2015), frequently resulted in their long-term impunity, including cases of theft and rape (Turrina et al., 2021). There are cases of monozygotic twins in which neither of the pair admitted to the crime, and the examination of STR profiles did not allow to identify the perpetrator. In such cases, courts will usually decide on the basis of other evidence, if it is available. These may be the results of analyses of fibres from clothing, as in the case of a pair of Polish twins refusing to cooperate in identifying the perpetrator of an accident (Szurek, 2022), or witness testimony, as in the case of establishing paternity or finding a rapist (Turrina et al., 2021). Often, due to the principle of the presumption of innocence and the court's inability to identify the perpetrator based on genetic STR profiles and the lack of evidence other than genetic tests, the perpetrator may remain unpunished for years, as in the case of a robbery and rape committed in Boston in 2004 (Turrina et al. al., 2021). There is another problem – because of the increasing number of artificial insemination procedures, the number of twin pregnancies is growing, which results in more STR profiles in DNA databases that are not unique to one person (Tvedebrink & Morling, 2015). The problem is particularly important in the case of adopted people who are not aware of the existence of twin siblings (Tvedebrink & Morling, 2015). Therefore, it is possible for identical STR profiles from different twins from the same pair to appear in databases (Tvedebrink & Morling, 2015).

Are next-generation sequencing technologies effective at differentiating between twins? Are they suitable for forensic applications? Can NGS results be used as evidence in the court? A review of the literature shows no clear answer to any of these questions. Therefore, it is worth returning to the case of the robbery and rape in Boston

committed by one of the McNair monozygotic twins. The examination of STR profiles did not allow to distinguish the brothers, and in 2004 this basically exhausted the possibilities of identification based on genetic tests. With the development of NGS technology, new possibilities emerged. Eurofins demonstrated differences in the genomes of the McNair twins, allowing to determine the culprit. Differences were found using ultra-deep next generation sequencing, the usefulness of which in differentiating monozygotic twins or determining the paternity of one of a pair of twins has been proven (Weber-Lehmann et al., 2014). However, the court did not accept the Eurofins results as reliable evidence (Turrina et al., 2021), although it stated the following: "testing and analysis were based on generally accepted, scientific and statistical principles". This also shows that until there is a consensus on how to validate and interpret NGS results, they may raise doubts in the courts. It is worth mentioning that in the McNair case the cost of NGS was over $100,000.

Interestingly, evidence from analyses of STR profiles, which are highly unlikely to differentiate between monozygotic twins, is very likely to be accepted by the court, while in numerous cases evidence from NGS tests allowing for the differentiation of monozygotic twins has not been recognized as reliable by the court (Rolf & Krawczak, 2021). In the case of the McNair brothers, the conviction was based on the testimony of a witness and occurred 14 years after the crime, despite the presentation of NGS results distinguishing the twins. In a paternity case in Brazil, the results of STR profile confirmed that two twin brothers had identical STR profiles and the test did not exclude the paternity of either of them. After assessing all the material, including the mother's testimony, the court ordered both brothers to pay alimony (Turrina et al., 2021). In a paternity case in Italy, the court found that the STR profile indicating the paternity of one of the twins is not evidence confirming that the tested twin is the father (Turrina et al., 2021). In both cases, in which the results of STR profile tests were presented to the court, they were accepted as reliable, but they neither excluded nor confirmed the paternity of one of the pair of twins, and the court made their decisions based on other evidence and premises. Below, we outline the ways in which courts have addressed evidence based on NGS results. In a rape case (Yuan et al., 2020) in which, similarly to the case of the McNair brothers, one of the pair of twins was the perpetrator (later shown to be the perpetrator of four rapes), and pleaded guilty, his brother had to be excluded as the perpetrator. Whole-genome sequencing was used, however, only the results of mitochondrial genome sequencing were considered sufficient for effective differentiation and further analyses of genomic DNA were omitted. The sequencing depth for the mitochondrial genome was 2000, and, as a rule, it is usually about 100 times higher than for genomic DNA (Davis et al., 2022). The average depth of sequencing coverage can be theoretically defined as LN/G, where L is the read length, N is the number of reads, and G is the length of the haploid genome (Sims et al., 2014). In simple terms, this gives an approximate number of reads of the sequence of interest, assuming that the coverage (the number of times the genome was sequenced, together with percentage expressing what part of the genome was sequenced) was high and the examined sequence was among those sequenced. High coverage and high sequencing depth increase the reliability of results and allow the detection of rare variants. These phenomena occurred in this case; the twins differed in one mutation in the mitochondrial genome, resulting from heteroplasmy, occurring at a level of 2.6% in one of the pair of twins (Yuan et al., 2020). The reliability of the result was confirmed using targeted next-generation sequencing, i.e. sequencing the region where the mutation was located. The results were successfully used as evidence in court, but it remains questionable whether finding one mitochondrial mutation, in the form of heteroplasmy at the level of 2.6%, distinguishing twins in a pair, is a rather favourable coincidence, or a method that allows for routine differentiation. The literature also described differentiation based on NGS results of the mitochondrial genome within 6 out of 16 pairs of twins, but not all detected differences would be reliable evidence in court (Chen et al., 2020). Similar reservations were formulated in another paper describing the use of sequencing the mitochondrial genomes in twins (Z. Wang et al., 2015). The authors of both studies (Chen et al., 2020; Z. Wang et al., 2015) express similar opinions: differentiating twins is possible based on NGS results of mitochondrial DNA, but some of the differences could be insufficient evidence in court. Both research groups agree that studies should be conducted on identical biological material taken from both twins and further works would be required before NGS of mitochondrial DNA can be routinely used in forensics (Chen et al., 2020; Z. Wang et al., 2015). In principle, it is possible to detect differences also in the genomic DNA of monozygotic twins from one pair (Jonsson et al., 2021). The average number of germline mutations (present in sperm and inherited) was 5.2 per pair of twins (Jonsson et al., 2021). Somatic mutations were also tested (blood and oral swabs) in 381 pairs of twins and in 2 monozygotic twin triplets. There were 39 pairs of twins differing in more than 100 mutations, and 38 pairs in which no differences were detected. However, when the coverage threshold was increased from 38-fold to 100-fold, the number of pairs differing by more than 100 mutations dropped from 39 to 5.

