论 著

Epidemiological characterization of drug-resistance and REP-PCR typing of *Shigella spp.* in Tianjin in the last two and half decades

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【Abstract】 Objective To compare the change of the dominant serogroup of *Shigella spp.* causing dysentery and their antimicrobial resistance for more than twenty years(between 1983 and 2009) in Tianjin, using molecular typing method to study their epidemiological characteristic. **Methods** 95 strains of *Shigella spp.* were selected and their serogroups were identified. In the same time, their characteristic of drug resistance were studied. Repetitive extragenic palindromic-PCR (REP-PCR) was used to study the homology of their genomic DNA. **Results** The isolated rate of *Shigella sonnei* in 2009 (55.38%) had obviously increased than that of in 1983(0%). Susceptibility results showed that the resistance rates of *Shigella flexneri* isolated in 1983 to the traditional antibiotics such as chloramphenicol, streptomycin and tetracycline were all higher than that of in 2009, but the differences had no statistical significance (P all> 0.05). The resistance rates of *Shigella flexneri* isolated in 2009 to ampicillin, piperacillin, cefazolin, cefotaxime, ceftriaxone and cotrimoxazole were all higher than that of in 1983 and the differences all had statistical significance(P all< 0.01).The resistance rate of *Shigella flexneri* to cefazolin, cefotaxime, ceftriaxone, cefepime, chloramphenicol and amikacin were all higher than that of *Shigella sonnei* isolated in 2009 and the differences all had statistical significance (P all< 0.01). The multidrug resistance (MDR) rates of *Shigella flexneri* in 2009 were higher than that of in 1983 and the difference had statistical significance (P< 0.05). There was better homology of *Shigella flexneri* genotype between 1983 and 2009 analyzed by REP-PCR. **Conclusion** The dominant serogroup of *Shigella* in Tianjin has changed from *Shigella flexneri* to *Shigella sonnei* and the antibiotics resistance has changed too. REP-PCR is a valid and rapid genotyping method for homological analysis and tracking the source of infection during epidemic outbreak.

【Key words】 *Shigella spp.*; Multidrug resistance; Repetitive extragenic palindromic PCR

天津近二十余年跨度志贺菌属耐药性流行病学特征和 REP-PCR 表型同源性分析

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【摘要】 目的 以细菌性痢疾致病菌为目标比较天津市 1983 年和 2009 年分离出的志贺氏菌优势血清型分布变化及耐药情况,并通过分子分型研究其流行病学溯源。**方法** 分离鉴定 95 株志贺菌血清型,研究其耐药性变化。使用重复序列 PCR 对志贺菌进行同源性分析。**结果** 2009 年宋内氏志贺菌的分离率(55.38%)高于 1983 年分离率(0%)。药敏结果显示 1983 年分离的福氏志贺菌对传统抗生素氯霉素、链霉素、四环素的耐药率均高于 2009 年分离的福氏志贺菌,但差异均无统计学意义(P均> 0.05)。2009 年分离的福氏志贺菌对氨苄西林、哌拉西林、头孢唑啉、头孢噻肟、头孢曲松、磺胺甲基异恶唑的耐药率均高于 1983 年分离的福氏志贺菌,且差异均具有统计学意义(P均< 0.01)。2009 年分离的福氏志贺菌对头孢唑啉、头孢噻肟、头孢曲松、头孢吡肟、氯霉素和阿米卡星的耐药率均高于宋内氏志贺菌且差异均有统计学意义(P均< 0.01)。2009 年分离的福氏志贺菌的多重耐药率高于 1983 年分离的福氏志贺菌,且差异具有统计学意义(P< 0.05)。通过 REP-PCR 分析,1983 年与 2009 年福氏志贺菌基因型具有较好的同源性。**结论** 天津市 2009 年志贺菌优势血清型已较二十余年前发生变迁,从福氏变为了宋内氏,耐药性也发生明显变化。REP-PCR 是一种有效快捷的基因分型法,可为细菌同源性分析以及爆发流行时追根溯源建立简便可行的方法。

