Multi-Gene Single Nucleotide Polymorphism Detection in Gastric Cancer Based on Ion Semiconductor Sequencing Platform

Kaihua Huang[1], **Xiao Yan**[1], **Zhicheng Huang**[1], **Huayuan Liang**[1], **Zhiwei Li**[1], **Minghao Wang**[1], **Yong Wan**[2], **Suihai Wang**[3,4], **Liying Zhao**[1]

[1] Nanfang Hospital, Southern Medical University [2] Research and Development Department, Guangzhou Darui Biotechnology Co., Ltd [3] Key Laboratory of Antibody Engineering of Guangdong Higher Education Institutes, School of Laboratory Medicine and Biotechnology, Southern Medical University [4] Provincial Key Laboratory of Immune Regulation and Immunotherapy, Southern Medical University

Corresponding Authors

Kaihua Huang
huangkh1999@163.com

Yong Wan
25766728@qq.com

Suihai Wang
wangsuihai@126.com

Liying Zhao
zlyblue11@163.com

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Abstract

Gastric cancer is a common heterogeneous tumor. Most patients have advanced gastric cancer at the time of diagnosis and often need chemotherapy. Although 5-fluorouracil (5-FU) is widely used for treatment, its therapeutic sensitivity and drug tolerance still need to be determined, which emphasizes the importance of individualized administration. Pharmacogenetics can guide the clinical implementation of individualized treatment. Single nucleotide polymorphisms (SNPs), as a genetic marker, contribute to the selection of appropriate chemotherapy regimens and dosages. Some SNPs are associated with folate metabolism, the therapeutic target of 5-FU. *Methylenetetrahydrofolate reductase* (*MTHFR*) rs1801131 and rs1801133, *dihydrofolate reductase* (*DHFR*) rs1650697 and rs442767, *methionine synthase* (*MTR*) rs1805087, *gamma-glutamyl hydrolase* (*GGH*) rs11545078 and *solute carrier family 19 member 1* (*SLC19A1*) rs1051298 have been investigated in different kinds of cancers and antifolate antitumor drugs, which have potential forecasting and guiding significance for application of 5-FU. The ion torrent next-generation semiconductor sequencing technology can rapidly detect gastric cancer-related SNPs. Each time a base is extended in a DNA chain, an H^+ will be released, causing local pH changes. The ionic sensor detects pH changes and converts chemical signals into digital signals, achieving sequencing by synthesis. This technique has low sample requirement, simple operation, low cost, and fast sequencing speed, which is beneficial for guiding individualized chemotherapy by SNPs.

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Introduction

Gastric cancer is a heavy burden in the field of global public health. According to the Global Cancer Statistics 2020, published by the International Agency for Research on Cancer (IARC), gastric cancer is the fifth most diagnosed cancer and the fourth leading cause of cancer-related death. Worldwide, the incidence of age-standardized rate in East Asia is the highest in both males and females[1]. The occurrence of gastric cancer is insidious, which means that patients often do not have any obvious and specific symptoms in the early stage. Among all gastric cancer patients, in countries without routine screening, 80%-90% of patients are either diagnosed at an advanced stage when the tumor cannot be operated on or relapses within 5 years after the operation[2].

For advanced or metastatic gastric cancer, chemotherapy is the main treatment, which can improve the survival rate and quality of life of patients. For the initial therapy of patients with metastatic gastric cancer, a platinum-fluoropyrimidine regimen is the principal choice for a first-line chemotherapeutic regimen[3]. Fluoropyrimidine mainly includes 5-fluorouracil (5-FU) and oral fluoropyrimidine derivatives, such as capecitabine and tegafur. The main target of 5-FU is folate metabolism-related enzymes, which inhibit DNA synthesis and slow the growth of tumor tissue. Adverse drug reactions limit their utility, with diarrhea, mucositis, myelosuppression, and hand-foot syndrome among the most frequent side effects. It has been reported that therapeutic response and adverse drug reactions are closely related to factors in the folate metabolic pathway. Notably, the homozygous mutation of rs1801131 has been identified as an indicator for hand-foot syndrome (p = 4.1 x 10^{-6}, OR =9.99, 95% CI: 3.84-27.8)[4]. Although fluoropyrimidines are extensively used in anticancer chemotherapy, their chemoresistance is a common emergency, causing therapeutic failure in gastric cancer treatment. For instance, the overall response rate is only 10%-15% among patients with advanced colorectal cancer treated with 5-FU only[5]. Also, fluoropyrimidines have toxicity that cannot be ignored. The toxicity reactions induced by 5-FU mainly include diarrhea, hand-foot syndrome, stomatitis, neutropenia, thrombocytopenia, neurotoxicity, and even death[6]. Severe treatment-related toxicity occurs in 10%-30% of patients treated with fluoropyrimidines, and fatal toxicity occurs in 0.5%-1% of these patients[7].

A quality-of-life study of patients with advanced gastric cancer found that the response rate was less than 50% for those who received 5-FU-based chemotherapy[8]. Therefore, understanding the factors related to the sensitivity of 5-FU-based chemotherapy is particularly important for precise treatment to maximize the response rate and effectiveness while minimizing toxicity. Considering that 5-FU is closely related to folate metabolism, the genetic variants of enzymes in folate metabolic pathway may be one of the factors. About 90% of human sequence variation is attributed to single base mutations in DNA, known as single nucleotide polymorphisms (SNPs)[9]. When SNPs change the enzyme properties of folate metabolism, it may lead to individual differences in efficacy, toxicity and chemoresistance to fluorouracil in gastric cancer patients.

