Correlation between genotype and phenotype in three families with Peutz-Jeghers Syndrome

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Abstract. Peutz-Jeghers syndrome (PJS) is a hereditary disorder characterized by mucocutaneous pigmentation, gastrointestinal (GI) polyposis and an increased risk of certain malignancies. Little is known about the causative genes of PJS, or their association with the clinical phenotypes of PJS. The present study reports the results of clinical and genetic analysis of three Chinese families with PJS. In addition, the medical histories and clinical manifestations of these families were compared. DNA was collected from the blood samples of patients with PJS and controls. Serine/threonine kinase 11 (STK11), olfactory receptor family 4 subfamily C member 45 (OR4C45) and zonadhesin (ZAN) were amplified by polymerase chain reaction, and analyzed by sequencing and cloning. Two PJS-affected members of one family had a de novo single base deletion (NM_00455.4:c.842delC) in the STK11 gene, and their clinical presentations reflected the quantity of mutant STK11 copies in a dose-dependent manner. No pathogenic variants of OR4C45 or ZAN were found in the patients with PJS, although a new single nucleotide polymorphism (NM_003386.2:c.5768delG) of ZAN was identified. The results of the current study identified that a STK11 mutation dose-dependent genotype-phenotype relationship exists in patients with PJS. In addition, an early onset and high severity of oral pigmentations in PJS was indicative of serious GI phenotypes. These findings may aid the diagnosis and treatment of PJS.

Introduction

Peutz-Jeghers syndrome (PJS) is a rare autosomal dominant disease, which is characterized by mucocutaneous pigmentation and gastrointestinal (GI) hamartomatous polyposis (1). The incidence of PJS has been estimated to be between 1/50,000 and 1/200,000 live births (2).

Mucocutaneous pigmentation are present in ~95% of patients with PJS, typically arising during the first decade of life, although there have been cases in newborns (3). These pigmentation may be the first sign of PJS (4). Mucocutaneous pigmentation primarily present as dark brown or blue-brown spots, which may fade during adolescence (5). They tend to accumulate on the lips, oral mucosa, such as the buccal and palate mucosa, and nostrils. In addition, they have been reported on the fingers, toes, palms, soles, anal area and intestinal mucosa.

Diverse GI complications are observed in patients with PJS, including GI bleeding, anemia and abdominal pain, due to intussusception, obstruction, infarction or pathological hamartomatous polyps. Polyps are frequently detected in patients with PJS, primarily in the small bowel (96%), followed by the colon and the stomach (6). In addition, patients with PJS have an increased risk of developing GI, pancreatic, breast and gynecological cancers (7). A number of cases of patients with sporadic PJS suffering from lung, testicular or ovarian cancers have been reported (5).

By the age of 10 years, >1/3 of patients with PJS begin to show initial symptoms, with half of these presenting with further symptoms by the age of 20-25 (5,7,8). Mucocutaneous pigmented lesions appear in infancy or early childhood, while GI polyps arise between the second and third decade of life in 80-100% of patients with PJS (6).
Mutations in a number of genes have been identified in patients with PJS, including serine/threonine kinase 11 (STK11), olfactory receptor family 4 subfamily C member 45 (OR4C45) (11) and zonadhesin (ZAN) (11). In addition, 19q13.4 (12), chromosome Ip and a pericentric inversion in chromosome 6 (13) are regarded as the locations of PJS-causative genes. Mutations in the STK11 gene are considered the primary cause of PJS (14). A previous study reported that 27% of STK11 mutations in patients with PJS were nonsense (15). Other STK11 mutations found in PJS include splice-junction alterations, insertions, and nucleotide or whole gene deletions (14). These mutations may cause frameshift or nonsense changes, leading to an abnormal truncated protein and the loss of kinase activity. Furthermore, the site and type of mutation has also been associated with cancer risk in various reports, as indicated in a recent review (14).

Correlations between STK11 mutations and clinical characteristics in PJS have previously been described. Missense mutations in STK11 typically lead to a later onset of PJS, while truncating mutations may cause an earlier onset, compared with nonsense or non-detectable mutations (15). In addition, the frequency of GI polyps and the occurrence of cancer appear to be positively correlated with truncating mutations of STK11 (16). Patients with PJS with STK11 mutations have a higher risk of developing cancer, with a risk of 81% at the age of 70 years (17). However, the genotype-phenotype correlation of PJS remains poorly understood.

