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Applications of gene-chip for the detection of mutations in cTnI gene associated with FHCM

Zhang Yuanying[1,3] Guo Huishi[1] He Nongyue[1,2] Gu Yu[1] Yang Di[2]
Wan Wenhui[2] Zhang Jinan[2]

([1] State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China)
([2] Research Institute of Cardiovascular Disease, First Affiliated Hospital, Nanjing Medical University, Nanjing 210029, China)
([3] Jiangsu Institute of Cancer Research, Nanjing 210009, China)

Abstract: Mutations in cardiac troponin I (cTnI) gene were assessed based on gene-chip technology. Special probes were designed to fabricate the low-density gene-chip, which could detect the mutations in exons 3, 5, 7, and 8 of the cTnI gene simultaneously. For each exon, two oligonucleotide sequences labeled with fluorescein at the 5′-end were designed, one (oligonucleotide Ⅰ) simulating the wild type and the other (oligonucleotide Ⅱ) simulating the mutant. Oligonucleotides Ⅰ and Ⅱ were mixed together to simulate the heterozygote. After optimizing the hybridization protocols, the fabricated gene-chip could detect the mutations in the exons of the cTnI gene with relative high sensitivity and specificity. The fully complementary probe gave a fluorescent signal almost 50% stronger than that of the one-base mismatched one, which is in accordance with the result from a theoretical estimate. An applicable special gene-chip is available to investigate and diagnose familial hypertrophic cardiomyopathy (FHCM) after further improvement.

Key words: hypertrophic cardiomyopathy (HCM); gene-chip; mutation; hybridization

The emergence of the Human Genome Project prompted various technological developments. One is the idea of using the gene-chip for sequence analysis. The gene-chip, also called DNA microarray, is an advanced technology which arose in the 1990s. It is composed of DNA probes immobilized on a solid substrate. It transfers many discontinuous analytical processes to chips, and makes it continuous and subminiature[1-3]. Based on the principle of molecular hybridization, the gene-chip can acquire a large amount of sequence information directly and simultaneously. In the past, most mutations were identified by conventional methods such as SSCP and DNA sequencing. These traditional gel-based sequencing methods are relatively time-consuming, laboursome, sequential processes. With the advantage of high throughput information, the gene-chip has become one of the best solutions to detect and analyze mutations in genes[4-6]. This newly developed technology has shown enormous potential and exciting foreground, representing a significant technical jump from previous technologies.

Hypertrophic cardiomyopathy (HCM) is a common cardiac muscle disease, which causes a high incidence of sudden death and heart failure. The prevalence reported is 0.2%. However, up to now, there is no simple and viable detection method for early diagnosis. Familial HCM(FHCM) accounts for up to 55% of all cases of HCM. It is transmitted as an autosomal dominant trait. Mutations in at least eight different genes contribute to HCM. The development of a low-density DNA chip hybridization assay for the identification of known point mutations causing HCM in MYH7, MYBPC3, TNNT2, and the a-tropomyosin gene has been reported. The cTnI gene, a member of the causes of HCM, has not been studied yet. About 3% to 5% of FHCM in patients is caused by cTnI (cardiac troponin I) mutations. Gene TNNI 3, which is located on the 19th chromosome, encodes cTnI, including eight exons. Several mutations associated with FHCM in exons 3, 5, 7 and 8 have been reported.

With the appearance of the gene-chip, fast genic diagnosis has become possible[7,8]. The purpose of this paper is to perform an assay of the mutations in the exons 3, 5, 7 and 8 of the cTnI gene associated with FHCM based on the gene-chip theory and technology. We studied whether the recently developed oligonucleotide microarray technique, the gene-chip assay, can be applied to sensitive detection of cTnI gene mutations from cardiac disease patients. When applying the fabricated gene-chip to detect the target pre-synthesized DNA sequence, the fluorescent intensity ratio could lead one to the result in accordance with the theoretical

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Biographies: Zhang Yuanying (1978—), female, master; He Nongyue (corresponding author), male, doctor, professor, nyhe1958@163.com.

estimate. Finally when applying the fabricated gene-chip to detect PCR product from a fresh blood sample, positive results were obtained. It is believed that an applicable special gene-chip can be developed to investigate and diagnose FHCM after further improvement.

1 Experimental

1.1 Reagents

MicroHyb hybridization buffer was purchased from Research Genetics. Target oligonucleotide and probes were purchased from Shanghai Shenyou Biotechnology Company.