Methylenetetrahydrofolate reductase (MTHFR) is mainly used to convert 5,10-methylenetetrahydrofolate (5,10-MTHF) into 5-methyltetrahydrofolate. 5-FdUMP,a metabolite of 5-FU, forms inactive ternary with 5,10-MTHF and thymidylate

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synthase (TS), inhibiting activity of TS and leading to deficiency of dTMP[10]. Accumulation of 5,10-MTHF can enhance the inhibition effect of 5-FU on TS, which is correlated with the activity of MTHFR. *MTHFR* rs1801131 and rs1801133 are the most common polymorphisms, which are related to lower enzyme activity (a decrease of 75% for rs1801133 and 30% for rs181131) and accumulation of 5,10-MTHF[11].

Dihydrofolate reductase (DHFR) is the key enzyme in folate metabolism and DNA synthesis. DHFR reduces dihydrofolate, using NADPH, to tetrahydrofolate (THF) which is used to carry one-carbon unit. SNPs of *DHFR* may affect its expression, change the activity and abundance of THF, and further affect folate metabolism and sensitivity of 5-FU. *DHFR* rs1650697 point mutation occurs in the major promoter of *DHFR* gene, which increases the DHFR expression[12]. A study found that rs442767 is associated with the efficacy and toxicity of antifolate antitumor drugs, such as pemetrexed and methotrexate. Regarding the SNP rs442767, a *GT* genotype signifies the inheritance of a G allele and a T allele at the same locus on the homologous chromosomes from each parent. Similarly, the genotypes *GG* and *TT* denote the inheritance of either two G alleles or two T alleles, correspondingly. Compared with genotype *GT+TT*, *GG* is related to decreased event-free survival and increased risk[13]. This suggests that rs442767 may lead to certain potential influence on 5-FU.

Methionine synthase (MTR) catalyzes the re-methylation of homocysteine to methionine, which plays an important role in folate metabolism. *MTR* rs1805087 is the most common polymorphism of *MTR* gene. *MTR* rs1805087 substitutes glycine for aspartic acid at the potentially functional site of protein, which may decrease the activity of MTR. Subjects with the G allele had increased plasma folate level and decreased plasma homocysteine level[14]. On the contrary, a study showed that rs1805087 has no statistically significant association with the efficacy of 5-FU. But this study focused on colorectal cancer and the sample size was small. The relationship between rs1805087 and the efficacy of 5-FU in gastric cancer patients remains to be explored[15].

Gamma-glutamyl hydrolase (GGH) is a lysosomal enzyme that regulates intracellular folate concentrations. Pteroylglutamic acid is the synonym of folic acid that is made up of pterin, p-aminomethylbenzoic acid and glutamic acid. There are two forms of folic acid in organisms, monoglutamate folate and polyglutamated folate. THF-polyglutamate is enzymatically converted to monoglutamic folate by GGH, successively releasing either mono-Glutamate (mono-Glu) or di-Glutamate (di-Glu)[16]. A study about GGH expression in patients with locally advanced gastric cancer, showed high GGH expression could reduce 5,10-MTHF and TS, which means that only a small dosage of 5-FU is needed to achieve the TS inhibitory effect in these patients[17]. GG is a prognostic biomarker in patients with locally advanced gastric cancer treated with postoperative adjuvant chemotherapy with S-1, a prodrug of 5-FU, and plays an important role in maintaining intracellular homeostasis of folic acid[18]. *GGH* rs11545078 is missense variant and alters Thr-127 to Ile-127. A study focusing on substrate specificity of GGH suggests that rs11545078, compared to wild-type, results in higher Km and lower catalytic efficiency for methotrexate and the structure is similar to folic acid[19]. Together, exploring the relationship between rs11545078 and clinical outcomes of 5-FU is a promising strategy to understand drug resistance.

Solute carrier family 19 member 1 (SLC19A1), also named reduced folate transporter, is a typical facilitative

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transmembrane protein that imports reduced folates that mammalian cells lack the ability to *de novo* synthesize, which is recognized to estimate the response of tumor to 5-FU[20]. However, only a few studies have been performed regarding the association between 5-FU and *SLC19A1* polymorphism[21]. In patients with non-small cell lung cancer receiving pemetrexed which is a folate analog, the rs1051298 on the *SLC19A1* gene contributed to increase the risk of all adverse drug reaction and decrease overall survival[22,23]. *SLC19A1* rs1051298, a 3'untranslated region variant about folate metabolism, may help explain some of the individual differences about 5-FU therapy. Here the aim is to evaluate the association between rs1051298 and 5-FU resistance among patients with gastric cancer.

