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Establishment of a fluorescent PCR melting curve method for detecting asthma susceptibility using gene SNP typing

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Abstract

Objective: To develop a detection method for single nucleotide polymorphisms (SNPs) of bronchial asthma (BA) susceptibility genes (*IL-13*, *IL-33*, and *GSDMA*) based on fluorescence PCR melting curves.

Methods: Peripheral blood samples from 33 patients with bronchial asthma were collected. DNA was extracted, and positive plasmids were constructed. Probes and primers for fluorescence polymerase chain reaction (PCR) were designed according to *IL-13*, *IL-33*, and *GSDMA* sequences, and the SNPs were separately detected by gene sequencing and fluorescence PCR melting curve.

Results: The system was successfully divided into 3 SNPs, including *IL-13*, *IL-33*, and *GSDMA*, and a comparison of sequencing methods showed that the results were completely consistent. The lowest detection limit was 1 ng/reaction, the sensitivity and specificity were 100%, and this method had high repeatability (CV=2.8%).

Conclusion: The fluorescence PCR melting curve method is suitable for the rapid and accurate classification of SNPs. The method is economical, simple, and efficient, and is suitable for the screening of the susceptible gene SNPs in a large-scale population of patients with BA.

Key Words: bronchial asthma; single nucleotide polymorphisms; genes; *IL-13*; *IL-33*; *GSDMA*

Bronchial asthma (BA) is one of the most common chronic respiratory inflammatory diseases in the world. China has the largest asthmatic population and is one of the countries with the highest asthma mortality rate[1]. With the development of molecular biology and genetics, more than 200 genes have been found to be associated with asthma, including the *ADAM33*, C-509T, and T869C polymorphisms of the *TGF- β 1* gene[2, 3], but the results were poorly reproducible in independent studies in various populations. The *IL-13*, *IL-33*, and *GSDMA* genes have been consistently identified as closely related to asthma by multiple studies[4-8]. However, little has been done regarding the detection of these loci. Therefore, the establishment of a simple, rapid, and high-throughput method for detecting asthma susceptibility gene polymorphisms with a larger sample size will greatly assist with the detection of disease occurrence. In the current study, the fluorescent PCR melting curve method was used to detect the SNPs of genes associated with asthma susceptibility (rs1295686, rs1342326, and rs3894194)[9]. The aim of this study is to verify the feasibility of this technique for

detecting SNP loci and to provide new ideas for the prevention, diagnosis, and treatment of asthma.

1. Subjects recruitment and Methods

1.1 Subjects recruitment

A total of 33 asthmatic patients from the Jining area were selected as subjects.. Grade 1 was comprised of 10 patients with intermittent symptoms only and no medication. Grade 2 was comprised of 19 patients whose symptoms were severe enough to require intermittent bronchodilators (BD). Grade 3 was comprised of 4 patients whose symptoms were severe enough to require daily BD. The average age was 24.12 years. All of the patients were of Han nationality, and they had no blood relationship with each other. The clinical information of 33 patients are shown in Table 1. All of the above cases met the diagnostic criteria formulated by Asthma Study Group of the Chinese Thoracic Society[10]. Informed consent from the patients was obtained. The study was approved by the ethics committee of Jining Medical University.

1.2 Methods

1.2.1 Nucleic acid extraction

A QIAamp DNA Mini Kit™ (Qiagen, Germany) was used to extract the DNA from peripheral blood. The purity and concentration of the extracted nucleic acid was determined with a NanoVue Plus™ micro-UV spectrophotometer (GE, USA).

1.2.2 Positive plasmid acquisition

1.2.2.1 Plasmid construction

The genomic DNA of wild-type HEK293T cells was used as the template and the target fragment was fused with plasmid pcDNA3.1/5Flag vectors by method of primer overlapping extension. To be specific, by using primers with complementary terminals, the products of PCR form overlapping chains, which in subsequent amplification reactions, overlapping fragments from different sources can be spliced together through

the extension of overlapping chains. The constructed plasmids were rs1342326 TT, rs1342326 GG, rs3894194 CC, rs3894 194TT, rs1295686 GG, and rs1295686 AA.

