# Detection of rare malignant cells in gastric lavage using Hexokinase 2 and single-cell sequencing for early-stage gastric cancer diagnosis

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**Research Article**

**Keywords:** Gastric cancer, early diagnosis, Warburg effect, Single cell sequencing (SCS), Copy number variation (CNV)

**Posted Date:** November 8th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-3494487/v1

# Abstract

**Objective:**Gastric cancer is a highly prevalent cancer. Endoscopy is the best way to diagnose gastric cancer at an early stage, but it relies on patient compliance and endoscopy physicians' experience, which makes it difficult to be used as a screening method for a large population. The aim of this study is to develop a novel method for early gastric cancer diagnosis by detecting exfoliated tumor cells in gastric lavage.

**Methods:** In our experiment, Hexokinase 2 (HK2) was firstly used as a metabolic function-associated marker to detect gastric exfoliated tumor cells engaging increased glycolysis in gastric lavage. And further the malignancy of HK2-derived high glycolytic tumor cells (hgTCs) was examinedby single-cell sequencing (SCS) by surveying genome-wide copy number variation (CNV).

**Results:** In a study of 60 individuals including 10 gastric cancer patients (9 IA and 1 IIA), 26 precancerous lesions patients, 15 patients with benign gastric diseases, and 9 healthy controls, the HK2 test showed diagnostic sensitivity and diagnostic specificity were 80% (8/10 patients with gastric cancer IA and IIA) and 96% (23/24 patients with benign gastric diseases and healthy controls), respectively. One point that is worth paying attention to is that the diagnostic sensitivity in patients with severe dysplasia was 57% (4/7), which showed promising application prospects in gastric cancer early diagnosis and prevention.

**Conclusions:** Thus, our results demonstrated a new approach using a gastric lavage-based HK2 assay combined with SCS validation. It has the great potential to be used for early gastric cancer detection with high accuracy, especially to improve the quality of gastroscopy at the early stage.


# 1. Introduction

Gastric cancer is the fifth most common tumor worldwide and the fourth leading cause of cancer-related death[1]. Early-stage gastric cancer lacks specific clinical symptoms. As a result, most gastric cancer patients are diagnosed at an advanced stage with a 5-year survival rate of < 30%[2, 3]. On the other hand, the gastric cancer patients diagnosed at an early and resectable stage will have the 5-year survival rate > 90%[4, 5]. Endoscopy followed by histologic biopsy is the gold standard for diagnosing gastric cancer. However, its diagnostic efficiency depends on the expertise of the endoscopy physicians. Early-stage lesions tend to show subtle changes in the mucosa and are covered by a scab, so the inexperienced endoscopy physicians can lead to missed diagnosis rates of gastric cancer and precancerous lesions ranging from 20 to 40 percent [6, 7]. In addition, endoscopy has a low patient acceptance as a screening tool because of the discomfort caused during gastroscopy[8]. The conventional serum biomarkers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19 − 9 (CA 19 − 9), carbohydrate antigen 72 − 4 (CA 72 − 4) have limited diagnostic value of early gastric cancer due to their low sensitivity and specificity[9]. To this end, the development of more sensitive and accurate non-invasive diagnostic methods that can assist endoscopists in the early detection of gastric cancer is of great value.

Over the past decades, research in liquid biopsy has developed rapidly, making it possible to achieve less invasive and more efficient diagnostic tools. However, accurate confirmation of malignant cells in body fluids remains an unsolved problem. Free cancer cells in the peritoneal cavity may induce or indicate peritoneal metastasis, which is a poor prognostic factor for advanced gastric cancer[10]. However, peritoneal lavage cytology carries a limited diagnostic sensitivity of only 20 to 30 percent, which is attributed to the rarity of free tumor cells in the peritoneal lavage taken from patients at an early stage. Although immunohistochemistry and Polymerase Chain Reaction (PCR) methods improved the diagnostic sensitivity, use of cytology alone is less than optimal[11–13].

Compared to other solid tumors, there are rare circulating tumor cells (CTCs) in peripheral blood of tumor patients, which are not easy to detect, especially at the early stage[14]. Because of the loosening of cell-to-cell junctions, cancer cells can easily detach from the lesion and exfoliate into the lumen of the stomach, while normal cells remain intact[15, 16]. As a direct up-close encounter with gastric lesions, the detection of exfoliated tumor cells in gastric lavage is straightforward and compelling. Gastric lavage can be obtained by washing the stomach with a saline solution during a routine endoscopy. It has been proposed that the detection of exfoliated tumor cells in gastric cancer would be a promising and non-invasive approach for the diagnosis of early-stage gastric cancer.

