

TP53, PIK3CA, FBXW7 and KRAS Mutations in Esophageal Cancer Identified by Targeted Sequencing

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Abstract. *Background/Aim: Esophageal cancer (EC) is a common malignancy with significant morbidity and mortality. As individual cancers exhibit unique mutation patterns, identifying and characterizing gene mutations in EC that may serve as biomarkers might help predict patient outcome and guide treatment. Traditionally, personalized cancer DNA sequencing was impractical and expensive. Recent technological advancements have made targeted DNA sequencing more cost- and time-effective with reliable results. This technology may be useful for clinicians to direct patient treatment. Materials and Methods: The Ion PGM and AmpliSeq Cancer Panel was used to identify mutations at 737 hotspot loci of 45 cancer-related genes in 64 EC samples from Chinese patients. Results: Frequent mutations were found in TP53 and less frequent mutations in PIK3CA, FBXW7 and KRAS. Conclusion: These results demonstrate that targeted sequencing can reliably identify mutations in individual tumors that make this technology a possibility for clinical use.*

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of death from cancer worldwide. In 2012, an estimated 456,000 new cases of esophageal cancer and roughly 400,000 esophageal cancer-related deaths were reported globally (1). Of those, China alone accounted for nearly 250,000 cases and close to 200,000 deaths (1). There are two major subtypes of esophageal cancer: adenocarcinoma (EAC) and squamous cell carcinoma (ESCC). EAC is much more common in Western countries where poor diet and obesity are major risk factors, whereas ESCC is more prevalent in Eastern countries and is strongly associated with alcohol consumption and smoking (2, 3). Similar to other cancer types, survival of patients with EC largely depends on disease stage and progression. More than 50% of EC patients have advanced, unresectable disease or present with distant metastases upon diagnosis, with an average of 8 to 10 months overall survival and a dismal 5%-17% 5-year survival rate (4, 5).

While treatment for EC varies with disease stage and subtype (ESCC or EAC), general treatment regimens for resectable tumors utilize broad-acting chemotherapeutic agents like cisplatin and fluorouracil; however, these drugs can have toxic effects, particularly in older patients who might be afflicted by comorbid conditions (6). Targeted therapies, based on DNA sequencing of cancer-associated gene mutations, have become the focus of current research. These targets include specific gene mutations in disrupted signaling pathways, such as those associated with vascular endothelial growth factor (*VEGF*), epidermal growth factor receptor (*EGFR*), receptor tyrosine-protein kinase *erbB-2* (*ERBB2*) and others (5). Many of the drugs targeting those mutations have shown promising results with minimal side effects in patients of other cancer types and are currently in clinical trials in EC patients (7-9).

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Key Words: Esophageal cancer, next-generation sequencing, Ion PGM, genetic mutations, targeted therapy, personalized medicine.

Individual cancer DNA sequencing is also useful to identify gene mutations that may interfere with drug effectiveness. For example, *KRAS* mutations, which are found in a small percentage of ECs, have been found to confer resistance to EGFR inhibitors, including tyrosine kinase inhibitors and monoclonal antibodies that slow or halt uncontrolled cell growth (10, 11). Hence, the identification of *KRAS* mutations may spare patients from unnecessary drug toxicity from an EGFR inhibitor rendered ineffective by the mutation. In addition to predicting drug resistance, personalized cancer sequencing may also reveal gene mutations with prognostic value. For example, *TP53* mutations, which are found in more than 40% of ECs, have been shown to correspond to poorer patient responses to the neoadjuvant chemotherapeutic agents fluorouracil and cisplatin, and patients with these mutations have reduced overall survival compared to those with wild-type *TP53* (12). Effective methods to identify such mutations may help clinicians guide treatment for EC patients.

