

Polymorphism of heat shock protein 70-2 and enterocutaneous fistula in Chinese population

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Abstract

AIM: To investigate whether the heat shock protein 70-2 (*HSP70-2*) polymorphism is associated with enterocutaneous fistulas in a Chinese population.

METHODS: This study included 131 patients with enterocutaneous/enteroatmospheric fistulas. Patients with inflammatory bowel disease or other autoimmune diseases were excluded from this study. All patients with enterocutaneous/enteroatmospheric fistulas were followed up for three months to observe disease recurrence. In addition, a total of 140 healthy controls were also

recruited from the Jinling Hospital, matched according to the sex and age of the patient population. Genomic DNA was extracted from peripheral blood from each participant. The *HSP70-2* restriction fragment length polymorphism related to the polymorphic PstI site at position 1267 was characterized by polymerase chain reaction (PCR). First PCR amplification was carried out, and then PCR products were digested with PstI restriction enzyme. The DNA lacking the polymorphic PstI site within *HSP70-2* generates a product of 1117 bp in size (allele A), whereas the *HSP70-2* PstI polymorphism produces two fragments of 936 bp and 181 bp in size (allele B).

RESULTS: The frequency of the *HSP70-2* PstI polymorphism did not differ between patients and controls; however, the A allele was more predominant in patients with enterocutaneous fistulas than in controls (60.7% vs 51.4%, $P = 0.038$, OR = 1.425, 95%CI: 1.019-1.994). Sixty-one patients were cured by a definitive operation, drainage operation, or percutaneous drainage while 52 patients were cured by nonsurgical treatment. There was no significant difference in the frequency of the *HSP70-2* PstI polymorphism between the patients who had surgery compared to those who did not ($P = 0.437$, OR = 1.237, 95%CI: 0.723-2.117). Moreover, 11 patients refused any treatment for economic reasons or tumor burden, and 7 patients with enterocutaneous fistulas (5.8%) died during the follow-up period. However, there was no significant difference in the frequency of the *HSP70-2* PstI polymorphism between the patients who survived compared to those who died ($P = 0.403$, OR = 0.604, 95%CI: 0.184-1.986).

CONCLUSION: The A allele of the *HSP70-2* PstI polymorphism was associated with enterocutaneous fistulas in this Chinese population.

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Key words: Enterocutaneous fistulas; Single nucleotide polymorphisms; Heat shock protein; *HSP70-2*

Core tip: Postoperative enterocutaneous fistulas are one of the most devastating abdominal complications after surgery. Currently, enterocutaneous/enteroatmospheric fistulas may be caused by trauma or surgery as a result of traumatic or iatrogenic bowel injury, infection, or anastomotic leakage. However, genetic or epigenetic factors may increase the risk for individual patients to develop enterocutaneous/enteroatmospheric fistulas. We found the frequency of the *HSP70-2* PstI polymorphism did not differ between patients and controls; however, the A allele was more predominant in patients with enterocutaneous fistulas than in controls. To our knowledge, this study was the first study about the enterocutaneous fistula and single nucleotide polymorphism.

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INTRODUCTION

Postoperative enterocutaneous fistulas (ECF) are one of the most devastating abdominal complications after surgery, which are defined as an abnormal communication between the intra-abdominal gastrointestinal tract (*i.e.*, the stomach, duodenum, small bowel, and colon) and skin^[1-3]. Postoperative ECF result from a wide variety of conditions and circumstances but caused a high mortality rate of 50%-60% before the 1970s^[4,5]. Treatment of patients with postoperative ECF requires a greater understanding of their metabolic and anatomic derangements. To date, with the development of nutritional support, interventional radiology, and wound management, the overall mortality rate due to postoperative ECF has declined to 3%-12%^[6]. However, with the introduction of damage control principles in surgery, open abdominal management has been used in patients undergoing complex surgery, who may have increased risk for the development of postoperative ECF^[7]. Surgical techniques may also cause a new type of enteric fistulas, enteroatmospheric fistulas, which is defined as a fistula between the lumen of the bowel or other hollow viscus and the atmosphere (open abdomen or chest)^[8,9]. Currently, enterocutaneous/enteroatmospheric fistulas may be caused by trauma or surgery as a result of traumatic or iatrogenic bowel injury, infection, or anastomotic leakage^[1,2,6,10-12]. The genomic composite score can be rapidly used to predict clinical outcomes in trauma patients^[13], and genome-wide expression studies have identified 12 plasma proteins as candidates for biomarker-based risk stratification in adults

with septic shock^[14]. So, genetic or epigenetic factors may increase the risk for individual patients to develop enterocutaneous/enteroatmospheric fistulas.

