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IGF2 and *IGF1R* mRNAs Are Detectable in Human Spermatozoa

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Purpose: Oligozoospermia is highly prevalent worldwide. Studies have reported a lower methylation rate in the *H19* differentially methylated region at the sperm level in oligozoospermic patients than in controls. *IGF2/H19* are the best-known pair of imprinted genes. However, no studies have yet evaluated whether they are transcribed in human sperm. To assess whether *IGF2* and *IGF1R* mRNAs are present in human sperm and if their levels are correlated with sperm concentration and total sperm count.

Materials and Methods: Sperm samples (n=22) underwent reverse-transcription quantitative polymerase chain reaction using specific primers to detect *IGF2* and *IGF1R* mRNA levels. They were then correlated with patients' conventional sperm parameters using the Spearman (τ) and Kendall (ρ) rank correlation coefficients.

Results: Statistically significant positive correlations were found between *IGF2* mRNA levels and sperm concentration (τ=0.403, $p<0.01$; ρ=0.587, $p<0.005$) and total sperm count (τ=0.347, $p<0.024$; ρ=0.509, $p<0.015$). *IGF1R* mRNA levels were positively correlated with sperm concentration (τ=0.595, $p<0.001$; ρ=0.774, $p<0.001$) and total sperm count (τ=0.547, $p<0.001$; ρ=0.701, $p<0.001$). Apart from *IGF1R* mRNA and sperm morphology (τ=0.325, $p<0.05$; ρ=0.461, $p<0.05$), no additional correlations were found between the levels of these transcripts and other conventional sperm parameters.

Conclusions: *IGF2* and *IGF1R* mRNAs were found to be present in human spermatozoa and their transcription levels were positively correlated with sperm concentration and total sperm count. Spermatozoa are the only source of *IGF2* mRNA since *IGF2* is a paternally-inherited gene. Further studies are needed to evaluate its role in human fertilization.

Keywords: H19, imprinted; *IGF2*; *IGF1R*; Infertility, male; Oligozoospermia; Sperm count

INTRODUCTION

We recently reviewed the epigenetics of male fertility, mainly focusing on DNA methylation, chromatin remodeling, histone modifications, and sperm RNAs [1]. Surprisingly, we found a great discrepancy between the tremendous amount of knowledge on DNA methylation and the low level of knowledge on sperm RNAs. Although several studies have investigated the impact of sperm DNA methylation on male fertility and sperm count [2], very little is known about sperm RNAs in this regard [1].

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Thousands of different RNAs (including coding and non-coding RNAs) have been detected in human spermatozoa [3]. As indicators of sperm transcriptional activity [4,5], RNAs in these cells likely derive from unmethylated or hypomethylated genes, which the transcriptional apparatus can access [1]. Several genes have been found to be differentially methylated in infertile patients compared to controls, and the most investigated pair of differentially methylated genes is *H19*/*IGF2* [2]. *H19* codes for a non-coding RNA involved in the suppression of *IGF1R* expression [6], while *IGF2* codes for the homonymous growth factor insulin-like growth factor 2 (IGF2). They are both located on chromosome 11p15.5, and represent the first historically characterized pattern of imprinted genes [7].

In greater detail, *H19* is a maternally-inherited gene (meaning that it is expressed by the maternal allele), while *IGF2* is a paternally-inherited gene (meaning that it is expressed by the paternal allele). *H19* and *IGF2* share common enhancers downstream to *H19*. Their transcription is regulated by the *H19* differentially methylated region (DMR), which is upstream to *H19*. In the maternal allele, the *H19* DMR is unmethylated. This prevents access to the *IGF2* enhancer, thereby allowing *H19* expression and inhibiting *IGF2* expression. Contrastingly, in the paternal allele, *H19* is methylated. This promotes *IGF2* expression and inhibits that of *H19* [1,8].

In humans, fetal spermatogonia are mostly unmethylated in the *H19* DMR, but this region is strongly methylated in mature spermatogonia [9]. Experimental findings have shown that the *H19* DMR methylation rate influences human sperm count, since a lower methylation rate occurs at the sperm level in oligozoospermic patients than in normozoospermic controls [10,11]. However, the mechanism underlying this effect is unknown.

