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# Genetic Mutations in Human Esophageal and Gastric Cardia Cancers Detected by Ampliseq Sequencing

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## Abstract

Esophageal and gastric cancers are two of the most common malignancies worldwide with particularly high mortality rates. Esophageal and gastric cardia cancers share certain environmental risk factors, but it is unclear if these cancers share similar gene mutation patterns. To improve patient diagnosis, treatment, and outcome, identification and characterization of the unique molecular mutation profiles of these cancers are needed to develop more effective target therapies. Until recently, personalized DNA sequencing to identify individual cancer mutations was unrealistic for clinical applications. But technological advancements in next-generation DNA sequencing, including the semiconductor-based Ion Torrent sequencing platform, have made DNA sequencing more cost and time effective with reliable results. Using the Ion Torrent Ampliseq Cancer Panel, we sequenced 737 loci from 45 cancer-related genes to identify genetic mutations in esophageal adenocarcinoma, esophageal squamous cell carcinoma, and gastric cardia cancer samples from Chinese patients. The sequencing analysis revealed frequent mutations in PIK3CA and TP53 genes, and less frequent mutations in several other genes. Thus, this study demonstrates the feasibility of using Ion Torrent sequencing on individual human cancers to detect patient-specific gene mutations with the goal of directing mutation-specific targeted therapies or aid in targeted drug development to more effectively treat cancer patients.

**Keywords:** Esophageal cancer; Genetic mutations; Ion Torrent sequencing; Targeted therapy; Personalized medicine

## Introduction

Esophageal and stomach cancers are two of the most common malignancies worldwide. In 2012, these two cancer types alone accounted for over 1,408,000 cases and 1,123,000 deaths globally [1]. Both esophageal and gastric cancer have the highest incidence and mortality in East Asia with roughly 42.5% of all gastric cancer cases and 48.9% of all esophageal cancer cases reported in China alone. Roughly half of esophageal cancer patients have advanced or metastatic disease upon diagnosis and natural overall survival time is less than 1 year [2]. The 5-year survival for all esophageal and specifically gastric cardia cancer patients remains less than 8% and surgical intervention only increases this to 18%-23% [3,4]. The prevalence and dismal survival rates of esophageal and gastric cancers indicate further efforts are needed for improved diagnostics and more effective treatment options.

The two major subtypes of Esophageal Cancer (EC) include adenocarcinoma (EAC), more commonly found in western countries and associated with poor diet and obesity, and squamous cell carcinoma (ESCC), which is more prevalent in Eastern countries and is strongly associated with smoking and alcohol consumption [5,6]. Gastric cardia cancers, virtually all of which are adenocarcinomas [7], share clinical symptoms with esophageal cancers, like dysphagia, but because most of these cancers are diagnosed at an advanced stage, it may be difficult to determine if the origin is esophageal or gastric [8]. While gastric cardia adenocarcinomas (GCA) also share similar risk factors with both EAC and ECC and gastric cancer, including dietary factors, obesity, smoking, and alcohol consumption, previous studies report that GCA has a greater tendency than other gastric cancers toward deeper gastric wall penetration, lymph node metastasis, and poor prognosis, suggesting that GCA may be more aggressive and increasing the need for proper diagnosis [9]. Increasingly, adenocarcinomas of the gastric cardia are thought to be distinct from adenocarcinomas of the esophagus or distal stomach, both biologically and epidemiologically [8].

As each cancer type and individual tumor contains a unique pattern of molecular mutations, genetic profiling in individual cancers will give further insight into the complex environmental and genetic interactions that contribute to the development and progression of the disease. Moreover, comparing the unique mutation profiles of esophageal and gastric cardia cancers, or further gastric cardia and non-cardia adenocarcinomas, may help to further clarify classifications and diagnoses, an issue which has been challenging in the past. Additionally, treatment of these diseases may be improved by targeting drugs to the specific molecular changes found in each tumor. Recent studies on whole-genome and -exome sequencing in esophageal and gastric cancers have uncovered numerous genes that are commonly mutated in these cancers, some of which have the potential to be used as drug

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targets [10-12]. The high cost of the instruments and assays used in these studies, however, largely prevent this technology from being used as a widespread diagnostic approach to individual cancer sequencing [13]. The possibility of personalized DNA sequencing for cancer treatment is becoming more feasible with the recent technological advancements in next-generation sequencing (NGS), such as the semiconductor-based Ion Torrent sequencing platform, which circumvents many of the previous hurdles of other NGS platforms like high cost and long assay times [14,15]. In the present study, we have used Ion Torrent sequencing technology to analyze clinical samples of esophageal cancer, including squamous cell carcinoma and esophageal adenocarcinoma, and gastric cardia cancer to identify the genetic mutations at 737 loci from 45 known cancer-related genes.

## Materials and Methods

### Ethics statement

The study has been approved by the Human Research Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University, China. For formalin-fixed, paraffin-embedded (FFPE) tumor samples from the tumor tissue bank at the Department of Pathology of the hospital, the institutional ethics committee waived the need for consent. All samples and medical data used in this study have been irreversibly anonymized.

### Patient information

Tumor samples used in the study were collected from the First Affiliated Hospital of XinXiang Medical University in China. A total of 35 FFPE tumor samples from gastric cardia cancer and 45 FFPE tumor samples from esophageal cancer patients were analyzed. Patients ranged from 43-79 years of age with a median age of 60 years (Tables 1,2). All 80 patients were categorized based on their gender and age. Tumor samples were categorized based on differentiation, the TNM staging system, and metastasis to regional lymph nodes. Esophageal cancer patients were further categorized based on pathological type (EAC vs. ESCC) (Table 3). Additionally, esophageal cancer patients reported histories of smoking and drinking alcohol (Supplemental Tables 1,2).

### DNA preparation

Xylene was used to deparaffinize 3-5 μm thick extracted sections of formalin-fixed, paraffin-embedded (FFPE) tissue samples. DNA was then isolated using the QIAamp DNA Mini Kit (Qiagen) following manufacturer's instructions.

| Characteristic | | n (frequency) |
|---|---|---|
| Gender | female | 16 (45.7%) |
| | Male | 19 (54.3%) |
| Age (years) | Average: 60 ± 8 | Median:60, Range:43-79 |
| Differentiation | high | 10 (28.6%) |
| | middle | 12 (34.3%) |
| | low | 5 (14.3%) |
| | unknown | 8 (22.8%) |
| Regional lymph node metastasis | N0 | 19 (54.3%) |
| | N1 | 16 (45.7%) |
| TNM | Ib | 1 (2.9%) |
| | II | 18 (51.4%) |
| | III | 1 (2.9%) |
| | IIIa | 10 (28.5%) |
| | IIIb | 4 (11.4%) |
| | IV | 1 (2.9%) |

**Table 1**: Clinical features of 35gastric cardia carcinoma patients.

| Characteristic | | n (frequency) |
|---|---|---|
| Gender | Female | 23 (51.1%) |
| | Male | 22 (48.9%) |
| Age (years) | Average: 60 ± 8 | Median:60, Range:43-79 |
| Pathological Type | EAC | 6 (13.3%) |
| | ESCC | 39 (86.7%) |
| Differentiation | high | 17 (37.8%) |
| | middle | 17 (37.8%) |
| | low | 4 (8.9%) |
| | unknown | 7 (15.5%) |
| Regional lymph node metastasis | N0 | 33 (73.3%) |
| | N1 | 12 (26.7%) |
| Location | Lower esophagus | 13 (28.9%) |
| | Middle esophagus | 28 (62.2%) |
| | Upper esophagus | 4 (8.9%) |
| TNM | 0 | 1 (2.2%) |
| | I | 7 (15.6%) |
| | IIa | 25 (55.6) |
| | IIb | 2 (4.4%) |
| | III | 10 (22.2%) |
| Smoking | No | 27 (60%) |
| | Yes | 18 (40%) |
| Alcohol consumption | Non drinking | 30 (66.7%) |
| | Drinking | 8 (17.8%) |
| | Unknown | 7 (15.5%) |

**Table 2:**Clinical features of 45 esophageal cancer patients.

### Ion Torrent PGM Library Preparation and Sequencing

We constructed an Ion Torrent adapter-ligated library using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Part #4475345 Rev. A) following the manufacturer's protocol and as described in our previous publications [16,17]. The Personalized Cancer Mutation Panel used for this study targets 737 loci to detect mutations in the following 45 cancer-related genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNAS, HNF1A, HRAS, IDH1, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, and VHL.

## Sequence Coverage

For the 45 esophageal cancer samples analyzed, the mean read length was 77 bp and the average reads were approximately 22 Mb of sequence per sample. With normalization to 30,000 reads per specimen, the average reads per amplicon was 1639 (range: 9 to 4346) (Figure 1A); 179/189 (94.7%) amplicons averaged at least 100 reads, and 168/189 (88.9%) amplicons averaged at least 300 reads (Figure 1B).

The mean read length for the 35 gastric cardia cancer samples analyzed was 76 bp and the average reads were approximately 17 Mb of sequence per sample. With normalization to 30,000 reads per specimen, the average reads per amplicon was 1639 (range: 10 to 6941) (Figure 2A); 175/189 (92.6%) amplicons averaged at least 100 reads, and 169/189 (89.4%) amplicons averaged at least 300 reads (Figure 2B).

