A Marker-based Method to Detect Phosphorothioated Oligonucleotides in Equine Plasma Using Spectroscopic Analysis

1. Teruaki Tozaki Genetic Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan Corresponding author: ttozaki@lrc.or.jp*

2. Kaoru Karasawa AB Sciex, 4-7-35 Kitashinagawa, Shinagawa-ku, Tokyo 140-0001, Japan Corresponding author: Kaoru.Karasawa@sciex.com*

3. Yohei Minamijima Drug Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

4. Hideaki Ishii Drug Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

5. Mio Kikuchi Genetic Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

6. Hironaga Kakoi Genetic Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

7. Kei-ichi Hirota Genetic Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

8. Kanichi Kusano Racehorse Hospital Ritto Training Center, Japan Racing Association, 1028 Misono, Ritto, Shiga 520-3085, Japan

9. Shun-ichi Nagata Genetic Analysis Department, Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan

** Teruaki Tozaki and Kaoru Karasawa contributed equally to this study*

Although there have been no known reports of gene doping in the horseracing industry to date, the relatively rapid advances and increasing accessibility of technologies for genetic modification may make this a reality. It is therefore prudent to investigate and develop methods to detect such practices. Therapeutic oligonucleotides technologies may be repurposed for gene doping. A common way of improving the structural integrity and stability of oligonucleotides to retard degradation when used for therapeutic applications is the addition of a phosphorothioated modification. This chemical moiety was employed as a viable marker for the detection of phosphorothioated oligonucleotides, using a method based on targeted and non-targeted spectroscopic analysis with liquid chromatography tandem mass spectrometry, including information-dependent data acquisition with dynamic background subtraction. It is proposed that this method be further investigated for use in the detection of equine gene doping.

Introduction

Maintaining the integrity and fairness of competitive sports has become an increasingly demanding effort. Since the establishment of the International Federation of Horseracing Authorities (IFHA) Gene Doping Control Subcommittee (GDCS) in March 2016 [1], several horseracing nations have decided to work together and invest in research to better understand this relatively new form of doping [2]. Although it is believed that gene doping has not been or is currently being misused to corrupt the sport, this investment demonstrates the commitment of these nations to stay ahead of potential cheating [2]. In Japan, the Laboratory of Racing Chemistry has been researching gene doping detection methods since 2017 and is currently developing nucleic acid medicine and transgene detection methods [3].

The doping could consist of the administration of oligomers or polymers of nucleic acid, nucleic acid analogues, or genetically unmodified or genetically modified cells, resulting in the genetic manipulation of racehorses to enhance their performance [4,5]. Although this has not been demonstrated in equine species, studies in other mammalian species have shown the feasibility of gene doping to enhance athletic performance [6,7]. Through its potential ability to not only affect the genetics of an animal long-term but also the genomes of its offspring, gene doping threatens both horse racing and breeding [2,4]. This was recognised by David Sykes, the director of equine health and welfare at the British Horseracing Authority (BHA), who stated in April 2019 [2], "This is new technology that is unravelling all the time; none of us here think that there has probably been a previous incidence of it, but that doesn't mean that we shouldn't be looking forward into the next five or 10 years and at least being able to identify if it is going to occur."

This technology came about originally in efforts to develop new medicines to treat previously undruggable, usually rare, human diseases, such as spinal muscle atrophy - a disease that is the most common hereditary cause of infant mortality [4,8,9]. The development of oligonucleotide medicines occurred in fits and starts with much hope and many setbacks over the last 30 years or so [10-12]. However, as the basic biology became better understood and chemistries improved, along with more sophisticated delivery systems, we are now seeing a renaissance in oligonucleotide therapies [10-12]. Several such rationally designed precision medicines have now

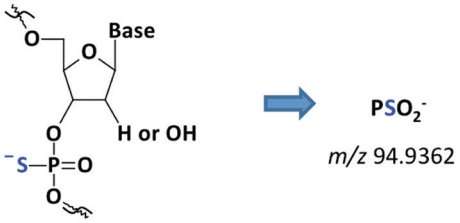

Figure 1: Chemical structure of phosphorothioated nucleic acids.