No study has so far evaluated whether the *H19* and *IGF2* genes are transcribed at the sperm level and if their level of transcription is related to sperm count. Therefore, the aim of the present study was to evaluate the presence, if any, of *IGF2* and *IGF1R* mRNAs in human spermatozoa and, if present, to assess possible correlations with conventional sperm parameters.

MATERIALS AND METHODS

1. Patients

Twenty-two semen samples from Caucasian patients attending the Andrology Section, Department of Clinical and Experimental Medicine, University of Catania for semen analysis were consecutively recruited. Since this was an exploratory study aimed at assessing whether *IGF2* and *IGF1R* mRNAs were detectable in human sperm, no exclusion criteria were adopted. The clinical history of each recruited patient was carefully collected.

2. Ethics statement

This study was approved by the Intradivisional Ethics Committee of the Andrology Section, Department of Clinical and Experimental Medicine, University of Catania. Written informed consent was obtained from all participants. All procedures were carried out in accordance with the Helsinki Declaration for medical research involving human subjects.

3. Sperm analysis

Semen samples were collected by masturbation into a sterile container after 2 to 7 days of sexual abstinence and were transported immediately to the laboratory. Each sample was evaluated for conventional sperm parameters according to the World Health Organization (WHO) criteria [12].

4. RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

Semen samples were diluted with phosphate-buffered saline to ~10^6 spermatozoa/mL and underwent osmotic shock to eliminate the non-gamete component, as described elsewhere [13]. Total RNA was extracted with TRIzol® Reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. RNA concentration and purity were determined using an Eppendorf Biophotometer. cDNA reverse transcription was carried out for each sample using a cDNA synthesis kit (Thermo Scientific Maxima First Strand cDNA Synthesis Kit for quantitative real-time polymerase chain reaction), according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA).

The reverse and forward primers were as follows: *IGF2*,

forward primer 5'-CCCGTGGGCAAGTTCTTCC-3', reverse primer 5'-CGCTGGGTGGACTGCTTC-3'; *IGF1R*, forward primer 5'-CAAGCCTGAGCAAGATGATTC-3', reverse primer 5'-GAACTTATTGGCGTTGAGGTATG-3'; actin, forward primer 5'-ACCTTCTACAATGAGCTGCG-3', reverse primer 5'-TCCATCACGATGCCAGTGGTA-3'.

Briefly, total RNA was extracted from samples using the TRIzol reagent (Life Technologies) and quantified by reading the optical density at 260 nm. In particular, 2.5 μg of total RNA was reverse-transcribed (RT) (Thermo Scientific) to a final volume of 20 μL. Quantitative polymerase chain reaction was performed using 25 ng of the cDNA prepared by RT and a SYBR Green Master Mix (Stratagene, Amsterdam, The Netherlands; Agilent Technologies, Santa Clara, CA, USA), in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. The mRNA level of each sample was normalized against β-actin mRNA and expressed as fold changes *versus* the levels in the sample with the lowest sperm count.

5. Statistical analysis

The statistical analysis was conducted using IBM SPSS ver. 22.0 for Windows (IBM Corp., Armonk, NY, USA). The Mann–Whitney test was used to compare the distribution of the variables. The Spearman (ρ) and Kendall (τ) rank correlation coefficients were used to investigate the associations between gene expression and conventional sperm parameters, as appropriate. A $p<0.05$ was considered to indicate statistical significance. The results are expressed as mean±standard deviation.

RESULTS

The patients enrolled in this study were 32.1±9.3 years (range, 15–54 years). They underwent semen analysis for the following reasons: an andrological

