Association of FPGS genetic polymorphisms with primary retroperitoneal liposarcoma

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Primary retroperitoneal liposarcoma is generally regarded as a genetic disorder. We have retrospectively genotyped 8 single nucleotide polymorphisms (SNPs) in 6 candidate genes (MDM2, CDK4, CDC27, FPGS, IGFN1, and PRAMEF13) in 138 patients and 131 healthy control subjects to evaluate the effects of genetic factors on individual susceptibility to primary retroperitoneal liposarcoma in Chinese population. Three SNPs (rs2870820, rs1695147, rs3730536) of MDM2 showed significant differences in single-loci genotypes and allele frequencies between case and control groups (p < 0.05). The minor allele G of SNP rs10760502 in FPGS (folylpolyglutamate synthase) gene was significantly associated with increased risk for primary retroperitoneal liposarcoma, compared with major allele A. Our data suggest that FPGS variant in Chinese population may affect individual susceptibility to primary retroperitoneal liposarcoma.

Soft tissue sarcoma accounts for about 1% of adult solid tumors, with 15–20% located in the retroperitoneum1. Although retroperitoneal soft tissue sarcoma is rare, it causes critical morbidity and mortality. Soft tissue sarcomas are composed of more than 50 different histological subtypes, each with specific pathogenesis and clinical outcome. Retroperitoneal liposarcoma is a subtype of liposarcoma, a malignant tumor of mesenchymal origin that may arise in any fat-containing region of the body. Liposarcomas are the 2nd most common (annually 2.5 cases per million) of all soft-tissue sarcomas following malignant fibrous histiocytomas. Primary retroperitoneal liposarcoma accounts for about 45% of primary retroperitoneal neoplasms. This tumor typically arises in persons 40–60 years of age, without any sex difference in incidence. There are 5 histological subtypes: 1) well-differentiated: ~54%, low grade; including lipoma-like; inflammatory and sclerosing; 2) myxoid: ~31%, low to intermediate grade; 3) pleomorphic: high grade; 4) round cell: high grade and 5) dedifferentiated: high grade. The pathological type of primary retroperitoneal liposarcoma determines the therapeutic outcome and likelihood of metastasis. Highly differential liposarcoma is classified as Grade 1 according to the Federation National des Centers de LutteContre le Cancer (FNCLCC) classification, and simple mucin-like liposarcoma is classified as Grade II3,4. A ring chromosome is indicated in many primary retroperitoneal liposarcomas. Altered p53 pathway may play a pathogenic role in tumor progression of myxoid malignant fibrous histiocytoma-like liposarcoma, a dedifferentiated subtype. Previous studies have focused on amplification of the chromosome region 12q13–15, and oncogenes MDM2 and CDK4. However, whether genetic variation in those genes affects primary retroperitoneal liposarcoma risk is unknown. To fill this gap in knowledge, we studied 138 patients and 131 healthy controls to evaluate possible associations of 8 single-nucleotide polymorphisms (SNPs) of 6 genes (MDM2, CDK4, CDC27, FPGS, IGFN1, and PRAMEF13) with primary retroperitoneal liposarcoma risk. Genetic susceptibility markers may be used to identify high-risk individuals for the early detection and prevention of primary retroperitoneal liposarcoma.
Methods

Study population. This study included 138 patients with primary retroperitoneal liposarcoma (experimental group) and 131 control subjects (control group) hospitalized at the Beijing Shijitan Hospital affiliated to Capital University between January 2009 and January 2013. Each patient in experimental group had a pathologically confirmed diagnosis of primary retroperitoneal liposarcoma. All subjects were Chinese Han ethnicity. Recruitment of patients was not restricted with respect to age or gender. During the same period control subjects were recruited from hospitalized patients with different diseases (including various types of cancer other than soft tissue sarcoma) at Shijitan Hospital. Experimental group and control group was frequency-matched by age at the time of enrollment (±5 years) and gender. All study participants were residents of Beijing. The inclusion criteria for this study were available DNA sample and risk factor information. Written informed consent for an interview and a blood sample donation has been obtained from each participant. The study has been approved by the Institutional Review Board of Beijing Shijitan Hospital, Capital Medical University and been conducted in accordance with all ethical guidelines. The following information was obtained by personal interview: smoking, drinking.