1.2.2.2 Cell culture, transient transfection and plasmid extraction

HEK293T cells were cultured in DMEM supplemented with 10% (v/v) FBS in a humidified atmosphere with 5% CO₂ atmosphere at 37°C. Transfection was performed by using Lipofectamine 2000[®] according to the manufacturer's instructions. Plasmid extraction was performed with the operation instructions of Plasmid Mini Preparation Kit.

1.2.3 Establishment of fluorescence PCR reaction system

Primer and probe were designed with the help of Primer Premier 5.0 and TM Utility v1.3 software. Three pairs of amplification primers (F1/R1, F2/R2, F3/R3) targeting IL33, GSDMA and IL13 genes and specific probes (P1, P2, P3) targeting target sites were designed respectively. The sequences are shown in Table 2. The expected amplified fragments were 221 bp, 142 bp, and 220 bp, respectively. The results are shown in Fig.1 Primers and probes with the designated sequences were synthesized by Shanghai Bioengineering Company.

The reaction procedures were as follows: 95°C pre-denaturation for 5 min, 95°C denaturation for 15 s, 58°C annealing for 15 s, 72°C extension for 20 s, for 40 cycles. Fluorescence signals of the corresponding detection channels were collected at 58°C annealing stage, 95°C denaturation for 1 min, 35°C holding for 3 min, and then the melting curve was analyzed by increasing the heating rate of the 0.5°C/step from 40°C to 85°C. The fluorescence signals of HEX, ROX, and CY5 detection channels were collected during the process of increasing temperature. The experiment was conducted using a Bio-Rad CFX96 real-time PCR machine (Bio-Rad, USA).

1.2.4 Repetitive study

Tests were conducted individually and separately at three different times.

1.2.5 Detection limit test

The established system was used to detect 10-fold gradient-diluted human genomic DNA templates to determine the minimum detection limit of the system. The genomic DNA templates added to each gradient were 10 pg, 100 pg, 1 ng, 10 ng and 100 ng.

1.2.6 Detection and sequencing of Samples

PCR amplification and melting curve analysis were carried out with a DNA sample volume of 5 µl. All of the amplified products were sent to Shanghai Meiji Biomedical Technology Co., Ltd. to perform the sequencing, and the results were compared with the melting curve analysis method.

1.2.7 Data analysis

CFX ManagerTM and OriginPro 8 software were used for data analysis.

2. Results

2.1 Analysis of fluorescence PCR melting curve data

The melting curves of 33 clinical samples were analyzed. The results are shown in Fig.2A. The melting diagram was divided into three parts: HEX signal, ROX signal and CY5 signal. The homozygosity of rs1342326 was TT in 33 samples; CC homozygosity, TT homozygosity, and CT heterozygosity were detected at rs3894194; and GG homozygosity, AA homozygosity, and GA heterozygosity were also detected at rs1295686. The results are summarized in Table 2. In order to verify the accuracy of this method, DNA samples were sent to a private company for sequencing, and the results are consistent with the melting curve results. Typical sequencing results are shown in Fig.2B.

2.2 Minimum detection limit

As shown in Fig.3, the height of the melting peak decreases with the decrease in the genomic DNA template concentration, but it does not affect the genotyping of the

sample. The sensitivity of rs1295686 was approximately 1 ng/reaction, and the sensitivity of rs1342326 and rs3894194 reached 10 pg/reaction.

2.3 Sensitivity and specificity detection

As shown in Table 3, this technique is highly sensitivity (100.0%) and specificity (100.0%) for the genotyping of SNPs.

2.4 Repeatability experiments

The results of three independent experiments were consistent, indicating that the experiment exhibited excellent repeatability (CV=2.8%).

3. Discussion

Nowadays, the incidence of BA is increasing worldwide, and this disease is becoming a more serious global health problem. People of all ages throughout the world are being affected by this chronic airway disease that, when uncontrolled, can severely restrict daily life, and it is sometimes fatal. The prevalence of asthma is increasing in most countries[11]. The burden of asthma is experienced not only in terms of health care costs, but also as lost productivity and reduced participation in daily activities[12]. Therefore, the early diagnosis of asthma has become an urgent problem.