1.2 Instrumentation

Ultrapure water was purified with a Milli-Q water purification system. DNA probes were spotted with a Pixsys5500 DNA spotting machine. Hybridization signals were recorded using the Scan-Array Lite Microarray Analysis System (Packard Biochip Technologies), and the intensity was analyzed using the scanner software.

1.3 Design of probes

For every mutation, we designed four probes. These probes were set to represent wild type, mutant and heterozygote of the exons. The mutational site was placed in the middle of the probe sequence (0 site). The first probe (probe 1) is completely complementary to the normal wild-type sequence. The second (probe 2) is completely complementary to mutation sequence. More specifically, the third probe (probe 3) had a common substitution position located at the +1 or −1 site and the rest bases were the same as probe 1, while the probe 4 had a common substitution position located at the +1 or −1 site and the rest of the bases were the same as probe 2. These two last probes can discriminate the heterozygosis. In all the designed probe arrays, the layout of probe 1 to 4 of each probe set were from top to bottom as described in Fig. 1. All the designed probe sequences are displayed in Tab. 1.

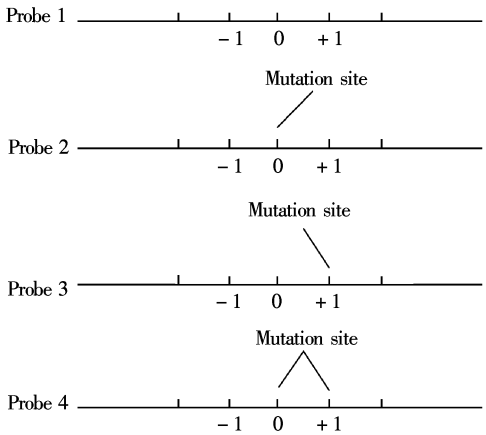

Fig. 1 Layout of designed probes

1.4 Preparation of gene-chip

Glass slides were used as substrates. After silanization with $(C_2H_5O)_3Si(CH_2)_3NH_2$ (APTES), the surfaces of the glass slides were coated with amido. After soaking in 5% glutaraldehyde for 2 h, aldehyde was linked on the surface. After washing with 10 mmol/L PB for 5 min and with pure water for 5 min twice, the glass was dried and was ready for use.

The probe solutions (80 μmol/L) were spotted on substrates and were left at room temperature through the night. Then the glass slides were incubated at 37 ℃ for 1 h in a water bath. The slides were washed with 0.1% SDS for 5 min and twice with pure water for 5 min. Thus, the fabrication of the gene-chip was completed. Then soaking the chip in the solution containing sodium borohydride (0.14 g sodium borohydride, 38 mL 10 mmol/L PB, 12 mL 95% ethanol) for 40 min, the aldehyde exposing at the surface was reduced to hydroxyl. After washing thoroughly, the chips were ready for hybridization.

1.5 Preparation of fluorescence-labeled DNA target

For each mutation investigated, we designed two sequences labeled with fluorescein at the 5′ end as target sequences to hybridize with probes on array. Oligonucleotides Ⅰ simulated the wild-type sequence and oligonucleotide Ⅱ simulated the mutant. The oligonucleotides Ⅰ and Ⅱ were mixed together to simulate heterozygote. For example, the oligonucleotides were synthesized for exon 8 as follows:

Oligonucleotide Ⅰ

5′-GGCGGAAGAACATCGATGCACTGAGTGG AATGGAGGGCCGCAAGAAAAAGTTTGAGAGCT3′

Oligonucleotide Ⅱ

5′-GGCGGAAGAACATCGACGCACTGAGTGG AATGGAGAGCCGCAAGCAAAAGTTTGAGAGCT3′

CY3-dUTP was incorporated into the target DNA chains by PCR. All the primers for PCR are listed in Tab. 2. 30 μL of PCR mixture was made by combining components such as 22.5 μL of DEPC-H_2O, 3 μL of 10 × PCR buffer (100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L $MgCl_2$), 1 μL of dNTP (5.0 mmol/L each of dATP, dTTP, dGTP, but 0.5 mmol/L of dCTP), 2 μL of CY3-dUTP (0.25 mmol/L), 1 μL of forward and reverse primers (5 μmol/L each), 0.5 μL of Taq DNA polymerase (5 U/μL) (DR100A, TaKaRa Biotechnology (Dalian) Co., Ltd.) and 1 μL of human DNA (extracted from a human blood sample). PCR was performed with one cycle at 94 ℃ for 4 min, then 35 cycles at 94 ℃ for 60 s, 64 ℃ for 60 s, 72 ℃ for 45 s, and an additional cycle at 72 ℃ for 7 min. The PCR products were purified and sequenced. The sequencing results were the same as those normal sequences. Taking it for example, the checked sequence of PCR product of exon 8 in cTnI gene is shown in Fig. 2.

