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Whole Exome Sequencing of Pediatric Gastric Adenocarcinoma Reveals an Atypical Presentation of Li-Fraumeni Syndrome

Vivian Y. Chang, MD, MS^{1,*}, Noah Federman, MD^{1,2}, Julian Martinez-Agosto, MD, PhD³, Sergei F. Tatishchev, MD⁴, and Stanley F. Nelson, MD^{1,4}

¹Department of Pediatrics, Division of Hematology/Oncology, David Geffen School of Medicine, University of California, Los Angeles, California

²University of California, Los Angeles, Jonsson Comprehensive Cancer Center

³Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, California

⁴Department of Pathology and Laboratory Medicines, and Psychiatry, David Geffen School of Medicine, University of California, Los Angeles, California

Abstract

Background—Gastric adenocarcinoma is a rare diagnosis in childhood. A 14-year old male patient presented with metastatic gastric adenocarcinoma, and a strong family history of colon cancer. Clinical sequencing of *CDH1* and *APC* were negative. Whole exome sequencing was therefore applied to capture the majority of protein-coding regions for the identification of single-nucleotide variants, small insertion/deletions, and copy number abnormalities in the patient's germline as well as primary tumor.

Materials and Methods—DNA was extracted from the patient's blood, primary tumor, and the unaffected mother's blood. DNA libraries were constructed and sequenced on Illumina HiSeq2000. Data were post-processed using Picard and Samtools, then analyzed with the Genome Analysis Toolkit. Variants were annotated using an in-house Ensembl-based program. Copy number was assessed using ExomeCNV.

Results—Each sample was sequenced to a mean depth of coverage of greater than 120×. A rare non-synonymous coding SNV in *TP53* was identified in the germline. There were 10 somatic cancer protein-damaging variants that were not observed in the unaffected mother genome. ExomeCNV comparing tumor to the patient's germline, identified abnormal copy number, spanning 6,946 genes.

Conclusion—We present an unusual case of Li-Fraumeni detected by whole exome sequencing. There were also likely driver somatic mutations in the gastric adenocarcinoma. These results highlight the need for more thorough and broad scale germline and cancer analyses to accurately inform patients of inherited risk to cancer and to identify somatic mutations.

*Correspondence to: Vivian Y. Chang, MD, MS, Department of Pediatrics, Division of Hematology-Oncology, David Geffen School of Medicine, University of California, Los Angeles, California, 10833 Le Conte Ave., MDCC A2-312, Los Angeles, CA 90095. Tel: 310-825-6708, vchang@mednet.ucla.edu.

Keywords

exome sequencing; pediatric gastric adenocarcinoma; Li-Fraumeni Syndrome

Introduction

Gastric adenocarcinoma (GA) is a rare diagnosis in childhood, making up much less than 1% of childhood cancers [1, 2]. Although there are environmental factors thought to influence the risk of developing GA, such as *Helicobacter pylori* infection and diet, these factors have not been well-documented in children [3–6]. Hereditary Diffuse Gastric Cancer (HDGC) is caused by an autosomal dominant germline mutation in E-cadherin (*CDH1*), which can be identified in about 40% of patients with a consistent clinical and family history [7, 8]. Less commonly, Familial Adenomatous Polyposis (FAP), caused by autosomal dominant germline mutations in APC, can result in the transformation of fundic polyps to gastric carcinoma [9]. Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is a familial colon cancer syndrome which is causal in 10–25% of cases, and can also include stomach cancer [10]. This is attributed to multiple different genes involved in DNA mismatch repair. Patients with Li-Fraumeni syndrome can rarely develop gastric carcinomas, representing up to 2.8% of families [11, 12]. Lastly, patients with Peutz-Jeghers syndrome and Juvenile Polyposis also have an increased risk of developing gastric cancers [13–16]. However, there are clearly other predisposing genetic factors that may result in the development of GA, especially in the young pediatric population.