### Variant calling

Data were initially processed using the Ion Torrent platform-specific pipeline software Torrent Suite to generate sequence reads and

| Genes | Number of GC samples with mutations (frequency in 35 samples) | Number of EAC samples with mutations (frequency in 6 samples) | Number of ESCC samples with mutations (frequency in 39 samples) |
|---|---|---|---|
| ABL1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| AKT1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| ALK | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| APC | 2 (5.7%) | 0 (0.0%) | 0 (0.0%) |
| ATM | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| BRAF | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| CDH1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| CDKN2A | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| CSF1R | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| CTNNB1 | 0 (0.0%) | 1 (16.7%) | 0 (0.0%) |
| EGFR | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| ERBB2 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| ERBB4 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| FBXW7 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| FGFR1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| FGFR2 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| FGFR3 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| FLT3 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| GNAS | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| HNF1A | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| HRAS | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| IDH1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| JAK3 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| KDR | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| KIT | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| KRAS | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| MET | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| MLH1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| MPL | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| NOTCH1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| NPM1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| NRAS | 1 (2.9%) | 0 (0.0%) | 0 (0.0%) |
| PDGFRA | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 2 (5.7%) | 0 (0.0%) | 2 (5.1%) |
| PTEN | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PTPN11 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| RB1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| RET | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| SMAD4 | 1 (2.9%) | 0 (0.0%) | 0 (0.0%) |
| SMARCB1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| SMO | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| SRC | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| STK11 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 6 (17.1%) | 0 (0.0%) | 13 (33.3%) |
| VHL | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |

**Table 3:**Mutation (including missense point mutations/deletion/insertion) frequencies of 45 genes (737 loci) in 35 gastric cardia cancer patients and 45 esophageal carcinoma patients with different pathological type. (ESCC: Esophageal squamous cell carcinoma; EAC: esophageal adenocarcinoma).

trim adapter sequences, as well as filter and remove poor signal-profile reads. Initial variant calling from the Ion AmpliSeq sequencing data was generated using Torrent Suite Software v3.4 with a plug-in "variant caller v3.4" program. Several filtering steps were then used to eliminate erroneous base calling in order to generate final variant calling: defining coverage depth and variant frequency, removing DNA strand-specific errors, defining variants within 727 hotspots, and eliminating variants in amplicon AMPL339432 (PIK3CA, exon13, chr3:178938822-178938906), as further described in our previous publications [16,17]. Finally, the JAK2 gene locus generated false deletion data from our sequencing runs; therefore, we excluded the sequencing data from this locus from further analysis.

## Somatic mutations

Detected mutations were compared to variants in the 1000 Genomes Project [18] and 6,500 exomes of the National Heart, Lung, and Blood Institute Exome Sequencing Project [19] to distinguish somatic mutations and germline mutations.

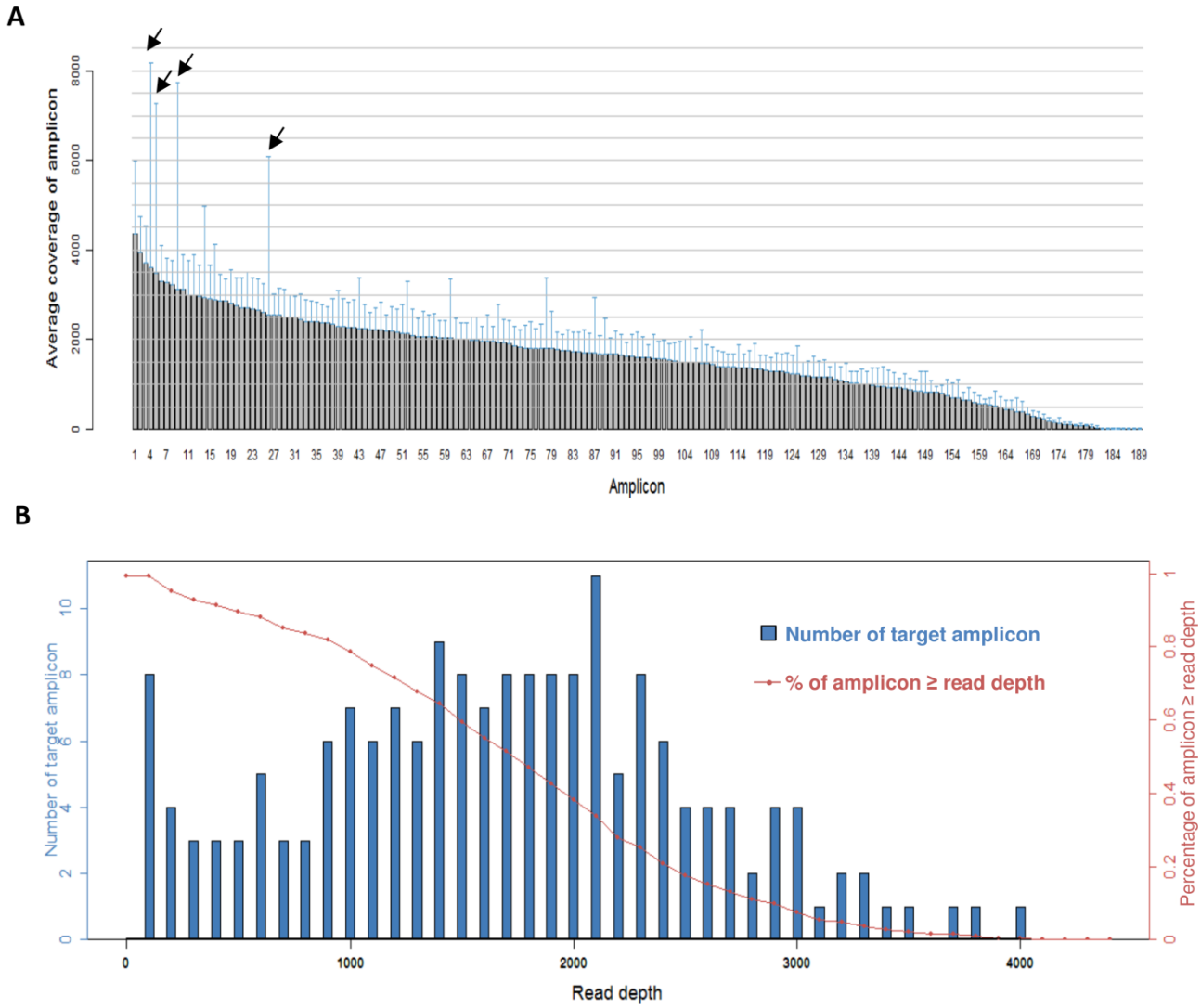



**Figure 1.** Sequence read distribution across 189 amplicons generated from 45 esophageal cancer samples, normalized to 300,000 reads per sample. Black arrows point to four amplicons of ERBB2.
A. Average number of reads observed for each amplicon.
B. Number of targets with a given read depth, sorted in bins of 100 reads.

## Bioinformatical and experimental validation

We used the COSMIC (version 64) [20], MyCancerGenome database (http://www.mycancergenome.org/), and additional publications to evaluate reappearing mutations in esophageal and gastric cardia cancers (Supplemental Tables 3,4). Additionally, some detected mutations were confirmed by Sanger's sequencing (Supplemental Table 5 and Supplemental Figure 1).

## Results and Discussion

A total of 80 patient samples were used for this study, including 35 GCA and 45 EC (6 EAC and 39 ESCC), and 45 oncogenes and tumor suppressor genes were sequenced with the Ion Torrent PGM. Overall, 25 of the 80 samples (31.3%) contained one mutation, and 3 samples (3.8%) contained two mutations. Specifically, 15 of the 45 (33.3%) ECs in our samples set had one mutation in various genes (Table 3), and one of these samples contained two mutations (Table 4). In the GCA samples, 10 out of 35 (28.6%) were found with one mutation (Table 3), and two samples contained two mutations (Table 5). Of the 45 genes sequenced in both cancer types, we detected the highest frequency of mutations in TP53 (28.9% of EC samples and 17.1% of GCA samples) and PIK3CA (4.4% of ECs and 5.7% of GCA samples). Additionally, four genes were found to have mutations at lower frequencies: one EC sample revealed a missense mutation in CTNNB1, two GCA samples contained mutations in APC, one GCA sample contained a mutation in NRAS, and one GCA with a SMAD4 mutation. Proportionally mutation rates were relatively similar between males and females in GCA samples (36.8% v. 31.3%, respectively), but in the EC samples, mutation rates were more than twice as high in females than males (47.8% v. 22.7%, respectively). Overall, a higher mutation rate was found in ESCC samples compared to EAC samples (38.5% v. 16.7%, respectively), which may partially be due to the small number of EAC samples in the study. No correlations could be made between EC mutations and smoking or alcohol history (Supplemental Tables