Tab. 1 Designed probe sequences

Exon	Condon	Type	Probe
Exon 3	16	Wild type	5′-NH_2-TTGGGGCTGGTGCAG-3′
		Mutant	5′-NH_2-TTGGGGCCGGTGCAG-3′
		One base mismatch	5′-NH_2-TTGGGGGTGGTGCAG-3′
		Two bases mismatch	5′-NH_2-TTGGGGGCGGTGCAG-3′
Exon 5	58	Wild type	5′-NH_2-CAGCTCTTGCTTTGCAATC-3′
		Mutant	5′-NH_2-CAGCTCTTGATTTGCAATC-3′
		One base mismatch	5′-NH_2-CAGCTCTTCCTTTGCAATC-3′
		Two bases mismatch	5′-NH_2-CAGCTCTTCATTTGCAATC-3′
	74	Wild type	5′-NH_2-AGAGCGCGCCCCTTC-3′
		Mutant	5′-NH_2-AGAGCGCTCCCCTTC-3′
		One base mismatch	5′-NH_2-AGAGCGGGCCCCTTC-3′
		Two bases mismatch	5′-NH_2-AGAGCGGTCCCCTTC-3′
	79	Wild type	5′-NH_2-TGGCAGCGGGTGCTC-3′
		Mutant	5′-NH_2-TGGCAGCAGGTGCTC-3′
		One base mismatch	5′-NH_2-TGGCAGCGCGTGCTC-3′
		Two bases mismatch	5′-NH_2-TGGCAGCACGTGCTC-3′
Exon 7	145	Wild type	5′-NH_2-CACTCTCCGCAGGGTGG-3′
		Mutant	5′-NH_2-CACTCTCCCCAGGGTGG-3′
		One base mismatch	5′-NH_2-CACTCTCCGGAGGGTGG-3′
		Two bases mismatch	5′-NH_2-CACTCTCCCGAGGGTGG-3′
	145	Wild type	5′-NH_2-TCACTCTCCGCAGGGTG-3′
		Mutant	5′-NH_2-TCACTCTCTGCAGGGTG-3′
		One base mismatch	5′-NH_2-TCACTCTGCGCAGGGTG-3′
		Two bases mismatch	5′-NH_2-TCACTCTGTGCAGGGTG-3′
	162	Wild type	5′-NH_2-TTAGCCCGGGCCCCC-3′
		Mutant	5′-NH_2-TTAGCCCAGGCCCCC-3′
		One base mismatch	5′-NH_2-TTAGCCCGCGCCCCC-3′
		Two bases mismatch	5′-NH_2-TTAGCCCACCCCCCC-3′
	179	Wild type	5′-NH_2-CTCGGTGTCCTCCTTCTTC-3′
		Mutant	5′-NH_2-CTCGGTGTCTTCCTTCTTC-3′
		One base mismatch	5′-NH_2-CTCGGTGTGCTCCTTCTTC-3′
		Two bases mismatch	5′-NH_2-CTCGGTGTGTTCCTTCTTC-3′
	183	Wild type	5′-NH_2-ACACTCACCTTCTCGGTG-3′
		Mutant(three base deletion)	5′-NH_2-CCCACACTCACCTCGGTG-3′
Exon 8	196	Wild type	5′-NH_2-CACTCAGTGCATCGATGTTCT-3′
		Mutant	5′-NH_2-CACTCAGTGCGTCGATGTTCT-3′
		One base mismatch	5′-NH_2-CACTCAGTGCAACGATGTTCT-3′
		Two bases mismatch	5′-NH_2-CACTCAGTGCGACGATGTTCT-3′
	203	Wild type	5′-NH_2-TTGCGGCCCTCCATT-3′
		Mutant	5′-NH_2-TTGCGGCTCTCCATT-3′
		One base mismatch	5′-NH_2-TTGCGGGCCTCCATT-3′
		Two bases mismatch	5′-NH_2-TTGCGGGTCTCCATT-3′
	206	Wild type	5′-NH_2-AAACTTTTTCTTGCGGC-3′
		Mutant	5′-NH_2-AAACTTTTGCTTGCGGC-3′
		One base mismatch	5′-NH_2-AAACTTTATCTTGCGGC-3′
		Two bases mismatch	5′-NH_2-AAACTTTAGCTTGCGGC-3′