【关键词】 志贺菌属;多重耐药性;重复序列聚合酶链式反应

Shigella spp. are Gram-negative bacteria belonging to the *Enterobacteriaceae*. These bacteria are responsible for morbidity and mortality in high risk populations such as children under five years old and the immunocompromized population[1]. In China, Shigellosis epidemics are mainly caused by *Shigella flexneri* and *Shigella sonnei* and there is a gradual increase in resistance of these *Shigella spp.* to the commonly used antibiotics in the last two decades. The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids, transposons, insertion sequences and genomic islands which has been the major factor in the development of multidrug resistance (MDR) strains[2]. In this study, we compare the serotype and PCR based molecular typing and antimicrobial resistance of *Shigella spp.* isolated from patients with diarrhea in 1983 and recent(2009) at the out-patient section to provide the basis for scientific control in Tianjin.

1 Materials and Methods

1.1 Source of strains 65 strains of *Shigella* were isolated and identified from clinical patients with diarrhea, by stool cultures between May and October 2009 at the Tianjin Children's Hospital, the First General Hospital of Tianjin Medical University and the Second Hospital of Tianjin Medical University. 30 strains of *Shigella spp.* which were isolated in 1983 and conserved by vacuum drying and stored at −70 ℃ were collected from the Tianjin Institute of Infectious Diseases.

1.2 Methods Bacterial isolation was done according to the Clinical Laboratory Procedures. Fresh stool specimens were inoculated into SS and MacConkey agar plates, and were incubated at 37 ℃ for 24 h in an incubator. Suspected *Shigella* colonies were selected from pure culture for biochemical with API and serological identification with standard antiserum typing kits. The strains in 1983 were resuscitated in LB nutrient broth and inoculated on blood agar for identification and serological typing. The *Shigella* antiserum was purchased from Lanzhou Institute of Biological Products. Serological test was done by slide agglutination, using normal saline as a control.

1.2.1 Bacteria susceptibility testing Antimicrobial susceptibility testing was carried out according to the Clinical Laboratory Standards Institute (CLSI) recommended criteria for disk diffusion method. The antimicrobial disks used include: ampicillin, piperacillin, cefazolin, ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, tetracycline, chloramphenicol, streptomycin, amikacin, gentamicin, cotrimoxazole, norfloxacin, and levofloxacin. The antimicrobial disks were purchased from Beijing Biological products Inspection. Strain ATCC25922 was used as a susceptibility quality control.

1.2.2 DNA extraction Bacteria DNA were extracted using the boiling method. The crude DNA was refined by extraction with equal volume of chloroform, and then 5 ul of the refined DNA template was used for repetitive extragenic palindromic-PCR(REP-PCR) analysis.

1.2.3 Polymerase chain reaction REP -PCR was carried out in Mygene™ Series Peltier Thermal Cycler machine. Briefly, the primer 5′ GCGCCGTCATGCGGCATT 3′ was used under the following conditions: an initial temperature of 94 ℃ for 2 min. with 30 cycles of 1 min. at 94 °C, 1 min. at 40 °C, and 8 min. at 65 °C, with a final extension of 16 min. at 65 °C. The PCR reaction was done in a total volume of 25 ul, with 5 ul of DNA template, 2 ul of primer, 12.5 ul of premix and 5.5 ul of sterilized water. The reaction was analyzed on a 1% agarose gel for 6 hours at 50 V. UV photos of electrophoresis were taken by UV scanning machine.

1.3 Statistical analysis Data was analyzed using SPSS 11.5 statistical software and the quantity one 4.5 software for statistical and band pattern analysis respectively.

2 Results

2.1 Serogroup distribution In 1983, 30 strains all were *Shigella flexneri*, while in 2009, 65 strains of *Shigella* were collected, including 29 strains of *Shigella flexneri*, accounted for 44.62%, 36 strains of *Shigella sonnei*, accounted for 55.38%. *S.dysentery* and *S.boydii* bacteria were not detected.

