Case Report

Cancer Comprehensive Analysis in Gastric Carcinoma: Benefits and New Perspectives

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Keywords

Gastrointestinal cancer · Array comparative genomic hybridization · Next-generation sequencing · Microarrays

Abstract

Gastric cancer is one of the most common and deadly cancers worldwide. Screening tests as well as tools for prediction of treatment outcomes and prognosis have been developed, but they have many limitations. The integration of liquid biopsy provided new aspects in screening and diagnosis of gastric cancer. In the present study, we used different techniques, studying the genetic and epigenetic profile of circulating tumor cells. We aimed to acquire all the available information, compare it with already existing studies, and evaluate the benefit of this approach. A blood sample was isolated from 2 gastric cancer patients at stages III–IV, followed by the isolation of CTCs. The circulating tumor cells were used for array comparative genomic hybridization, next-generation sequencing, and whole gene expression microarrays. Different variants were detected, while the microsatellite instability status was stable in both cases. The tumor mutational burden was low to medium. Gene expression assays revealed that >100 genes were overexpressed compared to noncancer samples. Amplifications of X chromosome were also observed in both cases, by using array comparative genomic hybridization. Although there are several techniques for cancer screening, prediction of therapy outcomes, and prognosis, the application of a complete comprehensive cancer panel, combining the study of variants, fusions, chromosomal abnormalities, and gene expression, is more appropriate. Information provided by the above techniques might contribute in designing more efficient

treatment protocols and screening tools. Despite the limitation of samples, the data are encouraging, and further study is needed so that they can be used at clinical level.

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Introduction

Gastric cancer, also known as stomach cancer, is one of the most common types of neoplasms worldwide. It is the third leading cause of cancer in the world, and over 1 million cases are diagnosed every year around the world [1]. This specific type of cancer is more prevalent in males, with approximately 2 times higher likelihood to affect males than females [2]. Five-year survival is highly associated with the stage of the disease, with patients at stage I having approximately 90% survival rate, while cases at stage III have only 18%. In addition, in Europe, the 5-year survival rate is lower than in the USA [3]. Different risk factors have been associated with gastric cancer, such as chronic gastritis, inflammation, gastroesophageal reflux disease, or *Helicobacter pylori*-caused gastritis [4]. Furthermore, genetic factors have also been implicated in gastric cancer pathogenesis. These include mutations in certain genes, polymorphisms in interleukin family genes, or other deletions and insertions [5, 6]. Other parameters that have been shown to increase the risk of gastric cancer are tobacco, alcohol, obesity, and chemicals. Smokers have 1.53-fold increased risk of gastric cancer, compared to nonsmokers, while alcohol increases the risk approximately by 40% [7, 8]. Increased BMI is highly associated with increasing risk of developing gastric cancer, since obesity contributes to inflammations through different factors (TNF- α and IL-6) [9]. Gastric cancer is a disease with higher heterogeneity both in molecular aspect and in phenotype. The treatment protocols differ depending on the stage of the disease and include endoscopic resection up to chemotherapy for advanced stages [10]. Recent studies demonstrated that the benefit after first-line therapy is limited, while second-line therapy is adequate for specific patients. Furthermore, the third- or later-line treatment is a potential solution mainly for metastatic gastric cancer; however, there are also a lot of limitations [11]. It is therefore clear that screening is of primary importance in gastric cancer. Most common screening methods involve contrast radiography and endoscopy and noninvasive testing like serum pepsinogen levels, serum trefoil factor 3, or *H. pylori* serology test [12]. Liquid biopsy, focusing in circulating tumor cells (CTCs), cancer stem cells (CSCs), circulating-free DNA (cfDNA), and extracellular vesicles, assists not only in early detection but also in prediction of treatment response and prognosis of the disease. In the present study, we examined 2 gastric cancer cases, performing a comprehensive analysis, including detection of mutations, detection of fusions using next-generation sequencing (NGS), chromosomal abnormalities via array comparative genomic hybridization (aCGH), and gene expression analysis through microarrays. Based on the above, we aim to assess whether this comprehensive analysis could reveal new data, beneficial for prediction or prognosis.

Methods

Samples

The first patient (coded as CS-CC-002) was a male, born in 1949, with gastric cancer, stage III. According to ECOG performance criteria [13], this patient is in status 1 (restricted in physically strenuous activity but ambulatory and able to carry out work of a light or

sedentary nature, e.g., light house work and office work). There is no family history, and the patient had partial gastrectomy in the October of 2020, followed by adjuvant chemotherapy. In addition, the patient is not a smoker, does not consume fatty foods, does not drink alcohol, and there is no history of inflammations, autoimmune colitis, and *H. pylori* infection.

