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INVESTIGATING THE EFFECT OF CIGARETTE SMOKING ON THE NKX3.1 AND TMPRSS2 GENES ASSOCIATED WITH MALE FERTILITY

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Abstract

Cigarette's smoking has a wide negative impact on human health, and it's have been related to many serious issues like cancer, heart disease, respiratory system, and number of health problems. Also, smoking can affect fertility in men by affecting the sperm on several levels. Our research will investigate the genetic risk factors in male by focusing on NKX3.1 and TMPRSS2 genes related to male fertility and investigate the correlation between the gene's polymorphisms of three groups

(smokers, non-smokers, and infertile men). The NKX3.1 or NK3 homeobox 1 located on chromosome 8 is the first known prostate epithelium-specific marker it is an androgen regulated transcriptional and tumor suppressor gene this gene encodes for a homeobox-containing transcription factor and the transcription factor involved in development of the testes and prostate. there are not enough research data about NKX3.1 single nucleotide's DNA variations and interaction with cigarettes smoking. The TMPRSS2 gene or transmembrane serine protease 2 is located on chromosome 21 is an endothelial cell surface gene encodes a protein that belongs to the serine protease family. TMPRSS2 is expressed in prostate epithelial cells and is needed for normal prostate function. It's also expressed across the gastrointestinal (digestive) tract, such as in intestinal epithelial cells and across the respiratory tract. This gives TMPRSS2 gene an advantage to study or investigate gene expression influenced by lifestyle habits such as cigarettes smoke. Most of today research is confined to respiratory and cardiovascular system affected by cigarettes smoke but this research will investigate the relation between cigarettes smoking and fertility problems in men by selecting two fertility related genes (NKX3.1 and TMPRSS2) and analyzing single nucleotide polymorphisms (SNP) From 75 blood samples collected from 25 smokers ,25 control and 25 infertile\sub-fertile men. To amplify the regions of interest within the applied gene from extracted DNA, a polymerase chain reaction (qPCR) will be used, and ELISA technique to measure serum hormones level (Testosterone and prolactin and Estrogen). We expect this research to provide new information from a new aspect about the effect of cigarettes smoking on those genes and their relation to male infertility.

INTRODUCTION

The failure of a couple to conceive after a year of regular, unprotected sexual contact is known as infertility. Male infertility, which is significantly contributing in roughly 50% of instances, is described as the inability of a male partner to make a fertile female partner pregnant. Male infertility can be brought on by a variety of conditions, including idiopathic, primary testicular abnormalities, endocrine problems, and sperm transport disorders. Infertility is linked to environmental factors, occupational hazards, and lifestyle factors such as smoking, alcohol consumption, and high body mass index (BMI) and the introduction of new pathogenic factors in the population. Also, several autosomal and sex chromosomal genes have critical effect on spermatogenesis.

Cigarette's smoking has a wide negative impact on human health, and it's have been related to many serious issues like, cancer, heart disease, respiratory system and number of health problems. Also, smoking can affect fertility in men by affecting the sperm on several levels. Cigarette’s smoking is widespread in societies and most of research is confined to respiratory and cardiovascular system. This research will investigate the relation between cigarettes smoking and NKX3.1 and TMPRSS2 gene modifications and provide new information from a new aspect about these two genes by applying Quantitative real-time PCR (qPCR) analysis and multiple biochemical tests. This is the first study of this kind in Saudi Arabia and will provide baseline information for future studies. According to a number of studies, smoking has a negative effect on sperm parameters, seminal plasma, and a number of other fertility factors. The effect that smoking exerts on male fertility is not clearly and completely revealed. Smoking increases the production of reactive oxygen species causing oxidative stress that affects sperm parameters like viability, morphology and function reducing male fertility. The results of various studies, however, have not all been consistent.