Next-generation sequencing has been used to identify causative genes in many Mendelian disorders, as well as somatic mutations in cancer [17–21]. About 85% of known disease-causing mutations occur within the 1% of the genome encoding for proteins [22]. Therefore, whole exome sequencing represents a powerful method to cost-effectively capture the majority of protein-coding regions for the identification of single-nucleotide variants (SNVs), small insertion/deletions (indels), and copy number abnormalities. When applied to paired tumor-normal samples, whole exome sequencing may be able to identify previously unknown causes of hereditary cancer syndromes, as well as fully characterizing exonic somatic mutations in rare pediatric cancers.

We applied this technology to a 14-year old male patient diagnosed with metastatic gastric adenocarcinoma. He had presented to an outside hospital with nausea, decreased appetite, intermittent fevers, and severe abdominal pain. The initial CT scan revealed multiple hepatic masses, the largest measuring 16×6×14 centimeters. He underwent a liver biopsy which showed a poorly differentiated adenocarcinoma of unknown primary (Figure 1). He then underwent a colonoscopy and upper endoscopy which confirmed metastatic gastric adenocarcinoma. The patient was started on chemotherapy with docetaxel, cisplatin, and oral capecitabine with near complete response by RECIST criteria and PET SUV metabolic uptake [23]. Unfortunately, the patient went on to have progressive disease and passed away about 18 months after diagnosis and two salvage therapies consisting of epirubicin, oxaliplatin, and capecitabine (EOX) followed by irinotecan monotherapy.

The patient's family history was significant for his father having died from colon adenocarcinoma at 18 years of age, and his paternal great-uncle having died from colon carcinoma in his early twenties (Figure 2). Clinical PCR-based genetic testing for *CDH1* and *APC* mutations were negative. Whole genome chromosome SNP microarray was performed to assess for imbalances in the genomic DNA and revealed no copy number gains or losses in *CDH1*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *PTEN*, *STK11*, or *APC*.

Methods

The institutional review board at University of California, Los Angeles approved this study and participants voluntarily provided written, informed consent.

DNA extraction and library construction

After obtaining informed consent, DNA was extracted from the patient's peripheral blood and the patient's mother's peripheral blood using the Autopure LS nucleic acid purification instrument (Qiagen, Valencia, CA). DNA was extracted from the primary tumor that was formalin-fixed, paraffin-embedded using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA).

Sequence libraries were constructed using standard Illumina protocols established in the UCLA Clinical Genomics Center for Clinical Exome Sequencing. In brief, DNA was sheared, end-repaired, A-tailed, and ligated with tagged adaptors. The ligated product was purified with the Agencourt AMPure XP kit and amplified. The sample library was hybridized with capture oligonucleotides from the Agilent SureSelect 50mb All Exon capture kit to enrich for exons (Agilent, Santa Clara, California). Each sample was sequenced on one lane of Illumina HiSeq2000 (Illumina, San Diego, California).

Bioinformatic analyses

Novoalign, using an index of the NCBI human genome reference build 37 (HG37), produced a binary SAM (bam) file of aligned reads. PCR duplicates for all files were marked using Picard's MarkDuplicates command and ignored in downstream analysis.

The Genome Analysis Toolkit (GATK) was used for variant calling [24, 25]. First, the quality scores generated by the sequencer were recalibrated to more closely represent the actual probability of mismatching the reference genome by analyzing the covariation among reported quality score, position within the read, dinucleotide, and probability of a reference mismatch. Second, local realignment around small indels was performed, using GATK's indel realigner to minimize the number of mismatching bases across all the reads. Statistically significant non-reference variants, SNVs and indels, were identified using the GATK UnifiedGenotyper. Using the GATK VariantAnnotator, each identified SNV and indel was annotated with various statistics, including allele balance, depth of coverage, strand balance, and multiple quality metrics. These statistics were then used to identify likely false positive SNPs, using the GATK VariantQualityScoreRealigner (VQSR). Variants with a low VQSR score were assumed to be false and filtered out, leaving a set of likely true variants.