In turn, among the pairs with no differences at 38-fold coverage, at 100-fold coverage no differences were detected only within 12 of 38 pairs (Jonsson et al., 2021). Therefore, the parameters and procedures used in NGS and WGS have a large impact on the estimation of the number of differences. Ultimately, it was found that a significant number of mutations specific for only one individual could be detected in 15% of twin pairs. The percentage of false positive differences depended on average coverage and verification method and ranged from 3% to 28%. The greatest reliability of results in the study conducted by Jonsson et al. (2021) was obtained at approximately 152x maximum coverage. The number of false positive differentiation results increased at lower – 38x coverage. This shows that this approach allows to indicate mutations that distinguish twins in a pair, but it would require significant modifications to be used in forensics. The importance of coverage in NGS is illustrated by the case of twins who had committed rape. Distinguishing them was only possible based on mitochondrial DNA sequencing (Yuan et al., 2020) at 2000-fold coverage, and impossible at 30-fold coverage using genomic DNA sequencing.

The research into the use of whole-genome sequencing to analyse the genomes of monozygotic twins for forensic purposes (Rolf & Krawczak, 2021) presented a list of WGS results applied as evidence in court. The summary reveals that the court accepted evidence in 4 out of 6 cases. One of these cases was incorrectly classified as evidence and accepted by the court. It concerned the previously mentioned case of the McNair brothers. In this case, the court found the methodology correct, but not meeting the formal and legal criteria allowing the evidence to be considered reliable (Rolf & Krawczak, 2021). It should be recalled that the reason the court did not recognize the results of Eurofins' WGS sequencing was that a combination of laboratory and statistical methods had never before been used in exactly the same way, for exactly the same purpose, i.e. the identification of a sperm donor within a pair of monozygotic twin brothers. The court made such a decision despite the existence of methods for calculating the likelihood ratio based on whole-genome sequencing (Krawczak et al., 2018). The court did not question the correctness of any element of the twin identification process, which was conducted separately. This example illustrates how important it is to create international standards for performing and interpreting the results of forensic examinations based on NGS and WGS technologies, so that they are accepted by courts as reliable evidence. At the time of writing this paper, no recommendations from recognized international organizations on WGS in forensics were available. The ENFSI database recommendations include the following statement: "A major issue for DNA database managers is that they cannot distinguish matches between monozygotic twins. Both epigenetic as well as next generation sequencing research is occurring, but the amounts of DNA which are necessary for these analyses must be reduced to enable analysis of forensic traces containing low amounts of DNA" (ENFSI, 2019). The above ENFSI statement applies to the use of NGS and epigenome analyses only in the context of managing DNA profile databases. The conclusions of the epigenome analyses described in the ENFSI recommendation are based on literature from over a decade ago (Li et al., 2011). Epigenome research and its applications in forensics are currently much more advanced than described in the literature (Li et al., 2011) cited in the ENFSI document.

A comprehensive overview of forensic technologies based on WGS, forensic genomics and related regulations can be found in a document issued by the European Commission. The Technical Report of the European Commission (Angers et al., 2021) is not a recommendation, but just a review of technologies, related recommendations and legal regulations.