A kit, based on semiconductor sequencing, is used for qualitative gene detection *in vitro* (**Figure 1**), which can detect 7 selected SNPs in 5 genes, the rs1801131 and rs1801133 mutations of *MTHFR* gene, rs1650697 and rs442767 mutations of *DHFR* gene, rs1805087 mutation of *MTR* gene, rs11545078 mutation of *GGH* gene and rs1051298 of *SLC19A1* gene in tumor tissue samples of gastric cancer patients. Firstly, the sample nucleic acid was extracted, and the target fragment was specifically amplified by PCR. A universal sequencing adapter was added at both ends of the DNA fragment to construct a library that can be used for sequencing. Then amplification of the sequencing library by PCR to form a sequencing template was done. The positive template was enriched to meet the sequencing requirements. Using the semiconductor sequencing system, by fixing the DNA strand in the tiny hole of the semiconductor chip. DNA polymerase takes the single-stranded DNA as the template and synthesizes the complementary DNA strand according to the principle of complementary base pairing. Each time a base is extended in a DNA chain, a proton will be released, causing local pH changes. An ionic sensor detects pH changes and converts chemical signals into digital signals, so that bases can be interpreted in real time, and finally the base sequence of each DNA segment can be obtained. Bioinformatics analysis was used to match these sequences to the reference map of the human genome. When gastric cancer related genes mutate, their corresponding DNA base sequences will change, so as to obtain the mutation information of related genes.

The result can display gene mutation status and provide reference for clinicians to select appropriate types and dosages of chemotherapy drugs and predict the drug resistance for gastric cancer patients. However, the test results are only for clinical reference and are not recommended to be the only basis for individualized treatment of patients. Clinicians should make a comprehensive judgment based on the patient's condition, drug indications, treatment reactions and other laboratory test indicators.

Protocol

All protocols and the tissue samples of gastric cancer (step 1), obtained from upper gastrointestinal endoscopy or surgery, used in this study were reviewed and approved by medical ethics committee on July 24th, 2023 (NFEC-2023-298). Besides, this protocol is designed exclusively to illustrate multi-gene detection in gastric cancer without engaging in cohort comparisons, therefore it imposes no specific criteria for including or excluding patients. The patients/participants provided their written informed consent to participate in this study.

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1. Preparation of embedded blocks of gastric cancer

1. Configure the tissue embedding system, comprising of a paraffin reservoir and dispenser, alongside warm and cold plates, following the prescribed operational guidelines.
2. Extract the prepared tissue for embedding from the dehydrator and deposit it into the embedding center's storage slot.
3. Select fitting embedding molds based on the tissue size, pour enough amount of melted paraffin to cover the tissue, and then position the mold on the warm plate.
4. Retrieve the tissue from the cassettes and place the gastric mucosa perpendicular to the base of the embedding mold to ensure the cross-sectional plane cuts through all tissue layers. Align the tissue in the mold.
5. Set the cassette on the top of the mold, followed by a secondary pouring of paraffin, filling the mold.
6. Place the embedded mold onto the cold plate, and following the paraffin's solidification, snap off the embedded blocks from the molds.

2. Extraction of nucleic acid from samples

1. Use nucleic acid extraction and purification kits to extract nucleic acids from paraffin-embedded tissue samples.
2. Use a nucleic acid quantifier to quantify the extracted nucleic acid. Nucleic acid concentration is recommended to be greater than 2 ng/μL.

   NOTE: Materials used here are available in the **Table of Materials**.

3. Preparation for sequencing library

1. Preparation before experiment
   1. Turn ON the UV lamp in the ultra-clean workbench, sterilize for 30 min. Then, turn OFF the UV lamp and turn ON the fan to ventilate for 10 min.
   2. Take the nucleic acid out of the -20 ± 5 °C. refrigerator, check and record the sample ID, nucleic acid barcode and specific label tag number assigned to the sample. Put it on the centrifuge tube rack for dissolution at room temperature (RT) after verification, and centrifuge it for 10 s, with a fixed 2,500 x *g* for standby.
2. PCR amplification of target fragment
   1. Take the fragment capture reaction solution, amplification primer of gastric cancer and fusion amplification primer from the custom-made gastric cancer multi-gene joint detection kit and put them on ice for melting. Shake and mix them after melting, centrifugate them for 10 s, and prepare nuclease-free water. Detailed primer information is shown in **Table 1**.

      NOTE: The kit is not commercially available yet, contact authotrs for further details.
   2. Prepare a 0.2 mL PCR reaction tube, add reagents into the tube in turn according to **Supplementary Table 1**, vortex and mix for 5 s, and centrifugate instantaneously for 10 s, with a fixed 2,500 x *g*, so that there are no obvious drops on the tube wall and cover. RNA samples shall be reverse transcribed into cDNA and then used for subsequent library construction. cDNA products shall be added into the tubes in turn according to **Supplementary Table 2**.

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3. Place each reaction tube on the thermal cycler. For the DNA samples, run the amplification program as detailed in **Table 2**. For cDNA products, run the amplification program as detailed in **Table 3**.

3. Digestion of primer sequence

1. Take out the primer digestion enzyme and put it on ice for melting. After amplification, take out the above reaction tubes, add 2 µL of primer digestion enzyme to each tube, and ensure the total volume is 22 µL.

2. Vortex and mix the reaction solution in the PCR tube and centrifuge instantaneously for 10 s, with a fixed 2,500 x *g*.

3. Put each reaction tube on the thermal cycler and run the program as detailed in **Table 4**.

4. Ligation of adapter

1. Put the adapter connecting reagents on ice to dissolve. Prepare 1.5 mL microcentrifuge tube, and then mix each component according to **Table 5**, and mark it as adapter mixture X.