It is well known that the occurrence of asthma is affected by genetic and environmental factors[13]. With the development of molecular biology and genetics, hundreds of genes have been found to be associated with asthma[6, 13-15]. For example, because of the increase of IL-33 factor, Th cells are induced to differentiate into Th2 cells. Activated Th2 cells secrete IL-13 factor and activate B lymphocytes. B lymphocytes produce specific IgE binding to IgE receptors on cells[4, 8, 16]. When the airway is stimulated by allergens or other stimuli, GSDMA factor is involved in airway epithelial lesions, resulting in increased IgE[17]. Therefore, *IL-33*, *IL-13* and *GSDMA* were selected in this study, even if they could not cover all the asthma susceptibility genes.

There are many SNP detection methods, including traditional polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), allele-specific polymerase chain reaction (AS-PCR), mass spectrometry, Sanger sequencing, and so on[18-21], but these methods are limited because of their complex operation, expensive cost, and other factors. Real-time PCR is one of the most important methods for RNA and DNA detection and is widely used in medical diagnostics and other medical research. It has been favored by many researchers in the field of gene detection because of its rapid, accurate, and simple detection results[22-26]. In view of this condition, we established a fluorescent PCR melting curve platform to detect SNPs of BA susceptibility genes.

In our study, 33 samples were detected by PCR melting curve and Sanger sequencing firstly. The results obtained by the two methods are identical (Fig.1 and Table 2). This result shows that our method can replace Sanger sequencing for sample detection. Moreover, we have studied the performance of the melting curve system of PCR. From Fig.2 and Table 3, we can see that the sensitivity and speciality of the fluorescent PCR melting curve platform have reached a high level (100%, 100%). We attribute this high recognition rate to the usage of target-specific probes. A single probe only binds to specific target fragments, which eliminates the production of other non-specific products in the amplification process. Then, we evaluated the lowest detection limit (LOD) of the fluorescent PCR melting curve system. We found that LOD at rs1342326 and rs3894194 loci could reach 10 pg/reaction, while LOD at rs1295686 loci could reach 1 ng/reaction. This results fully demonstrate that our platform can be used to detect samples with a small amount of DNA (amniotic fluid, blood spots, etc.), which greatly enhances the scope of application of our platform, as shown in Fig.3. Finally, good repeatability (CV = 2.8%) further reflects the advantages of this method.

The theory of genetic etiology of BA is a powerful measure to further understand its pathogenesis. Its ultimate goal is to achieve good results in disease prevention, diagnosis, and treatment measures. In the current study, the fluorescent PCR melting curve method was applied to detect the susceptibility gene SNPs for BA. The aim was to

identify the high-risk asthma patients according to the results of the study, formulate corresponding preventive measures to prevent the onset of asthma, or provide early diagnostic methods according to the results of the study, so as to reduce the incidence of asthma, as well as reduce the number of asthma attacks.

Of course, this study also has some limitations. First of all, the number of samples involved in the study is small, which may lead to one-sidedness in the evaluation of the melting curve system of PCR. We also plan to increase the sample size in future studies to make the results more convincing, so as to better assist clinicians in the detection of SNPs of asthma susceptibility genes. Nevertheless, compared with other detection technologies, the PCR melting curve platform built by us is superior to other detection technologies in terms of both performance and operation simplicity. Secondly, hundreds of genes have been reported to be susceptible to asthma, but it is not enough to select *IL13*, *IL33* and *GSDMA* only. Although these three types of genes represent some of the genes that cause asthma, many other genes to be missed. This is mainly due to the restriction of fluorescent labeling of probes, which can only label four kinds at most, which greatly reduces our detection flux. However, our next plan will be to introduce as many asthma susceptibility genes as possible, so as to maximize our clinical diagnosis positive rate.

There are few studies on the relationship between SNPs and severity of BA, and our future research will be in this direction. In addition, with the gradual globalization of research data, the integration of hundreds of asthma-related genes into a database specific to the Asian population is also the focus of our next research.

