# Association of *PFKM* Gene Polymorphisms and Susceptibility to Cryptorchidism in a Chinese Han Population

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# Abstract

Cryptorchidism is one of the most common congenital anomalies in newborn boys. There are various risk factors that have been verified to have relationship with cryptorchidism, including exogenous and genetic, but the pathogenesis of cryptorchidism remains unclear. *PFKM* gene is a critical gene encodes for a regulatory enzyme, which limits the rate in the pathway of glycolysis. In order to investigate the possible association between *PFKM* gene polymorphisms and cryptorchidism risk, 3 tag SNPs of *PFKM* gene, rs2228500 (A/G), rs4075913(A/G), and rs11168417(C/T) were selected and genotyped in a hospital-based case-control study involving 140 cryptorchidism patients and 227 healthy controls. The frequency of allele G of SNP rs2228500 is increased in cryptorchidism patients compared to that in controls ($p < 0.05$). Genotypic frequencies of rs2228500 are associated with the susceptibility of cryptorchidism in the codominant model ($p < 0.05$). And compared with G/G genotype in the dominant model, notable decreased frequencies of A carriers (A/G-A/A genotypes) were observed in cryptorchidism patients ($p = 0.0069$, OR = 1.80, 95% CI =1.17-2.75). This research firstly revealed that *PFKM* gene polymorphisms were associated with cryptorchidism in a Chinese Han population.


# Introduction

Cryptorchidism is a common congenital malformation in newborn boys, affecting 3–5% of the full-term-born male infants and up to 30% of preterm or low birth-weight male neonates (1). Cryptorchidism is one of the prevalent causes of azoospermia patients (2), and is also an important risk factor for testicular cancer (3). The conventional treatment for cryptorchidism is to reposition the cryptorchid testis into the scrotum through orchiopexy (4), which should be performed within one year of birth in cryptorchid neonates, whereas early childhood acquired cryptorchidism should be treated immediately (5). Cryptorchidism often occurs as an isolated disease, and the pathogenesis is not completely clear, since it is multifactorial and highly variable among individuals.

The complexity of the biological mechanisms driving testicular descent is probably a vital factor in the complexity of the etiology of the disease. However, several evidences proved environment and genetic factors may both play critical roles in the development of cryptorchidism (6).

The *PFKM* gene is located within a 41-kb region at chromosome 12q13.11. *PFKM* is the encoding gene for phosphofructokinase-M, which plays an important role in energy metabolism and muscle cell homeostasis. Phosphofructokinase-M is one of the key speed-limiting enzymes for glycolysis, and it is involved in the energy metabolism pathway through glycolysis (7). In human cells, phosphofructokinase (PFK) consists of 3 isozyme types, which are liver-type (PFKL), platelet-type (PFKP), and muscle-type (PFKM). These compositions vary depending on tissue types and PFKM is abundant mainly in skeletal muscles (8). In recent years, PFKM has attracted extensive attention in tumor research and many studies have shown that it is highly correlated with tumor cell growth (9–11). But in earlier in vivo studies have shown that PFK showed high activity in the rat testis during maturation (12, 13). Furthermore, PFK

activity is decreased in the testis in the early cryptorchidism (12). Hence, PFKM may participate in the normal growth and development of testis and testicular diseases.

We speculated that the *PFKM* gene may associate with cryptorchidism risk. To reveal whether *PFKM* is involved in the development of cryptorchidism, we conducted a case-control study to assess the role of 3 tag SNPs of PFKM (rs2228500, rs4075913, and rs11168417) in patients with cryptorchidism. To our knowledge, this is the first study to evaluate the correlation between *PFKM* gene polymorphisms and cryptorchidism risk.