Table 1. Conventional sperm parameters of the semen samples

Sample No.	Sperm concentration (million/mL)	Total sperm count (million/ejaculate)	Progressive motility (%)	Total motility (%)	Normal morphology (%)	Leukocyte concentration (million/mL)
ID1	130	390	32	62	9	1.3
ID2	85	425	30	63	4	0.85
ID3	100	230	15	59	8	10
ID4	75	300	18	53	5	0.75
ID5	45	135	33	55	4	1.35
ID6	20	120	34	61	2	0.6
ID7	20	30	18	58	5	0.2
ID8	33	99	25	68	8	0.99
ID9	60	180	33	62	7	0.6
ID10	45	103.5	25	60	10	0.9
ID11	50	100	20	60	8	0.5
ID12	50	100	25	65	10	3
ID13	18	45	11	60	7	0.18
ID14	6	27	28	63	9	0.06
ID15	2	9	10	45	3	3
ID16	9	36	4	48	1	10.8
ID17	2	10	9	36	2	0.02
ID18	26	65	5	54	3	2.08
ID19	44	154	37	65	7	1.32
ID20	200	400	18	70	3	6
ID21	26	117	29	65	4	0.26
ID22	35	140	15	60	3	1.4
Mean±SD	49.1±46.9	148.2±129	21.6±10.0	58.7±7.8	5.6±2.8	2.1±3.0
l.v.	>15	>39	>32	>40	>4	<1

SD: standard deviation, l.v.: lower value according to the World Health Organization criteria (2010).

check-up (n=14), varicocele (n=6), premature ejaculation (n=1), and infertility (n=1). Most of the patients were not interested in fertility at the time of counseling. None of them had genetic abnormalities.

The conventional sperm parameters of each patient and their mean values are shown in Table 1.

IGF2 and *IGF1R* mRNAs were both detectable at the sperm level. We found a positive correlation between *IGF2* and *IGF1R* mRNA levels (ρ=0.8, p<0.001; τ=0.6, p<0.001). *IGF2* and *IGF1R* mRNA levels were analyzed in relation to sperm concentration, total sperm count, sperm progressive and total motility, and sperm morphology. A statistically significant positive correlation was found between *IGF2* mRNA levels and sperm concentration (Fig. 1A) and between those levels and total sperm count (Fig. 1B). In addition, *IGF1R* mRNA levels were positively correlated with sperm concentration (Fig. 1C) and total sperm count (Fig. 1D). With the exception of *IGF1R* mRNA and sperm morphology, no additional correlations was found between the levels of these transcripts and other conventional sperm parameters (Table 2).

Among the entire cohort, on the basis of total sperm count, two groups could be identified: group 1 (n=5;

Table 2. Results of the correlation analysis

Variable	*IGF2* mRNA	p-value	*IGF1R* mRNA	p-value
Sperm concentration	τ=0.403	0.009	τ=0.595	<0.000
	ρ=0.587	0.004	ρ=0.774	<0.000
Total sperm count	τ=0.347	0.024	τ=0.547	<0.000
	ρ=0.509	0.015	ρ=0.701	<0.000
Progressive motility (%)	τ=-0.13	0.932	τ=0.181	0.246
	ρ=-0.18	0.936	ρ=0.221	0.323
Total motility (%)	τ=0.044	0.777	τ=0.115	0.461
	ρ=0.060	0.790	ρ=0.145	0.521
Normal morphology (%)	τ=0.226	0.155	τ=0.325	0.040
	ρ=0.312	0.158	ρ=0.461	0.031