In summary, our results showed that the SNP detection system based on the fluorescent PCR melting curve could accurately classify the susceptible genes of BA with high specificity, sensitivity, and reproducibility. We consider that many reasons to explain this phenomenon. First, the system has a single-step closed-tube detection, which greatly reduces the contamination when the cover of the PCR machine is opened; Second, the operation is relatively simple and fast, and it can be completed by a single

person, from the collection of samples to the output of the results of the entire process, reducing cross-contamination. This method can be used to economically screen the SNP genes associated with susceptibility to BA. With the development of molecular biology, the research on the genetics, immunology, and pathophysiology of BA has deepened day after day. It is helpful to reveal the genetic characteristics of BA from a higher level of research and approach, and we expect to control the inheritance of atopy and prevent the occurrence of BA through genetic screening in the future. This strategy can provide an objective basis for future gene therapy. Combining melting curve and amplification analysis in a single-tube reaction provides a powerful means for BA susceptibility gene SNP typing. This new method holds great promise for future applications not only in asthma but also as a simple solution for the diagnosis of other diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 1 The clinical information of 33 patients

ID	Gender	Age	Recruit	Situation	Progression of Disease	Family History	IgE (IU/ml)	Disease Staging
1	M	17	in-patient	co-infection	moderate	-	100.0	2
2	F	19	in-patient	acute	moderate	-	469.0	2
3	F	19	in-patient	acute	severe	-	8520.0	2
4	M	24	in-patient	acute	moderate	-	142.0	1
5	F	28	in-patient	acute	severe	-	598.0	3
6	F	28	in-patient	acute	moderate	-	115.0	2
7	F	30	in-patient	acute	moderate	-	469.0	2
8	M	30	in-patient	acute	moderate	-	41.7	2
9	F	21	in-patient	acute	mild	+	4.5	2
10	M	31	in-patient	co-infection	severe	-	244.0	3
11	F	32	in-patient	acute	mild	-	32.3	1
12	M	24	in-patient	acute	mild		83.3	1
13	M	34	in-patient	acute	mild	-	30.9	2
14	F	19	in-patient	co-infection	moderate	+	195.0	1
15	F	21	in-patient	acute	mild	-	331.0	2
16	F	18	in-patient	acute	mild	-	457.0	2
17	F	31	in-patient	acute	mild	-	16.4	1
18	M	19	in-patient	co-infection	moderate	+	56.1	1

19	F	20	in-patient	acute	mild	-	100.0	1
20	M	20	in-patient	acute	severe	-	2810.0	3
21	F	21	in-patient	co-infection	severe	-	8520.0	3
22	F	22	in-patient	co-infection	moderate	-	37.9	2
23	M	23	in-patient	acute	moderate	-	56.1	1
24	F	23	in-patient	acute	mild	+	96.2	1
25	F	23	in-patient	acute	mild	-	135.0	2
26	F	24	in-patient	acute	mild	-	11.0	2
27	M	24	in-patient	co-infection	moderate	-	80.0	2
28	F	24	in-patient	acute	mild	-	105.0	2
29	F	27	in-patient	acute	mild	-	7.1	1
30	F	29	in-patient	acute	mild	-	142.0	2
31	F	19	in-patient	acute	mild	-	628.0	2
32	M	23	in-patient	acute	mild	+	215.0	2
33	F	29	in-patient	acute	mild	+	105.0	2

Table 2. Primers and probe sequences

Name	Sequence (5'→3')	SNP sites	Amplified fragment length
F1	AGTTTCCTCCTCTGAGC	rs1342326	221 bp
R1	CTTTGACCCTTCAGAGCAC		
P1	HEX-CTCATGCTGGTGTCTTCATGAGA-BHQ1		
F2	GGCAGAAGGGATGGAAGC	rs3894194	142 bp
R2	CTCTCCTGTCTCCCA		

P2	ROX-TGGCCTCGAGGGTTTAGCTGTCTGGCCA-BHQ2		
F3	TTTGGTGTCTCGGACA	rs1295686	220 bp
R3	GCTCAAGGGCTCCTAACTC		
P3	CY5-CAGCGTTCTACTCACGTGCTGA-BHQ2		