Blood sample collection and DNA extraction. Peripheral blood samples (5 mL) were collected from each subject at the time of enrollment, immediately snap-frozen in liquid nitrogen and stored at −80 °C before DNA extraction. Portions of the tissues were treated in the same way as the blood while the residual tissues were paraffin-embedded for histopathological diagnosis according to WHO criteria. Genomic DNA from tumor tissues and peripheral lymphocytes was extracted using a QIAamp DNA

Table 1 | Demographic characteristics of the study population

| Index | Case (%) | Control (%) | p* |
|-------|----------|-------------|----|
| Age (years) |          |             |    |
| ≤40  | 36 (26.1) | 32 (24.5)   | 0.784 |
| 40–60 | 70 (50.7) | 70 (53.4)   |    |
| >60  | 32 (23.1) | 29 (22.1)   |    |
| Gender |          |             | 0.788 |
| Male | 62 (44.9) | 61 (46.5)   |    |
| Female | 76 (55.1) | 70 (53.5)   |    |
| Smoking |          |             | 0.815 |
| Yes | 60 (43.5) | 60 (45.8)   |    |
| No | 78 (56.5) | 71 (54.2)   |    |
| Drinking |          |             | 0.955 |
| Yes | 70 (50.7) | 75 (53.2)   |    |
| No | 68 (49.3) | 66 (46.8)   |    |

*p-value was from χ² test (2-sided).

Table 2 | Clinical characteristics of the patients with primary retroperitoneal liposarcoma

| Index | No. of patients (%) |
|-------|---------------------|
| Tumor size (cm) |          |
| ≤5  | 31 (22.5) |
| 5–10 | 54 (39.1) |
| >10 | 53 (38.4) |
| Tumor site |          |
| Left upper quadrant | 18 (13.0) |
| Right upper quadrant | 21 (15.2) |
| Hypogastrum | 44 (31.9) |
| Pelvic cavity | 15 (10.9) |
| Whole abdomen | 40 (29.0) |
| Surgical procedures |          |
| ≤1 | 51 (37.0) |
| >1 | 87 (63.0) |
| Pathological pattern |          |
| Highly differentiated | 79 (57.2) |
| Poorly differentiated | 40 (29.0) |
| Myxoid | 15 (10.9) |
| Pleomorphic | 4 (2.9) |
| Blood transfusion |          |
| Yes | 37 (26.8) |
| No | 101 (73.2) |
specifications. Briefly, 250 ng of genomic DNA was amplified at 37°C using the TruSeq Amplicon - Cancer Panel (TSACP) provided by Illumina Inc. All samples were genotyped on the Illumina miRNA beadchip assay, which includes >1000 SNPs of 400 tumor-related genes, and two other SNP500 tumor databases, which are provided by the National Institute of Health, USA (http://snp500cancer.nci.nih.gov/). These SNPs were selected from 1) Illumina TruSight Cancer panel, which includes >1000 SNPs, and 2) SNP500 tumor database, which is provided by National Institute of Health, USA (http://snp500cancer.nci.nih.gov/). These genes have been shown to correlate with the susceptibility to tumors of digestive tract.

Data analysis and functional annotation. The genotyping data for each SNP was analyzed using the Illumina SNP Golden Gate Assay Assay (Illumina, Inc., San Diego, CA, USA) according to the manufacturer’s specifications. Briefly, 250 ng of genomic DNA was amplified at 37°C for 20 hours, and then the amplified DNA was fragmented and precipitated. The dried pellet was resuspended and hybridized to the chip. The hybridized chips were incubated at 48°C for 20 hours, washed, and underwent a single-base extension step. After that, beadchips were stained, washed, coated, and dried. Finally, signal-intensity data was generated by an Illumina BeadArray Reader. We randomly selected 20% of the total samples and genotyped them in duplicate, and 99.8% concordance was observed. The inconsistent data were excluded from the final analysis.

Results
Demographic and clinical characteristics of the overall subjects and patients with primary retroperitoneal liposarcoma enrolled in the study are summarized in Table 1 and Table 2, respectively.