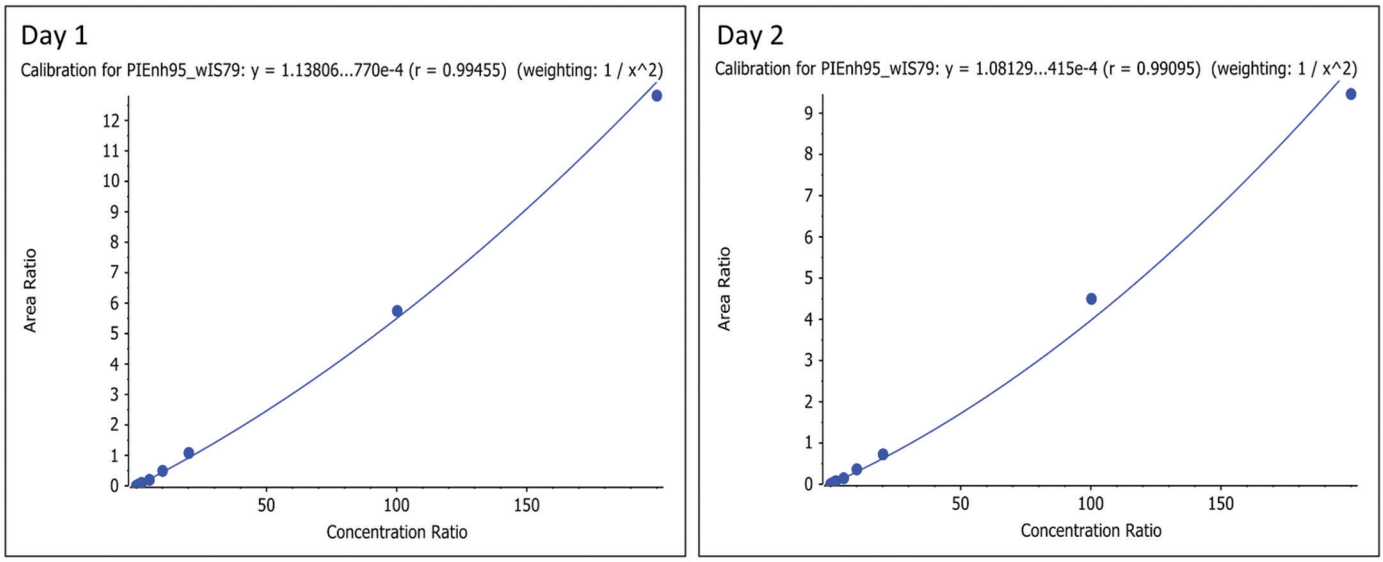

Figure 2: Quadratic calibration curves of PSOs in deproteined horse plasma on Day 1 and Day 2.

been approved by the US and EU drugs and medicines regulatory authorities (FDA and EMA respectively) [8]. However, with this powerful new technology comes the potential to misuse it [4].

Detection is essential to deterring gene doping

It is to counter of this potential abuse in sports, particularly in horse racing, that countries like the UK have already implemented research programs scheduled to span the next 5 years [2]. Genetic technologies could be used to enhance the performance of racehorses [4]. Genetic expression could be manipulated to improve muscle strength and sprinting ability, oxygen delivery or blood flow to muscles for enhanced endurance, energy metabolism in muscles to resist fatigue, pain alleviation or injury repair for post-race recovery [4,5]. Similarly, the performance of rival horses may be diminished by impairing any of these mechanisms through gene doping [4]. These genetic manipulations may also be applied to the germline of thoroughbreds, meaning that not only the doped horse is affected but also potentially all of its offspring [4,5]. This would massively disrupt the selective breeding of thoroughbred horses with pedigrees and heritage traceable for generations, some all the way back to the 18th century when Arabian stallions were first introduced and bred with mares in the UK [4,13]. Moreover, as this technology is still relatively new, there is a lot we still do not know about its adverse effects, especially in the long term, which may prove to be highly detrimental and irreversible [4,5].