NOTE: The adapter connecting reagents include P1 adapters and specific adapters X, numbered 1 through 48. These are designed to uniquely tag various samples. When adding a specific adapter, only open one tube at a time to prevent cross contamination of the specific adapter. The diluted specific adapter mixture can be stored at -20 ± 5 °C for standby. In sequencing workflows, samples are not processed individually, rather, multiple samples, each tagged with a specific adapter, are combined into a unified library, for sequencing. This method enables subsequent differentiation of samples based on their specific adapter sequences.

2. Take out the digested primer product (22 µL) from the thermal cycler. Add reagents into the tube in turn according to **Table 6**, vortex and mix for 5 s. Centrifuge at low speed for 10 s, with a fixed 2,500 x *g*, so that there is no obvious drop on the wall and cover of the tube.

3. Place the reaction tube on the thermal cycler. For the DNA samples, run the amplification program as detailed in **Table 7**. For cDNA products, run the amplification program as detailed in **Table 8**.

5. Purification of ligation product and amplification

1. Take the DNA purification magnetic beads out of the 2-8 °C refrigerator in advance, vortex them evenly and centrifugate them instantaneously for 10 s, with a fixed 2,500 x *g*. Equilibrate the magnetic beads at RT for 30 min.

2. Prepare a 1.5 mL low adsorption microcentrifuge tube and transfer the product of ligation reaction to the corresponding tube.

3. Add 45 µL of DNA purified magnetic beads into each tube, vortex and mix well, centrifugate instantaneously for 10 s, and incubate at RT for 5 min.

4. Place the tube on a magnetic rack for 3 min. Discard the supernatant and avoid pipetting the beads out.

5. Transfer 300 µL of newly prepared 75% ethanol into the microcentrifuge tube, and gently rotate the tubes 4x at 180°. After the solution is clear, quickly discard the supernatant. Avoid pipetting the beads out. Repeat the wash procedure 1x more.

6. Remove the 1.5 mL microcentrifuge tubes from the magnet rack, and centrifuge briefly (10 s, with a fixed

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2,500 x *g*). Place the tubes back into the magnet rack and pipette out the remaining liquid. Ensure that there is no residual liquid on the tube wall.

7. Open the cap of each 1.5 mL microcentrifuge tube and dry the beads at RT for 5 min. Pay attention to the dry-wet condition of the beads. After the magnetic beads are dried, check for no water stains on the surface. Extend the drying time appropriately if the magnetic beads are too wet. Close the lid immediately if any cracks emerge on the beads and continue the next step to amplify and purify the library.

6. Amplification and purification of library

1. Put PCR related reagents on ice for dissolution in advance, vortex them, and centrifuge them for 10 s. Remove the 1.5 mL microcentrifuge tube from the magnet rack, pipette the PCR regents into the tube according to **Table 9** and close the cap and vortex for 5s. Centrifuge briefly (10 s) to get no obvious liquid drop on the tube wall and cover.

2. Transfer the product above into a new PCR tube. Incubate the sample on a thermal cycler. For the DNA samples, run the amplification program as detailed in **Table 10**. For cDNA products, run the amplification program as detailed in **Table 11**.

3. Prepare a new 1.5 mL low adsorption microcentrifuge tube. Centrifuge the PCR tube for 10 s after incubation. Transfer the product from the PCR tube into the EP tube.

4. Add 25 µL of DNA purified magnetic beads into each tube. Vortex and mix well, centrifuge at low speed and incubate at RT for 5 min.

5. Place the tubes on a magnetic rack for 3 min and wait till the solution becomes clear. Transfer the supernatant into new microcentrifuge tubes. Avoid pipetting the beads out.

6. Add 60 µL of DNA purified magnetic beads into each tube. Vortex and mix well, centrifuge at low speed and incubate at RT for 5 min.

7. Place the tubes on a magnetic rack for 3 min and wait till the solution becomes clear. Discard the supernatant. Avoid pipetting the beads out.

8. Transfer 300 µL of the newly prepared 75% ethanol into the tubes, and gently rotate the tubes 4x at a 180°. After the solution is clear, quickly pipette and discard the supernatant. Avoid pipetting the beads out. Repeat the wash procedure 1x.

9. Remove the 1.5 mL microcentrifuge tubes from the magnetic rack, and centrifuge briefly (10 s). Insert the tubes back into the magnetic rack and pipette out the remaining liquid. Ensure that there is no residual liquid on the tube wall.

10. Open the caps of 1.5 mL tubes and dry the beads at RT for 5 min. Pay attention to the dry-wet condition of the beads. After the magnetic beads are dried, there is no water stain on the surface. Extend the drying time appropriately if the magnetic beads are too wet. Close the lid immediately if any cracks emerge on the beads.

11. Pipette 50 µL of eluent into the tubes, vortex and mix well. Centrifuge briefly (5 s, with a fixed 2,500 x *g*) and place the tubes at RT for 5 min.

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12. Place the tubes on a magnetic rack for 3 min and wait till the solution becomes clear. Carefully remove the liquid into a new tube and mark the library name.
13. Store the library in a refrigerator at 2-8 °C temporarily and wait for quantitative determination or store the library in a refrigerator at -20 ± 5 °C for long-term storage.