One of the hallmarks of cancer is that cancer cells rewire oxidative phosphorylation toward aerobic glycolysis, a phenomenon termed the “Warburg effect”, to rapidly proliferate and survive. This characteristic of malignant cells has already been applied to positron emission tomography (PET) for cancer diagnosis[17]. Hexokinase (HK) is the initial enzyme of glycolysis that phosphorylates glucose by ATP to glucose 6-phsphates. In previous studies, we have described HK2, an isoform of HK, as a novel biomarker for the detection of rare malignant cells that engage in elevated glycolysis in peripheral blood, urine, and malignant pleural effusion[18–21]. Similarly, elevated glycolytic activity and increased HK2 expression have also been found in gastric cancer[22–24].

Meanwhile, genome-wide copy number variation (CNV) is commonly considered the characteristic of malignancy of cells because it is rarely found in normal tissues. Correa in 1992 proposed a human model of gastric carcinogenesis, which is widely accepted to be a multi-step process: chronic atrophic; gastritis-intestinal; metaplasia-intraepithelial; and neoplasia-gastric cancer development[25]. In 1960s, Japanese scholars first confirmed the existence of precancerous lesions of gastric cancer from morphology, and used "atypia" to describe this type of lesion[26]. Since then, western scholars proposed the use of "dysplasia" in 1975 to specifically describe the precancerous lesions of gastric cancer[27]. A 15-year follow-up study of esophageal carcinoma patients showed that eight-year-old genomic copy number variations in some patients were associated with their prognosis[28].

To this end, we hypothesize that the HK2 assay could be used to diagnose gastric cancer by detecting rare exfoliated tumor cells in gastric lavage from patients with early-stage gastric cancer (IA and IIA) and patients with precancerous lesions. First, the HK2 assay allowed the detection of high glycolysis tumor

cells (hgTCs) in gastric lavage with a high accuracy. Second, single cell sequencing (SCS) was performed to validate selected putative tumor cells with a CNV profile analysis.

## 2. Materials and methods

## 2.1 Patient information and sample collection

This study was conducted at The Affiliated Wuxi People's Hospital of Nanjing Medical University between April 2021 to February 2022 according to the principles of the Helsinki Declaration and was approved by the Institutional Ethics Review Committee (KS202087). All participants in the study provided written informed consent. A total of 60 consecutive participants, including 10 patients with early-stage gastric cancer, 26 patients with precancerous lesions, 15 patients with benign gastric disorders, and 9 healthy individuals were enrolled.

All participants underwent either an endoscopic submucosal dissection or a gastroscopic examination. In our study, a saline solution was sprayed onto the surface of the gastric mucosa through an endoscopic working channel, and 50 ml of the resulting fluid in the stomach cavity was collected by endoscopic suction. All samples were sent to the laboratory at 4°C and processed within 12 hours. The clinical pathology parameters of the patients were independently confirmed by two pathologists based on the 8th edition of the International TNM Staging.

## 2.2 Fabrication and surface modification of polydimethylsiloxane (PDMS) chips

Microwell chips fabricated in PDMS refer to the standard microfabrication soft lithography techniques. A replica of the molded PDMS was obtained by patterning a silicon wafer using the photoresist SU-8 2050. The PDMS prepolymer was mixed at a ratio of 10:1, and subsequently cast on this lithographically patterned replica. After curing at 80°C for 2 h, the PDMS component was separated from the replicate. In order to make the hydrophobic surface of the PDMS chip hydrophilic, so that the cells could fall into the microwell, the PDMS chip was placed in a plasma cleaner and the surface was treated with oxygen plasma for 2 min.

## 2.3 Gastric lavage sample processing and the cell-based HK2 assay

Gastric lavage samples were firstly filtered through 10 μm cell strainer followed by centrifugation at 400 g for 10 min. The supernatant was removed and the cell pellets were resuspended in PBS (0.1% BSA). The cell suspension was then passed through a 5 μm cell strainer to remove red blood cells and white blood cells. After cells were recovered with PBS (0.1% BSA), approximately $10^5$ cells were added into prepared microwell chips for cell fixation and sedimentation. On-slide cells were permeabilized with 0.5% Triton X-100 for 15 min and blocked with 3% BSA and 10% normal goat serum for 1 h. Cells were incubated with anti-HK2 antibody (1:100) and anti-pan-CK antibody (1:100 dilution) overnight at 4°C. After washing with

PBS, cells were incubated with fluorescein-conjugated secondary antibodies (1:400 dilution) for 1 h and DAPI for 5 min. ImageXpress Micro XLS Widefield High Content Screening (Molecular Devices) imaged all cells in bright field and fluorescent colors to identify $HK2^+/CK^+/DAPI^+$ cells as putative high glycolytic tumor cells (hgTCs).