Tab. 2 Primers for exons 3, 5, 7 and 8 of cTnI gene

Exon	Forward (5′-3′)	Reverse (5′-3′)	Product size/bp
3	GGGTCCCCACTCCTCCTA	TCCGGCGCCTGTACTCTG	128
5	GTCTTTATCCTGAAGCCCCG	TCCGGGACTAGAAACCTCG	222
7	GTAGGATGGAGGAGTTGGGT	CCCCTCAGCATCCTCTTTCCT	287
8	AGACCCTAACCTCTGACTCATC	TAGGCAGGAAGGCTCAGCT	137

1.6 Hybridization of gene-chip

It should be noted that although the melting temperature (T_m) of oligonucleotides can be estimated by means of the relationship: $T_m = 4(G + C) + 2(A + T)$, where $(G + C)$ or $(A + T)$ are the $(G + C)$ or $(A + T)$ content in the oligonucleotide, usually the applied hybridization temperature is slightly lower than the estimated T_m. In this study, we first optimized the hybrid-

ization conditions including hybridization temperature, hybridization time, composition of hybridization solution, washing solution and washing time. Among these factors, the most important one is hybridization temperature. Actually, after a series of tests, we found that under hybridization at temperature 42 ℃ for 2 h the one-base mismatch can be detected. Therefore, the fluorescein-labeled target samples were then hybridized in a reaction solution containing MicroHyb hybridization buffer added to the probe arrays for 2 h at 42 ℃. To remove nonhybridized DNA fragments from the arrays, the probe arrays were then washed once with washing buffer A (2 × SSC, 0.1% SDS) for 12 min and buffer B (0.1 × SSC, 0.1% SDS) for 7 min, followed by rinsing in water quickly and drying. We performed the hybridization with each of the mixtures of oligonucleotides Ⅰ, Ⅱ, and PCR products according to the procedure. After completion of the hybridization-wash cycle, the arrays were scanned with a laser scanner. Target DNA binding to certain oligonucleotide probes at the surface was identified by detecting emission from a fluorophore attached to the target. The emitted light intensity is proportional to the bound DNA at each location in the probe array. The probes best matching the target sequences yielded the highest fluorescence intensity when they were exposed to the GeneArray scanner. The average signal intensities were calculated with the specialized software with the scanner for each probe cell. Since the positions and sequences of the specific cells in the arrays are known, the sequences of the target nucleic acids can be determined. A report sheet that listed fluorescence intensity ratio between probes was identified with respect to every mutation.

2 Results and Discussion

After hybridization, the microarray was scanned with a laser fluorescent scanner. The signal intensity was acquired using the software. The mutations of the investigated exons in the cTnI gene were identified by comparing the fluorescence intensity of sites. It was shown that the mutations could be clearly detected. As a representative, Fig. 3 shows the hybridization signals of the wild-type target, mutant target, and heterozygote target of exon 8 with the respective probe arrays. The columns from left to right in Figs. 3(a), (b) and (c) are the probe sets for condon 196, 203 and 206, respectively. The probes were assigned as described in Fig. 1 from top to bottom.

The detected hybridization signal intensity of each probe position can be ordered from high to low as follows: yellow > green > blue > black. The highest fluorescent site indicates the hybridization with the complementary nucleotide sequence. In Fig. 3 (a) (wild type), the green fluorescent signal identified in the first line represents the strongest hybridization of the normal sequence to probe at the queried mutation position. The blue weaker hybridization signal in the second line demonstrates lower signal intensity than that observed in the first line for the perfectly complementary hybridization, which shows a green signal. The signal in the third line is weaker than that in the first line but higher than those in the other two lines because of the departure of its single-base mismatch position of the probe from middle. The signal intensity in the last line is weaker than those in all the upper lines because of a two-base mismatch in the probe. In Fig. 3(b) (mutant), the highest fluorescent signal identified in the second line represents the strongest hybridization of the mutant sequence to probe at the queried mutation position. The order of signal intensity corresponds with the rule in Fig. 3(a). In Fig. 3(c) (heterozygote), the highest fluorescent green signals identified in the first and second lines represent the hybridization of the normal and mutational nucleotides in the mixture to probes. It is because that the mismatch situation in the first and second lines is similar to each other in the case of the heterozygote. The similar situation occurs in the third and the last lines. We find the signal intensities in the last two lines are almost equal.