The second patient (coded as CS-CC-004) was a male, born in 1963, with gastric cancer, stage IV. The ECOG status is 0 (fully active, able to carry on all pre-disease performance without restriction). There is no family history, and the patient receives therapy for the first time. He is a nonsmoker, nonalcohol consumer, and has obesity GII. Finally, there is no history of inflammations, autoimmune colitis, and *H. pylori* infection.

Blood Sample Preparation

Thirty milliliters of whole blood was incubated with 20 mL of ammonium chloride solution (154 mM NH₄Cl 31107; Sigma-Aldrich; 10 mM KHCO₃ 4854; Merck, Darmstadt, Germany; and 0.1 mM EDTA in deionized water) for erythrocyte lysis. Cells were incubated with the solution for 10 min under agitation and then centrifuged for 5 min at 2,000 rpm. Supernatant was discarded, and cell pellet was washed with 20 mL PBS. After centrifugation at 5 min at 2,000 rpm, the supernatant was discarded, and cell pellet was incubated with pancytokeratin beads recognizing CK4, CK5, CK6, CK8, CK10, CK13, and CK18 (5c-81714; Gentaur) at 4°C for 15 min. Following incubation, the samples were placed in a magnetic rack for pan-ck-positive fraction collection. Pan-ck-positive cells (cancer cells) were washed with 10 mL PBS for 5 min at 2,000 rpm. After centrifugation, the supernatant was discarded. The cell pellet was split in two. Half of the cells were used for RNA extraction, and half of the cells were used for DNA extraction.

Next-Generation Sequencing

Genomic DNA was isolated with the QIAamp DNA Mini Kit (51306; Qiagen, Hilden, Germany) and evaluated with Qubit Fluorometric Quantification and Qubit™ 1X dsDNA HS Assay Kit (Q33231; Invitrogen, Waltham, MA, USA). Libraries were prepared with TruSight Oncology 500 Kit (ILL.20032627; Illumina, San Diego, CA, USA), evaluated with qPCR (KK4844), and run on an Illumina's NextSeq 550 analyzer. The analysis was performed with Clinical Genomic Workspace, v6.15.1, powered by pieriandx.

Array Comparative Genomic Hybridization

Genomic DNA was isolated with QIAamp DNA Mini Kit (51306; Qiagen). The aCGH protocol was performed using the Sureprint G3 human CGH 8x60K platform (G4450A; Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Analysis was performed with Cytogenomics, v5.2.

Gene Expression Microarray Data

Total RNA was extracted using an RNeasy Mini Kit (74105; Qiagen). A reference sample (Universal Human Reference RNA, 740000-41; Agilent) was also used in the experiments. Preparation of samples for hybridization followed with Low Input Quick Amp Labeling Kit (5190-2305; Agilent) and hybridization continued with Gene Expression Hybridization Kit (5188-5242; Agilent), into SurePrint G3 Human Gene Expression v3 8x60K Microarray slides (G4851C; Agilent), according to manufacturer's instructions. Sample slides were washed with Gene Expression Wash Buffer Kit (5188-5327; Agilent) and were imaged on an InnoScan 710 (Innopsys) with 5-μm resolution. Scanned microarray image data were processed using Array-Pro Analyzer 6.3 Software. The microarray gene expression data were normalized using background subtraction.

Data Preprocessing

The raw data were assessed through the R statistical software and more specifically with the help of the VSN statistical package [14, 15], which implements a method for normalizing microarray data. In brief, it is a model that incorporates a data calibration step, a model for the dependence of the variance on the mean expression, and a variance stabilizing data transformation. The preprocessing procedure also involved background correction and data condensing. More specifically, the probe IDs were transformed into genes by eliminating those with no gene symbols, and areas without any expression were also removed from our study. In cases of multiple probes for the same gene, the mean value was evaluated, producing a single expression per gene. The result yielded 7,487 protein-coding genes.

DEGs Discovery and Classification

The differential expression patterns were analyzed and compared to the corresponding normal controls. Specifically, the significant p values for the DEGs were calculated by 2-sample t tests, defining as a level of statistical significance $\alpha = 0.05$ and $\log_2$ FC (Log fold change) ≥ 1.5 by using p value correction with the help of false discovery rate to minimize false positives [16]. Then, the significant DEGs were subjected to hierarchical clustering analysis, giving as a result 127 overexpressed genes.

Results

Next-Generation Sequencing

In patient CS-CC-002, a total of 1,062 variants were observed, with the majority of them being substitutions. There were no detected variants with strong clinical significance (tier I), but we observed variants with potential clinical significance (tier II), variants of unknown clinical significance (tier III), and benign or likely benign variants (tier IV). The tumor mutational burden (TMB) score was 0.8 mut/Mb, which is considered a low score, while the microsatellite instability (MSI) status was stable (0% of unstable sites).