The present study reports the results of clinical and genetic analysis of three Chinese families with PJS, in order to elucidate the genetic profiles of Chinese patients with PJS, and explore the association between gene variants and clinical phenotypes, such as mucocutaneous and GI features, in PJS.

Materials and methods

Clinical diagnosis and evaluation of PJS. Six patients with PJS in three families (F1, F2, and F3; 2 males and 4 females) were enrolled from the Clinic of Oral Rare Diseases and Genetic Diseases, School of Stomatology at the Fourth Military Medical University (Xi’an, China), between September 2012 and April 2014. Five family members (F1I, 40, male; F1II, 35, male; F1II, 32, male; F1II, 6, female; F1II, 2, female) without PJS also participated in this study. The diagnosis of PJS was made on the presence of mucocutaneous pigmentation, typical PJS polyps and a family history (4). Clinical symptoms of all participants were examined and assessed by three different clinicians. The following clinical features were measured: Medical and family histories; quantity, size and distribution of mucocutaneous pigmentation; and the presence of characteristic PJS polyps. Pedigrees of the three families are shown in Fig. 1. The study protocol was approved by the Ethics Committee of the School of Stomatology, Fourth Military Medical University (approval no. 2013-011). Informed consent was obtained from patients and healthy controls prior to participation. For those under the age of 18, consent was obtained from their legal guardians.

DNA preparation and amplification. Of the six patients who participated in this study, four were enrolled in genetic analysis, as one died prior to this stage and another refused to be enrolled in genetic analysis. Total genomic DNA was obtained from whole blood samples of four (F1I, F1II, F1II, F1II) patients with PJS and their family members using the QIAamp DNA Blood Mini kit (cat. no. 51106; Qiagen, Inc., Valencia, CA, USA), according to the manufacturer’s protocol. As a control, a total of 50 unrelated healthy controls were enrolled in this study in April 2014, which included 25 males and 25 females. The age of these controls ranged between 20 and 25 years, with a mean of 23.2 years.

The coding exons of STK11, OR4C45 and ZAN were then amplified using polymerase chain reaction (PCR). Primer sequences and PCR product sizes for individual amplicons are shown in Table I. Each PCR mixture (50 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 50 mM dNTPs, 0.25 µM of each primer, 200 ng genomic DNA and 1 unit Taq DNA polymerase (RUNDE Biotechnology Co., Ltd., Xi’an China). PCR thermal cycling conditions were as follows: Denaturation for 3 min at 93°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C; and 1 min at 72°C. PCR products were purified using the MicroElute DNA Clean-Up kit (cat no: D2500-01; Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturer’s instructions.

DNA sequencing and analysis. PCR products were sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sequence electropherograms produced were analyzed using Sequencher software (version 5.0; Gene Codes Corporation, Ann Arbor, MI, USA).

Plasmid constructs and sequence analysis. In order to examine the mutant copies of STK11 exon 6 identified in F1, purified PCR products were inserted into the pMD18-T vector (Takara Biotechnology Co., Ltd., Dalian, China), producing the recombinant vector pMD18-T-STK11-exon 6. Briefly, purified exon 6 of STK11 (150 ng) and pMD18-T vector (50 ng) were mixed in a final volume of 10 µl, incubated at 16°C for 30 min and then transformed into 100 µl competent DH5α Escherichia coli (Takara Biotechnology Co., Ltd.). For transformation, the DNA/E. coli mixture (150 ng/100 µl) was incubated on ice for 30 min, heat shocked at 42°C for 30 sec and placed on ice for 5 min. Lysogeny broth (LB) medium (400 µl; 1 l of LB mixture was spread onto LB plates containing ampicillin (60 µg/ml) and left to grow overnight at 37°C. For each patient, the successful transformation of E. coli was confirmed in 10 randomly selected colonies by PCR and the pMD18-T-STK11-exon 6 in these colonies was then isolated using the Plasmid Mini kit I (cat. no. D6943-01; Omega Bio-Tek, Inc.). The purified products were sequenced and analyzed as previously described.