Although there has not yet been a gene doping incident detected in horseracing or breeding, the possibility of this form of doping is very real [2]. The rapid development of this technology may mean the eventual availability of a do-it-yourself gene-doping kit in the not so distant future [2]. In order to get out in front of this potentially deeply persistent form of doping, it is vital that robust and reliable methods of detection are developed and implemented [2,4,5,14]. An analytical method has been developed to detect oligonucleotides synthesised for gene doping. This method relies on the detection of a key chemical moiety that has commonly been introduced to improve the structural integrity and stability of oligonucleotides, making them more resistant than native nucleic acids to degradation [15]. The moiety is that of phosphorothioate (PS) modification of nucleic acids, a modification that does not naturally occur in animals (see Figure 1) [14]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to analyse horse plasma, an ideal matrix for gene doping tests, to identify and quantify any PS oligonucleotides (PSOs) present.

Experimental

Sample preparation

To test the method developed to detect PSOs in equine serum, samples were prepared from equine plasma. One set of samples would be blank samples, serving as negative controls. Another set of samples would be spiked with PSOs, serving as the test samples. Horse plasma was obtained by centrifugal separation of whole blood collected with EDTA. The plasma was

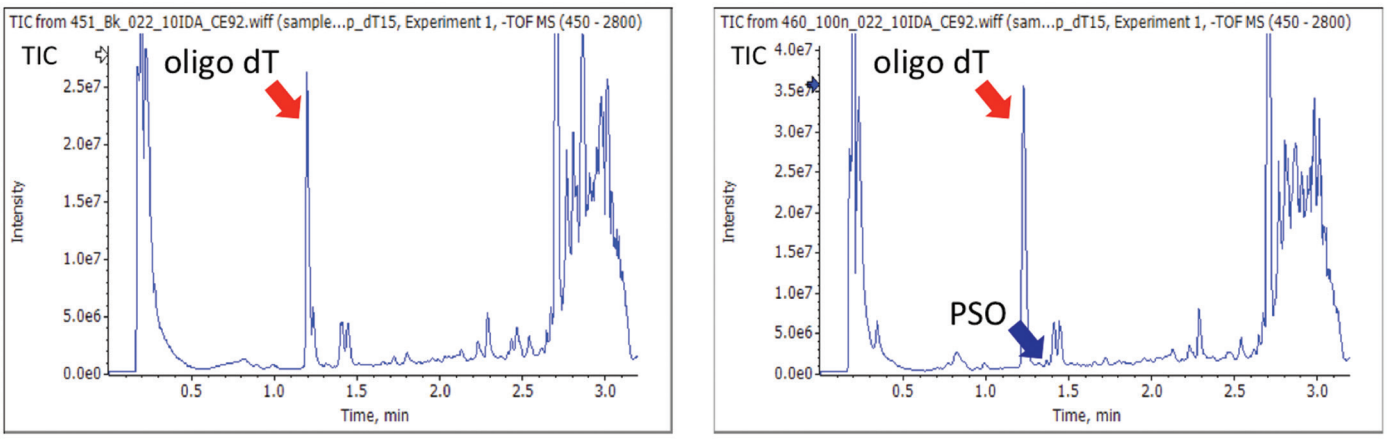

Figure 3: Total ion chromatogram (TIC) of non-targeted analysis of PSOs in deproteinated horse plasma with 4.5 µg/mL of oligo dT (blank sample control; left graph), and with 100 ng/ml PSO (spiked sample containing Oligo 1; right graph).

deproteinated by adding 30 mL acetonitrile (LC/MS grade, Wako, Osaka, Japan) to 15 mL horse plasma in 50 mL tubes, then mixing vigorously, storing at 4°C for 15 min, centrifuging at 3000xg for 15 min, and collecting 40 mL of the supernatant. Remaining solvent was removed using an evaporator, and the final volume was adjusted to 10 mL with Milli-Q water. The deproteinated horse plasma samples were then stored at –20°C until use.