MATERIALS AND METHODS

A total of 75 men between the ages of 19 and 60 years, were selected based on their health status from the Health Service Administration Department at King Abdulaziz University and the Health Plus Fertility and Women Health Center in Jeddah. Then they divided into three groups: Control (C) group, which are non-smokers and has no problem in fertility. 25 Infertile (Inf) group, which has problems in fertility, but they are not smokers. 25 Smokers (S) group, which they are smoking but they don’t have apparent fertility problems. samples were collected from both King abdul Aziz University Medical Services Center and Healthplus fertility & woman’s health center in jeddah,SA Sample are chosen depend on Criteria based on fertility history (duration of infertility, number of children) and Semen Analysis (the parameters were all below normal). All DNA sample concentrations were measured using the Thermo Scientific NanoDrop 2000 Spectrophotometer was used to determine the purity of the samples. The Spectrophotometer was adjusted by using nuclease free water as a blank then samples were put by Micro-Volume Pedestal mode. Samples absorbance and purity (ratio between 260 / 280) were recorded. The regions of interest within the applied gene (NKX3.1 and TMPRSS2) will be amplified using Polymerase Chain Reaction (PCR) technique.

DNA extraction and genotyping were performed at the Center of Excellence in Genomic Medicine Research (CEGMR) at King Fahd Medical Research Center (KFMRC), Jeddah, SA and Haven Scientific Innovation Center at King Abdullah University of Science and technology (KAUST), Jeddah ,SA.

RESULTS

Statistical analysis was delivered by Python 3.11.4. numeric variables are described by Mean±SD and Median [Min:Max] and compared using ANOVA and t-test if parametric assumptions met or Kruskal Wallis and Manwhitney tests in case of violation of parametric assumption. Moreover, categorical variables are described by count(percentage) and is compared by Chisquare test if expected count in a any cell is greater than 5 otherwise Fisher exact test was used. p-Value less than 0.05considered significant.

Table (1): Description of the SNPs of the whole samples.

SNP	Type		Count (%)
rs2228013	G/G	Normal	69(92%)
	G/A	Hetero	4(5%)
	Undetermined	---	2(3%)
rs12329760	C/C	Normal	45(60%)
	C/T	Hetero	28(37%)
	T/T	Homo	2(3%)

6.2.1 rs2228013

The rs2228013 in our sample has 92% the normal type whereas, 5% has the hetero type and finally there was 2 samples (constitute 3%) has a missing value (undetermined). The heterotype is examined whether it is correlated with infertility or smoking. It deserves noting that the heterotype did not appear in the smokers group and it appears only two times in the control as

well as in the infertile group. Because of the very low frequency of G/A, and the sample can be considered G/G then statistical comparing between G/A, and G/G (when possible) will not be valid and will not be accepted.

Small sample size, of the hetero-type. There is no further analysis that can be done.

Table (2): distribution of different SNP types among groups

		C	Inf	S	p-Values		
					C vs S	**C vs Inf**	**Inf vs S**
rs2228013	G/G	21 (91.3%)	23 (92.0%)	25 (100%)	0.224	>0.999	0.49
	G/A	2 (8.7%)	2 (8.0%)	0 (0.0%)			

6.2.2 rs12329760

The rs12329760 has three types where about 60% of the sample is the normal type C/C and about 37% is the Hetero type and finally only 2 samples have the homo type.

Table (3): comparing different population characteristics among rs12329760 SNPs.

Population Charachtersitics		C/C	C/T	T/T*	p-Value
Age	**19-29**	11(79.0%)	3(21.0%)	2(100.0%)	0.374
	30-39	25(60.0%)	17(40.0%)	0(0.0%)	
	>40	9(56.0%)	7(44.0%)	0(0.0%)	
BMI	**18.5- 24.9**	8(53.0%)	7(47.0%)	2(100.0%)	0.068
	25-29.9	29(74.0%)	10(26.0%)	0(0.0%)	
	>30	8(44.0%)	10(56.0%)	0(0.0%)	
Receive medication	**No**	37(63.0%)	22(37.0%)	2(100.0%)	>0.999

	Yes	8(62.0%)	5(38.0%)	0(0.0%)	
Receive Food supplements	**No**	37(62.0%)	23(38.0%)	2(100.0%)	>0.999
	Yes	8(67.0%)	4(33.0%)	0(0.0%)	
Fertility Problems	**No**	31(66.0%)	16(34.0%)	2(100.0%)	0.565
	Yes	14(56.0%)	11(44.0%)	0(0.0%)	
Receive fertility medication	**No**	40(61.0%)	26(39.0%)	2(100.0%)	0.400
	Yes	5(83.0%)	1(17.0%)	0(0.0%)	
Smokers	**No**	28(57.0%)	21(43.0%)	2(100.0%)	0.267
	Yes	17(74.0%)	6(26.0%)	0(0.0%)	
Chronic Disease	**No**	38(64.0%)	21(36.0%)	2(100.0%)	0.692
	Yes	7(54.0%)	6(46.0%)	0(0.0%)	

