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XIST and RPS4Y1 long non-coding RNA transcriptome as sex biomarkers in different body fluids

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Abstract

Background and objectives: Sex determination of an individual based on biological fluid evidence is a critical issue in forensic science. RNA has proved valuable in the identification of body fluids and the estimation of stain age. However, it still could not provide sufficient information about their donor. This study aimed to evaluate the potential use of the long non-coding XIST and RPS4Y1 markers in the identification of sex from different body fluids.

Methods: Saliva, semen, and peripheral and menstrual blood samples were obtained from apparently healthy volunteers. The expression of XIST and RPS4Y1 was assessed in these samples using real-time RT-PCR.

Results: XIST was detected in female saliva and peripheral and menstrual blood, while RPS4Y1 was detected in male blood and semen.

Conclusion: XIST and RPS4Y1 could be sex differentiation biomarkers in various body fluids.

Keywords: Sex identification, Body fluids, Long non-coding ribonucleic acid, X-inactive specific transcript, Ribosomal protein S4, Y-linked 1

Background

Accurate sex identification from biological samples is a vital issue in forensic casework (Borovko et al. 2015). The most commonly used method for human sex determination is based on the sex-typing marker amelogenin (Butler and Li 2014). The amelogenin gene is a single-copy gene, located on the X (AMELX) and Y (AMELY) chromosomes at Xp22.1–Xp22.3 and Yp11.2, respectively (Nakahori et al. (1991a); Nakahori et al. (1991b); Sasaki and Shimokawa 1995). In sex identification procedures, this gene is amplified for detection of the X and Y alleles. The presence of two amplified products indicates a male genotype, while a single amplicon indicates a female genotype (Giuliodori et al. 2011). Several studies have shown that the amelogenin is not a reliable tool for sex determination (Nakahori et al. (1991a); Nakahori et al. (1991b); Sasaki and Shimokawa 1995; Giuliodori et al. 2011; Maciejewska and Pawłowski 2009; Turrina et al. 2010). Mutations of AMELY may cause the failure of its amplification in male biological samples and their misidentification as female samples. Non-AMELY alleles' cases have been detected worldwide in different populations and with different percentages. Therefore, an additional reliable method is needed to accurately identify the sex of a sample donor (Borovko et al. 2015; Nakahori et al. (1991a); Nakahori et al. (1991b); Sasaki and Shimokawa 1995; Giuliodori et al. 2011; Maciejewska and Pawłowski 2009; Turrina et al. 2010).

The analysis of RNA has shown valuable results in the identification of body fluids (Roeder and Haas 2016) and the estimation of the age of body fluid stains (Alshehhi et al. 2017; Simard et al. 2012). However, it could not provide evidential data about the sex of the stain donor (van den Berge and Sijen 2017). Many studies searched for the sex differences in neuropsychiatric diseases (Ji et al. 2015), cancer, (Shi et al. 2013; Weakley et al. 2011), and other human disorders (Shi et al. 2013) using sex-specific RNA markers. In forensics, van den Berge

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and Sijen (2017) studied the use of XIST and RPS4Y1 in sex identification from body fluids and organ tissues where they reported promising results.

In this study, we aimed to further evaluate the potential use of these two markers (XIST and RPS4Y1) in sex identification from different body fluids.

X-inactive specific transcript (XIST) is a long non-coding RNA involved in X-chromosome inactivation (XCI). XCI is an essential biological process which is responsible for the inhibition of X-linked genes overexpression in women to maintain the same dosage between males and females (Ji et al. 2015). During development, one of the X chromosomes becomes randomly silenced. This silencing is automatically inherited all through the process of somatic cell division. The chief of this process is the XIST (Staedtler et al. 2013; Ng et al. 2007).

Ribosomal protein S4, Y-linked 1 (RPS4Y1) is one of the genes found on the Y chromosome that proved to have the greatest expression difference between both sexes (Vawter et al. 2004; Melé et al. 2015) and is expressed in all male human tissues (Planchard et al. 2009).