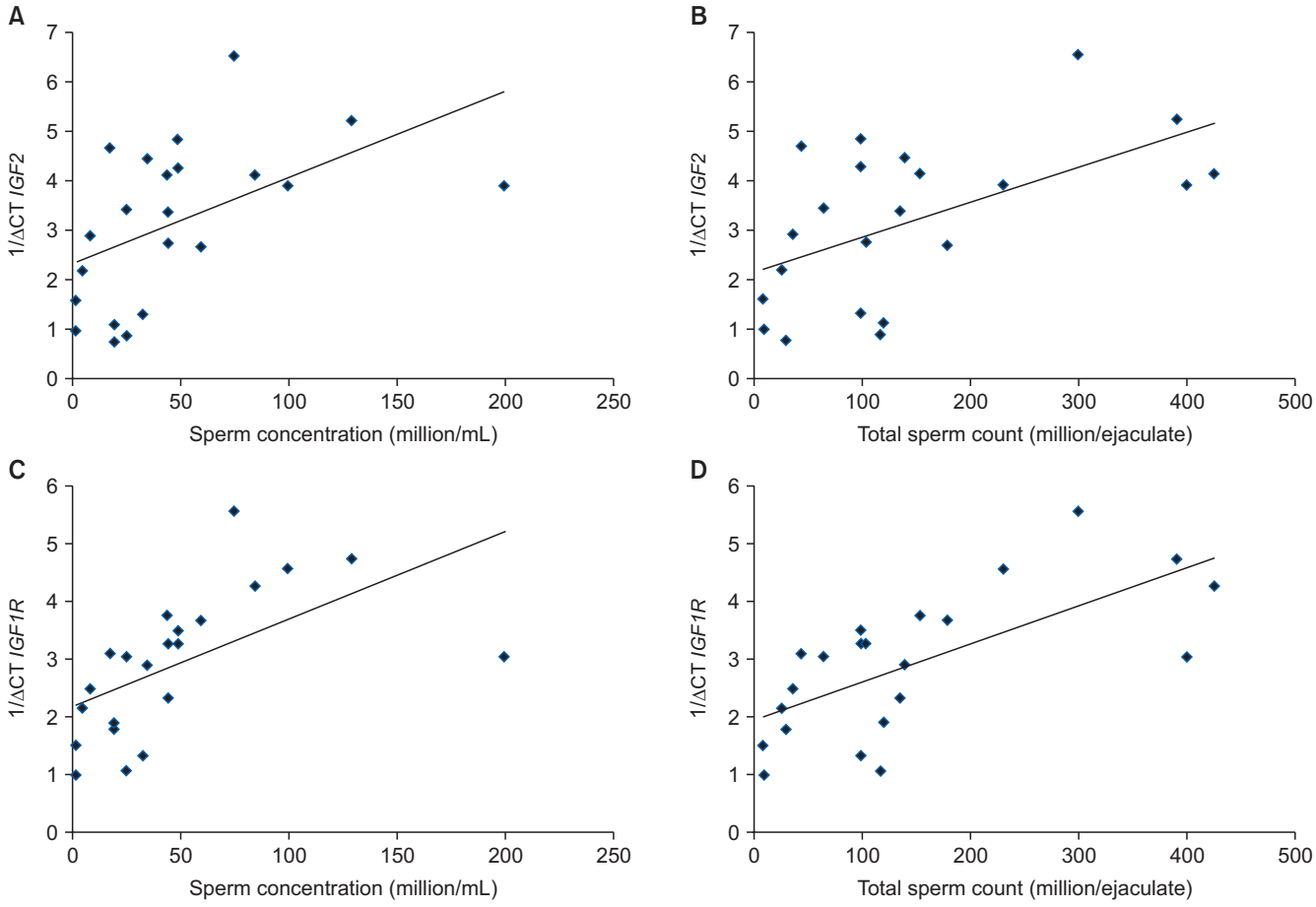

Fig. 1. Correlation analysis of sperm *IGF2* and *IGF1R* mRNA levels with sperm concentration and count. Sperm *IGF2* mRNA levels were positively correlated with (A) sperm concentration (τ=0.403, p<0.01; ρ=0.587, p<0.005) and (B) total sperm count (τ=0.347, p<0.024; ρ=0.509, p<0.015) (n=22). Sperm *IGF1R* mRNA levels were positively correlated with (C) sperm concentration (τ=0.595, p<0.001; ρ=0.774, p<0.001) and (D) total sperm count (τ=0.547, p<0.001; ρ=0.701, p<0.001) (n=22).

Table 3. Conventional sperm parameters of the semen samples

Variable	Group 1 (n=5)		Group 2 (n=17)	
	Mean±SD	Range	Mean±SD	Range
Sperm concentration (million/mL)	7.8±7.4*	2–20	61.3±48.7	18–200
Total sperm count (million/ejaculate)	22.4±12.2*	9–36	187.5±124.2	45–425
Progressive motility (%)	13.8±9.4*	94–28	23.8±9.2	5–37
Total motility (%)	50.0±10.7*	36–63	61.3±4.6	53–70
Normal morphology (%)	4.0±3.2	1–9	6.0±2.7	2–10

Group 1: patients with a total sperm count <39 million/ejacalate, Group 2: patients with a total sperm count ≥39 million/ejacalate, SD: standard deviation.
*p<0.05 *vs.* group 2.

age: 29.6±8.2 years), consisting of patients with a total sperm count <39 million/ejaculate and group 2 (n=17; age: 32.9±9.7 years), comprising those with a total sperm count ≥39 million/ejaculate. Group 1 showed lower sperm concentration, total sperm count, and sperm progressive and total motility compared to group 2 (Table 3). Both *IGF2* (1.7±0.9 *vs.* 3.6±1.5, p<0.05) and *IGF1R* (1.8±0.6 *vs.* 3.3±1.2, p<0.05) mRNA expression levels were significantly lower in group 1 than group 2.