As new targeted drug therapies are developed, and expanded clinical trials show promising results, the need to easily and reliably detect these mutations and identify new targets is heightened. Traditional Sanger sequencing, next-generation sequencing (NGS) platforms and whole exome sequencing have been used to identify mutations in ECs (13, 14), but these platforms are generally impractical for clinical use due to the high cost and lengthy run times. However, recent technological advancements have brought NGS to the benchtop, making affordable and time-efficient individual genome sequencing possible (15). Specifically, sequencing with the semiconductor-based Ion Personal Genome Machine (PGM) is able to circumvent many of the issues associated with other sequencing methods (16). In the current study, we used the Ion PGM and Ion AmpliSeq Cancer Panel to analyze 737 mutational hotspots from 45 known tumor-suppressor genes and oncogenes to identify genetic mutations in 64 esophageal cancer samples from Chinese patients.

Materials and Methods

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

Patient information. A total of 64 FFPE tumor samples were collected from the First Hospital of Qiqihar City, China, from esophageal cancer patients. Patients' characteristics can be found in Table I. For analyses, the patients were further categorized based on gender and tumors categorized by sub-types of esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC).

Table I. *Clinical features of 64 esophageal cancer patients.*

Characteristic	n (%)
Age (years)	
Median=64.1	
Range=45-85	
Gender	
Male	37 (57.8%)
Female	27 (42.2%)
Pathological diagnosis	
EAC	28 (43.8%)
ESCC	35 (54.7%)
ND	1 (1.6%)
Differentiation	
Low	3 (4.7%)
Middle	20 (31.3%)
High	0 (0.0%)
ND	41 (64.1%)

EAC: Esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma; ND: not determined.

DNA preparation, Ion Torrent PGM library preparation and sequencing. Sections of FFPE tissue samples (3-5 μm thick) were deparaffinized in xylene and DNA was then isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. The Ion AmpliSeq Library Kit 2.0 (Life Technologies, Carlsbad, CA, USA; Part #4475345 Revs. A) was used to construct an adapter-ligated library as per the manufacturer's protocol, while the Ion PGM 200 Sequencing Kit (Part # 4474004 Revs. B) was used for sequencing reactions according to the recommended protocol and as in our previous publications (17, 18). The AmpliSeq Cancer Panel used for this study is designed to target 737 mutational hotspot loci in the following 45 key 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*, *RBI*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL*. Detailed methods of Ion AmpliSeq Cancer Panel sequencing have been described previously (17).

Variant calling. The Ion Torrent platform-specific pipeline software Torrent Suite used data from the initial PGM runs to generate sequence reads, trim adapter sequences and filter and remove poor signal profile reads. Then, Torrent Suite Software v3.2, with a plugin "variant caller v3.2" program, was used to generate variant calling from the initial Ion AmpliSeq sequencing data. In order to eliminate base calling errors, several filtering steps were employed to generate final variant calling: the first filter was fixed at an average total coverage depth >100, each variant coverage >20, a variant frequency of each sample >5% and *p*-value <0.01; the second filter was visually inspecting the mutations using Integrative Genomics Viewer (IGV) software (<http://www.broadinstitute.org/igv>) or SAMtools software (<http://samtools.sourceforge.net>), along with eliminating possible DNA strand-specific errors; the third filter was set as variants within 737 hotspots, as per manufacturer's instructions; and the final filtering step eliminated variants in amplicon AMPL339432 (*PIK3CA*, exon13, chr3:178938822-