Heat shock proteins (HSPs) are highly conserved proteins in most mammalian and prokaryotic cells, and act as chaperones to other unfolded proteins and peptides during elevated temperatures or other stress, such as heat, ischemia-reperfusion, inflammation, and microbial infection^[15-17]. As the most highly inducible HSP, the HSP70 gene family contains HSP70-1, HSP70-2, and HSP70-Hom. This family has been mapped to the class III region of the human genome, which is the region in chromosome 6 that contains human leukocyte antigen (HLA) genes^[17,18]. The HSP70-2 gene polymorphism generates a PstI site due to an A to G substitution at position 1267. Previous studies have demonstrated that this *HSP70-2* polymorphism is associated with Crohn's disease (CD), especially with the perforating form, abscess formation, fistulas, and requirement for surgery in Japanese^[19], Caucasian^[20], Korean^[21], and Chinese^[22] populations. Thus, the aim of this study was to investigate whether the *HSP70-2* PstI polymorphism is associated with enterocutaneous/enteroatmospheric fistulas in a Chinese population.

MATERIALS AND METHODS

Patients

This study included 131 patients with enterocutaneous/enteroatmospheric fistulas. The diagnosis was based on the standard clinical and radiological criteria^[23]. However, patients with Inflammatory Bowel Disease or other autoimmune disease were excluded from this study. All patients with enterocutaneous/enteroatmospheric fistulas were followed up for three months to observe disease recurrence. In addition, a total of 140 healthy controls were recruited from Jinling Hospital, Healthcare System Center, during their routine health check-up. They did not have enterocutaneous/enteroatmospheric fistulas, any known IBD, or another autoimmune disease according to their medical history and a laboratory test. They were matched according to the gender and age of the cases in this study. All the cases and controls were Han Chinese and recruited between April 2011 and January 2012. Our institutional review board approved this study protocol, and each participant agreed to be recruited into this study.

Polymerase chain reaction analysis of the *HSP70-2* PstI polymorphism

Genomic DNA was extracted from peripheral blood from each participant according to the QIAamp® DNA Mini and Blood Mini Handbook (Qiagen, Dusseldorf, Germany). The *HSP70-2* restriction fragment length polymorphism related to the polymorphic PstI site at position 1267 was characterized by PCR. The primers spanning the polymorphic PstI site were designed according to the previously published sequences (5'-CATC-GACTTCTACACGTCCA-3' and 5'-CAAAGTCCTT-GAGTCCCAAC-3')^[9-12]. PCR amplification was carried out by 35 cycles of denaturing at 95 °C for 1 min, anneal-

Table 1 Clinical characteristics and the *heat shock protein 70-2* single nucleotide polymorphism of cases and controls

	Case (n = 131)	Control (n = 140)	P value OR (95%CI)
Sex (M/F)	94/37	95/45	0.510
Age (yr, mean \pm SD)	46.5 \pm 15.9	44.0 \pm 8.6	0.108
Genotype	AA	47	0.074
	AB	65	
	BB	19	
Allele	A	159	0.038 1.425 (1.019-1.994)
	B	103	

Table 2 *Heat shock protein 70-2* genotype and allele frequencies vs daily output of patients

	Low (n = 41)	Moderate (n = 46)	High (n = 44)	
Genotype	AA	12	15	20
	AB	20	27	18
	BB	9	4	6
P	0.000 ¹	0.000 ²	0.0033 ³	
Allele	A	44	57	58
	B	38	35	30
P	0.285	0.642	0.119	
OR	0.711	0.842	0.599	
95%CI	0.388-1.301 ¹	0.458-1.549 ²	0.323-1.112 ³	

¹Low output vs moderate output; ²Moderate output vs high output; ³Low output vs high output. Low-output, < 200 mL of effluent daily; moderate, between 200 and 500 mL of effluent daily; high output, > 500 mL of effluent daily.