For the PSO, a 22-mer antisense sequence of exon 2 of the equine myostatin gene (MSTN), 5'-GAG ATC GGA TTC CAG TAT ACCA-3', was synthesised, with all the nucleotides phosphorothioated (GeneDesign,Inc. Osaka, Japan). The PSO was purified using HPLC, then dissolved in 100 mmol/L triethylamine acetate (TEAA) buffer at a concentration of 20 µg/mL. One set of the equine plasma samples were then spiked with PSO to concentrations ranging from 1 ng/mL to 20,000 ng/mL. To evaluate any false positives, unphosphorothioated 15-mer oligo dTs, 5'-TTT TTT TTT TTT TTT-3' (GeneDesign, Inc.), were spiked into all the test and blank plasma samples to a concentration of 4.5 µg/mL.

High performance liquid chromatography

The blank and test equine plasma samples were analysed first with HPLC on a Prominence UFLC XR HPLC system (Shimadzu, Kyoto, Japan) using an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 30 mm, Waters, Milford, MA, USA). The column was maintained at 60°C, with a mobile phase consisting of Solvent A: 100 mM hexafluoro-2-propanol [HFIP] and 10 mM triethylamine (TEA), and Solvent B: methanol. The sample injected in 2 µL volumes, with the elution gradient as: 5 to 50 to 90% Solvent B in 2 and 0.01 min, then hold for 0.49 min, then 0.01 min in 5% Solvent B, then hold for 1.49 min, after pre-equilibrium for 0.5 min in 5% Solvent B at a flow rate of 0.4 mL/min.

Targeted and non-targeted spectroscopic analyses to selectively detect PSOs in equine plasma

After HPLC separation, the plasm samples were analysed with quadrupole-time-of-flight (Q-TOF) tandem mass spectroscopy (MS/MS) on a TripleTOF® 6600 System (SCIEX, Framingham, MA, USA) equipped with a DuoSpray™ ion source.

For targeted analysis, a product ion scan was performed for the precursor ion of mass-to-cha rge ration (m/z) of 785.18 corresponding to $[M–9H]^{9-}$, and the Q1 resolution was set to low to transmit the isotope ions and increase the sensitivity. Then, the product ion of m/z 94.9362 derived from the PS moiety was detected using high-resolution multiple reaction monitoring (MRM^{HR}) in high sensitivity and enhanced ion modes, which provided additional ion selectivity. The MS parameters used were a scan range of m/z 70–300, Gas 1 (nebuliser gas) at 60 psi, Gas 2 (heater gas) at 80 psi, curtain gas at 25 psi, at an ion source temperature of 600°C, an ion spray voltage of – 4000 V, a declustering potential of – 120 V, and collision energy (CE) of – 92 V. The mass spectrometer was calibrated with external standard solution before acquisition.

For non-targeted analysis, information- or data-dependent acquisition (IDA or DDA) was used to search for the PSOs that could produce the specific product ion of negatively charged PS. The product ion scan data was acquired by triggering the 10 most intense precursor ions within the DDA criteria every 0.2 s cycle. The same MS parameters were used as for the targeted analysis, except the scan ranges, which were m/z 450–2800 for the full scan and m/z 70–2800 for the product ion scan.

The data acquisition was information- or data-dependent (IDA or DDA). Dynamic background subtraction (DBS) was applied to the acquisition to minimise the collection of MS/MS spectra on background ions, thus increasing the identification of low-abundance analytes in the presence of background noise. Data acquisition, targeted analysis, and non-targeted analysis were performed using Analyst®TF 1.7.1, MultiQuant™ 3.0.2, and PeakView® 2.2 software (SCIEX), respectively.

Table 1: Accuracy and reproducibility of PSO detection.