Results

Clinical features. The pedigree plot revealed a typical autosomal dominant pattern of inheritance in F1 and F0 (Fig. 1). There was no obvious Mendelian inheritance of PJS in Fc (Fig. 1). The clinical characteristics of the six patients with PJS (4 females, F1I, F1II, F1I, and F1II; 2 males, F1I and F1II)
are summarized in Table II. Their initial symptoms appeared from between 2 and 30 years of age. Mucocutaneous pigmentations in the oral mucosa and perioral areas were observed in all patients. In Family I, Family II1 (Fig. 2A and B) had more severe and conspicuous hyperpigmentations compared with Family I2 (Fig. 2C and D). The most serious case, Family II1, exhibited a black lower lip (Fig. 2E and F). In addition, areas of pigmentation were present on the fingers and toes of two patients (Family I2 and Family II1; Fig. 3A and B).

Four participants exhibited GI polyps (Family I2, Family II1, Family III2 and Family I3). Three of these (Family I2, Family II1, and Family II2) had a history of recurrent masses, having had a total of <5 (Family I2), 15 (Family II1) and >20 (Family II2) polyps. The polyps occurred in the stomach, small intestine and colon (Fig. 3C and D). The most frequent GI symptom in these patients was abdominal pain (n=3). In addition, one patient with GI polyps (Family I2) complained of constipation, and two severe cases suffered from polypl-related intussusception and infarction. One patient succumbed to GI adenocarcinoma at 35 years of age.

Genetic analysis. Two patients with PJS in Family I (Family I2 and Family II1) showed a single base deletion at nucleotide 842 (NM_000455.4:c.842delC) in exon 6 of the STK11 gene, corresponding to amino acid 281 of the STK11 protein. This deletion causes a frameshift, resulting in a truncated protein due to a premature termination codon at amino acid 286 (Fig. 4).

In order to compare the proportion of copies of STK11 with this mutation, subcloning was carried out. Among the 10
randomly selected colonies that were analyzed, this deletion was found in 100% from FαII1 and 10% from FαII2, compared with 0% from the healthy control FαI1. The Fα member with more severe PJS symptoms, FαII1, showed a significantly higher proportion of mutant copies than FαII2.

Two previously reported PJS-associated mutations (OR4C45 c.767-768 and ZAN c.5767) were not found in any of the patients tested (Table III). A single base deletion at nucleotide 5768 (NM_003386.2:c.5768delG) of the ZAN gene was detected in four patients with PJS and three unaffected family numbers (3/5, 60%). This variant was also found in 30 out of 50 (60%) healthy controls. To the best of our knowledge, this variant is a novel single nucleotide polymorphism (SNP).

Table I. Primers used in PCR and product size.

| Gene/position | Forward primer (5'-3') | Reverse primer (5'-3') | Product size (bp) |
|---------------|------------------------|------------------------|------------------|
| STK11 Exon 1  | CGTTTGCTCCTGGGACAG     | GGTCAGTCTCAAGGTTT      | 969              |
| STK11 Exon 2  | GGTACGCCACTTCCACAG     | CTCGTGGCCAGTTCTCCT     | 302              |
| STK11 Exon 3  | GGGACTTGCTCTCTTGAAGGG  | GAAATACAGGAAAGAGACTG   | 612              |
| STK11 Exon 4  | GCCTGGACTCTGCTGTACCT   | ATGTCACCTTGAGAGCC      | 449              |
| STK11 Exon 5  | CCTGAAATCTCCGACC       | CTCGAGTGTGGTGCT       | 289              |
| STK11 Exon 6  | CGGCTTCAGGTTGGGACAT    | TCCTTGGTGCTGTGGGCT     | 294              |
| STK11 Exon 7  | GGACTGCAGTATCCACC      | AGGCACCTGCCCAGACAG     | 345              |
| STK11 Exon 8  | TGGGTCCGAAAACCTGGAC    | TTTGGGACGTTGGGATT      | 381              |
| STK11 Exon 9  | TAAGTGGCTCCCCCTGTGTT   | TGGCATCCAGCGTTGTC      | 355              |
| OR4C45 c.767-768 | ATGAACAATGTAACAGAATTCATCC | AATCACACATAAAGTGTCAGTAC | 526 |
| ZAN c.5767   | AAAGGCCACATCTTGAGT    | TGCAGCAGAGTTCACAGTC    | 574              |

PCR, polymerase chain reaction; STK11, serine/threonine kinase 11; OR4C45, olfactory receptor family 4 subfamily C member 45; ZAN, zonadhesin.