For patient CS-CC-004, the total number of variants were 1,098, consisting mainly of substitutions. No tier I variants were detected, and only one of tier II. The TMB score was 2.6 muts/Mb (medium) and stable MSI status (0% of unstable sites). The tier II and tier III variants are presented in Table 1.

Array Comparative Genomic Hybridization

Patient CS-CC-002 had an amplification in chromosome X, while CS-CC-004 had the same amplification, as well as a gain in the Y chromosome. The specific region and length are presented in Table 2.

Gene Expression Microarray Data

Genes' expression was normalized to reference RNA sample's expression and compared to the expression levels from normal individuals. More than 100 genes have been identified, which were overexpressed only in patients' samples and not in normal cells. These include transcription factors (ZNF family), receptors, metalloproteinases, and genes implicated in signaling transduction pathways.

The Comparative Toxicogenomics Database [17] was used for the genes that were related to gastrointestinal cancer, based on the information in the previously public literature. Subsequently, we performed relative analysis such as a set-based enrichment for collections of genes using correction of p values.

Table 1. Tier II–III variants detected with NGS analysis

Tier	CS-CC-002	CS-CC-004
II	FGFR4 p.G388R c.1162G>A	–
II	SMARCA4 p.Y372H c.1114T>C	–
II	TP53 p.P72R c.215C>G	TP53 p.P72R c.215C>G
III	ARID5B p.V651L NM_032199.2 c.1951G>C	BARD1 p.V507M NM_000465.2 c.1518_1519delinsCA
III	BARD1 p.V507M NM_000465.2 c.1518_1519delinsCA	DNAJB1 p.D167E NM_006145.1 c.501C>G
III	BARD1 p.I738V NM_000465.2 c.2212A>G	DNMT3B p.A384T NM_006892.3 c.1150G>A
III	CARD11 p.A21T NM_032415.4 c.61G>A	EML4 p.E257D NM_019063.3 c.771G>C
III	CUX1 p.A654T NM_001913.3 c.1960G>A	FAM46C p.E299K NM_017709.3 c.895G>A
III	FANCD2 p.I559V NM_033084.3 c.1675A>G	FANCF p.G225S NM_022725.3 c.673G>A
III	FAT1 p.D3317N NM_005245.3 c.9949G>	INHA p.G346R NM_002191.3 c.1036G>C
III	GNAS p.R600G NM_080425.2 c.1798C>G	INHA p.P256S NM_002191.3 c.766C>T
III	IGF1R p.R1337C NM_000875.3 c.4009C>T	KMT2A p.E502K NM_005933.3 c.1504G>A
III	LRP1B p.G935R NM_018557.2 c.2803 G>A	MST1 p.R400C NM_020998.3 c.1198C>T
III	MAP3K4 p.K1080R NM_005922.2 c.3239A>G	NOTCH1 p.T596M NM_017617.3 c.1787C>T
III	MGA p.T1645I NM_001164273.1 c.4934C>T	PLCG2 p.P522R NM_002661.3 c.1565C>G
III	NTRK1 p.H568Y NM_001007792.1 c.1702C>T	PTCH1 p.R682C NM_000264.3 c.2044C>T
III	NTRK1 p.G577V NM_001007792.1 c.1730G>T	SOX2 p.Q266E NM_003106.3 c.796C>G
III	RANBP2 p.T2602M NM_006267.4 c.7805C>T	
III	SDHD p.H50R NM_003002.3 c.149A>G	
III	SLX4 p.S1344P NM_032444.2 c.4030T>C	
III	SPTA1 p.A970D NM_003126.2 c.2909C>A	
III	ZNF217 p.E914_P915delinsDS NM_006526.2 c.2742_2743delinsTT	
III	ZNF703 p.H402Pfs*8 NM_025069.1 c.1205delA	

Table 2. Chromosomal abnormalities detected with aCGH

Patient	Chromosome	Cytoband	Size, kb	Start-stop position	Type
CS-CC-002	Chr X	p22.2-p11.21	44.216	13,715,582–57,932,274	Amplification
	Chr X	q11.1-q28	91.707	62,062,537–153,771,517	Amplification
CS-CC-004	Chr X	p22.31-p11.21	48.588	9,462,784–58,051,765	Amplification
	Chr X	q12-q28	87.878	66,656,771–154,535,178	Amplification
	Chr X	q21.33-q22.1	847	97,624,055–98,471,342	Amplification
	Chr Y	q11.21-q12	44.435	14,837,758–59,273,048	Amplification

Discussion

Gastric cancer has a high mortality rate, which is increased in males and affected by different risk factors, endogenous or exogenous. The treatment protocols differ depending on the characteristics of tumor and include surgery (gastrectomy) where it is operable, usually followed by adjuvant chemo/radio therapy or palliative chemotherapy

in nonoperable cases. The palliative chemotherapy is based on HER2-positive or HER2-negative, or other trials with novel agents. After that, second-line chemotherapy is a common treatment practice [18]. Early this year, FDA approved the use of trastuzumab deruxtecan, a second-generation antibody drug conjugate with DNA topoisomerase-I inhibitor, for gastric cancer [19].