2.2 Drug sensitivity tests analysis of isolated *Shigella* The results of the resistance rate of *Shigella* bacteria to the commonly used antibiotics were shown in table 1. From table 1: in 1983, the resistance rate of all *Shigella flexneri* to the traditional antibiotics(tetracycline, streptomycin, chloramphenicol) other than gentamicin were

higher than that of *Shigella flexneri* isolated in 2009 and varied from 76.70% to 100%, while the 2009 *Shigella flexneri* strains showed increased susceptibility to tetracycline, streptomycin and chloramphenicol and their resistance rate was slightly lower, but the differences all had no statistical significane(P all> 0.05). The resistance rate of *Shigella flexneri* to cotrimoxazole in 26 years increased significantly from 46.70% to 100% and the difference had statistical significance(P< 0.01). The *Shigella* bacteria in 1983 were 100% sensitive to amikacin, gentamicin, imipenem, the third and fourth generation cephalosporins, quinolones. While in 2009, *Shigella flexneri* showed different levels of increasing drug resistance to the third and fourth generation cephalosporins and fluoroquinolones. According to table 1, the resistance rate of *Shigella flexneri* to chloramphenicol, amikacin and the third and fourth generation cephalosporis and quindones were higher than *Shigella sonnei* in 2009. There was a rapid change of resistance rate especially for the third and fourth generation cephalosporins between the two *Shigella flexneri* groups and the differences all had statistical significance except ceftazidime (P all< 0.05). The resistance rates of the *Shigella flexneri in* 2009 to levofloxacin and norfloxatin were higher than that of the strains in 1983 in varying degrees, but there were no statistical significance (P all> 0.05). The differences between the resistance rates of the *Shigella flexneri* and *Shigella sonnei* in 2009 to amikacin and gentamicin all had statistical significance(P all< 0.01).

To enhance better explanation for the difference of MDR in this study, serogroups isolated in different years were classified into three groups. The *S. flexneri* in 1983 as group Ⅰ, *S.flexneri* in 2009 as group Ⅱ and the *S.sonnei* in 2009 as group Ⅲ. MDR was defined as the resistance of a microbe to more than 3 antibiotics. The results showed that group Ⅱ and group Ⅲ had higher MDR than group Ⅰ (table 2).

Table 2 Comparison of MDR of *Shigella spp.* in different groups[n(%)]

Groups	Total no. of Strains	Strains of MDR
Group Ⅰ	30	16(53.30)
Group Ⅱ	29	28(96.60)
Group Ⅲ	36	29(80.56)
Total	95	73(76.84)

Note: Ⅰ Vs Ⅱ: χ^2= 14.53, P< 0.05; Ⅱ Vs Ⅲ: χ^2= 2.47, P= 0.148

2.3 Typing of *Shigella* by REP-PCR technique 17 of the 30 strains of *S.flexneri* isolated in 1983 showed

Table 1 Comparison of antimicrobial resistance rates in different years and different serogroups(%)

Drugs	1983	2009			χ^2_1	P_1	χ^2_2	P_2
	S.flexneri(n= 30 st)	*S.flexneri*(n= 29 st)	*S.sonnei*(n= 36 st)	Total(n= 65 st)				
Ampicillin	13.30	96.60	91.70	93.85	41.14	0.000*	-	0.622
Piperacillin	13.30	51.70	86.10	70.77	9.95	0.002*	9.18	0.002*
Cefazolin	6.70	48.30	11.10	27.69	12.92	0.002*	11.07	0.001*
Ceftazidime	0.00	10.30	0.00	4.62	-	0.112	-	0.084
Cefotaxime	0.00	37.90	5.60	20.00	13.99	0.000*	10.52	0.001*
Ceftriaxone	0.00	41.40	5.60	21.54	15.58	0.000*	12.20	0.000*
Cefepime	0.00	20.70	0.00	9.23	4.83	0.028θ	5.92	0.015θ
Imipenem	0.00	0.00	0.00	0.00	-	-	-	-
Tetracycline	100.00	86.20	88.90	87.69	-	0.052	0.00	1.000
Chloramphenicol	83.30	62.10	2.80	29.23	3.37	0.066	27.30	0.000*
Streptomycin	76.70	75.90	72.20	73.85	0.01	0.940	0.11	0.740
Gentamicin	0.00	3.40	77.80	44.62	-	0.492	35.92	0.000*
Amikacin	0.00	24.10	0.00	10.77	6.07	0.014θ	7.39	0.007θ
Norfloxacin	0.00	13.80	0.00	6.15	-	0.052	-	0.035α
Levofloxacin	0.00	10.30	0.00	4.62	-	0.112	-	0.084
Cotrimoxazole	46.70	100.00	91.70	95.38	21.22	0.000*	-	0.247