## 2.4 Single-cell sequencing of genome-wide copy number alterations

The identified single cells were retrieved by a motorized micromanipulator (XenoWorks) for further single-cell sequencing. SutterP-2000 needle drawing instrument was used to pull the 1 mm capillary glass tube into a glass needle with a diameter of about 25 μm. Based on the location of the positive cells on the chip marked by the image analysis software, the XenoWorks hydraulic system and robotic arm were used to work together to extract the targeted positive cells from specific micropores on the chip and transfer them into a low-adsorption PCR tube pre-loaded with 5 μL cell lysate. Single-cell whole genome amplification (WGA) was conducted according to a protocol previously described[20]. Briefly, the genome amplification of the retrieved single cells was conducted with MALBAC Single Cell WGA Kit (Yikon Genomics).

## 2.5 Statistical analysis

GraphPad Prism 8 was used to analyze the data. Different experimental groups were compared by a two-tailed unpaired t-test. Fisher's exact probability test was used to analyze clinicopathological characteristics. Differences were considered significant at $p < 0.05$.

# 3. Results

## 3.1 Strategies for HK2 assay and single-cell sequencing based on gastric lavage

To investigate the feasibility and accuracy of gastric lavage-based HK2 assay in diagnosing gastric cancer, 60 candidates were recruited and grouped into 4 cohorts (early-stage cancer, precancerous lesions, benign diseases, and health) based on conventional histopathological results. Table S1 (Supporting information) lists the clinicopathological characteristics of patients with early gastric cancer and precancerous lesions in our study. The workflow of the gastric lavage-based HK2 assay and SCS validation is shown in Fig. 1. During an endoscopy, nurses assist the endoscopy physicians by injecting a sterile saline solution into the patient's stomach cavity through a special tube used for endoscopy. After rinsing the gastric cavity, the diluted lavage accumulated at the bottom of the gastric cavity, and then the endoscopy doctors draw back the lavage into a liquid tank linked with a collection tube (Fig. 1A). The lavage contains cells shed from the stomach and even the esophagus. In our study, the cells in the lavage solution were cleaned, negatively selected and placed in a microchip for immunofluorescence staining. Microwell chips were fabricated in PDMS. Each microwell chip contained 110,000 addressable wells with a diameter of 30 μm and a depth of 30 μm for holding cells. Cells placed in microwells could avoid cell

loss in immunofluorescence staining, while providing precise locations for easy extraction of suspected tumor cells for genome amplification and sequencing. The putative hgTCs in gastric lavage were assayed by on-slide fluorescence multi-immunostaining of HK2, pan-CK (exclusion of leukocytes) and DAPI (a nucleus stain). Based on the high specificity of hgTCs definition for malignant cell detection, the positive HK2 test was established when the number of hgTCs ($HK2^+/CK^+/DAPI^+$ cells) was greater than 1. To confirm the malignancy of putative hgTCs, these cells were retrieved individually and performed low depth whole-genome sequencing that characterized genome-wide CNV at the single-cell level (Fig. 1B).

# 3.2 HK2 protein expression and the genomic copy number variation in gastric cancer

First, the expression levels of HK2 between tumor tissues and normal tissues were comparatively analyzed in The Cancer Genome Atlas (TCGA) database. The expression of HK2 was significantly upregulated in gastric cancer tissues compared with that of the normal tissues, suggesting that HK2 was a malignancy-associated marker in gastric cancer (Fig. 2A). Next, we compared the HK2 levels in gastric cancer cell lines with human leukocytes (WBC) in the human protein atlas database. The results showed that the mean HK2 levels in gastric cancer cell lines were significantly higher than their WBC counterparts (Fig. 2B). And then, we directly measured the HK2 levels in gastric cancer cell lines (HGC27 and AGS) and leukocytes from a healthy donor by immunofluorescent staining. The results showed high HK2 levels in the gastric cancer cell lines (Fig. 2C), consistent with previous findings. Leukocytes are one of the types of common cells commonly found in gastric lavage. Our results represented a good separation between malignant gastric cancer cells and leukocytes by HK2 staining (Fig. 2D).

Somatic CNVs are widely present in tumors. Recurrent patterns of CNV in multiple cells are characteristic of malignant cells. Here, the CNV patterns of gastric cancer were mapped based on TCGA database. Large segments of the genome showed copy number increases or decreases in all four stages, whereas the CNV patterns were similar in stage T2, T3, and T4, indicating that genomic instability appears very early in the development of the gastric tumor (Fig. 2E).