Table 3 *Heat shock protein 70-2* genotype and allele frequencies vs etiology of fistulas

	Iatrogenic injury (n = 54)	Trauma (n = 42)	Malignancy (n = 34)	Infection (n = 1)	P value	
Genotype	AA	17	16	14	0	0.257
	AB	29	19	17	0	
	BB	8	7	3	1	
Allele	A	63	51	45	0	0.241
	B	45	33	23	2	

ing at 54 °C for 30 s, and extension at 72 °C for 1 min. PCR products were then digested with PstI restriction enzyme (Fermentas, Vilnius, Lithuania) at 37 °C for 1 h and electrophoresed in a 1.5% agarose gel. The DNA lacking the polymorphic PstI site within *HSP70-2* generates a product of 1117 bp in size (allele A), whereas the *HSP70-2* PstI polymorphism produces two fragments of 936 bp and 181 bp in size (allele B)^[15,20-22].

Statistical analyses

The data were summarized in terms of genotype and allele frequency, and Hardy-Weinberg equilibrium was used to confirm that the overall population was represented by the cases and controls in this study. Genotype and allele frequencies were analyzed by the χ^2 test using SPSS12.0 (SPSS, Chicago, IL), and $P < 0.05$ was established as the level of significance.

RESULTS

Comparison of the *HSP70-2* PstI polymorphism between cases and controls

One hundred and thirty-one patients with enterocutaneous/enteroatmospheric fistulas (94 males and 37 females) and 140 age- and sex-matched healthy controls (95 males and 45 females) were included in this study. The male to female ratio of the patients was 2.5:1. The mean age was 46.5 \pm 15.9 years old in cases vs 44.0 \pm 8.6 years old in the healthy controls (Table 1).

The genotype and allele frequencies in the cases and controls are shown in Table 1. Specifically, in patients with fistulas, the frequencies of allele A and B were 60.7% and 39.3%, respectively; while in controls, the frequencies of allele A and B were 51.4% and 48.6%, respectively. The most frequent genotype was the heterozygote AB (49.6% in cases vs 47.1% in controls); thus, the genotype frequency between cases and controls was not different ($\chi^2 = 5.199$, $P = 0.074$). However, the frequency of the *HSP70-2* allele was significantly greater in cases than in controls (60.7% vs 51.4%, $P = 0.038$; OR = 1.425, 95%CI: 1.019-1.994).

Association of the *HSP70-2* PstI polymorphism with patient clinicopathological data

Clinically, daily output was one of the classification schemes to determine the severity of the enterocutaneous/enteroatmospheric fistulas in patients; for example, low-output enterocutaneous/enteroatmospheric fistulas typically provide less than 200 mL daily of the effluent, moderate fistulas provide between 200 mL and 500 mL daily, whereas high output fistulas provide more than 500 mL daily^[2]. Our data showed that the *HSP70-2* BB genotype was slightly more prevalent in low output patients than in moderate or high output patients (22.0% vs 8.7% or 13.6%, respectively). In moderate output patients, the *HSP70-2* AB genotype was more frequent (58.7% vs 48.8% in low output patients and vs 40.9% in high output patients). In high output patients, the *HSP70-2* AA genotype was more prevalent than the others (45.5% vs 29.3% in low output patients and vs 32.6% in moderate output patients). Although these patients showed significant genotype differences among these three daily outputs, we did not find any significant differences in terms of allele frequencies among these patients (Table 2).

In this cohort of patients, iatrogenic injury was the main etiology of enterocutaneous/enteroatmospheric fistulas (54 patients, 41.2%); while trauma caused fistulas in 42 patients, which was the second most common etiology (32.1%). In addition, malignancies in 34 patients (26.0%) and tuberculosis infection in one patient caused fistula development. There were no significant etiological differences between *HSP70-2* genotypes and alleles among these patients (Table 3).