Epigenome studies in twins

As mentioned earlier, DNA methylation tests are used to assess chronological and biological age (Bell et al., 2019), phenotypic traits, and even lifestyle (Ryan et al., 2020). Due to greater variability in methylation patterns and levels than in genomic DNA sequences, the epigenome has become an interesting object of research in the context of twin differentiation (Du et al., 2015; Li et al., 2013; Planterose Jiménez et al., 2021b; Vidaki et al., 2018; Zhang et al., 2015). The number of DNA sequence differences between the twins' genomes is extremely small (Rolf & Krawczak, 2021), and therefore many authors have pointed out that epigenome examinations may allow for greater power to differentiate monozygotic twins compared to genome tests (Du et al., 2015; Planterose Jiménez et al., 2021b; Vidaki et al., 2018; Zhang et al., 2015). Researchers were searching for epigenetic markers to differentiate twins. The identification of such markers and their use to identify twins has been described, including a PCR panel using DNA melting curves to detect differences between twins (Marqueta-Gracia et al., 2018). The results of the study were questioned and the conclusions regarding the effectiveness of twin differentiation were invalid (Vidaki et al., 2018). This example is given to illustrate how important it is to be careful in assessing the reliability of twin differentiation tests in the era of dynamic development of this field. The possibility of developing a panel of markers

to differentiate twins needs clarification (Vidaki et al., 2018). In genomes of monozygotic twins, the mutations that differentiated them were unique and located in different regions of the genome (Jonsson et al., 2021), therefore they were specific to a given individual, and not to twins in general. Similarly, with regard to the epigenome, regions of the genome with high levels of methylation variability in the human population show variability in monozygotic twins (Planterose Jiménez et al., 2021b). Therefore, these are not regions of the genome with high variability in the pattern and level of methylation typical of twins, but regions of the genome with high variability in the pattern and level of methylation in the human population in general (Planterose Jiménez et al., 2021b). The gold standards for testing methylation status are whole genome bisulfite sequencing (WGBS) and methylation microarrays from Illumina (Planterose Jiménez et al., 2021b). Attempts have been made to use epigenome analysis to differentiate twins based on the biological trace from a cigarette butt (Vidaki et al., 2018). Two technologies were applied, Illumina 450K microarrays and MethyLight quantitative PCR, but both methods gave divergent results for some of the markers in the reference material (Vidaki et al., 2018). Furthermore, it was not possible to examine the trace material using microarrays due to the large amounts of DNA required by this technology.

In conclusion, Vidaki et al. indicate the limitations of the technologies and epigenome research itself in forensic applications (2018). It is unknown what is the minimum number of differentially methylated cytosines (markers) necessary to achieve reliable differentiation between individuals and how this number may vary depending on the tissue (Vidaki et al., 2018). Due to the high requirements for the quantity and quality of DNA, microarray technology is not suitable for trace analysis. In addition, the assessment of the cellular composition of biological material (based on the results of DNA analysis) would be required (Vidaki et al., 2018). Another problem was the normalization and analysis of the results obtained using Illumina 450K microarrays in a way that would eliminate artefacts (Vidaki et al., 2018). The last issue could be the source of some problems with the analysis of epigenetic markers in twins.

Methylation microarrays have been successfully used to assess human age for years, but there were limitations of this technology (Carmona et al., 2017; Logue et al., 2017; Maksimovic et al., 2015; Planterose Jiménez et al., 2021a; Price & Robinson, 2018). Recently, it turned out that the method of analysing the results from methylation microarrays caused errors in estimating the methylation level – it generated artefacts that distorted age prediction (Higgins-chen et al., 2022; Pang et al., 2022). These conclusions are consistent with the findings suggesting that targeted sequencing methods may be more adequate than microarrays in differentiating twins for forensic purposes based on DNA methylation patterns (Vidaki et al., 2018). Prediction of age and phenotypic traits has been successfully performed using next-generation targeted sequencing technology (Freire-Aradas et al., 2020; Kukla-Bartoszek et al., 2019; Pośpiech et al., 2020; Spólnicka et al., 2018; Zbieć-Piekarska et al., 2015).

Undoubtedly, variability in methylation patterns and levels has greater potential to discriminate between monozygotic twins than genetic diversity, because identical DNA sequences may differ in the level and status of cytosine methylation. Recently, hundreds of markers have been found to differentiate twins and unrelated individuals, which could potentially be used for forensic purposes in the future (Planterose Jiménez et al., 2021b). A new method for detecting methylated cytosines has been developed based on the enzymatic conversion of these bases. TAPS (TET-assisted pyridine borane sequencing) (Liu et al., 2020; Siejka-Zielińska et al., 2021) requires a smaller amount of DNA, which is extremely important in forensic applications, and will probably be used for these purposes in the future.

As in the case of genome research, the methods used to study epigenome variability will need to be adapted to the stringent requirements for forensic methods. It is expected that in the near future they will be included in the catalogue of techniques routinely used in forensics, but this will require their standardization and the appropriate legal regulations. In Poland, research into the forensic applications of epigenome testing is advanced (Freire-Aradas et al., 2020; Kukla-Bartoszek et al., 2019; Pośpiech et al., 2020; Spólnicka et al., 2018; Zbieć-Piekarska et al., 2015). Currently, the Central Forensic Laboratory of the Police is implementing the EPIGENOM project, number DOB-BIO10/06/01/2019, which also focuses on the differentiation of monozygotic twins based on the differences in patterns and levels of genomic DNA methylation.

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