Note: P_1 represents *S.flexneri* of 1983 and 2009 from the chi-square test P value; P_2 represents *S. flexneri* and *S.sonnei* of 2009 from the chi-square test P value; χ^2_1 represents the chi-square for P_1; χ^2_2 represents the chi-square for P_2; *= Pearson chi-square, θ= Yates' correction, α= Fisher's exact test

varying degree of drug resistance and 29 of the 65 strains of *S.flexneri* isolated in 2009 were typed by REP-PCR to produce optimal conditions of repeatedly legible fingerprint, and the results were analyzed using quantity one 4.5 similarity analysis software to obtain a dendrogram showing band similarity between strains. The strains were divided into 41 genotypes (Fig 1). In the dendrogram, it could be seen that strains 166(1983) and F2(2009) were identical with a similarity coefficient of approximately 97% to strain 181 (1983). Similarly, strains 184(1983), AB105, AB4, N1(2009) were identical with a partial similarity of approximately 96% to strain 1 (1983). Parts of strains UV photos in primary REP-PCR electrophoreses were in Fig 2.

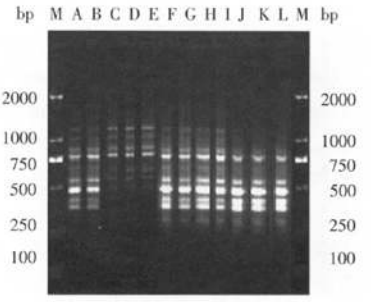

Fig.2 REP-PCR electro phoneses results

Note: Lane M, molecular size marker (DL 2000); Lanes A and B, *S. flexneri* isolated in 1983; lanes C, D and E *S. sonnei* isolated in 2009; lanes F, G, H, I, J, K and L, *S.flexneri* isolated in 2009

3 Discussion

Shigella spp. is one of the most common causes of shigellosis which can cause epidemics worldwide. Currently, bacillary dysentery caused by *Shigella* is still one of the most frequently-occurring diseases in chil-