After variants were identified, an in-house variant annotator based on Ensembl (ensembl.org) was used to additionally annotate each variant with information including dbSNP ID (for known common polymorphisms), gene names and accession numbers, variant consequences, such as missense mutation or frame shift deletion, protein positions and amino-acid changes, conservation scores, HapMap frequencies, Polyphen and Sift predictions on the effect of the variant on protein function, and clinical association. Novel (non-dbSNP) variants predicted to be protein damaging by Polyphen or Sift were included for further analyses.

Germline mutation analysis

The germline analysis assumed an autosomal dominant model and germline variants were filtered for damaging, heterozygous mutations found in the patient and absent in the unaffected mother. These were intersected with known germline mutations that predispose to cancer syndromes, found in Cosmic [26].

Somatic mutation analysis

Somatic mutations were filtered in a series of four steps. (1) Variants that were unique to the tumor and not present in the patient's germline were prioritized. (2) Variants that were also called in the unaffected mother's germline were removed. (3) Variants were removed if there were any non-reference reads both in the patient's and mother's germlines. (4) Lastly, Fisher's Exact test was performed with read counts and variants with $p < 0.0005$ with observation in the cancer and no observation in the normal genome were classified as somatic mutations.

Copy number analysis

ExomeCNV was used to detect somatic copy number abnormalities [27]. This is a statistical tool that uses coverage and alternative allele frequencies to estimate copy number variation and loss of heterozygosity. Circos was used to plot copy number abnormalities [28].

Results

Each sample was sequenced using 100 + 100 bases paired-end protocol in one lane of Illumina HiSeq2000. This resulted in each sample having greater than 88 million reads. Over 85% of all protein coding bases were covered at 20× or more. Average overall coverage was 123× reads for the tumor, 159× reads for patient's germline, and 149× reads for mother's germline (Table I).

There were about 23,000 single nucleotide variants (SNV) called in each of the three samples. 99.8% of alternate bases match the reported alternate allele in dbSNP132, in all three samples. After removing known common polymorphisms in dbSNP132, there were 1,398 novel SNVs in the primary tumor, 1,418 novel SNVs in patient's germline, and 1,381 novel SNVs in mother's germline (Table II). There were 1,800–1,900 small insertion/deletions (indel) in each sample. About 1,200 of these were novel (Table III).

There were 186 genes found to have heterozygous non-synonymous mutations in the patient's germline, and absent in the unaffected mother's germline. Of these, there was a non-synonymous coding SNV in *TP53*, codon 248 in the patient's germline, that has previously been associated with three families with GA in the TP53 database maintained by the International Agency for Research on Cancer (IARC) (www-p53.iarc.fr) [26]. There was a shift in the alternate allele frequency from the germline to the tumor, with 13 of 28 reads in the germline being non-reference and 19 of 28 reads in the tumor being non-reference. Accounting for almost 50% contamination of normal tissue in the tumor specimen, this likely represents a site of loss of heterozygosity.

There were no other mutated genes in the patient's germline that were in common with the known germline mutations that are associated with a predisposition to cancer. Interestingly, pathway analysis of all of the rare protein damaging heterozygous variants identified was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) on the 186 genes with heterozygous germline mutations [29, 30], and indicated that there was a greater than 3-fold enrichment for genes than expected by chance involved in "response to radiation" with $p < 0.05$ including the genes *RARG*, *TP53*, *MGP*, *TSHB*, *LRP2*, and *USF1*.

There were 10 novel, somatic, protein-damaging variants that were not present in the patient's unaffected mother, with Fisher Exact p -values < 0.0005 (Table IV). These were 2 nonsense, 3 frameshift, 1 splice-site, and 4 non-synonymous coding mutations. Four of these genes, *KBTBD10*, *SMC4*, *PANK1*, and *DSG3* have been previously reported to have somatic mutations in gastric cancer samples, highlight their potential role as driver mutations [31].