Materials and methods

Subjects

Fifty apparently healthy volunteers participated in this study (25 males and 25 females) after providing their informed consent. The male volunteers (aged 20–58 years) provided their saliva, peripheral venous blood, and semen samples. The female volunteers (aged 26–45 years) provided their saliva, peripheral venous blood, and menstrual blood samples. Exclusion criteria included the history of cancer and treatment with chemotherapy as long non-coding RNAs have abnormal gene expression in tumors (Weakley et al. 2011). The Ethical Committee of the Faculty of Medicine, Cairo University, approved this research.

Sample collection

Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes via venipuncture. Saliva was collected from participants who were asked to spit saliva into test tubes after withholding oral hygiene procedures, taking food or drinks, or smoking for an hour before collection. Semen was provided in plastic cups. Menstrual blood was collected from the vagina using sterile cotton swabs which were left to dry at room temperature for 24 h. All samples were stored frozen at − 20 °C until further processing.

RNA extraction

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and then was analyzed for both quantity and quality with Beckman dual spectrophotometer (USA).

Reverse transcription and real-time PCR

We used 10 ng of the total RNA from each sample for cDNA synthesis. The high capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA) was used for reverse transcription. Then, the cDNA was amplified with the Syber Green I PCR Master Kit (Fermentas) using the Step One instrument (Applied Biosystems, USA) as follows: enzyme activation for 10 min at 95 °C, then 40 cycles of 15 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C for amplification. For each gene, we used 1 μM of forward and reverse primers. Primer sequences for target and reference genes are shown in Table 1.

Calculation of relative quantification (relative expression)

After the RT-PCR run, the data were expressed in cycle threshold (Ct). The PCR data sheet included Ct values of the target genes and the housekeeping reference gene (β-actin). In order to measure the expression of a certain gene, a negative control sample was used. Therefore, the target gene expression was assessed and related to the reference gene as follows:

$$\Delta \text{Ct sample} = \text{Ct assessed gene} - \text{Ct reference gene}$$

$$\Delta\Delta \text{Ct} = \Delta \text{Ct sample} - \text{Ct control gene}$$

And finally, the relative expression (RQ) was calculated according to the following equation:

$$\text{RQ} = 2 - (\Delta\Delta \text{Ct})$$

Statistical analysis

Data were coded and entered using the statistical package SPSS version 25, then were summarized using mean

Table 1 Primer sequences specific for each gene

Gene	Primer sequence: 5′–3′	GenBank accession number
XIST (long non-coding RNA)	F: ATTTTAACTGATCCCATTGAAGATACCACGC R: TCAGAATGTCCAAGAGGAGCCTAAGG	NR_001564.2
RPS4Y1 ribosomal protein S4, Y-linked 1	F: TGGAAGAGGCAAAGTACAAGTTGTGC R: GGATTCCCTTCACTCCCACAGTAAT	NM_001008.3
β-actin	F: GGCGGCACCACCATGTACCCT R: AGGGGCCGGACTCGTCATACT	NM_001101.3

Table 2 Expression of XIST and RPS4Y1 in the studied body fluids of male and female participants

Body fluid	Number of samples		Number of XIST-positive samples	Number of RPS4Y1-positive samples
Peripheral blood	Male	25	0	25
	Female	25	25	0
Saliva	Male	25	0	0
	Female	25	25	0
Menstrual blood	Male	0	0	0
	Female	25	25	0
Semen	Male	25	0	25
	Female	0	0	0

and standard deviation. The non-parametric Kruskal-Wallis and Mann-Whitney tests were used to compare between quantitative variables (Chan 2003a), while the Spearman correlation coefficient was used to correlate between quantitative variables (Chan 2003b). Statistically significant results were considered if *P* value is less than 0.05.

Results

XIST is expressed in all studied female body fluid samples, with no expression in male samples, while RPS4Y1 is expressed in all male blood and semen samples, with no expression in male saliva or female samples (Table 2).

The mean expression values of XIST and RPS4Y1 in the studied body fluids are demonstrated in Table 3.