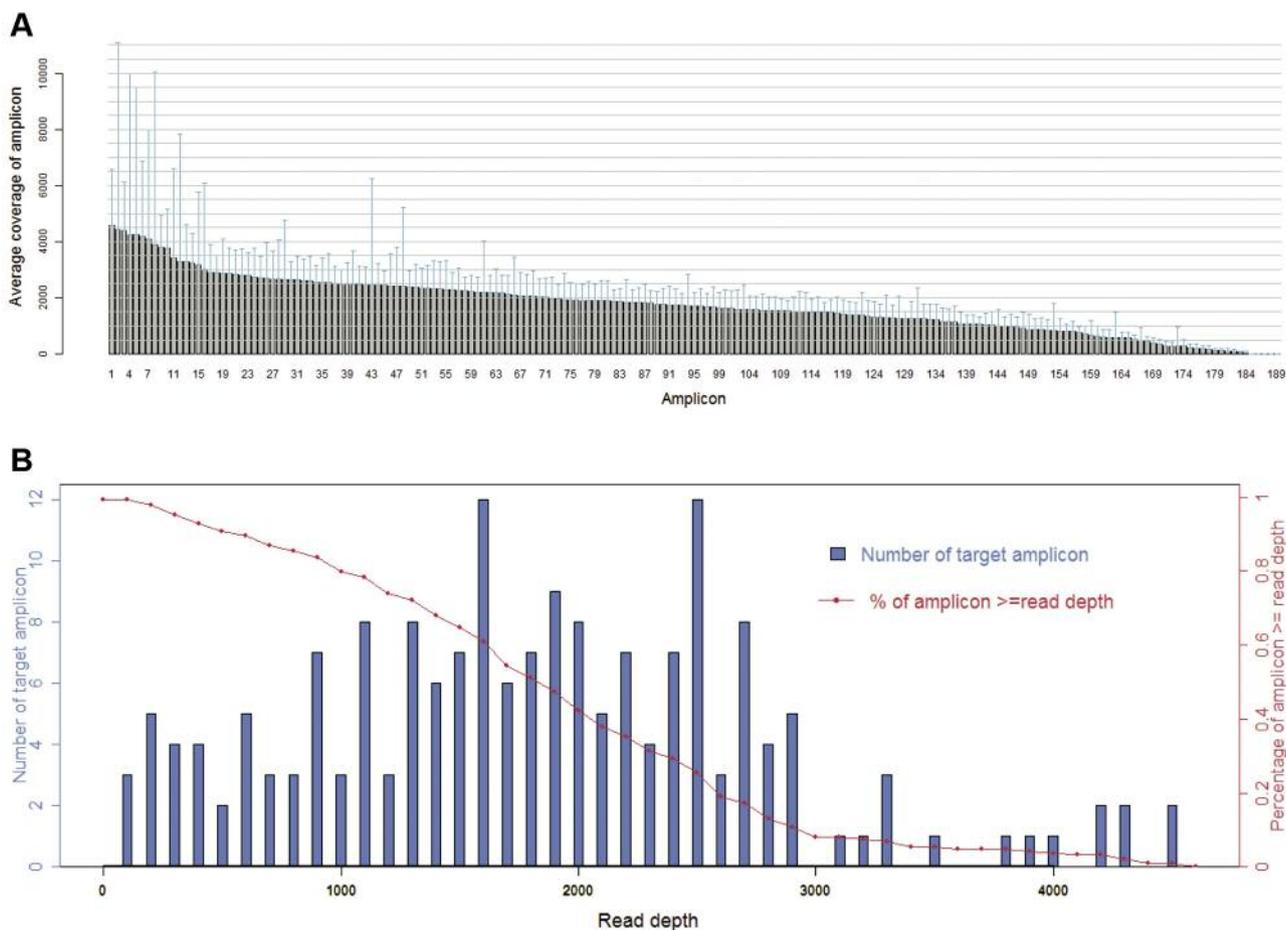


Figure 1. Sequence read distribution across 189 amplicons generated from 64 specimens, normalized to 300,000 reads per sample. A. Average number of reads observed for each amplicon. B. Number of targets with a given read depth, sorted in bins of 100 reads.

178938906), which is not uniquely matched in the human genome.

Bioinformatical and experimental validation. We used the COSMIC database (19) and MyCancerGenome database (<http://www.mycancergenome.org/>) to assess reappearing esophageal cancer mutations. Additionally, the accuracy of the Ion PGM was compared to the Sanger sequencing method when sufficient sample DNA was available.