## Material And Methods

# Study Subjects

The retrospective study was approved by the Ethics Committee of the West China Hospital and the written informed consents were obtained from all participants. 140 patients with cryptorchidism and 227 unrelated healthy volunteers were enrolled in this study, and all the patients were recruited from the West China Hospital from February 2017 to March 2020. The boys were examined shortly after birth and again at 3 months of age to determine. The examination technique and the definition of cryptorchidism developed by Scorer (14) were applied. All examinations for these children were performed in supine position under warm conditions. Testicle position was recorded after manipulation of testis to the farthest distal position along the pathway of normal descent by using firm and non-forced traction. Re-confirmation of the diagnosis before orchidopexy were conducted when the patient is around 1-year old. 105 cases manifested unilateral cryptorchidism and 35 cases were bilateral cryptorchidism in the case group. The testis position was decided by the higher one in bilateral cases. Among patients, the testis of 46 cases were in the superficial inguinal pouch while 34 cases in the pre-scrotal region, 31 cases in the external ring, 19 cases in the inguinal canal, and 10 cases in the internal ring. The subjects in the control group (mean age 4.8 ± 1.2 years, range 1 month-14 years) were admitted for resection of foreskin. For individuals in both groups, premature or low birth-weight infants and any personal or family history of cryptorchidism or serious disease were excluded to avoid multifactorial effects. All the subjects were Chinese Han population living in the Sichuan Province of southwest China.

# SNP selection and genotyping

Tag SNPs are representative SNPs in genome regions with high linkage disequilibrium. Three tag SNPs of the *PFKM* gene were selected out according to the data from tag SNPs genotyped in the CHB (Han Chinese in Beijing, China) population sample of the international HapMap project (http://www.hapmap.org/index.html.zh).

DNA isolation kit from BioTeke (Peking, China) was used to extract genomic DNA from each individual's blood sample according to the manufacturer's instructions and the DNA products were stored at -20℃ for further genotype. The rs2228500 (A/G), rs4075913 (A/G), and rs11168417 (C/T) SNPs in the *PFKM* gene were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

analysis. Primers for PCR were designed by the online software Primer3.0. The primer sequences and the information about restriction enzymes for digesting PCR products are shown in Table 1. About 10% of the samples were randomly selected for repeated genotyping, thus the results were 100% consistent.

Table 1
Information on primer and enzymes

| SNPs | Primer (5'-3') | Annealing temperature (°C) | Enzyme | Product length(bp) |
|---|---|---|---|---|
| rs2228500 | F: tgtctctggggagctgactt | 58 | Hpy188I | 122 |
| | R: acgcttcaccaggttgtagg | | | |
| rs11168417 | F: attctactggcattttatggatacaaaca | 60 | NdeI | 178 |
| | R: gcccctcacattactacatgc | | | |
| rs4075913 | F: aaggggcttggtgttaaggt | 58 | DdeI | 90 |
| | R: atggccattctatgggtttg | | | |
| SNP: single nucleotide polymorphism; bp: base pair. | | | | |

# Statistical analysis

SPSS ver. 26.0 (SPSS Inc., Chicago, IL, USA) was applied to analyze all the data. The allele and genotype frequencies of the 3 selected SNPs were obtained through directed computing and the Hardy-Weinberg equilibrium was evaluated by Chi-square test. The genotype associations between the *PFKM* gene and cryptorchidism susceptibility were calculated by SNPstats online analysis software (https://www.snpstats.net/start.htm), which assessed the frequency distributions within 4 genetic models (codominant, dominant, recessive, and overdominant) in cryptorchidism patients and healthy controls. Odds ratio (OR) and respective 95% confidence interval (95%CI) were reported to evaluate the statistical differences between alleles and genotypes. $P$ value < 0.05 was considered as statistically significant.

# Results

Three tag SNPs were genotyped in 140 cryptorchidism patients and 227 healthy control subjects. The genotype distributions of these polymorphisms all conformed to the Hardy-Weinberg equilibrium. The allele frequencies of the *PFKM* tag SNPs in patients with cryptorchidism and control individuals are summarized in in Table 2. The allele frequencies of rs4075913 and rs11168417 polymorphisms have no significant differences between cryptorchidism patients and healthy controls. However, the frequency of the G allele of the rs2228500 in patients with cryptorchidism is significantly higher than those in controls (78% *vs.*69%). By contrast, the A allele frequencies of rs2228500 decreased (22% *vs.*31%) in the case

group. A significantly increased cryptorchidism risk was found to be associated with the G allele of the rs2228500 ($p = 0.010$, OR = 1.58, 95%CI = 1.11–2.24).