Comparison between XIST mean values in female body fluids (peripheral blood, saliva, and menstrual blood) shows a statistically significant difference, with the maximum mean expression value in menstrual blood and the lowermost mean value in saliva (Table 4). Pairwise comparisons between these body fluids show statistically significant differences between the saliva and peripheral blood, saliva and menstrual blood, and peripheral blood and menstrual blood (Table 5).

Comparison between RPS4Y1 mean values in male blood and semen does not show any statistical significance (Table 6).

The Spearman correlation between participants' age and female XIST and between participants' age and male RPS4Y1 does not show any statistical significance (Tables 7 and 8).

Table 3 Relative expression (RQ) values of XIST and RPS4Y1 in the studied male and female body fluids

Body fluid		Sex	XIST	RPS4Y1
Peripheral Blood	Mean ± SD	Male	0.00	1.05 ± 0.29
		Female	1.57 ± 0.56	0.00
Saliva	Mean ± SD	Male	0.00	0.00
		Female	0.30 ± 0.11	0.00
Semen	Mean ± SD	Male	0.00	1.35 ± 0.81
Menstrual blood	Mean ± SD	Female	5.64 ± 2.20	0.00

Table 4 Comparison between XIST mean values in the studied female body fluids

Gene		Body fluids			*P* value
		Peripheral blood	Saliva	Menstrual blood	
XIST	Mean ± SD	1.57 ± 0.56	0.30 ± 0.11	5.64 ± 2.20	< 0.001*

**P value is statistically significant*

Discussion

Biological evidence has become extremely important in forensic science. Its presence at the crime scene may provide vital information regarding not only the details about the crime, but also the determination of its owner's identity (Zapata et al. 2015).

The XIST and RPS4Y1 markers have shown encouraging results in sex identification from body fluids and organ tissues (van den Berge and Sijen 2017; Guillén et al. 2014). In this study, we evaluated the use of these RNA markers in the sex identification of a trace donor and also the effect of age on these genes expression.

We chose real-time RT-PCR as the quantification technique because it is a sensitive, accurate, and reproducible method for the detection and quantification of mRNA (Lee et al. 2013; Pfaffl 2004).

We selected the blood, saliva, and semen as the targeted body fluids because of their constant presence at the crime scene. These body fluids are vital in the investigation of many crimes related to sexual abuse, assault and battery cases, and homicides (Zapata et al. 2015). We also added menstrual blood to our studied samples as distinguishing between peripheral and menstrual blood is highly challenging. It requires complex molecular procedures and a considerable degree of experience (Holtkötter et al. 2017).

In this study, we found that XIST is expressed in female body fluid samples (peripheral blood, saliva, and menstrual blood), with no expression in male samples (blood, saliva, and semen), while RPS4Y1 is expressed in male blood and semen samples.

A number of studies concerning human peripheral blood reported that XIST is expressed in females and RPS4Y1 in males. During the analysis of gene expression

Table 5 Pairwise comparisons between XIST mean values in the studied female body fluids

	P value
Saliva-peripheral blood	< 0.001*
Saliva-menstrual blood	< 0.001*
Peripheral blood-menstrual blood	< 0.001*

**P value is statistically significant*

Table 6 Comparison between RPS4Y1 mean values in male blood and semen

Gene		Blood	Semen	P value
RPS4Y1	Mean ± SD	1.05 ± 0.29	1.35 ± 0.81	1.000

profile using microarray, Guillén et al. (2014) found that RPS4Y1 was one of the highest expressed genes on the Y chromosome, while XIST was the highest expressed gene on the X chromosome, and they suggested that these genes can be used as sex-specific biomarkers. Moreover, Xu et al. (2011) found that XIST was highly expressed in females and RPS4Y1 was highly expressed in males with almost no RPS4Y1 expression in females or XIST expression in males. Additionally, Xu et al. (2011) reported that the ratio of XIST to RPS4Y1 was significantly greater in females which might signify important sex-linked biomarkers for future gene expression analysis in blood.