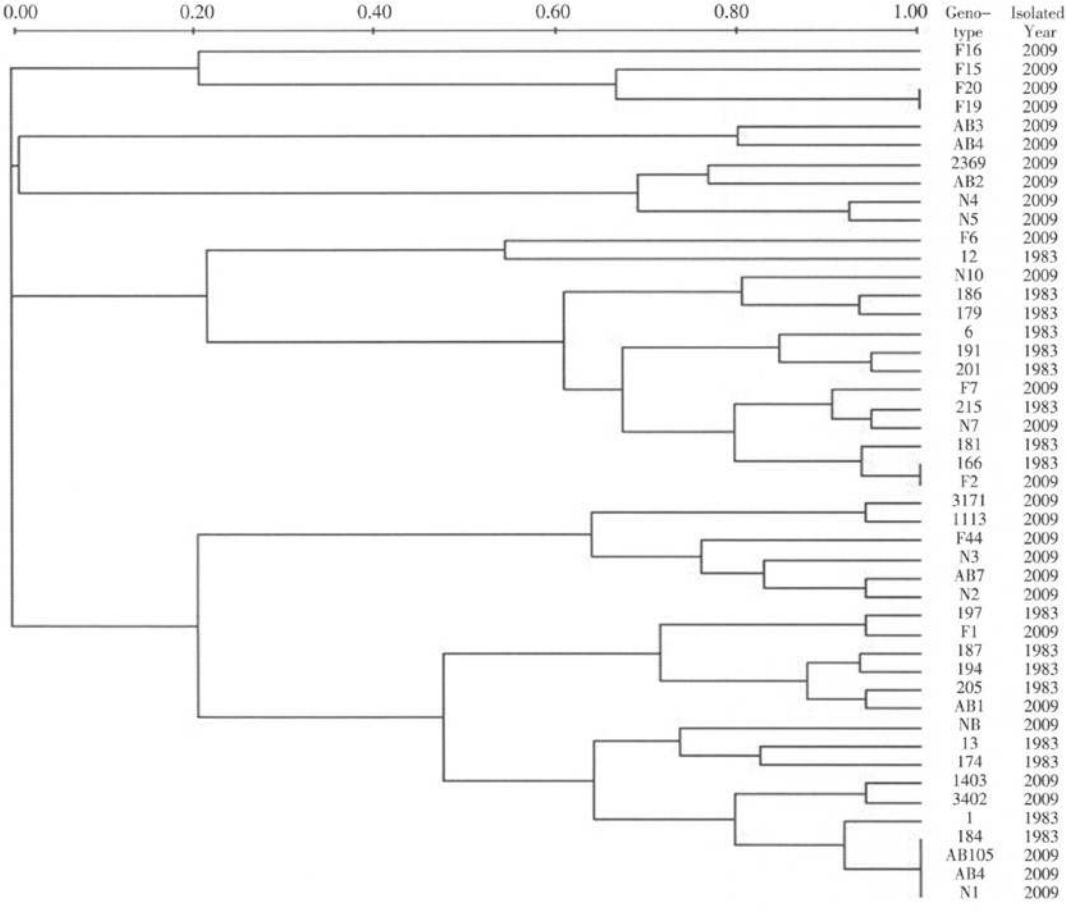

Fig.1 Dendrogram showing DNA similarity fingerprinting of *Shigella flexneri* isolated in 1983 and 2009 by REP-PCR typing technique

dren in China. The disease can occur all the year round, especially in summer and autumn. In developed countries, *S.sonnei* is the dominant specie, while in developing countries including China, *S.flexneri* has been the main pathogen causing dysentery in children and the immunocompromized[3]. In recent years, *S.sonnei* infection rate has increasing[4]. The statistical results showed that the dominant serogroup of *Shigella* in Tianjin had changed from *S.flexneri* to *S.sonnei*, contrary to Wang Xiaojuan's report [5]. The reason for the rapid increasing infection rate of *Shigella sonnei* in Tianjin may be related to the misused of antibiotics on one hand, and the broad use of disinfectant reagents on the other hand. The changes of the antibiotics using in treating dysentery and the improvement to health conditions might not be unrelated to the change of resistance and the dominating serogroup. In the 20th century, there was a gradual increasing resistance of *Shigella spp.* against the cheap and traditional antibiotics(cotrimoxazole, tetracycline and ampicillin etc.) frequently used in the late 80s for the treatment of *Shigella* infection. These drugs were no longer a suitable choice for patients with suspected *Shigella* infections, which was in the same view with Agasan[3]. In the last couple of years, with the application of the third and fourth generation cephalosporin, the drug resistant rate of *Shigella* against these drugs was increasing. The resistant rate against some kinds of the third generation cephalosporin was up to 40%. Because of the low usage of chloramphenicol, the resistant rate against it was decreased [6]. Does this suggest that some resistance can be avoided by rotation of antibiotics to resolve the problem, still remain to be a large and long-term observation.

In addition to that, between each group of *Shigella*, the resistance rate was somewhat different. The resistance rate of *Shigella flexneri* collected in 2009 was higher than that of *Shigella sonnei* to most of the antibiotics. Despite the large, extensive, and long-term use of quinolones, they still maintain a higher sensitivity to *Shigella*, suggesting that quinolones were better choice for diarrhea caused by *Shigella*. When we compare the distribution of MDR strains of *Shigella* from different years, the 2009 strains of *S.flexneri* shown significantly increasing MDR than that of 1983 strains ($P<0.05$), this prompted us that the increasing resistance may be resulted from the wide use of antibiotics. Physicians should request antimicrobial susceptibility test and choose drugs according to the results of the test. The change of dominant serogroup was a significant epidemiological study.

Compared with other DNA typing methods, REP-PCR typing offers a simple, rapid, and highly discriminatory means of identifying and comparing *Shigella* strains. The method can also be applied to other species responsible for nosocomial infection [7], making it a versatile and cost-effective tool for use in hospital diagnostic laboratories. In this paper, REP-PCR results remained us, even though the 1983 *S.flexneri* had been taken for 26 years, it still had genetic similarity to that of *S.flexneri* isolated in 2009 which caused frequent infection in Tianjin but with different resistance characterization. Because most of the cases studied here were caused by very similar or identical strains, which infer that these strains could survive in the environment either in contaminated water and food or in carriers such as humans and other animals[8,9].