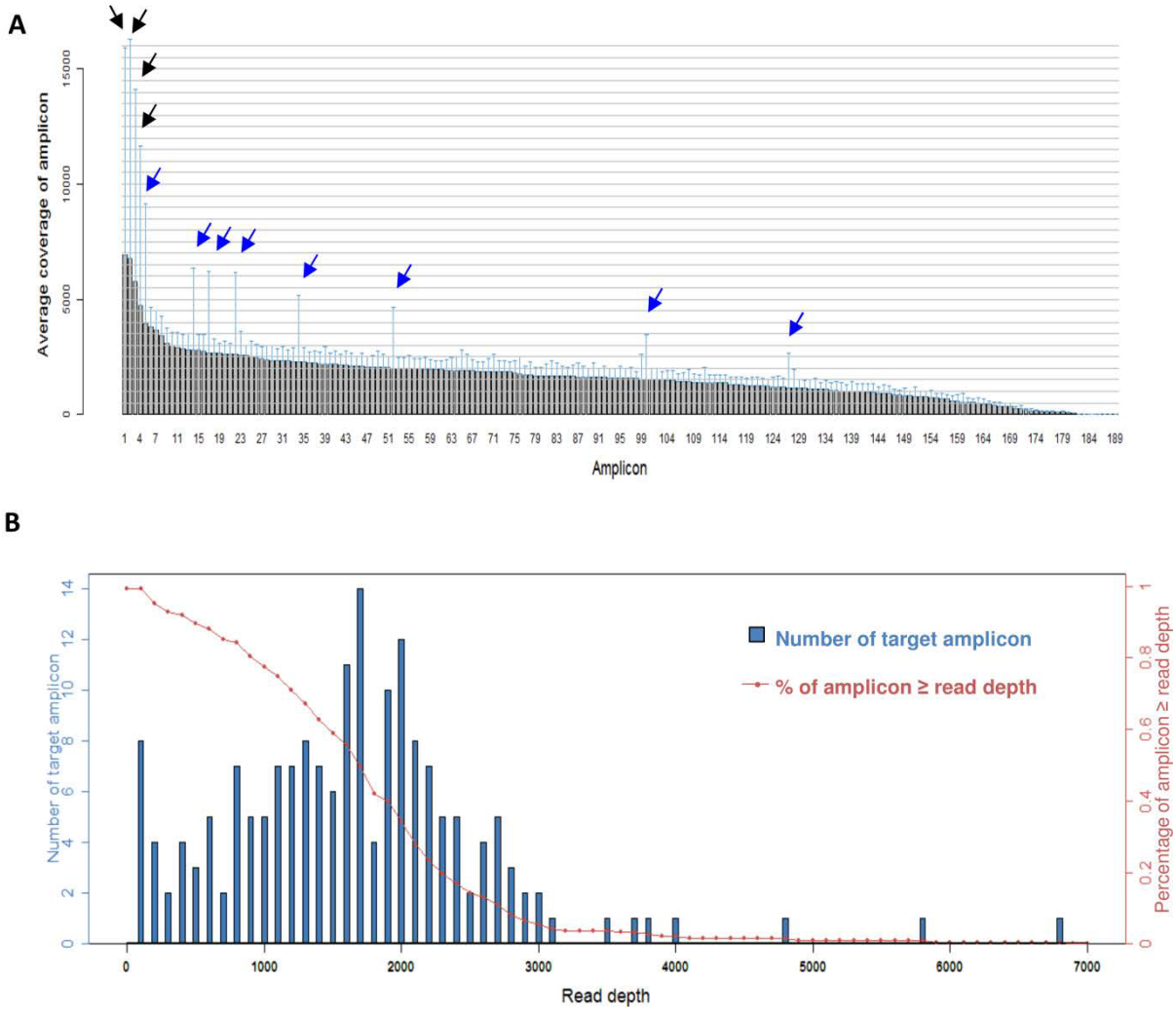



**Figure 2.** Sequence read distribution across 189 amplicons generated from 35 gastric cardia cancer samples, normalized to 300,000 reads per sample. Black arrows point to four amplicons of ERBB2. Blue arrows point to 8 amplicons of EGFR.
A. Average number of reads observed for each amplicon.
B. Number of targets with a given read depth, sorted in bins of 100 reads.

| Gene | Exon | Mutation | Mutation Type | n of samples with mutation (Frequency) | Sex | Differentiation | Pathological Diagnosis | TNM staging | Lymph node involvement |
|---|---|---|---|---|---|---|---|---|---|
| CTNNB1 | 3 | S37C | M | 1 (2.2%) | F | low | EAC | IIa | N0 |
| PIK3CA | 9 | E545K | M | 1 (2.2%) | F | high | ESCC | I | N0 |
| PIK3CA | 9 | Q546K[1] | M | 1 (2.2%) | F | high | ESCC | I | N0 |
| TP53 | 5 | A159V | M | 1 (2.2%) | M | middle | ESCC | I | N0 |
| TP53 | 5 | R175H | M | 2 (4.4%) | F | high/middle | ESCC | IIa/IIb | N0/N1 |
| TP53 | 5 | C176F | M | 1 (2.2%) | M | high | ESCC | IIa | N0 |
| TP53 | 6 | R196* | N | 1 (2.2%) | F | middle | ESCC | IIa | N0 |
| TP53 | 6 | R213* | N | 1 (2.2%) | F | middle | ESCC | III | N1 |
| TP53 | 7 | C242F | M | 1 (2.2%) | F | low | ESCC | IIa | N0 |
| TP53 | 7 | R248W | M | 1 (2.2%) | M | high | ESCC | IIa | N0 |
| TP53 | 8 | V272M | M | 1 (2.2%) | M | high | ESCC | IIa | N0 |
| TP53 | 8 | P278S | M | 2 (4.4%) | F | unknown | ESCC | I | N0 |
| TP53 | 8 | R306*[1] | N | 1 (2.2%) | F | high | ESCC | I | N0 |
| TP53 | 10 | R342* | N | 1 (2.2%) | M | high | ESCC | IIa | N0 |

[1]Mutations found within the same sample; *Nonsense mutations resulting in STOP codon; M: Missense mutation; N: Nonsense mutation; N0: noregional lymph node metastasis; N1: metastasis in 1-2 regional lymph nodes

**Table 4:**Specific point mutations detected among 45 esophageal cancer samples.

| Gene | Exon | Missense point mutations/deletion/insertion counts (Percentage in all mutation counts of this gene) | Missense point mutations (including coding silent) counts (Percentage in all mutation counts of this gene) | Deletion counts (Percentage in all mutation counts of this gene) | Insertion counts (Percentage in all mutation counts of this gene) |
|---|---|---|---|---|---|
| TP53 | 2 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 4 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 5 | 4 (30.8%) | 4 (30.8%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 6 | 2 (15.4%) | 2 (15.4%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 7 | 2 (15.4%) | 2 (15.4%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 8 | 4 (30.8%) | 4 (30.8%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 10 | 1 (7.7%) | 1 (7.7%) | 0 (0.0%) | 0 (0.0%) |

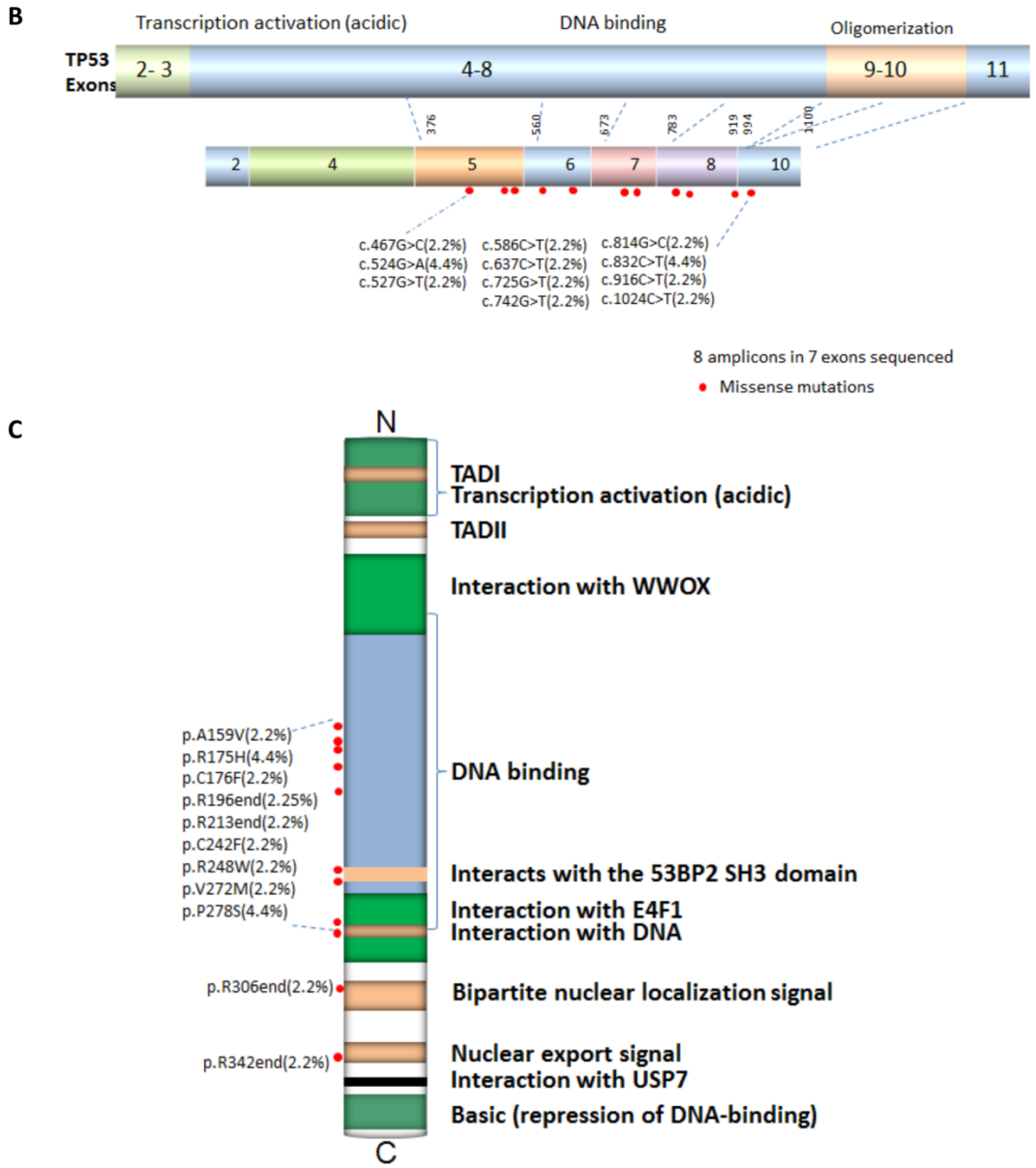



**Figure 3.** Missense mutation distribution in the exons and function domains of TP53 in esophageal cancer samples.
A. Frequencies of detected mutations in different exons.
B. Mutation distribution in exons.
C. Mutation distribution in functional domains.