Table II. Clinical features of six patients with PJS in three families.

| Features                        | Patients          |
|--------------------------------|-------------------|
|                                | FβI2  | FβII1 | FαI2  | FαII1 | FαI2  | FαII2  |
| Age at onset of PJS (years)     | 15    | 10    | 11    | 7     | 2     | 24     |
| Age at diagnosis of PJS (years) | 40    | 23    | 25    | 7     | 2     | 30     |
| Mucocutaneous pigmentation     | +     | ++    | ++    | +++   | +     | +      |
| Number of GI polyps             | <5    | 15    | >20   | 0     | 0     | 1      |
| Constipation                    | Y     | N     | N     | N     | N     | N      |
| Abdominal pain                  | Y     | Y     | Y     | N     | N     | N      |
| GI bleeding                     | N     | Y     | Y     | N     | N     | N      |
| GI intussusception              | N     | N     | N     | N     | N     | N      |
| Anemia                          | N     | Y     | Y     | N     | N     | N      |
| Cancer                          | N     | N     | Y     | N     | N     | N      |

+, small and few; ++, medium and many; ++++, large (cover >1/2 the lower lip). Y, yes; N, no; PJS, Peutz-Jeghers syndrome; Fα, family a; Fβ, family b; Fc, family c; STK11, serine/threonine kinase 11; OR4C45, olfactory receptor family 4 subfamily C member 45; ZAN, zonadhesin; GI, gastrointestinal.

Table III. Summary of the genetic analysis of four PJS patients.

| Gene/mutation     | Patients          |
|-------------------|-------------------|
|                   | FβI2  | FβII1 | FαI2  | FαII1 | FαI2  | FαII2  |
| STK11 c.842delC   | +     | +     | -     | -     | -     | -      |
| OR4C45 c.767-768  | -     | -     | -     | -     | -     | -      |
| ZAN c.5767        | -     | -     | -     | -     | -     | -      |
| ZAN c.5768delG    | +     | +     | +     | +     | -     | -      |

PJS, Peutz-Jeghers syndrome; Fα, family a; Fβ, family b; Fc, family c; STK11, serine/threonine kinase 11; OR4C45, olfactory receptor family 4 subfamily C member 45; ZAN, zonadhesin; GI, gastrointestinal.
Discussion

Since the first PJS case was described in 1921 (4), there has been an increasing awareness of this disease. The characteristic symptoms of PJS are mucocutaneous pigmentations and GI disorders. GI symptoms, such as intussusception or bleeding, typically occur in early life and there is an increased risk of cancer in later life. The majority of genetic analyses in patients with PJS have focused on the patterns of alteration, malignancy risk, and the type and locus of potential causative genes. In the present study, all patients with PJS exhibited mucocutaneous pigmentations and 66.7% had a history of GI hamartomatous polyps.

The primary causative gene of PJS has been identified as the STK11 gene located on chromosome 19p13 (1). Germline mutations in STK11 are considered to cause a predisposition to PJS (4). STK11 mutations have been detected in 80-94% of patients with PJS, with ~150 different mutations in the STK11 gene associated with PJS (4). A recent survey in 133 Chinese patients with PJS from 28 families found that STK11 is the primary causative gene of PJS in the Chinese population (18). Consistent with these reports, 2 out of 4 (50%) patients with
PJS in the present study were identified to have germline STK11 mutations.