The fluorescence intensities ratio of different target hybridization signals are shown in Fig. 4. The signal intensities from left to right in Fig. 4 for each condon are corresponding to the spots in Fig. 3. It can be seen that the fully complementary probe gave a fluorescent signal almost 50% stronger than that of the one-base mismatched one, which was in accordance with the result from the theoretical estimate.

To check the applicability of the fabricated probe arrays, we detected the hybridization of the whole probe array interrogated by PCR products. The sequences of the PCR product for exons 3, 5, 7 and 8, which were prepared from the DNA template extracted from a fresh health blood sample, completely matched the wild type sequences. Fig. 5 displays the hybridization signal pattern of the integrated probe array which contains all the designed probes. From left to right the probes were assigned in the sequence listed in Tab. 1. In each column the probes were assigned as described in Fig. 1. Then the array was hybridized with the mixture of PCR products to interrogate mutations in exons 3, 5, 7 and 8. Taking exon 8 for example, it is demonstrated that the distribution of signal intensities of probe positions is similar to that

shown in Fig. 3(a). So it is proved that the sample we tested has no mutation in exon 8 of cTnI gene. The hybridization results of PCR products of other exons also accord with those observed for the hybridization of the synthetic oligonucleotide of relative exons. That the signal intensities of exon 7 are relatively weak may be due to the actual difference in melting point temperatures (T_m) of different probes for different exons, even if the theoretically estimated T_m values for different probes are similar to each other. These results mean that the fabricated gene-chip can serve as a good tool to detect the mutations in the cTnI gene.

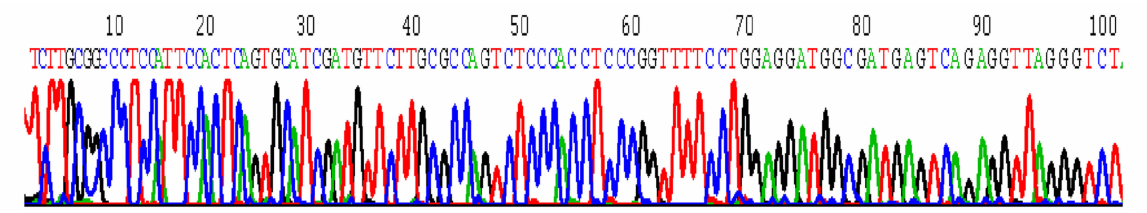

Fig. 2 Checked sequence of the anti-sense strand of the PCR product of exon 8

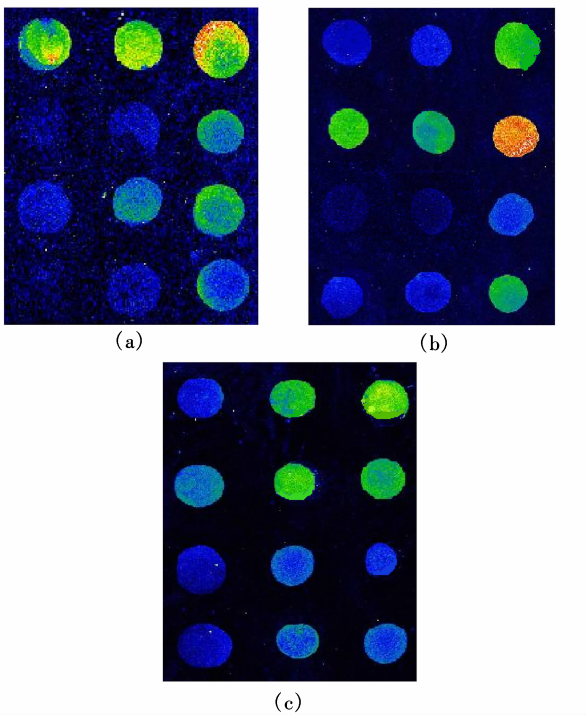

Fig. 3 Images of hybridization signals. (a) Oligonucleotide Ⅰ target (wild type); (b) Oligonucleotide Ⅱ target (mutant type); (c) Mixture of oligonucleotide Ⅰ and Ⅱ (heterozygote)