Liquid biopsy enabled improvement in treatment decision and/or prognosis of the disease. CTC number and immunophenotype are associated with survival and response. Patients with CTCs ≥ 5 in 7.5 mL had shorter disease-free survival and overall survival, compared to cases with lower CTC number [20]. In patients with CTCs expressing CD133, the prognosis was significantly poorer, than in CTCs without expression of this marker, while expression of cytokeratins and CD144 was associated with metastasis [21, 22]. Based on our gene expression assays, the genes that were found to be overexpressed are not limited to those observed in previous studies, but we were able to identify many other overexpressed genes that are involved in various signaling transduction pathways and other cellular processes.

Apart from studying the expression profile of CTCs, detection of variants and fusions could also be beneficial, since they provide useful information for targeted therapies or immunotherapies. The detection of mutations, the calculation of TMB, and evaluation of MSI status have already been evaluated in gastrointestinal cancer [23]. In our analysis, we expanded the number of genes tested and included RNA genes, in order to incorporate any fusions associated with gastric carcinoma. The data revealed plenty of variants, however the majority with unknown or no clinical significance. Furthermore, no fusions were detected as the one of DUS4L-BCAP29, previously reported by Kim et al. [24]. As far as the TMB and MSI status, there were no unstable sites observed, while the TMB score was relatively low, decreasing the possibilities of efficient immunotherapy [25]. In addition, among the different variants detected in both cases, only TP53 p.P72R c.215C>G and BARD1 p.V507M NM_000465.2 c.1518_1519delinsCA are common in patients.

The strategy of comprehensive analysis also includes aCGH, for detection of chromosomal abnormalities [26]. According to previous studies, gains at 1p, 5p, 7q, 8q, 11p, 16p, 20p, and 20q and deletions at 1p, 2q, 4q, 5q, 7q, 9p, 14q, and 18q are observed [27]. In the cases we tested, it is noteworthy that all the abnormalities were observed in the X and Y chromosomes. These alterations were observed in both cases, indicating a potential pattern. Genetic alterations in the X chromosome have been implicated in cancer formation and progression [28], while reactivation of X-linked tumor suppressor genes has been a treatment challenge for the last decade [29]. The gained regions of X chromosome are similar to Klinefelter syndrome (XXY), but they do not have a complete extra copy of X, which could contribute to cancer [30]. Their phenotype is also noncompatible with Klinefelter syndrome, making things more complicated.

Based on all the above, it is clearly demonstrated that scientists are able to provide a wide range of information needed for diagnosis and treatment response. On the other hand, physicians need to be educated also in the principles of these techniques, so to know what information could earn in each case. This study is another example of how powerful is the combination among different sciences, including but not limited to biology and medicine. Implementation of mathematics could also enhance the power of this comprehensive panel. We strongly are in favor of using all fields of science to extract all the valuable information that could improve patients' survival and quality of life.

This study is focused on a very limited number of cases; however, the patterns revealed by aCGH and the common genes expressed in both of them, as well as the common mutations, could be used for further studies. Despite the low number of patients, they constitute a representative group of patients, sharing characteristics common in the majority of gastric cancer

cases. Furthermore, the comprehensive cancer panel used could counteract the small number, by providing a wide variety of information, which could not be extracted just using specific panels, even in a higher number of patients. However, we totally agree that a higher number of samples is required, including long-term information about the progression of the disease and any treatment response. Both the suggested panel, with an appropriate number of cases, could be an ideal proof of this concept.

Conclusions

Based on the above data, we conclude that one method of screening or diagnosis could not reveal the whole profile of a tumor. The majority of the commonly used methods have advantages and disadvantages. Many of them focus on variants at DNA level, but not all variants affect gene expression, while changes in gene expression levels could be caused not only by mutations but also from many different other parameters, such as activation by other genes or extracellular signals. Therefore, the establishment of a complete cancer comprehensive panel might be more beneficial, since it reveals plenty of information.

Statement of Ethics

This study is not a clinical trial and does not include intervention in patients. All procedures were conducted according to the standards of Safety, Bioethics, and Validation. The study was reviewed and approved by the Bioethical Committee of the Research Genetic Cancer Centre Group. Each patient has consented in writing for the use of their sample in the present study and for publication. The patients retained the right to withdraw their sample until the date when the sample was received at the laboratory and tested.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

V.P. supervised the study. P.A. carried out the molecular biology assays and drafted the manuscript. G.B. performed analysis of data and drafted the manuscript. E.H. performed cell culturing assays. I.P. supervised the assays and the manuscript.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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