Sample Type	Concentration (ng/mL)	Accuracy (%)	
		Day 1	Day 2
Standard	0.1	103.0	108.5
	0.2	103.5	93.4
	0.5	82.5	82.0
	1.0	89.8	86.6
	2.0	91.6	94.2
	5.0	98.7	90.9
	10.0	113.1	117.4
	2.0	114.7	117.2
	100.0	104.0	109.8
	200.0	97.4	95.4
Quality Control	20.0	111.5	119.3
	200.0	96.1	102.4

Results and Discussion

Targeted and non-targeted spectroscopic analyses selectively detected PSOs in equine plasma

In the targeted spectroscopic analysis, the MRM^{HR} enhanced ion mode enabled the target product ions to reach the detector 16 times more efficiently. The target analyte was detected with a limit of detection (LOD) of 0.1 ng/mL. In quantitative analysis, the calibration curves generated using quadratic regression demonstrated excellent linearity in the range of 0.1–200 ng/mL with a correlation coefficient (R) of ≥ 0.990 (see Figure 2). Accuracy measures of 82–118% were observed and intra- and inter-day reproducibility were confirmed (see Table 1). The sensitivity of the quantitation also indicates that the product ion of m/z 94.9362 could serve as a good marker for pharmacokinetic studies of PSOs.

In the non-targeted spectroscopic analysis, the specific product ion of m/z 94.9362 was detected in the PSO-spiked plasma and not in the blank samples (see Figure 3). Therefore, the detected product ion of m/z 94.9362 did not originate from the endogenous components of the plasma. The PSO analyte was detected at a concentration of 100 ng/mL at a retention of approximately 1.4 minutes. No interfering peaks were detected at or around 1.4 minutes in the blank samples. Moreover, when filtered to the product ion of m/z 94.936, peaks were detected in the spiked samples, while no peaks were detected in the blanks (see Figure 4). Phosphorothioated oligomers were thus selectively detected in a heavy biological matrix containing a 45 times higher concentration of a non-PS oligomer without any sequence information.