Genotypic results. The genotypes and allele frequencies of SNPs in case and control groups are summarized in Table 3. All genotype distributions were in HWE, which is a genetic balance test (Table 4).

Genotype | Hardy-Weinberg equilibrium genetic balance testing |
--- | --- |
rs2870820 | 0.467 |
rs1695147 | 0.0718 |
rs3730536 | 0.199 |
rs2069502 | 0.327 |
rs74348171 | 0.931 |
rs10760507 | 0.327 |
rs11803067 | 0.199 |
rs71183793 | 0.0632 |

Figure 1 | Linkage disequilibrium (LD) of 3 SNPs (rs2870820, rs3730536 and rs1695147).
Table 5 | Association of genotypes with primary retroperitoneal liposarcomas

| Genotype                  | Case/Control (%) | OR* (95% CI)       | P*    |
|---------------------------|------------------|-------------------|-------|
| MDM2 rs2870820            |                  |                   |       |
| CC vs. CT/TT              | 77/65 vs. 23/35  | 1.082 (1.046–3.103)| 0.034 |
| MDM2 rs1695147            |                  |                   |       |
| GG vs. GT/TT              | 59/70 vs. 41/30  | 0.584 (0.347–0.982)| 0.042 |
| MDM2 rs3730536            |                  |                   |       |
| AA vs. AG/GG              | 68/55 vs. 32/45  | 1.762 (1.065–2.916)| 0.028 |
| CDK4 rs2069502            |                  |                   |       |
| AA vs. AG/GG              | 80/82 vs. 20/18  | 0.876 (0.472–1.626)| 0.675 |
| CDC27 rs74348171          |                  |                   |       |
| AA vs. AG/GG              | 70/73 vs. 30/27  | 0.884 (0.511–1.530)| 0.659 |
| FPGS rs10760502           |                  |                   |       |
| AA vs. AG/GG              | 46/68 vs. 54/32  | 0.396 (0.24–0.656) | <0.001|
| IGFN1 rs11803067          |                  |                   |       |
| AA vs. AG/GG              | 59/55 vs. 41/45  | 1.171 (0.715–1.917)| 0.532 |
| PRAMEF1 rs71183793        |                  |                   |       |
| CC vs. TT/CT              | 77/65 vs. 23/35  | 0.642 (0.391–1.056)| 0.081 |

*OR (95% CI) and P value were calculated from logistic regression model adjusted for age, gender, smoking and drinking.

(Table 5). As shown in Figure 1, the genotyping result has been confirmed by sequencing (Fig. 2).

**Protein function prediction.** As shown in Figure 2, SAMtools\(^{12}\) (http://samtools.sourceforge.net/) software was used for spatial analysis of two-dimensional structure of proteins. The FPGS\(^{13,14}\) protein contains 587 amino acids, having a molecular weight of 64609.1 Da. The overall mean hydrophilic coefficient of native FPGS protein is −0.155. The mutated FPGS protein has a molecular weight of 64595.0 Da, with a total average hydrophilic
The coefficient of $-0.156$. The native FPGS has 203 $\alpha$-helix, accounting for 34.58% of the total secondary structure, and 302 random coils, accounting for 51.45% of the secondary structure. The mutated FPGS has 202 $\alpha$-helix, accounting for 34.41% of the total secondary structure, and 303 random coils accounting for 51.62% of the secondary structure.

**Discussion**

In the current study, we have found that genetic polymorphism of FPGS rs10760502A > G is associated with susceptibility to primary retroperitoneal tumor. The genotype GG, compared to AG or AA, is significantly associated with reduced risk for primary retroperitoneal liposarcomas. Patients carrying rs10760502 GG genotype have lower risk compared to AG or AA, indicating that rs10760502 polymorphism is a potential biomarker for primary retroperitoneal tumor. This mutation is within the first exon of FPGS, encoding the 21st amino acid isoleucine. As a missense mutation, ATA $\rightarrow$ GTA, it replaces isoleucine with valine ($I$ [Ile] $\rightarrow$ $V$ [Val]). Based on protein structure predications, it has changed the protein 2D structure (conformation), adding an alpha helix and a random coil, without changing functional domains.