4. Quantification of the constructed library

1. Use the nucleic acid quantifier to quantify the library. If the library concentration is ≥ 0.2 ng/μL, it is qualified. Otherwise, rebuild the library.
2. Mix equal volume of DNA (or RNA) and quantify the solution. According to the quantitative result, dilute the mixed solution to 100 pmol/L by using the following formula.

$$dilution\ mutiple = \frac{library\ concentration \times 10^7}{660 \times 200}$$

NOTE: An alternative is to dilute the library to 100 pmol/L according to the formula, and then quantify by fluorescent quantitative PCR. Mix DNA(or RNA) libraries equally according to quantitative results of PCR instrument.

3. Mix 100 pmol/L DNA library and RNA library in a ratio of 4:1. Use the mixed solution of DNA and RNA library for computer sequencing.

5. Sequencing

1. Perform sequencing by referring to the manual of the universal kit of sequencing reaction[24,25] (semiconductor sequencing method).

6. Quality control of samples

1. Positive quality control of gastric cancer DNA: Take the DNA positive control of gastric cancer and perform the test according to the instructions of the kit. The control is provided with the kit. The result shows that *MTHFR*, *DHFR*, *MTR*, *GGH* and *SLC19A1* gene mutants are detected.
2. Negative quality control of gastric cancer DNA: Take the DNA negative control of gastric cancer and perform the test according to the instructions of the kit. The control is provided with the kit. The result shows that the wild types of *MTHFR*, *DHFR*, *MTR*, *GGH* and *SLC19A1* genes are detected.

   NOTE: Ensure criteria are met in both the cases, otherwise, re-detection is required.

7. Data analysis

1. Run the corresponding plug-in (the Variant Caller, Coverage Analysis, and Ion Reporter Software) in the Torrent Suite Software to get the analysis results of the samples. Judge the sample detection results according to the analysis results of the plug-in.

Representative Results

The determination of test results relies on the positive judgement value, which is also recognized as the reference interval. Use semiconductor sequencing method to detect the collected clinical samples. When the mutation frequency value of *MTHFR*, *DHFR*, *MTR*, *GGH* and *SLC19A1* genes is ≥ 5%, the detection result is the mutant of corresponding gene. When the mutation frequency value is < 5 %, the detection result is the wild type of the corresponding gene[26].

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The following criteria can be used to determine whether the detection results are credible. Firstly, if the average coverage of DNA sequencing results is ≥ 500, and the mapped reads of RNA sequencing results are ≥ 20000, the test results are credible[27]. Otherwise, it is recommended to retest. Secondly, the average coverage of DNA positive quality control of gastric cancer is ≥ 500, and the test result should conform to the rs1801131 and rs1801133 mutations of *MTHFR* gene, rs1650697 and rs442767 mutations of *DHFR* gene, rs1805087 mutation of *MTR* gene, rs11545078 mutation of *GGH* gene and rs1051298 mutation of *SLC19A1* gene as positive. Otherwise, it is recommended to retest. Thirdly, the average coverage of DNA negative quality control of gastric cancer is ≥ 500, and the detection results should show that all the sites within the detection range of this kit are wild type. Otherwise, it is recommended to retest. Lastly, the library concentration is lower than 0.2 ng/μL, which may be due the degradation of DNA or RNA in the sample, or the failure to strictly follow the experimental process or use of expired reagents during the experiment. The above conditions may cause the sequencing quality to decline or fail. It is recommended to rebuild the library.

With this kit, a series of steps are followed to build a sequencing library from clinical samples. The library is then sequenced on an ion semiconductor sequencing platform, with ANNOVAR used for annotating the results. After sequencing, a document is used to summarize the mutant types for each sample. The absence of a mutant type indicated that the sample did not undergo that specific mutation. **SupplementaryTable 3** provides a typical example of this. **Table 12** includes basic information about the SNPs detected here. Analyzing each SNPs using filter-based annotation in various database, such as 1000 Genomes Project (1000g2015aug; https://www.internationalgenome.org/; **Table 13**) and the Exome Aggregation Consortium (ExAC; https://gnomad.broadinstitute.org/; **Table 14**), can reveal that different SNPs are significantly present in different populations.