### 3.3 Results of the gastric lavage-based HK2 assay for the diagnosis of gastric carcinoma in this study

We first measured 9 gastric lavage samples from healthy donors for the cell-based HK2 test. No samples showed HK2 positive counts (Fig. 3A and Table 1). The healthy cohort provided a baseline for gastric cancer patients measurement. And then, we used the cell-based HK2 assay to detect the samples from benign group, only 1 of 15 benign samples showed HK2 positive counts, which demonstrated that HK2 was a highly specific marker (Fig. 3A and Table 1). Next, 26 patients with precancerous lesions and 10 patients with early-stage gastric cancer were enrolled. The detection rate of HK2 positive cells using the gastric lavage-based HK2 assay was 0%, 43% and 57% in the mild, moderate, and severe dysplasia groups, respectively, across different precancerous lesions (Fig. 3A and Table S1), while the detection rate of HK2 positive cells in the early-stage gastric cancer was remarkably high (80%, Fig. 3A and Fig. 3B). Remarkably, the sizes of $HK2^+$ malignant cells were statistically larger than the leukocytes from the

gastric lavage samples (Fig. 3C). We further analyzed the correlation between HK2 assay results and clinicopathological characteristics in patients with early-stage gastric cancer and precancerous lesions. The HK2 assay results were not correlated with sex, age, location, or tumor/lesion size (Fig. 3D and Table S1).

Table 1
Detection rates of HK2 positive cells in gastric lavage of different groups in this study.

| Groups | Detection rate |
|---|---|
| Early-stage gastric cancer | 8/10 (80%) |
| Precancerous lesions | 10/26 (38%) |
| Mild dysplasia | 0/5 (0%) |
| Moderate dysplasia | 6/14 (43%) |
| Severe dysplasia | 4/7 (57%) |
| Benign diseases | 1/15 (7%) |
| Healthy control | 0/9 (0%) |
| **Supplementary data**: | |
| Doc S1: Supplementary figures and table. | |

# 3.4 Single-cell sequencing validated the malignancy of HK2-derived hgTCs

The genome of tumor cells is extremely unstable and prone to genome rearrangement. The size of the rearranged genome is larger than 1 kb, and the submicroscopic structure shows amplification or deletion. In our previous studies, we have demonstrated that CNV profiles can be used to identify cancer cells. Gastric precancerous lesions are a well-known risk factor for the development of intestinal-type gastric adenocarcinomas in multistep models of gastric carcinogenesis. Our HK2 assay revealed the presence of epithelial-derived hgTCs in the gastric lavage of patients with moderate and severe dysplasia (Table S1). In a patient with moderate dysplasia (Patient 25), there were at least 13 $HK2^{+}/CK^{+}/CD45^{-}/DAPI^{+}$ cells (additional CD45 staining excluded leukocytes) identified as hgTCs in the gastric lavage sample (Fig. 3E and Figure S2). To verify the malignancy of hgTCs, we randomly selected six of them for SCS based on the laboratory established route for CNV analysis. Four of the six hgTCs had concordant gains and losses in CNV patterns with those of primary tissue, which provided compelling evidence of the lesion origin (Fig. 3F). Further, it was noticed that the amplification of chromosome 8 in CNV profile is common in gastric cancer, demonstrating the malignant nature of cells (Fig. 3F). These results demonstrate that the HK2 assay combined with SCS validation can detect malignant cells in gastric lavage with a high specificity and accuracy for gastric cancer cells, even at the precancerous stage.

# 4. Discussion

Gastric cancer is a major malignancy with a poor prognosis. Endoscopy as the “gold standard” diagnostic tool of gastric cancer is an invasive method that has low patient acceptance and a certain rate of missed detection. Liquid biopsy holds potential as a less invasive and more cost-effective diagnostic tool. The FDA-approved CellSearch system enables standardized enrichment and enumeration of CTCs, but it does not verify the malignant nature of the cells[14]. As cancer cells from the mucosal layer are easily exfoliated into the gastric lumen, especially by endoscopic irrigation[24], the identification of cancer cells in gastric lavage is a high level of evidence for the detection of neoplasia. Several studies have shown that positive gastric cytology is associated with neoplastic aggressiveness[29]. Methylation analysis of gastric wash-derived DNA and gastric juice-derived exoDNA can be used for early detection of gastric cancer[30]. How to accurately identify the benign and malignant cells in body fluids has not been fully addressed. Gastric lavage cytology identifies malignant cells based on their morphological features but suffers from poor diagnostic accuracy. Therefore, gastric lavage cytology has not been applied to routine clinical diagnosis and screening for early-stage gastric cancer.