The most frequent sites of fistulas in these patients were located in the small bowel, which accounted for approximately 42.0% of cases. Other fistula sites included the stom-

Table 4 Heat shock protein 70-2 genotype and allele frequencies *vs* fistula location

		Stomach (<i>n</i> = 5)	Duodenum (<i>n</i> = 21)	Small bowel (<i>n</i> = 55)	Colon/rectum (<i>n</i> = 16)	Internal (<i>n</i> = 4)	Complex (<i>n</i> = 30)	<i>P</i> value
Genotype	AA	1	5	21	6	0	14	0.410
	AB	3	11	27	9	2	13	
	BB	1	5	7	1	2	3	
Allele	A	5	21	69	21	2	41	0.119
	B	5	21	41	11	6	19	

Table 5 Heat shock protein 70-2 genotype and allele frequencies *vs* patient survival

		Survived (<i>n</i> = 113)	Deceased (<i>n</i> = 7)	<i>P</i> value OR (95%CI)
Genotype	AA	40	4	0.484
	AB	56	2	
	BB	17	1	
Allele	A	136	10	0.403
	B	90	4	

Table 6 Heat shock protein 70-2 genotype and allele frequencies *vs* surgical and nonsurgical treatment

		Surgery (<i>n</i> = 61)	No surgery (<i>n</i> = 52)	<i>P</i> value OR (95%CI)
Genotype	AA	24	16	0.601
	AB	29	27	
	BB	8	9	
Allele	A	73	59	0.437
	B	45	45	

Table 7 Heat shock protein 70-2 genotype and allele frequencies *vs* recurrent and nonrecurrent fistulas after surgery

		Recurrence (<i>n</i> = 51)	No recurrence (<i>n</i> = 10)	<i>P</i> value OR (95%CI)
Genotype	AA	20	4	0.949
	AB	24	5	
	BB	7	1	
Allele	A	64	13	0.848
	B	38	7	

Table 8 Multivariate analysis using heat shock protein 70-2 frequency as a dependent variable

	<i>P</i> value
Sex	0.073
Age	0.910
Daily output	0.259
Fistula etiology	0.299
Fistula location	0.271
Treatment	0.729

ach (5 patients, 3.8%), the duodenum (21 patients, 16.0%, including 2 with a pancreatic fistula and 1 with a biliary fistula), and the colon/rectum (16 patients, 12.2%). Furthermore, there were 4 patients with internal fistulas, consisting of 2 recto-vaginal fistulas and 2 enterocolic fistulas. Overall, 30 patients had fistulas in more than one location (complex fistulas, 22.9%). However, there were no significant differences in fistula location between HSP70-2 genotypes and alleles among these patients (Table 4).

Eleven patients refused any treatment due to economic reasons or tumor burden, while seven patients died during the follow-up period (5.8%). There was no significant difference in the frequency of the HSP70-2 PstI polymorphism between the patients who survived and died ($P = 0.403$, OR = 0.604, 95%CI: 0.184-1.986, Table 5). In the patients who survived, 61 patients were cured by a definitive surgery, drainage operation, or percutaneous drainage; but 52 patients were cured by nonsurgical treatment. There was also no significant difference in the frequency of the HSP70-2 PstI polymorphism between the patients who received surgery or not ($P = 0.437$, OR = 1.237, 95%CI: 0.723-2.117, Table 6). In patients who received surgery, 10 had recurrent fistulas after surgery (16.4%). There was still no significant difference between the patients who received surgery or not ($P = 0.848$, OR = 0.907, 95%CI: 0.333-2.472, Table 7). Furthermore, multivariate analysis showed that there was no significant

The median age of the patients was 45 years old and used as a cut-off point.

association between the HSP70-2 PstI polymorphism and clinicopathological factors from these patients (Table 8).

DISCUSSION

Heat shock protein was discovered by Ritossa *et al.*^[16] in 1962; subsequently, HSPs have been detected in almost all organisms^[17]. They are divided into different classes according to their molecular weights, *i.e.*, HSP100 (approximately 100 kDa), HSP90, HSP70, and so on^[17]. In humans, the HSP70 family of genes is mapped to the short arm of the sixth chromosome, close to HLA; thus, they may play a role in the regulation of immune responses. For example, HSP70 expression can be induced by a variety of infectious and inflammatory stimuli^[24,25] (Christians, 2002 #26) and functions to protect cell homeostasis^[26,27]. The extracellular HSP70 protein can induce a proinflammatory response through toll-like receptor-2 and toll-like receptor-4^[28]. However, the HSP70-2 PstI polymorphism is a nonfunctional single nucleotide polymorphism (SNP) that does not cause a change in the derived amino acid^[29,30], although HSP70-2 SNPs have been considered as candidates for increased susceptibility to inflammatory diseases. Tahara *et al.*^[31] have found that the HSP70-2 BB genotype is associated with a reduced risk of gastric premalignant conditions in *H. pylori*-infected older individu-

als. In addition, Partida-Rodríguez *et al.*^[32] have shown that an *HSP70-2* SNP is significantly associated with both gastric cancer and duodenal ulcers. Thus, in the current study, we determined the association between the *HSP70-2* PstI polymorphism and a patient's susceptibility to enterocutaneous/enteroatmospheric fistulas. We found that the genotype frequency of the *HSP70-2* PstI polymorphism did not differ between patients and controls, but the A allele was more prevalent in patients with enterocutaneous fistulas than in controls, which may indicate that the *HSP70-2* A allele is more susceptible to enterocutaneous/enteroatmospheric fistula development.