A marker-based test proposed for the detection of gene doping

These findings indicate that the product ion

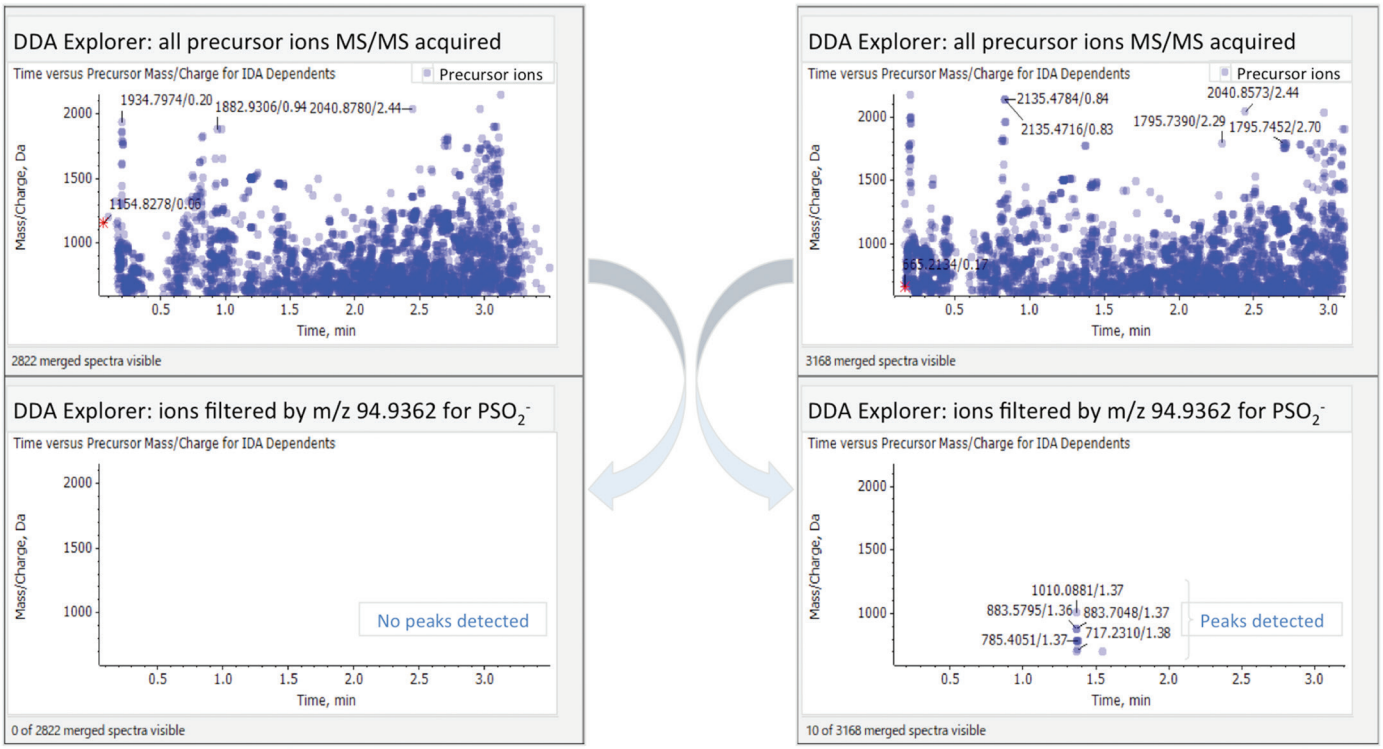

Figure 4: DDA Explorer (time versus precursor MS/charge for DDA dependents) and DDA Explorer filtered by m/z 94.936 with 10 ppm mass tolerance of deproteinated plasma with 4.5 µg/mL oligo dT (blank sample), and with 10 ppm mass tolerance of 100 ng/mL PSOs in deproteinated plasma with 4.5 µg/mL oligo dT (spiked sample).

of m/z 94.9362 would be a good marker for detecting and monitoring PS compounds, such as those that may potentially be used in the gene doping of racehorses. The analytical method described may be of use in the testing of racehorses for gene doping, particularly as it can be applied using either a targeted or non-targeted analysis. This is especially pertinent as the oligonucleotides being used for gene doping may not be certified, and comprise unknown genetic sequences depending on the gene being manipulated, or any number of nucleic acids or nucleic acid analogues, making it difficult to identify the analyte for a targeted analysis. Another key factor for this methodology is the need for adequately rapid spectroscopic scan speeds, as well as the capability to capture comprehensive data with an information-dependent acquisition (IDA) and dynamic background subtraction (DBS). Therefore, the scan was able to identify all 95 phosphate groups without missing any detectable analytes, thus forming a complete study. The use of the SCIEX TripleTOF® system was therefore critical to the study design and the feasibility and overall success of this study.

Although it has yet to be investigated, this method may also perhaps be adapted for use in the detection and monitoring of gene doping in other sports, such as athletics, since PS compounds also do not naturally occur in humans. The direct detection of PSOs would also be a useful method to develop but until then, the use of this method to detect the PS moiety as a surrogate marker for actual PSOs should be of some value. Further validation studies will need to be performed on samples from horses that have been doped in vivo but it is hoped that this would not be necessary because gene doping will not be realised in the sport of horseracing.

In conclusion, LC-MS/MS is a useful method for the detection of known and unknown compounds such as PSOs that may be used for doping, and the product ion of m/z 94.9362 is an effective marker for the detection of PSOs in equine plasma.

For those looking for more information, September's Gene Doping Workshop in Japan (https://biz.knt.co.jp/tour/2019/09/aorc/about.html) will look further into genetic laboratories used for gene doping detection, along with analyst and instrument requirements, and additional testing methods. The workshop will be organised and chaired by Dr Tozaki.

References

1. International Federation of Horseracing Authorities (IFHA). About IFHA. Committees – Gene Doping Control Subcommittee. https://www.ifhaonline.org/default.asp?section=About%20IFHA&area=101.

2. G. Wood. Talking Horses: racing attempts to get out in front of gene doping. The Guardian. 24 April 2019. https://www.theguardian.com/sport/blog/2019/apr/24/talking-horses-bha-commits-1m-gene-doping.