In this study, we developed an assay for high-throughput screening of rare metabolically active tumor cells in gastric lavage samples, depending on the characteristics of tumor cells with elevated glucose metabolism compared to benign cells. A total of 60 endoscopic patients were enrolled and grouped into four cohorts, including those with early gastric cancer, precancerous lesions, benign gastric diseases, and healthy individuals. The detection rate of cellular metabolism based on gastric lavage in early gastric cancer (stage I/II) was 80%, while the overall detection rate in patients with precancerous lesions was 38%, among which the detection rates of patients with mild, moderate and severe dysplasia were 0%, 43%, and 57%, respectively. These results suggest that highly metabolically active epithelial-derived cells can be detected in gastric lavage in patients with moderate and severe dysplasia, further suggesting enhanced glycolytic metabolism in these precancerous tissues. Gastric cancer development is a complex process involving multi-stage, multi-step, multi-gene mutation accumulation caused by multiple environmental pathogenic factors, and can take up to 10 years from the earliest inflammatory ulcer to irreversible gastric cancer lesion[31].

To verify the malignancy of HK2-positive hgTCs, SCS was used to provide a genome-wide CNV pattern. The sequencing results of epithelial-derived hgTCs of a patient with moderate dysplasia showed that it had chromosome 8 amplification, which are commonly reported as a CNV mutation site in gastric cancer[32], indicating that the detected hgTCs did come from the diseased tissue of moderate dysplasia, and their genomes had significant chromosomal variations. The question of whether precancerous lesions already have the characteristics of malignant cells is a subject of great interest and of great clinical value. Most importantly, our approach demonstrated the malignancy of gastric precancerous lesions at the single-cell level, rather than morphological features, and provided more genomic characterization for informative diagnosis.

As the gold standard for gastric cancer diagnosis, endoscopy is highly dependent on a professional endoscopist and has a certain discomfort that results in poor patient compliance. As a result, it is difficult to use as a means of large-scale screening of patients, and it also has a certain rate of missed detections[7]. The development of a simple and non-invasive method for the detection of early-stage gastric cancer with high sensitivity and specificity has become a research hotspot. This project, as an original validation of this approach, directly used the discarded gastric lavage from endoscopy for detection and analysis. After preliminary verification of the feasibility of the detection of gastric lavage, we have developed an independent and simpler equipment for collecting gastric lavage that is not based on endoscopy (Patent No. CN113143328-A). The process is simpler and more automated than endoscopy, using thinner, softer tubes, which makes it more comfortable for patients, less expensive, and can be performed by nurses, thus facilitating mass adoption and future use for community screening.

# 5. Conclusions

In this study, we developed a test for exfoliated tumor cells in gastric lavage and used it to diagnose early-stage gastric cancer and precancerous lesions. Through clinical studies and single-cell sequencing, it has been demonstrated that assays based on the detection of elevated malignant cell metabolism can be used to screen for rare exfoliated tumor cells in gastric lavage with high sensitivity and specificity for the diagnosis of early gastric cancer and precancerous lesions. This approach is nearly non-invasive and is expected to be used as a pre-screening tool prior to routine endoscopic exploration and biopsy.

# Declarations


### Acknowledgements

We acknowledge the support of the Department of Gastroenterology, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Jiangsu Province, China.

### Grant support

This work was supported by the National Natural Science Foundation of China (82172366) and a project from the Health Commission of Minhang District, Shanghai, China. (2021MHZ080).


### Conflict of Interest Statement

No potential conflicts of interest are disclosed.

### Author contributions

Conceived and designed the study: Peiyu Qian, Liu Yang and Peihua Lu.

Collected clinical samples: Jie Sun.

Performed experiments, collected and analyzed data: Peiyu Qian, Jie Sun and Liu Yang.

Performed bioinformatic analysis: Yining Tao and Haoran Mu.

Wrote the manuscript: Peiyu Qian and Liu Yang.