***T/T group was excluded when caculateing the p-value because of its low frequency.**

Basic characteristics of population under study was compared among different rs12329760 SNPs. It was clear from table (3) than different SNPs were not statistically significant among population characteristics

Moreover, It was clear that around 60% of the groups has the normal type C/C, this percentage is a bit more in the S group. Moreover, C/T constitutes the rest of the groups. Only the control group has the homotype T/T and it was only two observations. The comparison of distribution of different rs12329760 types among each group showed a non-significant results. Because of the too small sample size the homo group, the T/T will be excluded for any further analysis.

Table (4): distribution of different SNP types among groups

		C	Inf	S	p-Values*		
					C vs S	C vs Inf	Inf vs S
rs12329760	C/C	14(56.%)	14(56.%)	17(68%)	0.831	0.961	0.56
	C/T	9(36%)	11(44%)	8(32%)			
	T/T	2(8%)	0(%)	0(%)			

*** T/T values was ignored when calculating P-value.**

When hormones are compared among different rs12329760 types there were no significant difference among groups. The testosterone was a bit lower in the C/T group. However, this decrement was not significant.

Table (5): comparing hormones levels among different SNP type

Num_Parameter	types	Mean±STD	Median[Min:Max]	p-Value
Testosterone	C/C	20.05±7.63	18.14[6.6:36.54]	0.062
	C/T	16.42±7.94	14.99[3.52:38.72]	
Prolactin	C/C	10.51±4.67	9.67[4.93:27.54]	0.734
	C/T	10.07±4.45	8.54[5.34:26.98]	
FSH	C/C	3.74±2.63	3.2[0.8:14.73]	0.654
	C/T	5.54±8.91	3.34[0.52:48.24]	
LH	C/C	3.44±1.35	3.24[1.28:7.64]	0.386
	C/T	3.85±1.76	3.66[1.38:10.55]	

A two way ANOVA was proposed to check the difference when different groups (C, Inf and S) considered along with the SNP types the interaction effect wasn't significant in all hormones (p-values of interaction are Testosteron:0.863 , Prolactin: 0.616, FSH: 0.188, LH:0.804).

DISCUSSION

Reproduction in males is regulated by hormones via complicated mechanisms. It is regulated by the main endocrine axis, HPG axis, via positive and negative feedback regulations by testicular hormones. Alterations and deficiencies in any of these hormones or their receptors leads to subfertility or infertility (Singupta et al., 2022).

SNPs are used as molecular markers in studying diseases. This can be done by case-control studies including large-scale SNP genotyping in a patient group and healthy control group to compare differences in genotype for the studied disease. Then the relationship between a specific genotype and a phenotype is used to characterize susceptibility genes that are associated with the disease (Kim & Misra, 2007). The results of the current study showed no significant differences in hormone levels between the individuals of the two different SNPs of NKX3.1 meaning that genetic variations of this gene did not affect the hormone levels in males. Also, the results revealed no significant differences in the ratios of the two SNPs among the three studied groups. Our results show no linkage of NKX3.1 gene to hormonal regulation, smoking effect or infertility. The results of the current study are not in line with the findings of Voeller et al. (1997) that C3T polymorphism at nucleotide 154 (C154T) coded for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of NKX3.1 (Voeller et al., 1997). Ouyang et al. (2005) revealed that loss of protection against oxidative damage is linked to loss-of-function of Nkx3.1. Nkx3.1 mutant mice had problems in the expression of many antioxidant and prooxidant enzymes, including glutathione peroxidase 2 (GPx2), Glutathione peroxidase 3 (GPx3), peroxiredoxin 6 (Prdx6), and sulfyhydryl oxidase Q6 (Qscn6). The role of Nkx3.1 could be differentiation of prostate epithelium for protection against oxidative damage (Ouyang et al., 2005). Bethel et al., (2006) and Khalili et al. (2010) suggested that Nkx3.1 gene deletion causes oxidative stress and initiates cancer. It regulates antioxidant and pro-oxidant genes in the prostate in a direct manner and the prostate glands of Nkx3.1−/− mice shows increased oxidative stress.