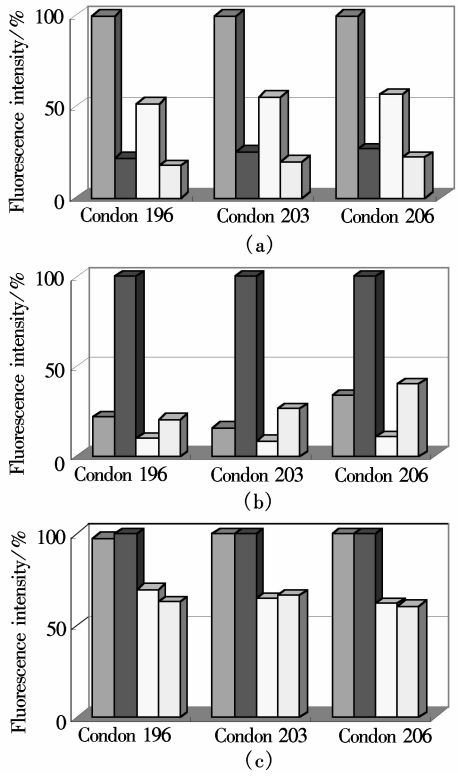

Fig. 4 Fluorescence intensity ratios of the hybridization results. (a) Oligonucleotide Ⅰ target (wild type); (b) Oligonucleotide Ⅱ target (mutant type); (c) Mixture of oligonucleotide Ⅰ and Ⅱ (heterozygote)

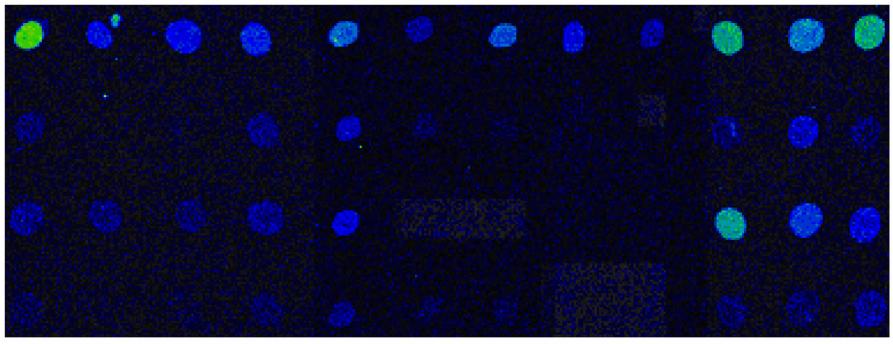

Fig. 5 Hybridization signal pattern of the integrated probe array

3 Conclusion

This study was designed to assess the sensitivity, specificity, and accuracy of DNA microarray for diagnosis of FHCM. Summarily, we demonstrated that this new gene detection system could detect the mutations in exons 3, 5, 7 and 8 of the cTnI gene with relatively high sensitivity and specificity. When applying the fabricated gene-chip to detect the target DNA sequence, we found that the fully complementary probe gave a fluorescent signal almost 50% stronger than that of the one-base mismatched one, which was in accordance with the result from a theoretical estimate. It is believed that an applicable special gene-chip can be developed for investigating and diagnosing FHCM after further improvement.

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家族性肥厚型心肌病相关 cTnI 基因突变检测芯片的应用

张元颖[1,3] 郭会时[1] 何农跃[1,2] 谷 宇[1] 杨 迪[2] 万文辉[2] 张寄南[2]

([1] 东南大学生物电子学国家重点实验室,南京 210096)
([2] 南京医科大学第一附属医院心血管病研究所,南京 210029)
([3] 江苏省肿瘤防治研究所,南京 210009)

摘要:基于基因芯片技术对心肌钙蛋白(cTnI)基因上的突变进行了分析. 针对外显子上的突变特征设计了特异性探针,制备了可以同时检测 cTnI 基因上第 3,5,7,8 外显子突变的低密度基因芯片. 对每一个外显子,设计了 2 条 5′端标记荧光的寡核苷酸链,一条模拟野生型序列,另一条模拟突变型序列,将二者混合起来模拟杂合子序列. 经过实验条件的优化,制作的芯片可以灵敏、特异地检测 cTnI 基因外显子上的突变. 结果表明,该芯片检测突变正配错配区别明显,荧光强度比值符合理论估计(正中碱基错配的信号强度是完全正配信号强度的 50% 左右). 经进一步对芯片优化后,该芯片有望在家族性肥厚型心肌病的研究和诊断中得到应用.

关键词:肥大型心肌病;基因芯片;突变;杂交

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