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## Figures

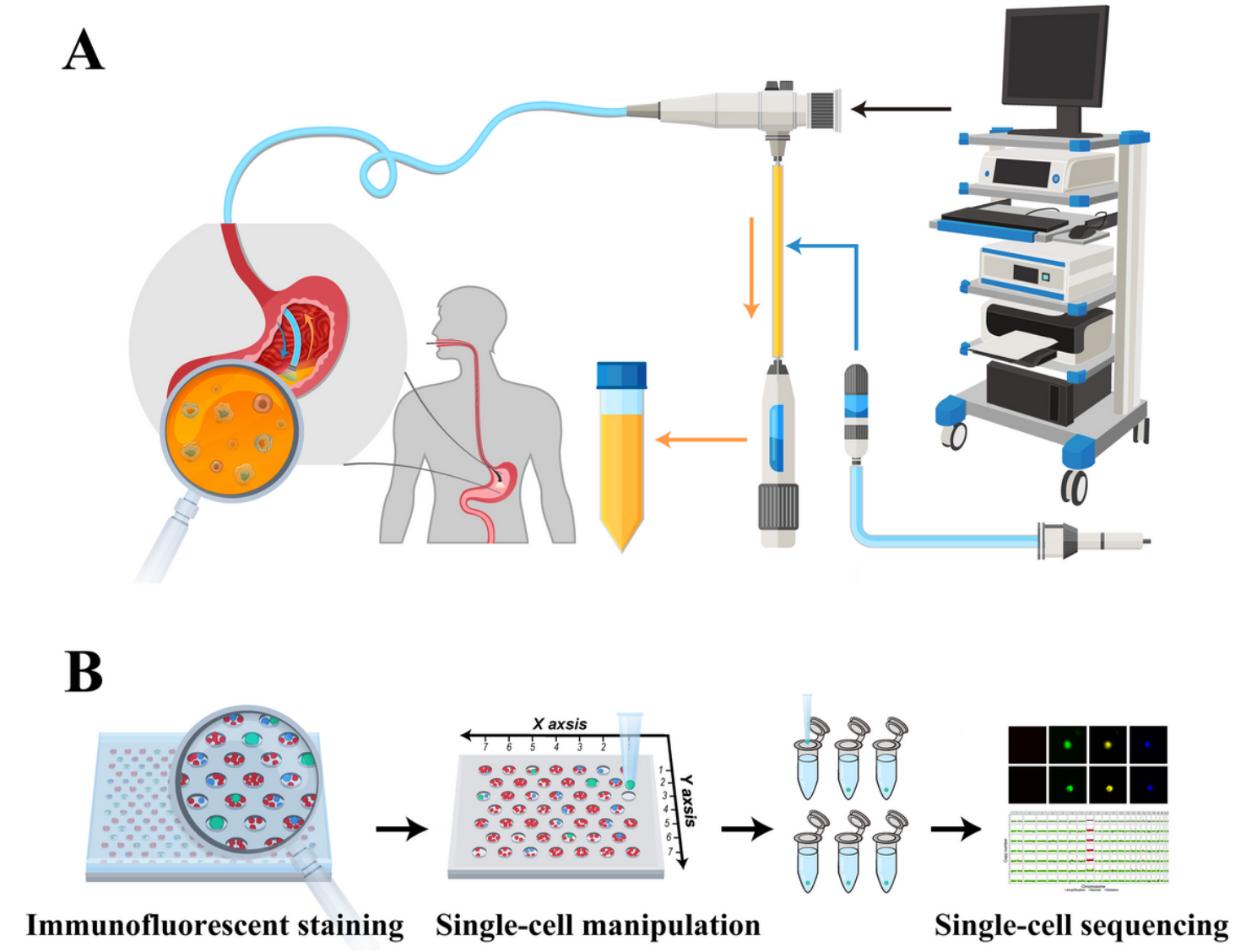
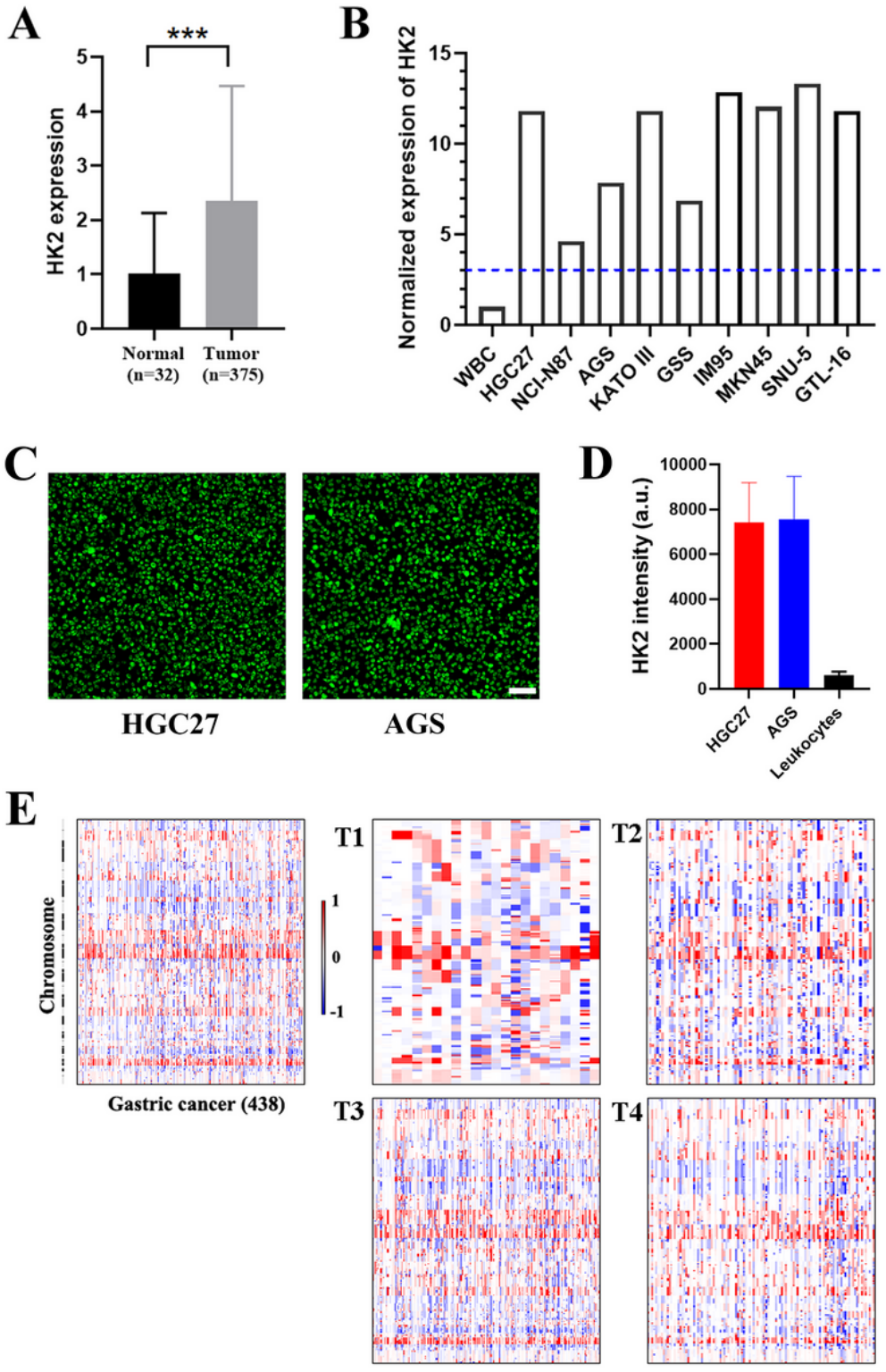