| Gene | Exon | Mutation | Mutation Type | n of samples with mutation (Frequency) | Sex | Differentiation | TNM staging | Lymph node involvement |
|---|---|---|---|---|---|---|---|---|
| APC | 15 | R876*[1] | N | 1 (2.9%) | M | high | IIIb | N1 |
| APC | 15 | R1450*[2] | N | 1 (2.9%) | F | high | II | N0 |
| NRAS | 3 | Q61H[1] | M | 1 (2.9%) | M | high | IIIb | N1 |
| PIK3CA | 9 | E542K[2] | M | 1 (2.9%) | F | high | II | N0 |
| PIK3CA | 9 | E542Q | M | 1 (2.9%) | M | middle | II | N0 |
| SMAD4 | 8 | R361H | M | 1 (2.9%) | M | unknown | II | N0 |
| TP53 | 5 | V173L | M | 1 (2.9%) | M | unknown | IIIa | N1 |
| TP53 | 5 | C176F | M | 1 (2.9%) | F | unknown | IIIa | N1 |
| TP53 | 6 | R213* | N | 1 (2.9%) | M | unknown | III | N1 |
| TP53 | 7 | R248W | M | 2 (5.7%) | F/M | low/middle | II/IIIb | N0/N1 |
| TP53 | 7 | R249S | M | 1 (2.9%) | F | high | IIIa | N1 |

[1]Mutations found within the same sample; [2]Mutations found within the same sample; *Nonsense mutations resulting in STOP codon; M: Missense mutation; N: Nonsense mutation; N0: noregional lymph node metastasis; N1: metastasis in 1-2 regional lymph nodes

**Table 5:** Specific point mutations detected among 35 gastric cardia cancer samples.

1,2). The detailed list of mutations detected in the 737 loci of 45 tumor suppressor and oncogenes in 45 esophageal and 35 gastric cardia cancer samples is listed in Tables 4 and 5, respectively.

## TP53 mutations in esophageal and gastric cardia cancers

TP53 mutations were identified in TP53 mutations were found in 17.1% of GCA samples and 28.9% of esophageal tumors, all of which were found in the ESCC type samples (Table 3). Compared to previous data which suggests that 38% - 50% of all human cancers and up to 70% of esophageal cancers have TP53 mutations [10,21], the mutation rate in our study was somewhat less than expected; however, mutation rates tend to vary by population and geographic location. Additionally, gastric cancers that are positive for the Epstein-Barr virus (~9%) have been found to have a much lower incidence of TP53 mutations [11]. While some differences in GCA and EC samples with TP53 mutations in our sample set could be observed, including staging (83.3% GCA vs. 7.7% EC at stage III or higher) and regional lymph node involvement (16.7% GCA vs. 84.6% EC without lymph node involvement) (Tables 4,5), the limited sample size and relatively low number of all TP53 mutations detected preclude convincing comparisons between these two cancer types. Regardless, there is still some supportive evidence here that cancer in the esophagus is more often detected sooner than the gastric cardia, possibly due to surveillance, but also that esophageal cancers presents with dysphagia sooner than tumors of the gastric cardia[22]. Also, the advanced stage of nearly all TP53-mutated EC and GCA in our study supports that T53 mutations are typically an early event in esophageal and gastric neoplasms [23], and mutations in TP53 may be a predictable marker for cancer development.

Because of its high mutation rate in various cancer types, TP53 is an important prognostic marker in carcinogenesis. One clinical study showed that TP53-mutated EACs were more often of advanced stage with poorer differentiation than EACs with no TP53 mutation, indicating disruption of this gene is associated with more aggressive tumors [24]. Another study found that after curative resection, esophageal cancer patients without TP53 mutations survived nearly twice as long as those harboring TP53 mutations [25]. While this study did not conclude that treatment response or patient survival depends on specific TP53 mutations, other research indicates that specific TP53 mutations may in fact play a role in patient outcome or response to treatment [26].

TP53 has multiple important biological functions, both nuclear and cytosolic, and is involved in tumor suppression, cell cycle arrest, apoptosis, and more [27,28]. TP53's major role is as a transcription factor occurs via a localized DNA binding domain in the core of the protein, where exons 4-8 encompass the DNA binding domain and exon 10 encodes for the oligomerization domain [29]. Mutations within these exons, which result in the loss sequence-specific DNA binding and defects in p53-dependent transcription, cell-cycle arrest, and apoptosis are the most common TP53 mutations in human cancers [24,30]. Over 85% of TP53 mutations cluster between codons 125 and 300, which mainly corresponds to the DNA binding domain [21]. Accordingly, 89.5% of the TP53 mutations in our study were found within these codons. Of the TP53-mutated ECs in our study, 30.8% were in exon 5 (A159V, R175H, and C176F), 15.4% in exon 6 (R196* and R213*), 15.4% in exon 7 (C242F and R248W), 30.8% in exon 8 (V272M, P278S, and R306*), and 7.7% in exon 10 (R342*) (Figure 3). In the GCA samples, 33.3% were found in exon 5 (V173L and C176F), 16.7% in exon 6 (R213*), and 50.0% in exon 7 (R248W and R249S) (Figure 4). All of the mutations identified are in known hotspot locations. Additionally, several mutations are located within the critical L2 and L3 zinc binding domains (R175H, C176F, C242F, R248W, V173L, and R249S). Three of these specific amino acid substitutions (C176F, R213*, and R248W) were found in both EC and GCA samples, and these three mutations account for 36.8% of all TP53 mutations identified.

## PIK3CA mutations in esophageal and gastric cardia cancers

PIK3CA mutations were identified in 5.1% of ESCC samples and 5.7% of GCAs in our sample set (Table 3). Mutations in PIK3CA are a common event in various human cancers. The greatest PIK3CA mutation frequencies are found in breast (27%), colon (23%), and endometrial (36%) cancers [31], with lower frequencies observed in gastric cancers (13%) and esophageal cancers (4.5%) [32,33]. Despite PIK3CA mutations occurring in multiple cancer types, research indicates these mutations may confer different outcomes depending on cancer type. For instance, clinical studies have shown PIK3CA mutations to correlate with poor prognoses in colorectal cancer patients [34] but are associated with a better prognosis in certain ESCC patients [35,36].

The phosphatidylinositol 3-kinase (PI3K) pathway is known to be important in cancer development and progression. PI3Ks are a ubiquitous family of lipid kinases capable of activating a variety of downstream targets that regulate numerous important cellular processes like cell proliferation, migration, survival, and oncogenic transformation. PIK3CA encodes for the catalytic subunit p110α of class IA PI3Ks [37,38]. Roughly 80% of oncogenic PIK3CA mutations are located in hotspots in exon 9 (E542K and E545K), which encodes for the helical domain, and exon 20 (H1047R), which encodes for the kinase domain [31]. In agreement with previous research, all of these

**A**

| Gene | Exon | Missense point mutations/deletion/insertion counts (Percentage in all mutation counts of this gene) | Missense point mutations (including coding silent) counts (Percentage in all mutation counts of this gene) | Deletion counts (Percentage in all mutation counts of this gene) | Insertion counts (Percentage in all mutation counts of this gene) |
|---|---|---|---|---|---|
| TP53 | 2 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 4 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 5 | 2 (33.3%) | 2 (33.3%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 6 | 1 (16.7%) | 1 (16.7%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 7 | 3 (50.0%) | 3 (50.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 8 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| TP53 | 10 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |

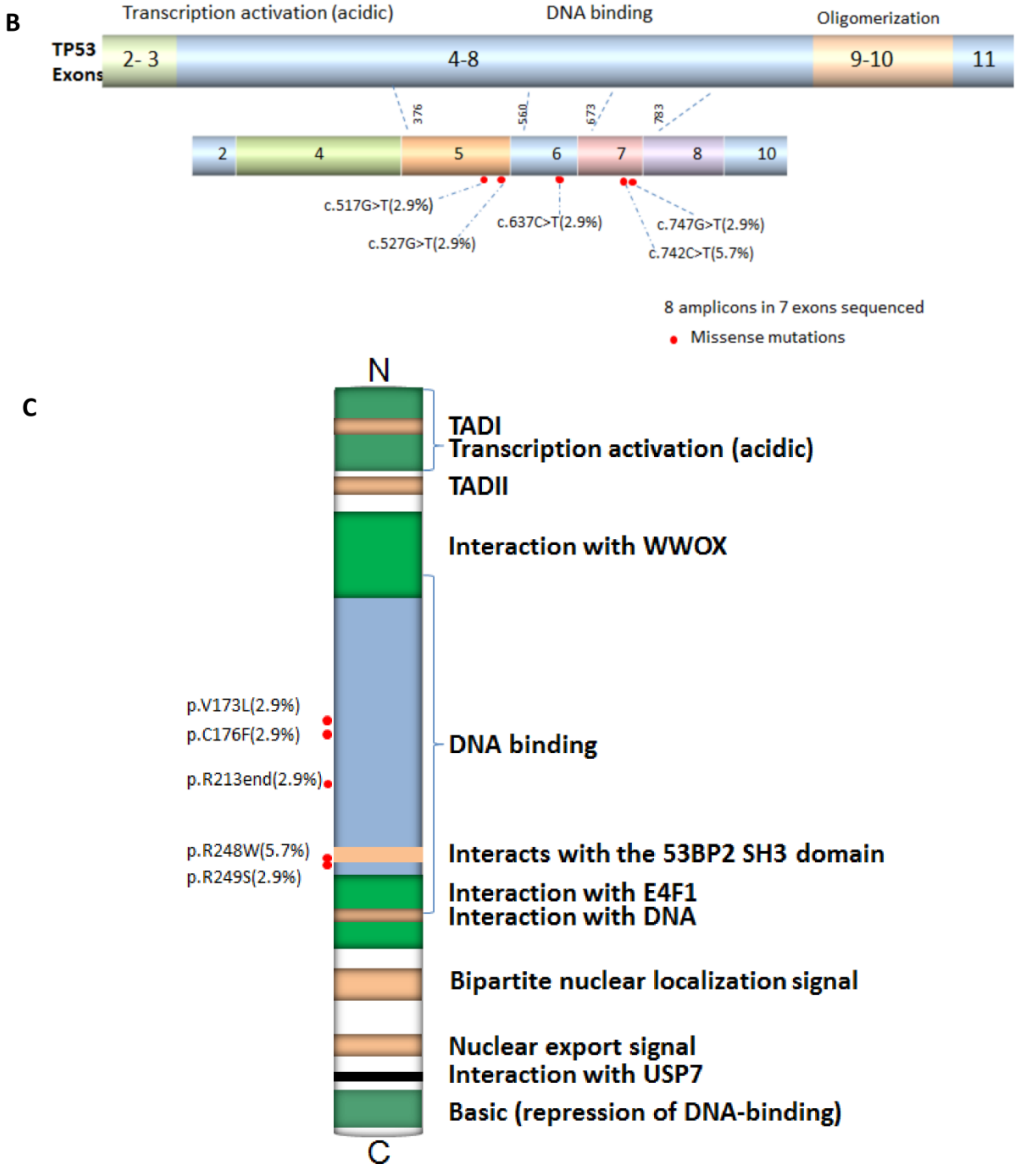
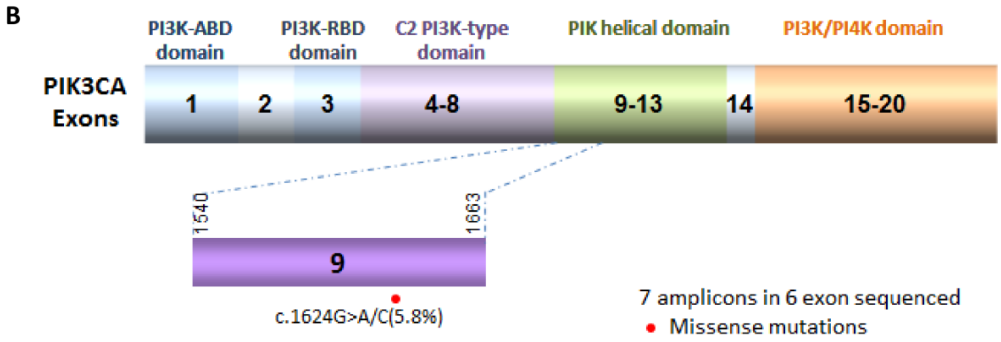



**Figure 4.** Missense mutation distribution in the exons and function domains of TP53 in gastric cardia cancer samples.
A. Frequencies of detected mutations in different exons.
B. Mutation distribution in exons.
C. Mutation distribution in functional domains.

**A**

| Gene | Exon | Missense point mutations/deletion/insertion counts (Percentage in all mutation counts of this gene) | Missense point mutations (including coding silent) counts (Percentage in all mutation counts of this gene) | Deletion counts (Percentage in all mutation counts of this gene) | Insertion counts (Percentage in all mutation counts of this gene) |
|---|---|---|---|---|---|
| PIK3CA | 1 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 4 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 8 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 9 | 2 (100.0%) | 2 (100.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 13 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| PIK3CA | 20 | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |



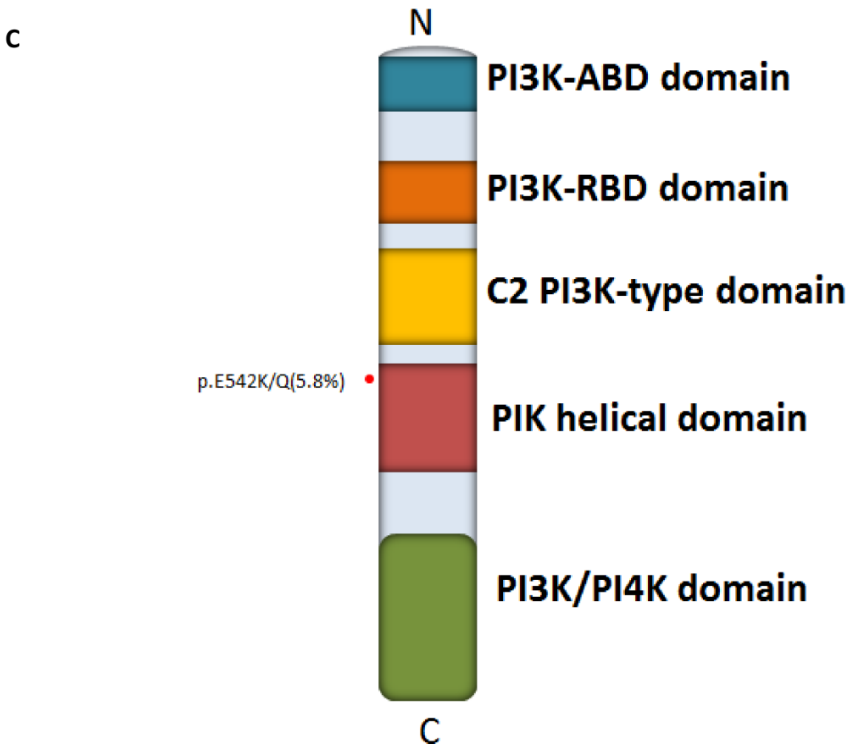




**Figure 5.** Missense mutation distribution in the exons and function domains of PIK3CA in gastric cardia cancer samples
**A.** Frequencies of detected mutations in different exons.
**B.** Mutation distribution in exons.
**C.** Mutation distribution in functional domains.

missense mutations detected in our study were located in exon 9, but the specific point mutations differed between cancer type: E545K and Q546K in EC versus E542K and E542Q in GCA (Figure 5). Mutations in these residues cause the protein's surface charge potential to change, which may alter interactions with other regulatory proteins like RAS and p85 [39]. The increase in lipid kinase activity and activation of downstream Akt signaling caused by these mutations interferes with other signaling pathways that regulate cell survival, proliferation, apoptosis, and others, thus contributing to oncogenicity [40-42].

## APC mutations in gastric cardia cancers

We detected APC mutations in two highly-differentiated GCA samples both in exon 15 of with the amino acid substitution resulting in a stop codon (R876* and R1450*) (Table 5 and Figure 6). APC is a tumor suppressor gene that plays a significant role in the negative regulation of epithelial cell growth. The APC gene product directly modulates

**A**

| Gene | Exon | Missense point mutations/deletion/insertion counts (Percentage in all mutation counts of this gene) | Missense point mutations (including coding silent) counts (Percentage in all mutation counts of this gene) | Deletion counts (Percentage in all mutation counts of this gene) | Insertion counts (Percentage in all mutation counts of this gene) |
|---|---|---|---|---|---|
| APC | 15 | 2 (100.0%) | 2 (100.0%) | 0 (0.0%) | 0 (0.0%) |