Statistical analysis. The Fisher's exact test was used to calculate *p*-values in the detected mutated genes and total variants using GraphPad QuickCalcs Online Calculator for Scientists (<http://www.graphpad.com/quickcalcs>). All *p*-values are two-sided and statistical significance was defined as $p < 0.05$.

Results

Sequence coverage. For the 64 samples analyzed, the mean read length of each sequence was 78 bp and the average sequence was approximately 24.4 Mb per sample. With reads

normalized to 329,000 per specimen, there was an average of 1,788 reads per amplicon (range=44-4,574) (Figure 1A), 181/189 (95.8%) amplicons averaged at least 100 reads and 172/189 (91.0%) amplicons averaged at least 300 reads (Figure 1B).

TP53, PIK3CA, FBXW7 and KRAS gene mutations. Sequencing with the Ion PGM revealed that 18 of the 64 (28.1%) esophageal cancers in our sample set had one mutation in various genes and one of these samples contained a combination of three missense mutations. Of the 45 genes sequenced, we detected the highest mutation frequency in *TP53* (20.3%) and lower frequencies of mutations were found in *PIK3CA* (4.7%), *FBXW7* (3.1%) and *KRAS* (1.6%). A detailed list of individual point mutations can be found in Table II. There was no statistical difference between mutation rates in males vs. females (32.4% vs. 22.2%, respectively; $p=0.370$). A higher mutation

rate was found in ESCC samples compared to EAC samples (31.4% vs. 21.4%, respectively), but again this difference was not significant ($p=0.374$). Figure 2 summarizes the detected mutations based on pathological type of EC and patient sex and age ranges in years.

We identified *TP53* mutations in 20.3% (13/64) of samples at known hotspot locations in exon 5 (p.A159V, p.R175H, p.C176F, p.C275Y and p.H179R), exon 7 (p.S241F), exon 8 (p.C275Y, p.P278S and p.E298*) and exon 10 (p.R342*). While more *TP53* mutations were found in ESCCs vs. EACs (22.9% vs. 14.3%, respectively), this difference was not significant ($p=0.523$). Additionally, *TP53* mutations occurred at roughly equal proportions between males and females (18.5% vs. 21.6%, respectively).

PIK3CA mutations were identified in 3/64 samples (4.7%): one EAC and two ESCC, all from male patients. These were all missense mutations located in exon 9 at the known hotspot residues p.E542K and p.E545K.

Two samples (3.1%) contained a mutation in the *FBXW7* gene: one in exon 8 (p.R465C) and one in exon 9 (p.R505L). Interestingly, the EAC sample with the *FBXW7* p.A465C mutation also contained two *KRAS* mutations, one in exon 2 (p.G13D) and the other in exon 3 (p.A59T).

Bioinformatical and experimental validation. Our detected mutations were compared to those in esophageal cancer from the COSMIC database (19) and MyCancerGenome database; we found that two of our mutations (*FBXW7* p.R505L and *KRAS* p.A59T) have not been previously reported in esophageal cancers. Additionally, the accuracy of the Ion PGM was compared to the Sanger sequencing method for nine samples with sufficient DNA available. All nine samples gave consistent results between the Ion PGM and Sanger sequencing (Table III).

Discussion

In the current study we used the high-throughput Ion PGM and AmpliSeq Cancer Panel to sequence 64 esophageal cancers from Chinese patients by which we identified mutations in *TP53*, *PIK3CA*, *FBXW7* and *KRAS* in the sample population. While we did not have access to patient information regarding disease stage, treatments or patient outcome, many of these mutations have already been identified as biomarkers in EC patients (14, 20). Esophageal cancer DNA sequencing has previously been performed with Sanger sequencing and on a variety of NGS platforms (13, 14). We used Sanger sequencing to confirm our mutations when sufficient sample DNA was available, and all of these samples had consistent results between both methods. While the AmpliSeq Cancer Panel may only provide information on a pre-defined set of genes, it is useful for identifying known point mutations associated with disease. Additionally, the Ion PGM has been demonstrated to have greater sensitivity than

Table II. Specific point-mutations detected among 64 esophageal cancer samples.