Indeed, other studies have shown that HSP70 allele frequencies are associated with the pathogenesis of autoimmunity^[33]. Their data show that different *HSP70-2* allele frequencies are associated with spondyloarthropathies^[34], rheumatoid arthritis^[35], systemic lupus erythematosus^[33], celiac disease^[18], insulin-dependent diabetes (IDDM)^[36,37], (Caplen, 1990 #37; Pociot, 1993 #36) type 2 diabetes and obesity^[38], and autoimmune thyroid disease^[39]. Moreover, recent studies have associated an *HSP70-2* SNP with IBD^[19,21,29,30]. In these studies, the *HSP70-2* genotype BB of the PstI polymorphism or the *HSP70-2* allele B was associated with the perforating form of CD or an indication for surgical treatment of CD, respectively, although the role of *HSP70-2* in the pathogenesis of CD is still unknown. However, another study^[15] did not confirm these data. In our previous study^[22], we showed that the *HSP70-2* A allele of the PstI polymorphism is associated with susceptibility to CD and surgical treatment of perforating CD. Therefore, in the current study, we excluded patients with CD, which may comprise up to 20% of patients with fistulas^[2,10].

ECF are an uncommon but not well-studied postoperative complication, but they may have been first described as early as 450 BC^[1,2]. The "modern" history of ECF began with William Beaumont and his patient, Alexis St. Martin in the 18th century; during this time period, ECF were a challenging surgical complication with a high mortality rate because of a combination of factors, including sepsis, electrolyte imbalance, and malnutrition^[1,2,40]. In 1969, Dudrick *et al.*^[41] reported the advent of parenteral nutrition, which was a "game changer" for surgeons to prevent malnutrition and to provide patients with time to heal; therefore, the mortality rate due to fistulas has decreased ever since. In 1993, Rotondo *et al.*^[42] described the term "damage control laparotomy." Next, an "open abdomen" has become more and more common in current clinical practice^[8,9,11,43]. Moreover, enteroatmospheric fistulas are one of the most common and potentially devastating complications after surgery^[5]. But in our current study, there were not many cases of patients with enteroatmospheric fistulas.

In conclusion, in our current study, we found that the *HSP70-2* A allele of the PstI polymorphism was associated with ECF, while the *HSP70-2* BB genotype was slightly more prevalent in low output patients and the *HSP70-2* AB genotype was more prevalent in moderate output patients. In high output patients, the *HSP70-2* AA

genotype was more common than the others. To the best of our knowledge, the current study is the first report on an *HSP70-2* PstI polymorphism in patients with ECF. However, our current study does have some limitations; for example, the small sample size, lack of a validation cohort, and the loss of statistical significance of the PstI polymorphism after the multivariate analysis. Thus, future studies with a larger sample size are needed to verify our current findings.

COMMENTS

Background

Postoperative enterocutaneous fistulas are one of the most devastating abdominal complications after surgery.

Research frontiers

Currently, enterocutaneous/enteroatmospheric fistulas may be caused by trauma or surgery as a result of traumatic or iatrogenic bowel injury, infection, or anastomotic leakage. However, genetic or epigenetic factors may increase the risk for individual patients to develop enterocutaneous/enteroatmospheric fistulas.

Innovations and breakthroughs

The authors found the frequency of the heat shock protein 70-2 (*HSP70-2*) PstI polymorphism did not differ between patients and controls; however, the A allele was more predominant in patients with enterocutaneous fistulas than in controls.

Applications

In high output patients, the *HSP70-2* AA genotype was more common than the others.

Peer review

There are important limitations of the genetic study such as the small sample size, the need for a validation cohort and the loss of statistical significance of PstI polymorphism after the multivariate analysis.

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