It should be noted that the relationships shown in the dendrogram represent statistical relationships between banding patterns. REP-PCR is fast becoming the most widely used method of DNA typing. The technique is easy to perform, cheaper and can be applied to larger or smaller numbers of isolates. REP-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting [10]. Finally, several studies have shown REP-PCR to have good correlation with PFGE results which, in general, with slightly less discriminatory power.

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状，既往有多年偏头痛史。入院查体及常规辅助检查均无阳性改变。考虑此次患者的症状表现与既往偏头痛的表现一致，症状不典型，无其他伴随症状，且查体及辅助检查无阳性改变。因此，初步诊断为“偏头痛”并按此治疗 2 d，效果不佳，请上级医师会诊。

4.2 会诊医师分析 以上所述的病史，查体及辅助检查，认为本例确实具有一定的隐匿性、误导性，导致初诊误诊主要是过分依赖影像学诊断。该患者血栓的范围较局限，侧支循环建立迅速，没有造成大范围的实质性病损，因而导致了头颅 MRI 的假阴性，临床表现不典型，比较少见。脑血管造影对本病是最可靠的检查，但由于脑血管造影检查相对复杂、耗时长、费用高，且易导致造影剂过敏，使之还不能成为对患者的常规检查手段。

4.3 检验医师分析 常规行凝血功能检查提示血 PT 正常，但对于微小血栓还应进行 D-D 检测。血 D-D 检测是血栓性疾病及弥漫性血管内凝血诊断中的一个极敏感的重要的指标。因为在凝血过程最后阶段，纤维蛋白原在凝血酶的作用下转变为可溶性纤维蛋白单体，然后进一步形成不可溶的交联纤维蛋白（血栓形成），与此同时也启动了纤溶系统，纤溶酶将交联的纤维蛋白水解，D-D 是这些降解产物中的一种。血浆中 D-D 含量升高，表明体内有血栓形成及溶解发生[2]。临床医生应高度重视血浆 D-D 的检测结果。近年来，D-D 检测逐渐拓展到许多领域，尽管其诊断特异性不高，但该试验所具有的高度敏感性和极佳的阴性预测能力使其在许多疾病中，特别是在血栓形成（高凝状态）等疾病的鉴别诊断和治疗监测方面具有较好的应用价值，D-D 对深静脉血栓和肺栓塞具有排除诊断价值。另外，有学者[3]研究认为 D-D 的检测对于疑似颅内静脉窦血栓形成患者也是有效的，并且阳性的 D-D 测定结果是患者进行影像学检查的指征。因此建议临床医生对于此类疾病在患者就诊初期即应进行 D-D 测定，以防误诊或漏诊。

5 小结

脑静脉窦血栓形成病因复杂，一般分感染性和非感染性。上矢状窦血栓形成多属非感染性，常见病因有：①血液成份的改变，如高凝状态、高脂血症、口服避孕药、血液病等；②血液动力学改变，如脱水、心衰、高热、全身衰竭等，导致血流缓慢而形成血栓；③机械因素、外伤、肿瘤或血肿压迫等。本病临床表现多样，与血栓部位、范围及梗阻程度有关，常见症状有：①颅内压增高：头痛、恶心、呕吐、视乳头水肿等；②意识障碍：疾病的不同阶段，可出现不同程度的意识障碍，如嗜睡、朦胧甚至昏迷；③癫痫发作与运动障碍：根据受累部位不同，可出现局限性癫痫发作以及下肢瘫痪，排尿障碍、黑朦或偏盲等。本病的脑脊液表现：压力多增高，有时 WBC、蛋白增高，如合并蛛网膜下腔出血，亦可见红细胞及黄染。

脑静脉窦血栓的确诊主要是影像学方法，如头颅 CT，增强 CT 或脑血管造影（静脉期），但由于静脉窦距头皮很近，CT 有时不易发现。脑血管造影检查相对复杂、耗时、费用高，且存在造影剂过敏等风险，还不能成为对患者的常规检查手段。而 D-D 检测，方便、快捷、敏感，是很好的筛查指标，如果本例患者早期即进行了 D-D 检测，注重其变化的提示作用，可能不会造成该患者的早期误诊。

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