Gene	Exon	Mutation	Gender	Age (years)	Pathological diagnosis
<i>FBXW7</i>	8	p.R465C#	F	55	EAC
<i>FBXW7</i>	9	p.R505L	M	58	ESCC
<i>KRAS</i>	2	p.G13D#	F	55	EAC
<i>KRAS</i>	3	p.A59T#	F	55	EAC
<i>PIK3CA</i>	9	p.E542K	M	69	EAC
<i>PIK3CA</i>	9	p.E545K	M	71	ESCC
<i>PIK3CA</i>	9	p.E545K	M	79	ESCC
<i>TP53</i>	5	p.A159V	M	72	EAC
<i>TP53</i>	5	p.R175H	F	64	ESCC
<i>TP53</i>	5	p.R175H	F	70	ESCC
<i>TP53</i>	5	p.C176F	M	75	EAC
<i>TP53</i>	5	p.C176Y	M	66	EAC
<i>TP53</i>	5	p.H179R	M	61	ESCC
<i>TP53</i>	7	p.S241F	M	49	ND
<i>TP53</i>	8	p.C275Y	M	ND	ESCC
<i>TP53</i>	8	p.C275Y	F	70	ESCC
<i>TP53</i>	8	p.P278S	F	62	EAC
<i>TP53</i>	8	p.E298*	M	67	ESCC
<i>TP53</i>	10	p.R342*	M	65	ESCC
<i>TP53</i>	10	p.R342*	F	68	ESCC

#Mutations found within the same sample. *Nonsense mutations resulting in STOP codon; EAC: esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma; ND: not determined.

the Sanger method: the Ion PGM can detect an allele variant frequency of 5%, whereas Sanger sequencing has been shown to miss mutations where the allele variant frequency is less than 10% (21, 22). Of further clinical relevance, the Ion PGM is considerably more cost- and time-effective than other NGS platforms (23).

Of the mutations identified in our study, *TP53* was most commonly mutated with 20.3% of samples containing a mutation in this gene. *TP53* plays many roles as a tumor suppressor gene and its protein product p53 works in cell cycle regulation, DNA repair, maintaining genomic stability and apoptosis (24-26). Additionally, *TP53* mutations significantly impair the regulatory tumor suppressor activity of p53. Although an estimated 80% of *TP53* mutations are missense resulting in a stable full-length protein (27), most mutant p53 proteins lose their DNA-binding activity, leading to faulty growth inhibition and apoptotic properties (28). *TP53* mutations have been widely studied, as these are some of the most common gene mutations present in greater than 50% of all patients with various types of cancer, and *TP53* mutations are specifically present in 36-80% of esophageal cancers (29-31). The *TP53* mutation rate found in our sample set was lower than previous reports, which may reflect our relatively small sample size and the tendency for mutation rates to vary greatly depending on the population and geographic location.