Pathogenic roles of STK11 variants have previously been explored. Mutations in exon 6 of STK11 were found to be associated with a higher cancer risk in patients with PJS (19). Schumacher et al (20) determined that missense mutations in STK11 were primarily located in the C terminus and VIB-VIII regions. In-frame deletions and splice site mutations were rarely associated with malignancies (20). However, in a survey of 419 patients with PJS, cancer risks were similar in patients with PJS and STK11 mutations and those without detectable mutation, suggesting that the type or site of STK11 mutation did not have a significant influence on the risk of malignancy (21). In the current study, neither of the patients with STK11 mutations (FαI2 and FαII1) had cancer of GI or any other system, while the patient with PJS who had GI cancer exhibited no genotype-phenotype correlation between STK11 mutations and carcinogenesis.

The association between other clinical features of PJS and STK11 mutations was investigated. A missense mutation in STK11 has been connected with a later onset of PJS symptoms (15). In the current study, two patients with the same mutation (NM_000455.4:c.842delC; FαI1 and FαII1) in STK11 showed oral and GI manifestations of PJS to varying degrees of severity. Compared with FαI2, FαII1 developed oral and GI symptoms earlier, presented an increased quantity and size of oral pigmentation, had an increased quantity of GI polyps, and more frequent and severe GI complications. Notably, FαII1 showed a markedly higher quantity of mutant STK11 copies (100%) than FαI2 (10%), suggesting that there is a correlation between the severity of PJS phenotypes and the number of copies of mutated STK11 in the present study. To the best of our knowledge, this is the first study to report a STK11 mutation dose-dependent genotype-phenotype correlation in patients with PJS, which may have future applications in risk assessment and genetic counseling for PJS.

The STK11 gene is ubiquitously expressed in adult and fetal human tissues (9,22). The human STK11 protein consists of an N-terminal nuclear localization signal domain, a highly conserved serine/threonine kinase domain and a C-terminal regulatory domain (14). STK11 is important in chromatin remodeling, cell polarization, metabolism, cell

Figure 4. Sequencing results of the mutation in STK11 in Fα with genomic DNA. Sequencing analysis of (A) FαI1, (B) FαI2 and (C) FαII1. FαI2 and FαII1 showed a single base deletion (NM_000455.4:c.842delC) in exon 6 of STK11, indicated by the red arrow. STK11, serine/threonine kinase 11; Fα, family α.
The results of the present study indicate that the severity of GI phenotypes may reflect the severity of GI symptoms. In the patients with PJS studied, and that the severity of these symptoms, such as oral pigmentations, were found in one of the PJS families studied, with the typical clinical manifestations of PJS, did not carry any STK11, OR4C45 or ZAN mutations, suggesting that genetic heterogeneity and other causative factors of PJS exist.

Patients with PJS typically present heterogeneous phenotypes with regards to oral and GI lesions, including different onset times, disease severity, distribution of GI symptoms and level of malignancy (4). In order to investigate phenotypic heterogeneity in the present study, the clinical features of participants with PJS were compared, including onset time, duration, severity and prognosis. The earliest initial manifestations of PJS, such as oral pigmentations, were found in one participant at the age of 2 years. The most severe PJS case, F1_2, suffered serious GI complications and succumbed to a malignant tumor at 35 years of age. The patient's two young children (F2_II and F3_II) exhibited severe oral hyperpigmentation during childhood. This early onset (pre-puberty) of PJS may be an indicator for the development of serious GI complications, and will be followed up in the future. The present study found that oral lesions appeared prior to GI symptoms in the patients with PJS studied, and that the severity of these may reflect the severity of GI phenotypes. F1_II had a high quantity and large size of oral pigmentations and a severe GI phenotype. GI symptoms are the primary complaints of patients with PJS. If mild oral lesions are ignored in the early stages of PJS, misdiagnosis may occur, delaying treatment. The results of the present study indicate that the severity of oral pigmentations may be used as a prognostic and diagnostic marker of PJS.

In conclusion, the present study employed cloning and sequencing techniques for the molecular study of inherited PJS, revealing a dose-dependent genotype-phenotype relationship in a PJS family with a STK11 gene mutation. In addition, it was found that an early onset time and severe oral pigmentations in patients with PJS may indicate more serious GI phenotypes. These findings may aid the diagnosis and treatment of PJS.

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