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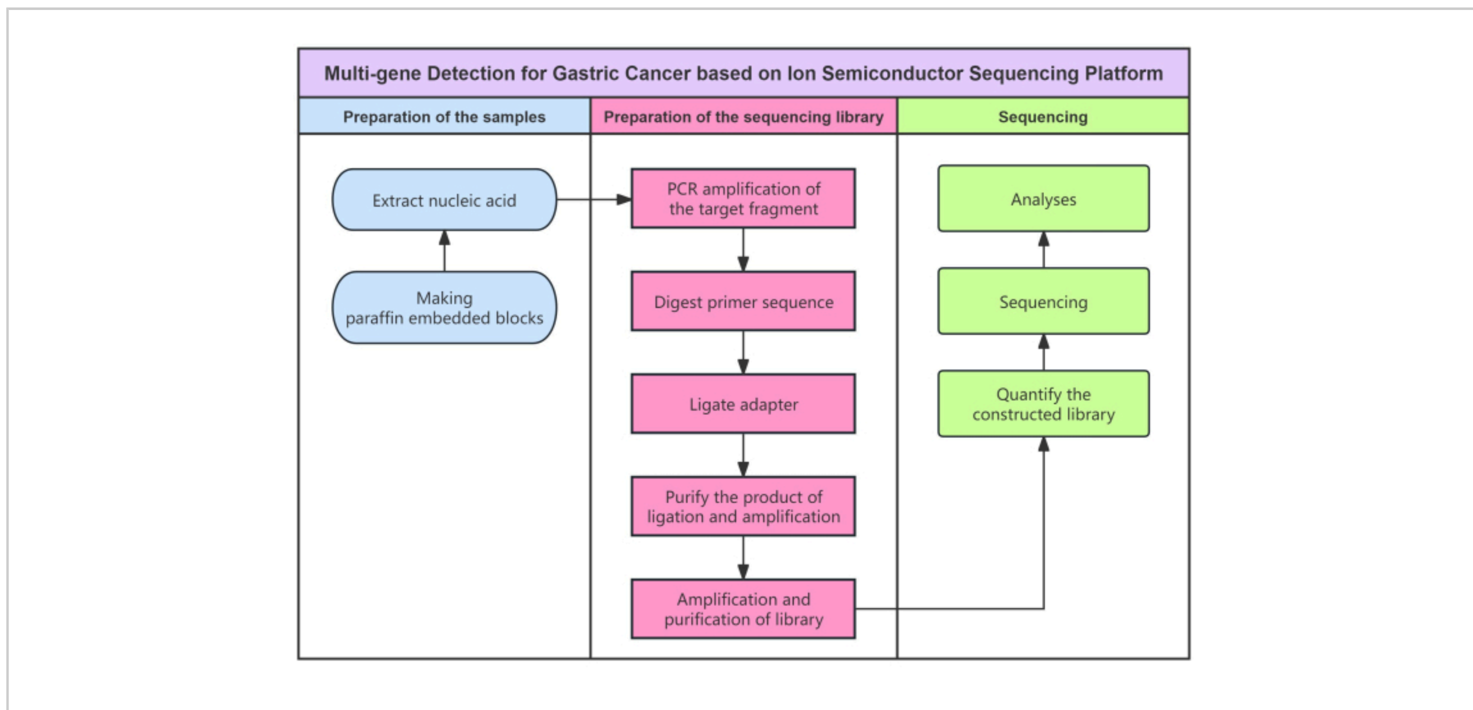

Figure 1: Flow diagram for the protocol. Flowchart of multi-gene detection for gastric cancer using semiconductor sequencing method. Please click here to view a larger version of this figure.

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Chromosome	Location	Primer design interval	Left primer	Right primer
chr1	11854475	chr1:11854175-11854775	ACAGGATGGG GAAGTCACAG	AAACCGGAAT GGTCACAAAG
	11856377	chr1:11856077-11856677	CTTCAGGTCA GCCTCAAAGC	TCCCTGTGGT CTCTTCATCC
chr5	79950780	chr5:79950480-79951080	CGCCGCACAT AGTAGGTTCT	CTTCCTCCTC CAGCCCTATC
	79951495	chr5:79951195-79951795	CTTGGGTCAC CTGCACAGTA	ATTTTGAAGC ACCCAAGCTG
chr1	237048499	chr1:237048199-237048799	GTCAAAGGCC AGTCCCTTCT	CTCCCTTCAC CAACTGTGCT
chr8	63938763	chr8:63938463-63939063	CAGTGAAGTT CAGCGGCAT	TATTTTCCTGT GTGGGGCAC
chr21	46934825	chr21:46934525-46935125	CCAACCTGAG ATGGCTTTTC	TCCTTGGTGC TCTTGCTTTT

Table 1: Primers used for the seven selected SNPs in five genes. This sheet displays the information about the location of the primers of SNPs associated with 5-FU resistance in gastric cancer.

Temperature	Time	No. of cycles
99 °C	2 min	1 cycle
99 °C	15 s	22 cycles
60 °C	4 min	
10 °C	Hold	1 cycle

Table 2: Thermal cycle program for DNA amplification. Thermal reaction conditions such as temperature, time, and cycles are shown.

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Step	Temperature	Time	No. of cycles
Activate enzymes	99 °C	2 min	1 cycle
Denature	99 °C	15 s	30 cycles
Anneal and extend	60 °C	4 min	
Preserve	10 °C	Hold	1 cycle

Table 3: Thermal cycle program for cDNA amplification. Thermal reaction conditions such as temperature, time, and cycles are shown.

Temperature	Time	No. of cycles
50 °C	10 min	1 cycle
55 °C	10 min	1 cycle
60 °C	20 min	1 cycle
10 °C	Hold	1 cycle

Table 4: Thermal cycle program for digestion of primer sequence. Thermal reactions conditions such as temperature, time, and cycles are shown.

Component	Volume
P1 adapter	1.5 µL
Specific adapter X	1.5 µL
Nuclease-free water	3 µL
Total volume of reaction system	6 µL
X: Indicates the specific adapter number	

Table 5: Sequence of adding reagents for preparation of adapter mixture. Add reagents into the tube in the order provided here.

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Component	Volume
Connecting buffer	4 μL
Adapter mixture X	2 μL
DNA ligase	2 μL
Total volume of reaction system	30 μL

Table 6: Sequence of adding reagents into the digested primer production. Add reagents into the tube in the order provided here.