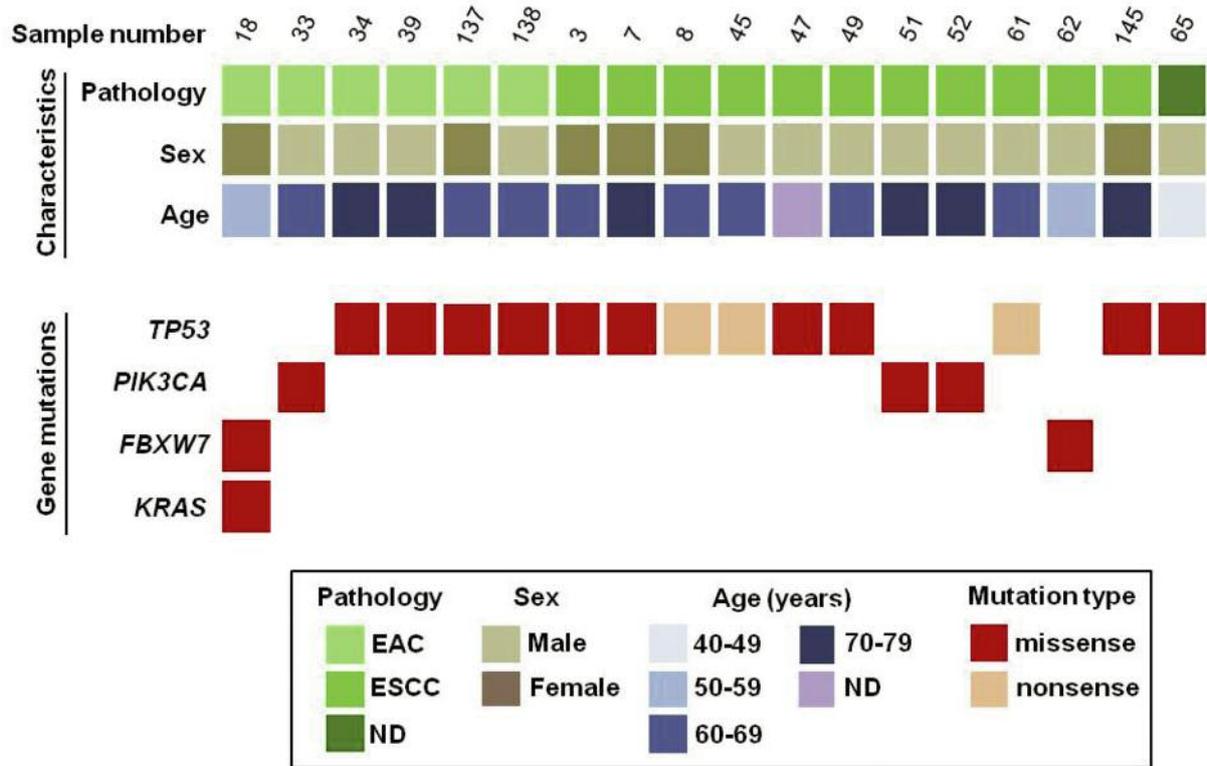


Figure 2. Summary of mutated genes detected in 64 esophageal cancer samples. Eighteen (18) samples harbor mutations in *TP53*, *PIK3CA*, *FBXW7* and *KRAS*. Samples are classified by three methods: (i) Pathological type (EAC or ESCC); (ii) sex (M or F); (iii) age (years). EAC: esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma; ND: not determined.

Table III. Ion PGM versus Sanger sequencing results for 9 esophageal cancer samples.

#	Sample ID	Pathological diagnosis	Gene	Mutation AA	Ion PGM Variant Frequency (%)	Sanger result Consistent?
1	7	ESCC	<i>TP53</i>	p.R175H	47.01	YES
2	39	EAC	<i>TP53</i>	p.A159V	30.53	YES
3	34	EAC	<i>TP53</i>	p.C176F	39.57	YES
4	3	ESCC	<i>TP53</i>	p.R175H	35.16	YES
5	49	ESCC	<i>TP53</i>	p.H179R	20.20	YES
6	33	EAC	<i>PIK3CA</i>	p.E542K	11.14	YES
7	45	ESCC	<i>TP53</i>	p.R342*	66.92	YES
8	47	ESCC	<i>TP53</i>	p.C275Y	12.92	YES
9	8	ESCC	<i>TP53</i>	p.R342*	14.4	YES

*Nonsense mutations resulting in STOP codon; EAC: esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma.