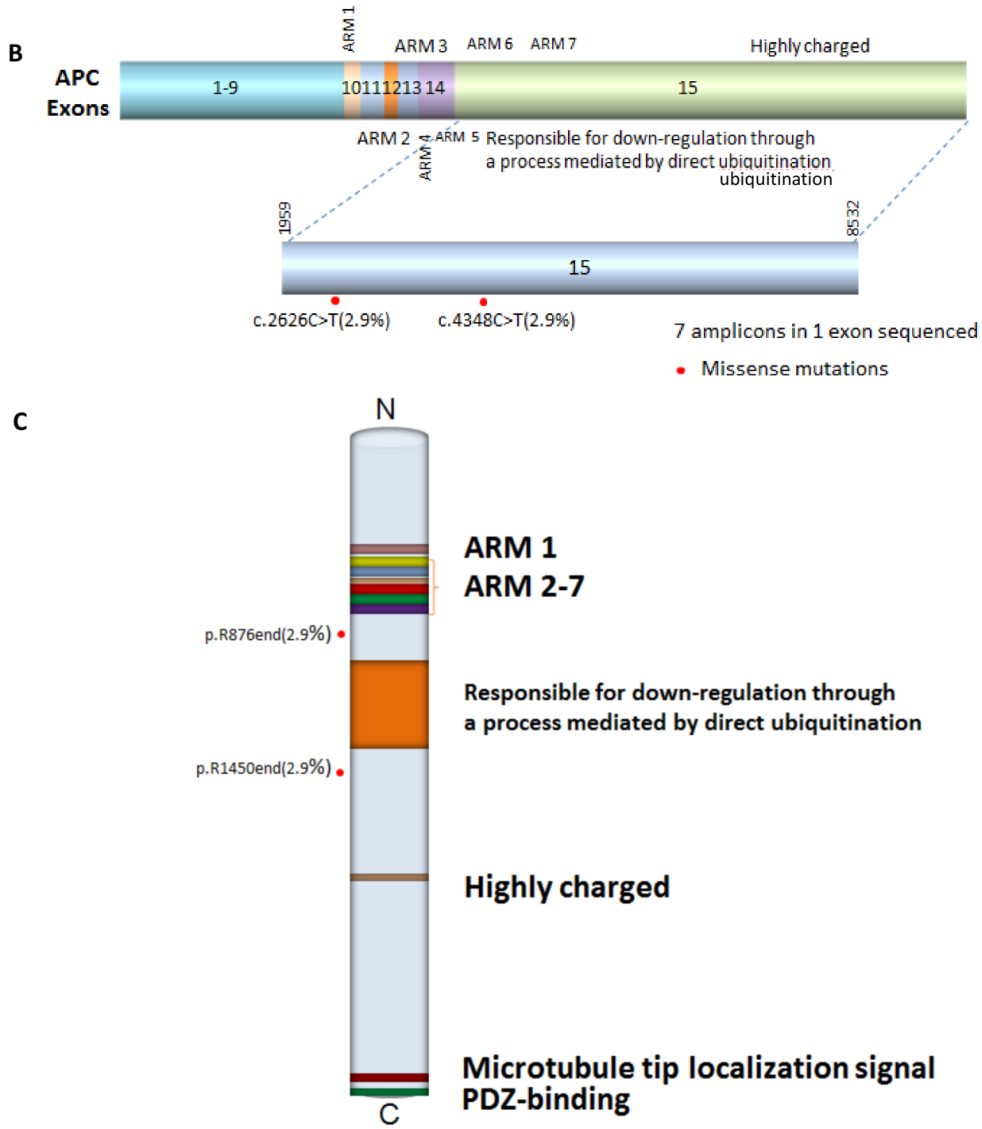



**Figure 6.** Missense mutation distribution in the exons and function domains of APC in gastric cardia cancer samples
**A.** Frequencies of detected mutations in different exons.
**B.** Mutation distribution in exons.
**C.** Mutation distribution in functional domains.

the dual roles of β-catenin in cell adhesion and gene transcription. Additionally, APC mediates β-catenin degradation, and loss of a functional APC protein deregulates β-catenin turnover, resulting in an accumulation of transcriptionally active β-catenin–Tcf-LEF complexes and uncontrolled transcriptional activation of Tcf-responsive genes, which may contribute to cancer progression [43].

APC mutations are most common in colorectal cancers, where up to 60% of tumors harbor an APC mutation [44]; however, APC mutations in gastric cancers, especially gastric cardia, are much less common and found in ~4% of these tumors [9,45]. When found in gastric cancers, mutated APC has been found to significantly correlate to depth of tumor invasion and is associated with advanced, well-differentiated tumors [46]. Regardless of cancer type, the most common APC mutations occur in exon 15, which accounts for 77% of the coding sequence [47]. In accordance, both mutations we identified were located in this exon. Mutation R876* results in increased accumulation of nuclear β-catenin. Although this mutation is relatively rare and accounts for roughly 2.5% of all APC mutations [48], it has been associated with the formation of aggressive and invasive colorectal carcinomas [49]. Mutation 1450* is within the Mutation Cluster Region (MCR) (codons 1286-1513), which represents about 8% of APC's entire coding sequence [50]. This mutation been identified in colorectal adenocarcinomas detected at very early stages, supporting evidence that APC mutations occur early in tumorigenesis [51,52].

## Less frequent mutations in esophageal and gastric cardia cancers

**CTNNB1:** One EAC sample in our study harbored a missense mutation in CTNNB1, and was the only mutation detected in all of the 6 EAC samples sequenced (Table 3). The CTNNB1 gene encodes for β-catenin, a ubiquitous intracellular protein that plays a vital role in the APC/β-catenin/Tcf signaling pathway. The APC protein can form a complex with glycogen synthase kinase 3β (GSK-3β) and controls degradation of β-catenin through NH2 terminus phosphorylation of GSK-3β. Mutations in the APC or CTNNB1 genes, particularly in GSK-3β's phosphorylation region, can cause β-catenin to accumulate in the nucleus and also to interact with TCF/Lef transcription factor to activate target genes, an interaction that hinders cell growth regulation and contributes to tumorigenesis [53]. Previous studies found that most mutations in CTNNB1 occur in exon 3, as was the mutation identified in our study. Specifically, we identified that a cysteine was substituted for a serine at codon 37 (S37C) (Table 4), a mutation that affects the phosphorylation sites of GSK-3β, making it resistant to degradation [54].

CTNNB1 mutations are fairly common in many different cancer types, including gastric cancers (8-27%) [54], but is relatively rare in esophageal cancers occurring in ~2% of patients [45,53]. Several clinical studies have shown that reduced β-catenin expression in EC patients did not correlate with disease stage, but rather correlated with poor tumor differentiation and shorter overall survival, regardless of histological type [55,56]. As aberrant expression β-catenin is disease stage-independent, expression levels of the protein could be used as a predicative factor of poor prognosis for EC patients or to identify patients who run a higher risk of disease recurrence [57].

**NRAS:** One GCA sample contained a missense mutation in exon 3 of NRAS (Q61H) (Table 5). An estimated 20% of all human tumors contain activating RAS mutations, where KRAS mutations account for roughly 85% of these and 15% are NRAS mutations [58]. NRAS mutations are most commonly found in lymphoid malignancies and up to 30% of melanomas, and more recently have been identified in a small subset of colorectal cancers (4%) [59]. A recent study on RAS mutations in colorectal cancers found NRAS mutations to have a strong association with WT KRAS, and NRAS mutations were more often found in metastatic cancers [60]. In agreement, the sample in our study with the NRAS mutation was found to have WT KRAS and metastases in 1-2 regional lymph nodes.

The family of RAS proteins plays crucial roles in controlling multiple signaling pathways to regulate cellular proliferation. Activating RAS mutations significantly contribute to a malignant phenotype by dysregulation of cell growth, invasiveness, and blood vessel formation [58]. Nearly all RAS mutations in cancerous cells are a result of an amino acid substitution in codons 12, 13, and 61 [58]. These common mutations interfere with the intrinsic GTPase activity of RAS and confer resistance to GAPs, which stabilize the transition state of the RAS–GTP hydrolysis reaction, resulting in the accumulation of active, GTP-bound RAS proteins. The glutamine at codon 61 is required for GTP hydrolysis, and an amino acid substitution other than glutamic acid at this position blocks this reaction [61]. The mutation found in our study was in codon 61 with a histidinesubstituted for the glutamine.

**SMAD4:** One GCA sample contained a missense mutation in exon 8 of SMAD4 (Table 5). SMAD4, a member of the SMAD family of transcription factors, regulates transduction of TGF-β and inhibits cell proliferation. An inactivating mutation in SMAD4 leads to interference with TGF-β signaling and a loss of cell growth regulation which contributes to carcinogenesis [62]. SMAD4 mutations are most common in pancreatic cancers (roughly 50%) and are also found in a smaller percentage of breast, ovary, and colon cancers [63], but are relatively rare in gastric cancers [64]. Dulak et al. found SMAD4 mutations in 8% of EC and GCAs, but this study involved Caucasian patients and did not distinguish between the cancer types [10]. Studies that have focused on SMAD4 mutations in GCAs found that SMAD4 expression was related to tumor depth and cancer progression, and loss of SMAD4 expression correlated with male sex, poorer prognoses, and decreased survival [65,66].

SMAD4 function largely depends on its ability to form a heterocomplex with R-SMAD, and D351 and R361 in the loop–helix region of SMAD4's MH2 domain are key residues in this process. A mutation to either of these residues to histidine prevents normal interaction between SMAD4 and phosphorylated SMAD1 and SMAD2. Mutations at these two codons are associated with a loss of the TGF-β response and are found in cancer cells at higher frequency than other SMAD4 missense mutations [67,68]. Accordingly, the SMAD4 mutation detected in our study was a histidine substitution at codon 361, and in agreement with previous studies, was found in a sample from a male patient.

## Combination mutations in esophageal and gastric cardia cancers

Two of the GCA samples in our study contained two mutations: one highly differentiated sample with lymph node metastasis harbored mutations in both APC and NRAS (Table 5), two genes with a strong tendency toward co-occurrence in liver cancers [69]. The second GCA was also highly differentiated and contained an APC and PIK3CA mutation, a combination that is found in ~20% of colorectal cancers [70]. One ESCC patient had two mutations of PIK3CA and TP53 (Table 4), a combination which also found in some colorectal cancers [70]. Accumulating evidence indicates that effective treatment for the majority of most malignancies requires combination therapies instead of administration of a single agent [71]. Identifying mutation

combinations in individual cancers, such as in our study, will better allow for administering a combination of targeted agents against the detected mutations, which may have greater benefits clinical success for cancer patients.