Temperature	Time	No. of cycles
22 °C	30 min	1 cycle
72 °C	10 min	1 cycle
10 °C	Hold	1 cycle

Table 7: Thermal cycle program for connecting adapter to DNA. Thermal reactions conditions such as temperature, time, and cycles are shown.

Temperature	Time	No. of cycles
22 °C	30 min	1 cycle
68 °C	5 min	1 cycle
72 °C	5 min	1 cycle
10 °C	Hold	1 cycle

Table 8: Thermal cycle program for connecting adapter to cDNA. Thermal reactions conditions such as temperature, time, and cycles are shown.

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Component	Volume
Reaction solution of library amplification	50 µL
Library primer mixture	2 µL
Total volume of reaction system	52 µL

Table 9: Sequence of adding amplification reagents. Add reagents into the tube in the order provided here.

Temperature	Time	No. of cycles
98 °C	2 min	1 cycle
98 °C	15 s	5 cycles
60 °C	1 min	
10 °C	Hold	1 cycle

Table 10: Thermal cycle program for amplifying DNA library. Thermal reactions conditions such as temperature, time, and cycles are shown.

Temperature	Time	No. of cycles
98 °C	2 min	1 cycle
98 °C	15 s	5 cycles
64 °C	1 min	
10 °C	Hold	1 cycle

Table 11: Thermal cycle program for amplifying cDNA library. Thermal reactions conditions such as temperature, time, and cycles are shown.

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	rs1801131	rs1801133	rs1650697	rs442767	rs1805087	rs11545078	rs1051298
Ref	T	G	A	G	A	G	G
Alt	G	A	G	T	G	A	A
Func.ref GeneWithVer	exonic	exonic	UTR5	upstream	exonic	exonic	UTR3
Gene.ref GeneWithVer	MTHFR	MTHFR	DHFR	DHFR	MTR	GGH	SLC19A1
GeneDetail .refGeneWithVer	.	.	NG_023304.1: g.5020T>G	.	.	.	NM_001205206.1: c.*64C>T; NM_001205207.1: c.*746C>T; NM_194255.2:c. *746C>T
ExonicFunc .refGeneWithVer	nonsynonymous; SNV	nonsynonymous; SNV	.	.	nonsynonymous; SNV	nonsynonymous; SNV	.
AAChange.ref GeneWithVer	MTHFR:NM_001330358.1:exon8:c.A1409C:p.E470A;MTHFR:NM_005957.4:exon8:c.A1286C:p.E429A	MTHFR:NM_001330358.1:exon5:c.C788T:p.A263V;MTHFR:NM_005957.4:exon5:c.C665T:p.A222V	.	.	MTR:NM_001291939.1:exon25:c.A2603G:p.D868G;MTR:NM_001291940.1:exon25:c.A1535G:p.D512G;MTR:NM_000254.2:exon26:c.A2756G:p.D919G	GGH:NM_003878.2:exon5:c.C452T:p.T151I	jin
cytoBand	1p36.22	1p36.22	5q14.1	5q14.1	1q43	8q12.3	21q22.3

Table 12: Relevant information of the SNPs. Annotating the samples using ANNOVAR.

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	MTHFR		DHFR		MTR	GGH	SLC19A1
	rs1801131	rs1801133	rs1650697	rs442767	rs1805087	rs11545078	rs1051298
1000g2015aug_all	0.249401	0.245407	0.76857	0.290136	0.218251	0.085463	0.524361
1000g2015aug_afr	0.1513	0.09	0.9349	0.0318	0.2844	0.056	0.5772
1000g2015aug_amr	0.1513	0.4741	0.755	0.3991	0.1772	0.0403	0.4323
1000g2015aug_eas	0.2192	0.2956	0.6607	0.5853	0.1052	0.0873	0.5635
1000g2015aug_eur	0.3131	0.3648	0.7555	0.3191	0.173	0.0924	0.4493
1000g2015aug_sas	0.4172	0.1186	0.6779	0.228	0.3211	0.1483	0.5552

Table 13: Filter- based annotation of SNPs in 1000 Genomes Project.

	MTHFR		DHFR		MTR	GGH	SLC19A1
	rs1801131	rs1801133	rs1650697	rs442767	rs1805087	rs11545078	rs1051298
ExAC_ALL	0.295	0.3037	.	.	0.2091	0.0936	.
ExAC_AFR	0.1588	0.1124	.	.	0.2666	0.0545	.
ExAC_AMR	0.1555	0.5141	.	.	0.1864	0.0361	.
ExAC_EAS	0.2148	0.3052	.	.	0.1132	0.0824	.
ExAC_FIN	0.3128	0.2227	.	.	0.1892	0.0581	.
ExAC_NFE	0.3191	0.345	.	.	0.1919	0.0969	.
ExAC_OTH	0.304	0.3062	.	.	0.2108	0.0973	.
ExAC_SAS	0.4153	0.1409	.	.	0.316	0.165	.

Table 14: Filter- based annotation of SNPs in the Exome Aggregation Consortium.

Supplementary Table 1: Sequence of adding reagents for DNA amplification. Add reagents into the tube in the order provided here. Please click here to download this File.