TP53 mutations have previously been used as prognostic markers for patient survival in various cancers. In one clinical study, EC patients without *TP53* mutations who underwent curative resection survived nearly twice as long as those who had *TP53* mutations (30). While this study did not find any correlation between treatment response or patient survival and specific *TP53* mutations, other research

suggests that different *TP53* point mutations may indeed influence the patient outcome or response to treatment. One such clinical study found that patients with *TP53* mutations in the zinc-binding domains (L2 and L3, amino acids 163-195 and 236-251, respectively (32)) were more resistant to chemotherapy or radiation and had significantly poorer prognoses compared to patients without *TP53* mutations or

with *TP53* mutations outside L2 or L3 (33). Additional studies indicate that L2-L3 mutations are correlated with decreased survival time in patients with breast and colorectal cancer (34, 35). Five of the *TP53* mutations identified in our study (p.R175H, p.C176F, p.C176Y, H179R, p.S241F) were found within the L2-L3 zinc binding domain. Knowledge of such mutations may help to better predict a patient's response to treatment or outcome, thus highlighting the importance of genetic sequencing for each patient.

The *PIK3CA* gene encodes for the catalytic subunit p110 α of class IA phosphatidylinositol 3-kinases (PI3Ks) (36) and mutations in this gene, while common in many cancers, including breast and colon, are only found in roughly 5% of ECs (19, 37). The two *PIK3CA* mutations we identified at p.E542K and p.E545K are known hotspot mutations in the *PIK3CA* helical domain that have previously been identified in various cancers (20, 37). These mutations alter interactions with other regulatory proteins like p85 and RAS and elevate lipid kinase activity that leads to an activation of downstream Akt signaling (38), which in turn regulates several signaling pathways controlling, among others, cell survival, proliferation and apoptosis (39, 40). Mutations in *PIK3CA* may offer valuable prognostic information as recent clinical studies indicate that these mutations are associated with a better prognosis in certain ESCC patients (20, 41). *PIK3CA* mutations have been found to interfere with anti-EGFR therapy (42) and, as some of these drugs are currently being tested in some EC patients (43, 44), identifying these mutations prior to drug administration may save a number of patients from unnecessary toxicities from treatments rendered ineffective by the mutations.

In addition to the common mutations found in this study, other less frequent mutations in ECs, such as *FBXW7* and *KRAS*, may serve as prognostic markers or have clinical implications in directing patient treatment. *FBXW7* is a *TP53*-dependent tumor suppressor gene that encodes for a subunit of a ubiquitin protein ligase that regulates levels of Cyclin E, Notch and other proteins. Mutations in *FBXW7* impair Cyclin E degradation and are associated with decreased genetic stability and impaired growth regulation (45, 46). A recent clinical study found EC patients with low *FBXW7* expression to have a significantly poorer overall survival than those with higher expression levels (47). RAS proteins are critical components of signaling pathways that help regulate cell proliferation, differentiation, cell cycle regulation and angiogenesis (48), while mutations in *KRAS* lead to constitutive activation and impaired regulatory functions (49). While *KRAS* mutations are uncommon events in ECs that are found in only 2-3% of samples (19, 50), they are nonetheless clinically relevant as *KRAS* mutations cause resistance to currently used anti-EGFR therapies in various cancers, such as colorectal and lung (51, 52).

In conclusion, individualized cancer sequencing may be the next critical step in improving patient treatments and outcomes by guiding therapy for those with disease. Our current study supports the applicability of the Ion PGM and AmpliSeq Cancer Panel to sequence esophageal cancer samples in a clinical setting to potentially provide patient-specific information that could help make personalized medicine a feasible option for cancer patients.

Acknowledgements

The Authors would like to thank Rong Shi at the Wu Jieping Foundation, Haibo Wang, Ying Li and other members of San Valley Biotechnology Inc. Beijing, for their assistance in sample and data collection. We would also like to thank the staff at the First Hospital of Qiqihar and Beijing Military Hospital for their generous support for DNA sequencing and data collection. This research was supported by an institutional grant and grants from the Wu Jieping Medical Foundation and the National Institutes of Health (R01 CA90427 & R01 AI084811 to SYC).

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Received February 11, 2016

Revised March 18, 2016

Accepted March 21, 2016