Menstrual blood is composed of blood, vaginal secretions, and the endometrial cells of the uterine wall (Yang et al. 2012). Since XIST is expressed in female peripheral blood, it would be expressed in the menstrual blood as a cumulative effect. Our results are similar to the previously reported results of van den Berge and Sijen (2017) who observed XIST in four studied menstrual secretion samples with no expression of RPS4Y1.

Saliva is formed and secreted into the mouth by the exocrine salivary glands. Humans possess three main sets of these glands (parotid, sublingual, and submandibular) and numerous minor glands. Most of the saliva (> 80%) is produced by the parotid and submandibular glands (Turner and Sugiya 2002; Cook et al. 1994). Srivastava et al. (2008) studied the gene expression of parotid gland using microarray, and their results demonstrated the much higher expression of XIST in females and the exclusive expression of RPS4Y1 in males. In this study, we did not detect RPS4Y1 in the male saliva. This is in accordance with the van den Berge and Sijen (2017) study in which they observed XIST only in female saliva and did not observe XIST or RPS4Y1 in the single studied male saliva sample.

Table 7 Spearman's correlation between participants' age and female XIST in the studied body fluids

	Body fluid		XIST
Age	Peripheral blood	Correlation coefficient	− 0.212
		P value	0.310
	Saliva	Correlation coefficient	0.049
		P value	0.815
	Menstrual blood	Correlation coefficient	0.196
		P value	0.347

Table 8 Spearman's correlation between participants' age and male RPS4Y1 in the studied body fluids

	Body fluid		RPS4Y1
Age	Blood	Correlation coefficient	0.101
		P value	0.631
	Semen	Correlation coefficient	− 0.058
		P value	0.782

Similar results were reported regarding the positive expression of RPS4Y1 and the negative expression of XIST in semen by van den Berge and Sijen (2017).

Comparisons and pairwise comparisons were made between XIST mean values in the studied female body fluids. These comparisons show statistically significant differences between the saliva and peripheral blood, saliva and menstrual blood, and peripheral and menstrual blood. This can be explained by the facts that gene expression varies extensively among tissues and that some markers are differently expressed between body fluids (Park et al. 2013; Whitehead and Crawford 2005).

In this study, the Spearman correlation between participants' age and female XIST and between participants' age and male RPS4Y1 does not show any statistical significance.

Age has an evident effect on gene expression (Srivastava et al. 2008; Lu et al. 2004). However, studies which examined age influence on gene expression reported that age-biased genes are genes responsible for several biological functions such as blood coagulation, inflammatory response, transcription, cellular metabolic process, signaling pathway, cell death, and apoptosis, which reflect the cellular aging process in the nucleus and cytoplasm (Xu et al. 2011; McLean and Le Couteur 2004). In addition, Jansen et al. (2014) reported that the fold changes between males and females of the identified sex-specific genes in peripheral blood were highly concordant between age ranges suggesting that the identified sex effects are present in all ages.

Conclusions

Real-time RT-PCR was successfully applied to all donor samples. The observed results suggest that XIST can be used as a female-specific marker in peripheral blood, saliva, and menstrual blood and RPS4Y1 can be used as a male-specific marker in blood and semen. Further studies including other body fluids and dried and mixed samples should be addressed to consider these markers as universal for sex identification from different body fluids.

Abbreviations

AMELX: Amelogenin X-linked; AMELY: Amelogenin Y-linked; cDNA: Complementary deoxyribonucleic acid; Ct: Cycle threshold; EDTA: Ethylenediaminetetraacetic acid; RNA: Ribonucleic acids;

RPS4Y1: Ribosomal protein S4, Y-linked 1; RQ: Relative expression; RT-PCR: Reverse transcription polymerase chain reaction; XCI: X-chromosome inactivation; XIST: X-inactive specific transcript

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FH and HA are responsible for the interpretation of results and the writing and final revision of the paper. DA and MW are responsible for the experimental work of the research. All authors read and approved the final manuscript.

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The Ethical Committee of the Faculty of Medicine, Cairo University, approved this research.

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Not applicable.

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The authors declare that they have no competing interests.

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