3. Tozaki T, Gamo S, Takasu M, Kikuchi M, Kakoi H, Hirota KI, Kusano K, Nagata SI. Digital PCR detection of plasmid DNA administered to the skeletal muscle of a microminipig: a model case study for gene doping detection. BMC Res Notes. 2018 Oct 10;11(1):708

4. T. Wilkin, A. Baoutina, N. Hamilton. Equine performance genes and the future of doping in horseracing. Drug Test Anal. 9 (2017) 1456–71.

5. E.W.I. Neuberger, P. Simon. Gene and Cell Doping: The New Frontier – Beyond Myth or Reality. Med Sport Sci 62 (2017) 91–106.

6. A. Macedo, M. Moriggi, M. Vasso, S. De Palma, M. Sturnega, G. Friso, C. Gelfi, M. Giacca, S. Zacchigna. Enhanced Athletic Performance on Multisite AAV-IGF1 Gene Transfer Coincides with Massive Modification of the Muscle Proteome. Human Gene Therapy 23 (2012) 146–57.

7. M. Le Page. Gene doping in sport could make the Olympics fairer and safer. New Scientist. 5 August 2016. https://www.newscientist.com/article/2100181-gene-doping-in-sport-could-make-the-olympics-fairer-and-safer/.

8. C.A. Stein, D. Castanotto. FDA-Approved Oligonucleotide Therapies in 2017. Molecular Therapy 25 (2017) 1069–75.

9. M. Krishnan. Drugging the

'undruggables.' PharmaTimes. June 2019. http://www.pharmatimes.com/magazine/2019/june_2019/drugging_the_undruggables.

10. Bentley University. Thirty years of innovation pays off as oligonucleotide therapeutics come to market. Medical Xpress. 25 January 2018. https://medicalxpress.com/news/2018-01-years-oligonucleotide-therapeutics.html.

11. J.M. Beierlein, L.M. McNamee, F.D. Ledley. As Technologies for Nucleotide Therapeutics Mature, Products Emerge. Mol Ther Nucleic Acids 9 (2017) 379–86.

12. Editorial. The commercial tipping point. Nat Biotech 35 (2017) 181.

13. M.A. Bower, M.G. Campana, M. Whitten, C.J. Edwards, H. Jones, E. Barrett, R. Cassidy, R.E. Nisbet, E.W. Hill, C.J. Howe, M. Binns. The cosmopolitan maternal heritage of the Thoroughbred racehorse breed shows a significant contribution from British and Irish native mares. Biol Lett 7 (2011) 316–20.

14. T. Tozaki, K. Karasawa, Y. Minamijima, H. Ishii, M. Kikuchi, H. Kakoi, K. Hirota, K. Kusano, S. Nagata. Detection of phosphorothioated (PS) oligonucleotides in horse plasma using a product ion (m/z 94.9362) derived from the PS moiety for doping control. BMC Res Notes 11 (2018) 770–5.

15. Eckstein F. Phosphorothioates, essential components of therapeutic oligonucleotides. Nucleic Acid Ther 24 (2014) 374–87.

Author biography

Teruaki Tozaki PhD is the Technical Advisor at the Genetic Analysis Department, Laboratory of Racing Chemistry, Japan; Visiting Lecturer at the Showa University School of Medicine; and Visiting Professor of Veterinary Medicine at the Faculty of Applied Biological Sciences, Gifu University. Dr Tozaki is also a Sports Pharmacist.

Authors' contributions

Conception and design: T. Tozaki, K. Karasawa

Experiment implementation and data collection: T. Tozaki, K. Karasawa, H. Ishii, Y. Minamijima

Manuscript content and review: T. Tozaki

Data collection and analysis supervision and study design support: M. Kikuchi, H. Kakoi, K. Hirota, K. Kusano, S. Nagata

Declaration of interest

This article was written with the support of SCIEX, a Danaher operating company and a global leader in the accurate quantification and characterisation of molecules.

www.sciex.com

Disclaimer

For In Vitro Diagnostic Use. Rx Only. Product(s) not available in all countries. For information on availability, please contact your local sales representative or refer to https://sciex.com/diagnostics.

IVD-MKT-19-9983-A