We found 3 SNPs located in introns of the MDM2 gene. Since they did not change the amino acid sequence, there is no evidence that they can affect protein translation process of MDM2. However, we propose that these genetic variants may be in linkage with other functional SNPs or germline mutations within the gene (area), and may serve as tags/markers for hot-spot changes within the genomic region highly associated with the development of primary retroperitoneal tumor. This hypothesis needs to be further investigated. We plan to collect more samples and will examine the association...
between functional SNPs in MDM2 and other FPGS related genes in a large sample size in the near future.

Two previous studies reported that the allele C of rs1544105 was associated with poor response to methotrexate in patients with rheumatoid arthritis in north India15,16. Previous research reported that rs1544105 is the independent risk factors of childhood leukemia. This variant was also associated with poor therapeutic outcome for ALL in children13. FPGS rs1544105C > T polymorphism may modify FPGS expression and affect treatment outcome in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) patients17. Compared to the CT/TT genotypes, the CC genotype was an independent prognostic factor for poor relapse-free survival (RFS) and individuals with the T allele had lower levels of FPGS transcripts. These observations, taken together, provide supporting evidence that FPGS may be involved in tumor genesis. To the best of our knowledge, our study is the first report that FPGS rs10760502 is associated with primary retroperitoneal liposarcoma. Previous studies have demonstrated that downregulation of FPGS expression was observed in anti-folate-resistant cell lines13,14,15; however, to date, there is no convincing evidence that primary retroperitoneal liposarcoma is related to abnormal metabolism of folate. To examine the genotype-phenotype association, we are collecting tissue samples to measure the FPGS protein expression profile in primary retroperitoneal tumor using immunohistochemistry (IHC) assays in our ongoing study, which focuses on identification of biomarkers with prognostic and predictive value for those patients.

FPGS encodes the folylpolyglutamate synthetase enzyme, which plays a central role in establishing and maintaining both cytosolic and mitochondrial folylpolyglutamate concentrations and is essential for folate homeostasis and the survival of proliferating cells. Dysregulation of FPGS directly results in deficiency of folate synthesis. Patients in the absence of folate supplementation have increased risk of DNA breakage, and therefore, may be susceptible to tumorogenesis. There is evidence to support that the lack of folate acid and tumor occurrence has the relevance in leukemia and colon cancer. Folate is required for de novo synthesis of nucleotides A, T, and G (3 of 4 nucleotides required for DNA synthesis). Tumor cells are addicted to folate in order to synthesize DNA prior to cell division.

Antitumor drugs can block tumor cell growth by terminating the folate supply or inhibiting the folate metabolism19-21. Folate deficiency results in inappropriate incorporation of uracil into DNA in place of thymine, due to insufficient methylation of DUMP to dTMP. When uracil is removed in the process of DNA repair, transient nicks are formed, leading to chromosome breakage. In addition, A to G mutation occurs more frequently when the folates are deficient, which may eventually promote neoplastic transformation. Another study, however, demonstrated that abundant folates is a double-edged sword, and that timing can dictate whether it is beneficial or harmful16. Early folate supplementation may be helpful in the prevention of tumor development; however, after DNA damage and resultant neoplastic transformation have occurred, folate supplementation can accelerate tumor progression.

It has been reported that the incidence of colon cancer is decreasing in US and Canada; however, when folate supplementation was implemented, the incidence of colon cancer showed a transient increase, and then began to decrease17. One explanation of this observation is that folate supplementation can increase pre-cancer growth and tumor progression, and therefore, may result in a transient increase in the incidence of colon cancer. Over time, folate supplementation exerts a protective effect, decreases tumor formation, and eventually reduces the incidence of colon cancer.

Our study demonstrated that patients with primary retroperitoneal liposarcomas harbor a mutation in FPGS, causing a functional abnormality of folylpolyglutamate synthase, impaired folate synthesis, and resultant DNA breakage. This study suggests that folate supplementation may be used to decrease tumorogenesis and prevent postoperative tumor recurrence.

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Author contributions
C.M. and F.Z. wrote the main manuscript text; D.L. Y.W. and Y.Z. Did data collection; D.L. and Y.Z. participated in data analysis; Z.Z., B.L., Y.J., G.L. and X.L. Did study conception and design. All authors reviewed the manuscript.
Additional information

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