DISCUSSION

Oligozoospermia is defined as an abnormally low sperm concentration and total sperm count. According to the WHO (2010) criteria [12], the cut-offs for oligospermia are 15 million spermatozoa/mL and 39 million spermatozoa/ejaculate, respectively. Oligozoospermia is highly prevalent worldwide. Recently, a retrospective study esteemed its prevalence to be up to 22% and that of azoospermia to be as high as 12%, among a cohort of 117,979 cases [14]. Furthermore, meta-regression studies have shown a drastic decrease in sperm count with increasing age [15]. The reasons for this decline are not fully understood.

We found that *IGF2* and *IGF1R* mRNAs were detectable in human spermatozoa and that their levels were associated with human sperm count. Indeed, they were both correlated positively with sperm concentration and total sperm count. In addition, although our analysis was limited to a small cohort, patients with oligozoospermia (group 1) had lower *IGF2* and *IGF1R* mRNA expression levels than those with a normal sperm count (group 2). These results seem to be in reasonable agreement with evidence showing a lower *H19* DMR methylation rate in oligozoospermic patients than in controls [10,11]. In fact, lower levels of methylation are known to promote *H19* transcription (with a negative impact on *IGF1R* expression [6]) and to halt transcription of the *IGF2* gene [1,8]. In turn, this inevitably decreases both *IGF2* and *IGF1R* mRNA sperm levels [1]. However, we did not evaluate *H19* DMR methylation in our samples. Therefore, whether this is the mechanism underlying our findings has yet to be demonstrated.

IGF2 and *IGF1R* mRNAs may influence sperm count through the action of their derived proteins as growth factors. IGF2 is an IGF1R stimulatory ligand [16]. Growth factors belonging to the IGF family are secreted by Sertoli cells [17] and may be involved in the promotion of progression through the cell cycle, thereby impacting the number of spermatogonia and, therefore, the final sperm number [18,19].

IGF1 was found to promote the differentiation of spermatogonia to primary spermatocytes in cultures from newt testis [20]. In a recent analysis of seminal concentrations of human growth hormone (GH) and IGF1, a positive correlation between the IGF1-GH coefficient and sperm morphology was reported [21]. This finding suggests that IGF1 may play a role in human spermatogenesis and is in accord with the positive correlation between *IGF1R* mRNA and sperm morphology found in the present study.

Several studies have investigated the protein composition of the seminal plasma and of ejaculated germinal cells in recent years, resulting in the identification of thousands of proteins, the function of which is not always entirely clear [22-24]. IGF1R protein expression in human spermatozoa has been reported, and this protein is known to be involved in sperm capacitation [20]. In contrast, IGF2 protein expression in spermatozoa has never been investigated, and properly designed studies should therefore be carried out to evaluate its expression. Irrespective of protein expression, sperm RNA has been suggested to play a role in human embryogenesis, since it is carried into the oocyte and then transcribed, thereby influencing embryo growth [25-27]. In agreement with this hypothesis, murine studies demonstrated that in the absence of RNA, sperm cells failed to fertilize oocytes [28-30]. This finding reinforces

the role of sperm RNAs in fertility, especially of RNAs coming from paternally imprinted genes. Since *IGF2* is paternally inherited, the only source of *IGF2* mRNA is the spermatozoon, and low levels may therefore compromise fertilization.

CONCLUSIONS

We found that *IGF2* and *IGF1R* are transcribed in human spermatozoa and that the levels of their transcripts are positively related to the amount of sperm. Further studies are needed to investigate whether *IGF2* mRNA levels may have an impact on fertility. These findings may shed light on new diagnostic and/or therapeutic targets in the field of male infertility.

Conflict of Interest

The authors have nothing to disclose.

Author Contribution

Conceptualization: RC, AEC. Data curation: RAC. Formal analysis: CB. Investigation: SLV. Project administration: AEC. Resources: AEC, GL, RC. Software: CB. Supervision: AEC. Writing–original draft: RC. Writing–review & editing: RC, AEC.

Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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