Supplementary Table 2: Sequence of adding reagents for cDNA amplification. Add reagents into the tube in the order provided here. Please click here to download this File.

Supplementary Table 3: Example of clinical specimen sequencing results. Only mutations present in the

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specimens will be recorded in the documents. Please click here to download this File.

Discussion

Clinical experts unanimously concur that patients, even with the same type and stage of gastric cancer, can have markedly different responses to an identical treatment approach. Years of research have revealed to scientists that the individual variations are chiefly attributed to gastric cancer's nature as a heterogeneous, polymorphic, and diversely differentiated cellular population, leading to significant individual disparities in treatment responses[28]. Consequently, the acquisition of gastric cancer samples through upper gastrointestinal endoscopy or surgery and blood samples, coupled with high-throughput sequencing for genetic analysis, enables personalized gastric cancer treatment. This strategy is designed to enhance clinical treatment efficacy and reduce the risk of severe toxic side effects. The progress in ion semiconductor sequencing technology has turned personalized treatment into a practical reality[29].

Here are some limitations of this test method. The kit used here is primarily for *in vitro* diagnosis, so it is limited to detecting the mutation of rs1801131 and rs1801133 of *MTHFR* gene, rs1650697 and rs442767 of *DHFR* gene, rs1805087 of *MTR* gene, rs11545078 of *GGH* gene and rs1051298 of *SLC19A1*. Mutation in other sections cannot be detected. Due to significant heterogeneity in tumor tissue, different sampling locations may affect the detection results. For paraffin embedded tissue samples stored for longer duration, DNA and RNA may be degraded to a certain extent, affecting the test results. Unreasonable sample collection, transportation, and processing, as well as improper experimental operation and experimental environment may lead to false negative or false positive results. The detection result cannot be guaranteed if the nucleic acid concentration is lower than 2 ng/μL. The test results of the kit are only for clinical reference. The selection of personalized treatment for patients should be considered in combination with their symptoms/signs, medical history, other laboratory tests and treatment reactions. Negative results cannot completely exclude the existence of target gene mutation. Negative results can also be caused by too few tumor cells in the sample, excessive degradation of nucleic acid or the concentration of target gene in the amplification reaction system below the detection limit.

Some performance indexes of the kit used are as described. Analytical sensitivity: For DNA samples, the minimum detectable amount of the total nucleic acid in this kit is 10 ng, and 5% mutation rate can be detected. For RNA samples, the minimum detectable amount of the total nucleic acid in this kit is 10 ng. Positive and negative coincidence rate: The positive and negative coincidence rate reaches 100%. Limit of detection (LOD): A total of 14 LOD references, numbered L1-L14 can be used. L1-L11 is LOD references of *MTHFR*, *DHFR*, *MTR*, *GGH* and *SLC19A1* genes, and their detection results should be that the mutation types of the corresponding genes sites are positive, and the coincidence rates are 100%. Repeatability: A total of five repetitive reference materials, numbered R1-R5 can be used. R1 is a strong positive repetitive reference material (rs67376798 mutation of *DPYD* gene). R2, which contains this mutation in lesser number, is a weak positive repetitive reference material (rs67376798 mutation of *DPYD* gene), R3 is a negative repetitive reference material (6 sites of the *DPYD*, *MTHFR* and *ABCB1* genes are detected as wild type). Each reference sample must be tested 10 times, ensuring that the outcomes of these repeated assessments correspond with their predefined classifications.

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Data volume: The effective data volume of DNA and RNA samples should be controlled above 0.05 M. The sequencing depth of DNA samples shall be controlled above 500, and the Mapped Reads of RNA samples shall be controlled above 20000. Interference test: This kit is not affected by endogenous interference substances (triglycerides and albumin) and exogenous interference substances (formalin and dehydrated alcohol).

Some precautions must be observed during the experiment. The kit used here can only be used for *in vitro* testing. Please read this manual carefully before the experiment and use it within the validity period. Components of the kit in different batches cannot be used interchangeably. It is recommended to use disposable consumables for this kit to prevent contamination. During the use of this kit, it is recommended to use a suction head with a filter element. In order to avoid any potential biological hazards in the sample, the test sample should be considered as infectious substances to avoid contact with skin and mucous membrane. It is recommended to handle the samples in a biosafety cabinet that can prevent the outflow of aerosols. Test tubes and suckers used in the operation should be sterilized before being discarded. The operation and disposal of samples shall meet the requirements of relevant laws and regulations: General Guidelines for Biosafety of Microbial Biomedical Laboratories and Medical Waste Management Regulations of the Ministry of Health[30,31]. The experimental personnel must receive professional training, operate in strict accordance with the instructions, and strictly separate the areas according to the experimental process. Special instruments and equipment shall be used at each stage of the experimental operation, and the articles at each stage of each area shall not be used interchangeably. The experimental personnel must strictly separate the areas according to the experimental process. Special instruments and equipment shall be used at each stage of the experimental operation. Take protective measures as required, such as gloves, work clothes, etc. Waste disposal shall comply with relevant national regulations.

Although the focus of this article is on the seven SNPs within five genes related to gastric cancer, the sequencing in practical applications is not confined to these five genes alone. This paper definitively establishes a significant correlation between the seven SNPs and the sensitivity to 5-FU chemotherapy in gastric cancer.

Disclosures

The authors have nothing to disclose.

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