Figure 1

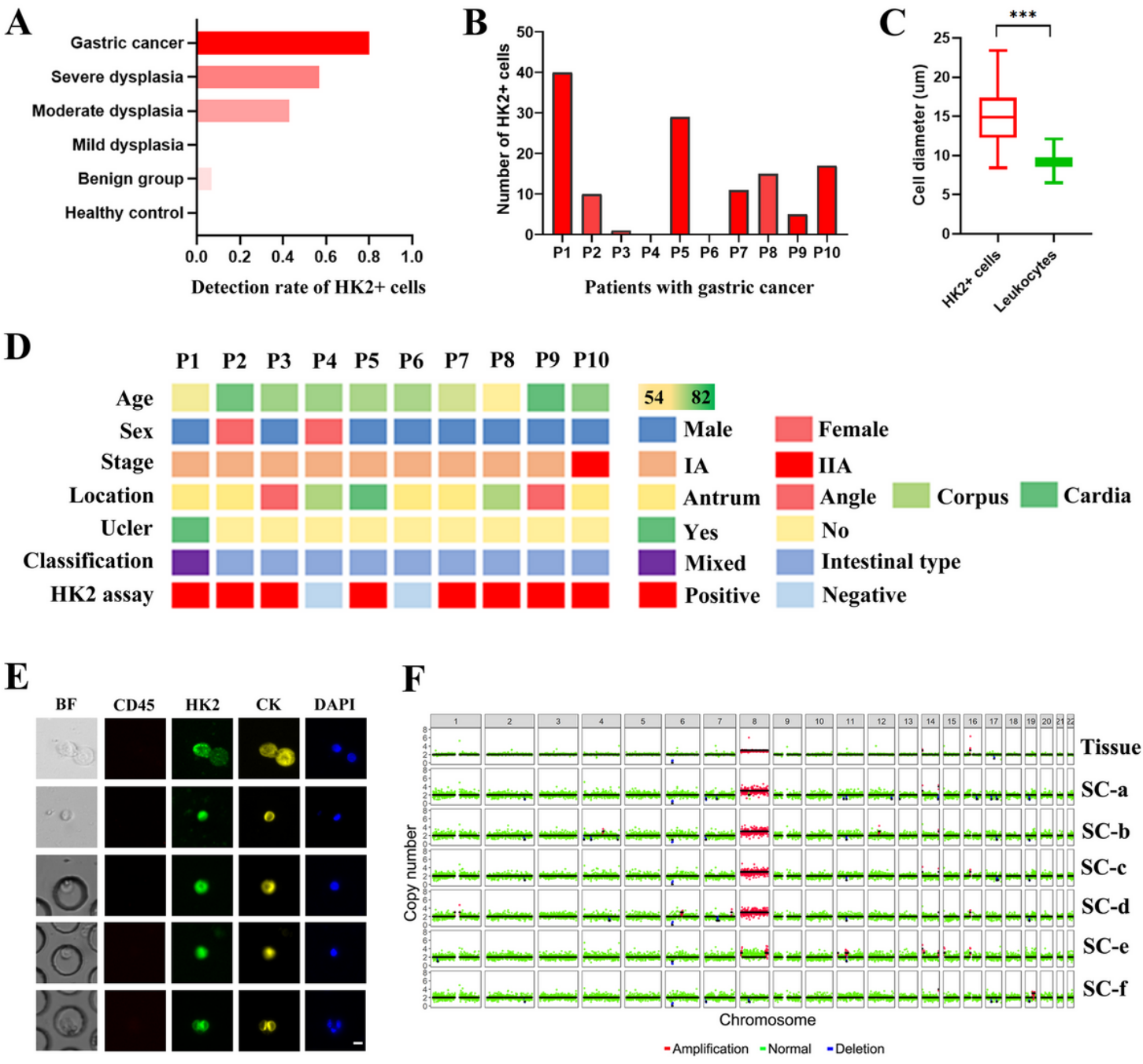
The workflow of the gastric lavage-based HK2 assay and single-cell sequencing. **(A)** Gastric lavage collection. The surface of the tumor was sprayed with a sterile saline solution through the endoscopic working channel, and after the cells were washed down and deposited in stomach cavity, the endoscopic tube was connected to a negative pressure pump and a collection device, then the gastric lavage was collected and transferred into a centrifuge tube. **(B)** HK2 assay and SCS validation. The processed cell suspension of gastric lavage was added to the microwell chips containing 110,000 small chambers with 30 μm depth and 30 μm diameter for single cell embedded. Following the immunofluorescence staining, the (HK2+/CK+/DAPI+ cells) were marked and imaged by High Content Screening system (ImageXpress Micro XLS  Molecular Diagnose, USA). Each cell could be retrieved and collected by micromanipulation, and malignant cells were identified using single-cell genome amplification and sequencing. The results of gastric lavage were compared with clinical diagnosis to calculate the diagnostic efficiency and detection rates in different groups.




## Figure 2

Verification of rationality. **(A)**HK2 expression levels in gastric cancer tissues (n=375) and normal tissues (n=32) in The Cancer Genome Atlas (TCGA) database. **(B)** The protein expression of HK2 in different tumor cell lines and healthy human leukocytes (data from the human protein atlas database). **(C)** Representative fluorescence images of HGC27 and AGS cells stained with HK2. Scale bar: 100 μm. **(D)** Single-cell measurements of HK2 levels in HGC27 and AGS cells, as well as leukocytes from a healthy

donor. **(E)** Heat map of genomic copy number variation of gastric cancer and each stage obtained from the analysis of gastric cancer sequencing data (n=438) in the TCGA database, where red represents amplification, blue represents deletion, and white represents constant copy number.




## Figure 3

Detection of HK2 positive cells in different groups and the malignancy of HK2 positive cells detected by the cell-based HK2 assay. **(A)** Detection rate of HK2 positive cells based on HK2 cell metabolism assay in gastric cancer, precancerous lesion group, benign group, and healthy controls. **(B)**HK2 positive cell counts of 10 gastric cancer patients whose gastric lavage samples were analyzed in this study. **(C)** Comparison of cell sizes between HK2+ cells and WBC from gastric lavage samples. **(D)** Schematic representation of

clinicopathological characteristics of ten early-stage gastric cancer patients. **(E)** Representative immunofluorescence staining images of high metabolically active epithelial-derived cells (HK2 + /CK + /CD45 - /DAPI +) from patients, Scalebar: 10 um. **(F)** Single cell genome copy number variation profile of six HK2 positive cells from gastric lavage sample of patient 25 and cells from the primary lesions (Tissue, patient 25), in which red represented amplification, blue represented deletion, and green represented unchanged copy number.

## Supplementary Files

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