As shown in Table 3, the genotype distributions of G/G, A/G, and A/A of rs2228500 were 60.7%, 33.6%, 5.7% in the case group, and 46.3%, 44.9%, and 8.8% in the control group, respectively. Obviously, significance could been observed in the codominant model ($p = 0.025$). In the dominant model, compared with the G/G genotype, A/G-A/A genotypes were associated with a significantly decreased risk of cryptorchidism ($p = 0.0069$, OR = 1.80, 95% CI = 1.17–2.75). No significant correlation was observed in any genetic models of rs4075913 and rs11168417 polymorphisms with the risk of cryptorchidism.

Table 2
Allele frequencies of tag SNPs in PFKM gene among cryptorchidism patients and controls and their associations with cryptorchidism risk.

| SNPs | Allele | Patients N = 140(%) | Controls N = 227(%) | *p* |
|---|---|---|---|---|
| rs2228500 | G | 217(78) | 312(69) | 0.010 |
| | A | 63(22) | 142(31) | |
| rs4075913 | A | 190(69) | 190(62) | 0.084 |
| | G | 86(31) | 86(38) | |
| rs11168417 | C | 252(0.9) | 418(0.92) | 0.334 |
| | T | 28(0.1) | 36(0.08) | |

N: the number of individuals; $p < 0.05$ was statistically significant and bold values indicate a significant difference.

# Discussion

Cryptorchidism refers to undescended testis, is one of the most frequent urogenital abnormalities in newborn boys (15), and it is the best-characterized risk factor for reduced fertility in males and testicular neoplasia (6). In human, the testosterone and insulin-like factor 3 (INSL3) are the main regulators for testicular descenting from the abdominal cavity to the bottom of the scrotum (16, 17). Mutations in the genes encodes for INSL3 and its receptor and androgen receptors were considered to be the main cause of some forms of cryptorchidism (18–20). Although some cases of cryptorchidism in humans can be attributed to known genetic defects and several pathogenetic mechanisms for cryptorchidism have been described, the exact cause of cryptorchidism in most patients remains unknown.

*PFKM* gene maps on chromosome 12q13.11 and its coding region consist of 2340 bp nucleotides, which encodes approximately 780 amino acids (21). A key glycolytic regulatory enzyme, PFKM, which is concerned as an energy activator of muscle glycolysis, is encoded by the *PFKM* gene. Recently, there have been several researches focused on the association between *PFKM* mutations and different types

of cancers, such as bladder cancer (22), breast cancer (23), and ovarian cancer (24), etc. The funny fact that most of these cancers are also highly correlated with hormones (25–27). The occurrence of cryptorchidism was also thought to be influenced by the hormones coincidentally. What is even more interesting is that earlier research in rat embryos indicated that growth retardation and congenital defects could be caused by interfering with glycolysis, which is an important source of ATP production (28). As is known to us, PFKM is a pivotal regulator of cellular glycolysis by catalyzing the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. Taken together, there may be a significant role of the *PFKM* gene to play in the pathogenesis of cryptorchidism. Among the 3 tag SNPs we have chosen in *PFKM*, one SNP is located in the intron region and was observed to be associated with cryptorchidism risk, and the other two SNPs are located in the synonymous codon regions and both of them have no significant correlation with cryptorchidism risk. Julia S *et al.*(29) identified the muscle patterning defection is association with cryptorchidism in the rat fetal. Therefore, we suspected that the tag SNP rs2228500 may be affecting protein structure by influencing coding amino acids. In addition, it may affect the energy metabolism in muscle of glycolysis pathway, which may be a key factor in the lack of testicular descent motivation.

In summary, we investigated the impact of the *PFKM* gene polymorphism on cryptorchidism, and we observed significant differences in the frequency of alleles and genotypes at 1 tag SNP between patients and controls. We have offered primary evidence that the G allele and the G/G genotype of rs2228500 SNP in the *PFKM* gene are more frequent in patients with cryptorchidism than healthy controls. It implies that the polymorphism of the *PFKM* gene locus (rs2228500) may be a new genetic marker for cryptorchidism susceptibility and these alleles and genotypes may be risk factors for this disease.

Although we detected the association between the *PFKM* SNPs and cryptorchidism, there were certain limitations in this study. The sample size and ethic types of this study were small and these results needs to be further confirmed in a larger cohort. Moreover, the function and the underlying signal transduction mechanisms of *PFKM* gene in cryptorchidism development need to be clarified.