Table 3. Statistics of 33 clinical samples

SNP sites		Sample number	Frequency (%)	Sensitivity (%)	Specificity (%)
rs1342326	T/T	33	100	100	100
	G/G	0	0	-	-
	T/G	0	0	-	-
rs3894194	C/C	5	15.2	100	100
	T/T	9	27.2	100	100
	C/T	19	57.6	100	100
rs1295686	G/G	8	24.2	100	100
	A/A	11	33.4	100	100
	G/A	14	42.4	100	100

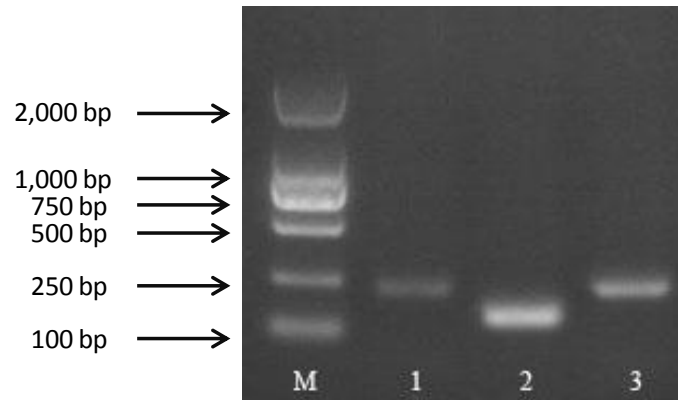


Fig.1 Result of agarose gel of target fragment

Note: From left to right, M represents 2000 bp marker, Lane 1 represents rs1342326 site amplification product (221 bp), Lane 2 represents rs3894194 site amplification product (142 bp), Lane 3 represents rs1295686 site amplification product (220 bp).

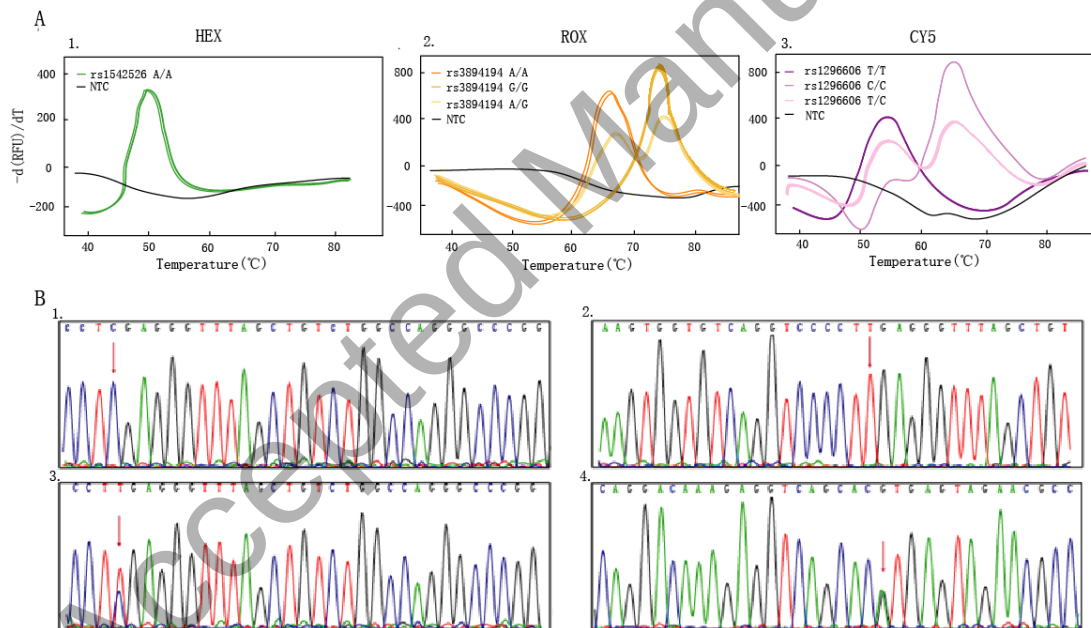


Fig. 2 Detection of melting curve and sequencing results

Note: Figure A shows the results of the fusion curves of 33 specimens. Figure B shows partial sequencing results, with red arrows pointing to SNP loci, B1:rs3894194, CC homozygous; B2:rs3894194, TT homozygous; B3:rs3894194, CT heterozygous; and B4:rs1295686, GA heterozygous.