## Conclusions and future directions

Of the 35 gastric cardia and 45 esophageal cancer samples sequenced in our study, some mutations were shared between cancer types: TP53 had three identical mutations in both ECs and GCAs (C176F, R213*, and R248W), and PIK3CA mutations were found in both cancer types, albeit the point mutations were not the same. The remaining mutations we identified were unique to the cancer type. While this supports previous research suggesting that esophageal and gastric cardia cancers have distinct molecular profiles, and thus potentially different prognoses or patient outcomes, our limited sample size and low TP53 mutation rate necessitates follow-up studies with larger sample sets to further investigate the genetic profiles of both types of cancer and to identify additional molecular targeted therapy options for patients. Furthermore, because cancers often exhibit a high degree of intratumoral heterogeneity [72], additional studies utilizing multiregion sequencing may help to more intricately define the mutation profile for these cancers and for each patient. Fortunately the affordable cost of Ion Torrent sequencing may facilitate such a follow-up study.

Accumulating evidence suggests that not only does the gene that contains the mutation have prognostic power in various types of cancer, but that also the specific amino acid substitution may have an important impact on disease treatment and progression. For example, several clinical studies have found that esophageal, breast, and colon cancer patients with TP53 mutations within the zinc-binding domains (L2 and L3) were more resistant to chemotherapy or radiation, and had significantly poorer prognoses and decreased survival times compared to patients without TP53-mutatated cancers or with TP53 mutations outside L2 or L3 [26,73,74]; hence, importance in patient treatment and prognosis lies not only in what gene is mutated, but also where in the gene and what point mutation the gene has incurred.

As further knowledge of molecular gene mutations is gained, therapeutic drugs can be designed to target the particular mutation. For example, the critical GTP-binding site at codon 61 of RAS found mutated in our study is thought to be a suitable target for drug therapies. Here, small molecule inhibitors that bind to the GTP site on RAS and inhibit its interaction with GTP would maintain RAS in its inactive conformation, an approach which has been successful on the ATP-binding site of various protein kinases. An alternative strategy may be the development of target drugs that specifically interact with residue 61 to restore GTPase activity in mutant RAS. Such drugs could convert oncogenic RAS proteins to normal molecules without affecting other cellular functions [58]. While still under investigation, such targeted drugs could eventually be appropriate for clinical development and personalized cancer treatments for patients with RAS mutations. Additional research on other point mutations in other genes is warranted to develop mutation-specific therapies, and identifying these mutations is a critical step.

The next obstacle in improving cancer patient treatments and outcomes is individualized DNA cancer sequencing, not only to guide drug therapies for those with disease or predict disease progression, but also for genetic screening to determine susceptibility for cancer development. Cancer patients tend to have better responses to targeted therapies versus generalized treatments, and as such, individualized tumor sequencing is a critical step to direct effective, patient-specific treatments. The standard of care for most patients with EC or GCA is one or more non-specific chemotherapeutic agents like cisplatin or fluorouracil, but as evidenced in the high mortality rate of these diseases, these drugs are not highly effective and can have significant side-effects. By knowing which gene mutations a patient has, specific drugs targeting these mutations can be administered for potentially more effective results with fewer or more tolerable side-effects. Certain drugs have already been developed to target common mutations in VEGF, EGFR, and Her2/Neu [75]; but because these are only effective in patients with these specific mutations, additional effort is needed to expand the treatment options for esophageal and gastric cardia cancer patients with different gene mutations.

Improved personalized medicine hinges on expanding the current knowledge base of gene mutations in various cancers by identifying new molecular drug targets or fine tuning existing treatments based on specific point mutations to offer greater therapeutic benefits and improved outcomes for patients with cancer. Technological advancements in next generation sequencing (NGS) has facilitated this in recent years, although assay cost and time has prevented the needed transition to clinical personalized sequencing. By circumventing some of the cost and complexity associated with four-color optical detection used in other NGS platforms like 454 Pyrosequencing, HelicosHeliscope, IlluminaHiSeq, and SOLiD Sequencing [76-78], the semi-conductor-based Ion Torrent sequencing technology is allowing for highly cost- and time-effective high-throughput screening with reliable results [79]. Our current study supports the use of the Ion Torrent sequencing platform for clinical individual cancer genome sequencing, making personalized, targeted drug therapies a possibility for each patient in the near future.

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### References

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, et al. (2013) Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. International Agency for Research on Cancer.
2. Wiedmann MW, Mössner J (2013) New and emerging combination therapies for esophageal cancer. Cancer Manag Res 5: 133-146.
3. Peters CJ, Rees JR, Hardwick RH, Hardwick JS, Vowler SL, et al. (2010) A 4-gene signature predicts survival of patients with resected adenocarcinoma of the esophagus, junction, and gastric cardia. Gastroenterology 139: 1995-2004.
4. Allum WH, Stenning SP, Bancewicz J, Clark PI, Langley RE (2009) Long-term results of a randomized trial of surgery with or without preoperative chemotherapy in esophageal cancer. J Clin Oncol 27: 5062-5067.
5. Lin Y, Totsuka Y, He Y, Kikuchi S, Qiao Y, et al. (2013) Epidemiology of esophageal cancer in Japan and China. J Epidemiol 23: 233-242.
6. McManus DT, Olaru A, Meltzer SJ (2004) Biomarkers of esophageal adenocarcinoma and Barrett's esophagus. Cancer Res 64: 1561-1569.
7. Ekström AM, Signorello LB, Hansson L-E, Bergström R, Lindgren A, et al. (1999) Evaluating Gastric Cancer Misclassification: a Potential Explanation for the Rise in Cardia Cancer Incidence. Journal of the National Cancer Institute 91: 786-790.
8. Wang LD, Zheng S, Zheng ZY, Casson AG (2003) Primary adenocarcinomas of lower esophagus, esophagogastric junction and gastric cardia: in special reference to China. World J Gastroenterol 9: 1156-1164.

9. Tajima Y, Yamazaki K, Makino R, Nishino N, Masuda Y, et al. (2007) Differences in the histological findings, phenotypic marker expressions and genetic alterations between adenocarcinoma of the gastric cardia and distal stomach. Br J Cancer 96: 631-638.

10. Dulak AM, Stojanov P, Peng S, Lawrence MS, Fox C, et al. (2013) Exome and whole-genome sequencing of esophageal adenocarcinoma identifies recurrent driver events and mutational complexity. Nat Genet 45: 478-486.

11. Cancer Genome Atlas Research Network (2014) Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513: 202-209.

12. Gao YB, Chen ZL, Li JG, Hu XD, Shi XJ, et al. (2014) Genetic landscape of esophageal squamous cell carcinoma. Nat Genet 46: 1097-1102.

13. Glenn TC (2011) Field guide to next-generation DNA sequencers. Mol Ecol Resour 11: 759-769.

14. Hadd AG, Houghton J, Choudhary A, Sah S, Chen L, et al. (2013) Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. J Mol Diagn 15: 234-247.

15. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, et al. (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 30: 434-439.

16. Bai X, Zhang E, Ye H, Nandakumar V, Wang Z, et al. (2014) PIK3CA and TP53 gene mutations in human breast cancer tumors frequently detected by ion torrent DNA sequencing. PLoS One 9: e99306.

17. Cai X, Sheng J, Tang C, Nandakumar V, Ye H, et al. (2014) Frequent mutations in EGFR, KRAS and TP53 genes in human lung cancer tumors detected by ion torrent DNA sequencing. PLoS One 9: e95228.

18. 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, et al. (2010) A map of human genome variation from population-scale sequencing. Nature 467: 1061-1073.

19. Server EV (2013) NHLBI Go Exome Sequencing Project (ESP). Seattle, WA.

20. Bamford S, Dawson E, Forbes S, Clements J, Pettett R, et al. (2004) The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br J Cancer 91: 355-358.

21. Olivier M, Hollstein M, Hainaut P (2010) TP53 mutations in human cancers: origins, consequences, and clinical use. Cold Spring Harb Perspect Biol 2: a001008.

22. Lagarde SM, ten Kate FJ, Reitsma JB, Busch OR, van Lanschot JJ (2006) Prognostic factors in adenocarcinoma of the esophagus or gastroesophageal junction. J Clin Oncol 24: 4347-4355.

23. Rivlin N, Brosh R, Oren M, Rotter V (2011) Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer 2: 466-474.

24. Madani K, Zhao R, Lim HJ, Casson AG (2010) Prognostic value of p53 mutations in oesophageal adenocarcinoma: final results of a 15-year prospective study. Eur J Cardiothorac Surg 37: 1427-1432.

25. Ribeiro U Jr, Finkelstein SD, Safatle-Ribeiro AV, Landreneau RJ, Clarke MR, et al. (1998) p53 sequence analysis predicts treatment response and outcome of patients with esophageal carcinoma. Cancer 83: 7-18.

26. Kihara C, Seki T, Furukawa Y, Yamana H, Kimura Y, et al. (2000) Mutations in zinc-binding domains of p53 as a prognostic marker of esophageal-cancer patients. Jpn J Cancer Res 91: 190-198.

27. Farnebo M, Bykov VJN, Wiman KG (2010) The p53 tumor suppressor: A master regulator of diverse cellular processes and therapeutic target in cancer. Biochem Biophys Res Commun 396: 85-89.

28. Green DR, Kroemer G (2009) Cytoplasmic functions of the tumour suppressor p53. Nature 458: 1127-1130.

29. Soussi T, Wiman KG (2007) Shaping genetic alterations in human cancer: the p53 mutation paradigm. Cancer Cell 12: 303-312.