Nkx3.1 transcription factor directly targets the oxidative stress regulatory genes Gpx2, Prdx6, and Qsox1 and impairs their regulation mouse and human prostate and affects oxidative stress. Loss of Nkx3.1 expression in the prostate causes dysregulation of antioxidant and pro-oxidant direct target gene resulting high ROS levels in the hyperplastic Nkx3.1−/− prostate. These ROS interfere with proliferation in the Nkx3.1−/− prostate by inhibiting expression of pro-proliferative genes (Martinez et al., 2012). Loss of NKX3.1 expression was detected in 34% of hormone-refractory localized prostate cancer, and 78% of metastases (Bowen et al., 2000). Decreased expression of NKX3.1 protein decreases the proliferation, differentiation, and polarity of epithelial prostate cells. Deletion of NKX3.1 disturbs the expression of antioxidants and increases the chance of DNA damage and oncogenic predisposition (Abate-Shen et al., 2008).

In NKX3.1 gene, a C3T polymorphism at nucleotide 154 (C154T) codes for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of NKX3.1 protein. The polymorphism lay NH2-terminal to the homeodomain in a region of the protein that was not conserved between mouse and human. Voeller et al. (1997) found that C3T polymorphism at nucleotide 154 (C154T) coded for a variant protein with a substitution of cysteine for arginine at amino acid 52 (R52C) of NKX3.1 (Voeller et al., 1997).

On the other hand, most studies reported that tobacco smoke impairs spermatogenesis and cause defective sperm function (Martini et al. 2004; La Maestra et al. 2015) due to increased reactive oxygen species (ROS) and oxidative stress (OS), DNA damage, and germ cell apoptosis. Kumar et al. (2015) found that smoking men had increased levels of seminal ROS, increased sperm DNA fragmentation index and increased 8-hydroxy-2-deoxyguanosine (8-OHdG) levels. Another study revealed that smoking men had decreased activity of sperm glutathione peroxidase (GPx-1, 4) and reduced in the mRNA expression of glutathione reductase in spermatozoa (Viloria et al. 2010). Many studies have reported that nicotine can cause genetic defects in testes and affect (Kumar et al. 2015). Nicotine acts as an oxidizing agent that disrupts the sperm plasma membrane (Omolaoye et al., 2022).

The results of the current study showed no significant differences in hormone levels between the individuals of the two different SNPs of TMPRSS2 meaning that genetic variations of this gene did not affect the hormone levels ion males. Also, the results revealed no significant differences in the ratios of the two SNPs among the three studied groups. Our results show no linkage of TMPRSS2 gene to hormonal regulation, smoking effect or infertility. TMPRSS2 gene

contains an enhancer13 kb upstream of the transcription start site. This enhancer is pivotal for the androgen regulation of the TMPRSS2 gene. The androgen response element is situated next to two binding sites. The enhancer activity involves the androgen response element and the GATA-2 binding sites. Single nucleotide polymorphism (rs8134378) within this androgen response element reduces binding and transactivation by the androgen receptor. The presence of this SNP might have implications on the expression and/or formation levels of TMPRSS2 fusions because both have been shown to be influenced by androgens (Clinckemalie et al., 2013). These results are not in line with our results as ours revealed no correlation between TMPRSS2 SNPs and infertility. However, this study provides valuable information regarding the effect of smoking on these two gene SNPs in Saudi Arabian. Further studies can help us better understand male infertility in Saudi Arabian men and have valuable implications on infertility treatment in Saudi Arabia.

CONCLUSION

Smoking is considered a major factor leading to male infertility. In this study, we focus on understanding the effect of cigarette smoking on the NKX3.1 and TMPRSS2 genes associated with male fertility in males of Saudia Arabia. Our results show no linkage of NKX3.1, TMPRSS2 gene to hormonal regulation, smoking effect, or infertility. This study can have important implications for understanding the effects of smoking on infertility in Saudi Arabia.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

QPCR, Quantitative real time polymerase chain reaction; DNA, Deoxyribonucleic acid; SNP, Single-nucleotide polymorphism; NKX3.1,Homeobox protein Nkx-3.1; TMPRSS2,Transmembrane serine protease 2; ANOVA, Analysis of variance; , HPG axis, hypothalamic–pituitary–gonadal axis.

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