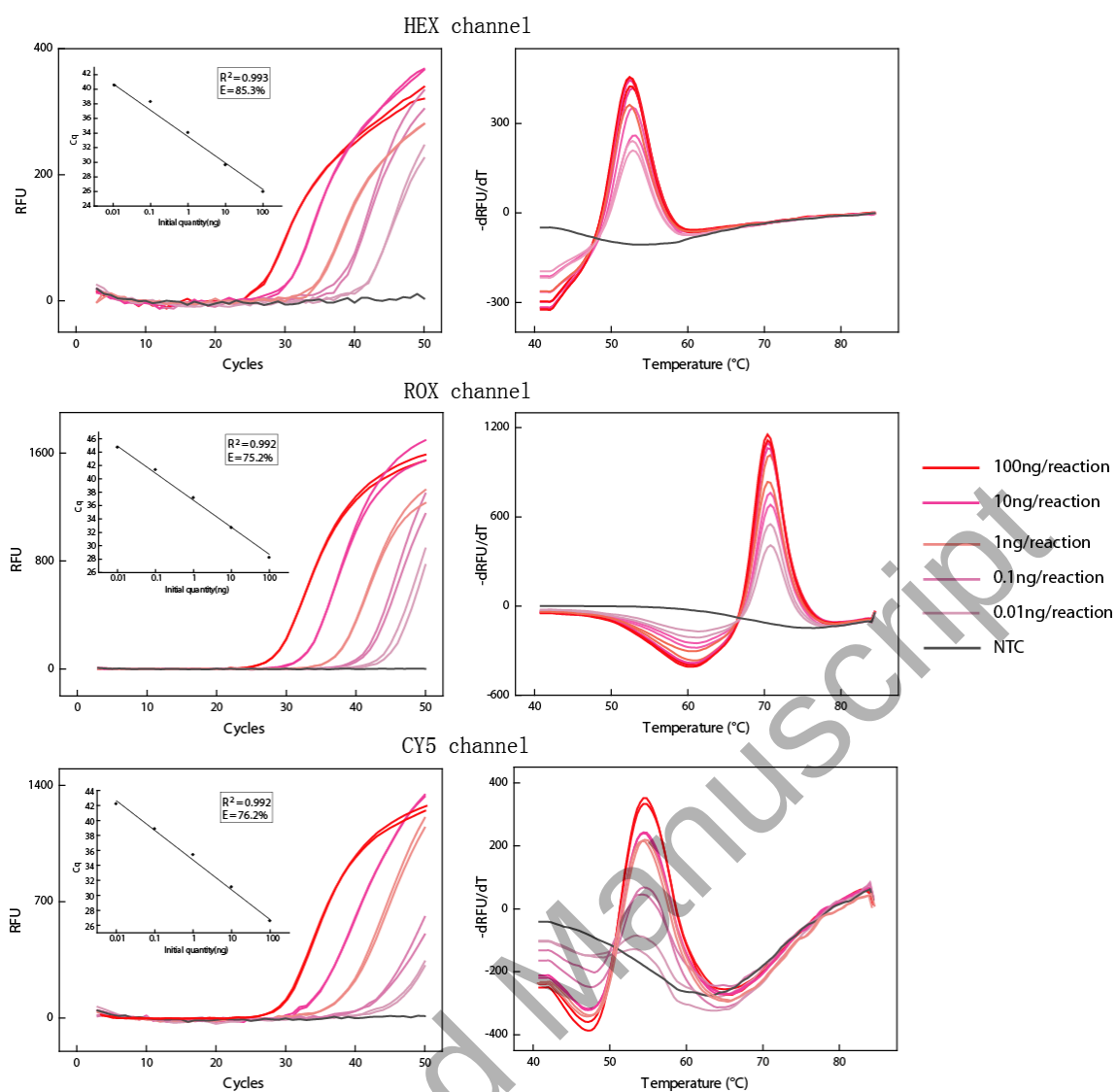


Fig. 3. Results of the sensitivity experiment

Note: The HEX channel is the rs1342326 site, the ROX channel is the rs3894194 site, and the CY5 channel is the rs1295686 site.