30. Vazquez A, Bond EE, Levine AJ, Bond GL (2008) The genetics of the p53 pathway, apoptosis and cancer therapy. Nat Rev Drug Discov 7: 979-987.

31. Vogt PK, Kang S, Elsliger MA, Gymnopoulos M (2007) Cancer-specific mutations in phosphatidylinositol 3-kinase. Trends Biochem Sci 32: 342-349.

32. Nagarajan N, Bertrand D, Hillmer AM, Zang ZJ, Yao F, et al. (2012) Whole-genome reconstruction and mutational signatures in gastric cancer. Genome Biol 13: R115.

33. Song Y, Li L, Ou Y, Gao Z, Li E, et al. (2014) Identification of genomic alterations in oesophageal squamous cell cancer. Nature 509: 91-95.

34. Ogino S, Nosho K, Kirkner GJ, Shima K, Irahara N, et al. (2009) PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. J Clin Oncol 27: 1477-1484.

35. Hou J, Jiang D, Zhang J, Gavine PR, Xu S, et al. (2014) Frequency, characterization, and prognostic analysis of PIK3CA gene mutations in Chinese esophageal squamous cell carcinoma. Hum Pathol 45: 352-358.

36. Shigaki H, Baba Y, Watanabe M, Murata A, Ishimoto T, et al. (2013) PIK3CA Mutation Is Associated with a Favorable Prognosis among Patients with Curatively Resected Esophageal Squamous Cell Carcinoma. Clin Cancer Res 19: 2451-2459.

37. Zhao L, Vogt PK (2008) Class I PI3K in oncogenic cellular transformation. Oncogene 27: 5486-5496.

38. Phillips WA, Russell SE, Ciavarella ML, Choong DY, Montgomery KG, et al. (2006) Mutation analysis of PIK3CA and PIK3CB in esophageal cancer and Barrett's esophagus. Int J Cancer 118: 2644-2646.

39. Kang S1, Bader AG, Zhao L, Vogt PK (2005) Mutated PI 3-kinases: cancer targets on a silver platter. Cell Cycle 4: 578-581.

40. Bader AG, Kang S, Vogt PK (2006) Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc Natl Acad Sci U S A 103: 1475-1479.

41. Kang S, Bader AG, Zhao L, Vogt PK (2005) Mutated PI 3-kinases: cancer targets on a silver platter. Cell Cycle 4: 578-581.

42. Zhao L, Vogt PK (2008) Class I PI3K in oncogenic cellular transformation. Oncogene 27: 5486-5496.

43. Mei JM, Hord NG, Winterstein DF, Donald SP, Phang JM (1999) Differential expression of prostaglandin endoperoxide H synthase-2 and formation of activated ß-catenin–LEF-1 transcription complex in mouse colonic epithelial cells contrasting in Apc. Carcinogenesis 20: 737-740.

44. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, et al. (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359: 235-237.

45. Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, et al. (2014) COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res .

46. Wang JY, Hsieh JS, Chen CC, Tzou WS, Cheng TL, et al. (2004) Alterations of APC, c-met, and p53 genes in tumor tissue and serum of patients with gastric cancers. J Surg Res 120: 242-248.

47. Fearnhead NS, Britton MP, Bodmer WF (2001) The ABC of APC. Hum Mol Genet 10: 721-733.

48. Drew DA, Devers TJ, O'Brien MJ, Horelik NA, Levine J, et al. (2014) HD Chromoendoscopy Coupled with DNA Mass Spectrometry Profiling Identifies Somatic Mutations in Microdissected Human Proximal Aberrant Crypt Foci. Mol Cancer Res 12: 823-829.

49. De Rosa M, Scarano MI, Panariello L, Morelli G, Riegler G, et al. (2003) The mutation spectrum of the APC gene in FAP patients from southern Italy: detection of known and four novel mutations. Hum Mutat 21: 655-656.

50. Nagase H, Nakamura Y (1993) Mutations of the APC (adenomatous polyposis coli) gene. Hum Mutat 2: 425-434.

51. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, et al. (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359: 235-237.

52. Nishimura K, Yokozaki H, Haruma K, Kajiyama G, Tahara E (1995) Alterations of the apc gene in carcinoma cell-lines and precancerous lesions of the stomach. Int J Oncol 7: 587-592.

53. Choi YW, Heath EI, Heitmiller R, Forastiere AA, Wu TT (2000) Mutations in beta-catenin and APC genes are uncommon in esophageal and esophagogastric junction adenocarcinomas. Mod Pathol 13: 1055-1059.

54. Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 1653: 1-24.

55. Krishnadath KK, Tilanus HW, van Blankenstein M, Hop WC, Kremers ED, et al. (1997) Reduced expression of the cadherin-catenin complex in oesophageal adenocarcinoma correlates with poor prognosis. J Pathol 182: 331-338.

56. Zeng R, Duan L, Kong YK, Wu XL, Wang Y, et al. (2014) Prognostic significance of beta-catenin expression in patients with esophageal carcinoma: a meta-analysis. Asian Pac J Cancer Prev 15: 6103-6108.

57. Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, et al. (1996) Beta-catenin expression in human cancers. Am J Pathol 148: 39-46.

58. Downward J (2003) Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3: 11-22.

59. Omholt K, Platz A, Kanter L, Ringborg U, Hansson J (2003) NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. Clin Cancer Res 9: 6483-6488.

60. Shen Y, Wang J, Han X, Yang H, Wang S, et al. (2013) Effectors of Epidermal Growth Factor Receptor Pathway: The Genetic Profiling of KRAS, BRAF, PIK3CA, NRAS Mutations in Colorectal Cancer Characteristics and Personalized Medicine. PLoS ONE 8: e81628.

61. Schubbert S, Shannon K, Bollag G (2007) Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 7: 295-308.

62. Steeg PS1, Theodorescu D (2008) Metastasis: a therapeutic target for cancer. Nat Clin Pract Oncol 5: 206-219.

63. Ali S, Cohen C, Little JV, Sequeira JH, Mosunjac MB, et al. (2007) The utility of SMAD4 as a diagnostic immunohistochemical marker for pancreatic adenocarcinoma, and its expression in other solid tumors. Diagn Cytopathol 35: 644-648.

64. Wang LH, Kim SH, Lee JH, Choi YL, Kim YC, et al. (2007) Inactivation of SMAD4 tumor suppressor gene during gastric carcinoma progression. Clin Cancer Res 13: 102-110.

65. Xiangming C, Natsugoe S, Takao S, Hokita S, Ishigami S, et al. (2001) Preserved Smad4 Expression in the Transforming Growth Factor ß Signaling Pathway Is a Favorable Prognostic Factor in Patients with Advanced Gastric Cancer. Clin Cancer Res 7: 277-282.

66. Zizi-Sermpetzoglou A, Myoteri D, Arkoumani E, Voultsos M, Marinis A (2014) A study of Smad4 and Smad7 expression in surgically resected samples of gastric adenocarcinoma and their correlation with clinicopathological parameters and patient survival. J BUON 19: 221-227.

67. Lim SK, Hoffmann FM (2006) Smad4 cooperates with lymphoid enhancer-binding factor 1/T cell-specific factor to increase c-myc expression in the absence of TGF-beta signaling. Proc Natl Acad Sci U S A 103: 18580-18585.

68. Shi Y, Hata A, Lo RS, Massagué J, Pavletich NP (1997) A structural basis for mutational inactivation of the tumour suppressor Smad4. Nature 388: 87-93.

69. Jang S, Chun S-M, Hong S-M, Sung CO, Park H, et al. (2014) High throughput molecular profiling reveals differential mutation patterns in intrahepatic cholangiocarcinomas arising in chronic advanced liver diseases. Mod Pathol 27: 731-739.

70. Yeang CH, McCormick F, Levine A (2008) Combinatorial patterns of somatic gene mutations in cancer. FASEB J 22: 2605-2622.

71. Kahn M (2014) Can we safely target the WNT pathway? Nat Rev Drug Discov 13: 513-532.

72. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, et al. (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 366: 883-892.

73. Børresen A-L, Andersen TI, Eyfjörd JE, Cornelis RS, Thorlacius S, et al. (1995) TP53 mutations and breast cancer prognosis: Particularly poor survival rates for cases with mutations in the zinc-binding domains. Genes Chromosomes Cancer 14: 71-75.

74. Børresen-Dale AL, Lothe RA, Meling GI, Hainaut P, Rognum TO, et al. (1998) TP53 and long-term prognosis in colorectal cancer: mutations in the L3 zinc-binding domain predict poor survival. Clin Cancer Res 4: 203-210.

75. Tew WP, Kelsen DP, Ilson DH (2005) Targeted therapies for esophageal cancer. Oncologist 10: 590-601.

76. Schuster SC (2008) Next-generation sequencing transforms today's biology. Nat Methods 5: 16-18.

77. Voelkerding KV, Dames SA, Durtschi JD (2009) Next-generation sequencing: from basic research to diagnostics. Clin Chem 55: 641-658.

78. Meyerson M, Gabriel S, Getz G (2010) Advances in understanding cancer genomes through second-generation sequencing. Nat Rev Genet 11: 685-696.

79. Chen S, Li S, Xie W, Li X, Zhang C, et al. (2014) Performance comparison between rapid sequencing platforms for ultra-low coverage sequencing strategy. PLoS One 9: e92192.