Table 3
Distributions of PFKM SNPs among cryptorchidism patients and controls as well as their associations with cryptorchidism susceptibility.

| Genetic model | Genotype | Patients | Controls | Logistic regression | |
|---|---|---|---|---|---|
| | | N = 140(%) | N = 227(%) | OR(95%CI) | *p* |
| rs2228500 | | | | | |
| Codominant | G/G | 85(60.7%) | 105(46.3%) | 1.00 | 0.025 |
| | A/G | 47(33.6%) | 102(44.9%) | 1.76(1.12–2.75) | |
| | A/A | 8(5.7%) | 20(8.8%) | 2.02(0.85–4.82) | |
| Dominant | G/G | 85(60.7%) | 105(46.3%) | 1.00 | 0.0069 |
| | A/G-A/A | 55(39.3%) | 122(53.7%) | 1.80(1.17–2.75) | |
| Recessive | G/G-A/G | 132(94.3%) | 207(91.2%) | 1.00 | 0.27 |
| | A/A | 8(5.7%) | 20(8.8%) | 1.59(0.68–3.72) | |
| Overdominant | G/G-A/A | 93(66.4%) | 125(55.1%) | 1.00 | 0.03 |
| | A/G | 47(33.6%) | 102(44.9%) | 1.61(1.04–2.50) | |
| Log-additive | | | | 1.58(1.11–2.24) | 0.0089 |
| rs4075913 | | | | | |
| Codominant | A/A | 65(47.1%) | 87(39.5%) | 1.00 | 0.21 |
| | A/G | 60(43.5%) | 101(45.9%) | 1.26(0.80–1.98) | |
| | G/G | 13(9.4%) | 32(14.6%) | 1.84(0.89–3.78) | |
| Dominant | A/A | 65(47.1%) | 87(39.5%) | 1.00 | 0.16 |
| | A/G-G/G | 73(52.9%) | 133(60.5%) | 1.36(0.89–2.09) | |
| Recessive | A/A-A/G | 125(90.6%) | 188(85.5%) | 1.00 | 0.15 |
| | G/G | 13(9.4%) | 32(14.6%) | 1.64(0.83–3.24) | |
| Overdominant | A/A-G/G | 78(56.5%) | 119(54.1%) | 1.00 | 0.65 |
| | A/G | 60(43.5%) | 101(45.9%) | 1.10(0.72–1.69) | |
| Log-additive | | | | 1.32(0.96–1.82) | 0.084 |
| rs11168417 | | | | | |
| Codominant | C/C | 111 (79.3%) | 194 (85.5%) | 1.00 | 0.21 |
| | C/T | 28 (20%) | 30 (13.2%) | 0.61 (0.35–1.08) | |

| Genetic model | Genotype | Patients | Controls | Logistic regression | |
|---|---|---|---|---|---|
| | | N = 140(%) | N = 227(%) | OR(95%CI) | *p* |
| | T/T | 1 (0.7%) | 3 (1.3%) | 1.72 (0.18–16.70) | |
| Dominant | C/C | 111 (79.3%) | 194 (85.5%) | 1.00 | 0.13 |
| | C/T-T/T | 29 (20.7%) | 33 (14.5%) | 0.65 (0.38–1.13) | |
| Recessive | C/C-C/T | 139 (99.3%) | 224 (98.7%) | 1.00 | 0.57 |
| | T/T | 1 (0.7%) | 3 (1.3%) | 1.86 (0.19–18.07) | |
| Overdominant | C/C-T/T | 112 (80%) | 197 (86.8%) | 1.00 | 0.087 |
| | C/T | 28(20%) | 30(13.2%) | 0.61(0.35–1.07) | |
| Log-additive | | | | 0.78(0.46–1.30) | 0.21 |
| CI: confidence interval; OR: odds ratio. Bold Values indicate a significant difference at the 5% level. | | | | | |

# Declarations

### Conflicts of Interest

All authors declare that there is no conflict of interest regarding the publication of this paper.

### Author contributions

Data Collection and Curation: Siyu Long, Bin Zhou, Ran Zhang.

Formal analysis: Siyu Long.

Investigation: Siyu Long, Yaping Song, Qinni yang.

Methodology: Siyu Long, Ran Zhang.

Supervision: Bin Zhou, Yanyun Wang, Zhang Lin.

Validation: Zhang Lin.

Manuscript writing: All authors

Final approval of manuscript: All authors

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