ExomeCNV comparing tumor to the patient's germline, identified 12,077 somatic segments with abnormal copy number, corresponding to 6,946 genes, consistent with chromosomal aneuploidies (Supplemental figure 1). There were several large regions of abnormal copy number such as amplification of 3q, 7p, 8q, 20q, and 20p that have previously been reported in gastric carcinoma samples by comparative genomic arrays [32]. There were also novel regions of abnormal copy number in our sample such as amplifications of 12q, 17p, 18p, and 22p.

There were 5 genes with somatic abnormal copy number that also had concomitant somatic variants. *SMC4* had a frameshift mutation and also had increased copy number to 3. *KBTBD10* harbored a nonsense mutation and loss of copy number to 1, with loss of the reference allele. *DSG* had a nonsynonymous coding mutation and increased copy number to 3. *ITGAX* had a nonsynonymous coding mutation and loss of copy number to 1. *LAMA1* had a frameshift mutation and had two different segments with increased copy number to 3 and 5. Interestingly, *ERBB2IP*, also known as ERBB2 interacting protein or Erbin, had three segments with amplification, involving exons 3, 10, and 17.

Discussion

Pediatric gastric adenocarcinoma is extremely rare, and it is unclear if it has the same biology as adult adenocarcinoma, especially in the context of germline predisposition. In

this case, exome sequencing of the patient's tumor and germline allowed us to screen for mutations and copy number changes in almost all protein-coding genes simultaneously allowing comprehensive search for a presumed inherited risk allele. Having the unaffected mother's germline sequencing allowed significant filtering of shared mutations that are likely to be non-pathogenic polymorphisms. Unfortunately, both the father and paternal great-uncle were unable to be tested because they died from disease progression more than a decade before the proband presented to our clinic.

Germline *TP53* mutations are associated with Li-Fraumeni Syndrome (LFS) [33]. This patient's germline *TP53* mutation involves R248W, which is the same amino acid change that has been reported in many families with Li-Fraumeni Syndrome, including at least two individuals with GA [34]. While the observation of other heterozygous potential mutations in genes involved in radiation damage response, have the potential to contribute to the familial risk of cancer, the observed role of the variant detected favors an unusual presentation of Li-Fraumeni.

Although there are a few reports in the literature about the association of GA and LFS [11, 35], it is not a common cancer associated with LFS, and thus was not considered for high priority sequencing. Thus, this patient was not thought to have LFS initially because he did not have the more typical tumors associated with LFS such as sarcomas, brain tumors, breast and adrenal cortical carcinomas [33, 35]. Given the complexity of identification of causal inherited variants for cancer risk, exome sequencing led to the quickest and most cost-effective method of discovering the *TP53* mutation. Though it could be argued that the diagnosis of Li-Fraumeni syndrome would eventually have been made through traditional single-gene testing, this would have led to delays in diagnosis and excess costs. Research exome sequencing at the time these data were generated cost \$2,000 per genome including data generation and variant calling in a 6 week period. This single test assays nearly 95% of all protein-coding sequence of current clinically-offered disease genes. *APC* and *CDH1* single-gene testing cost around \$2,000 per gene and sequential testing for just those two genes would take over 8 weeks. This highlights the need for more thorough and broad scale germline and cancer analyses to accurately inform patients of inherited risk to cancer, as it is not always obvious based on clinical history alone.

The somatic mutation analysis revealed 10 damaging somatic variants, three of which were previously described [35]. Half of these (5/10) genes had concomitant copy number abnormalities. *LAMA1* has been associated with overexpression in brain tumors and had two segments with increased copy number [36].

ERBB2IP is an adaptor for the Her2/Neu receptor and is involved in the location and signaling function of Her2/Neu [37]. Although our patient tested negative by fluorescent in situ hybridization for the amplification of Her2/Neu, we found an amplification of 3 exons of *ERBIN*. The use of trastuzumab, a monoclonal antibody against Her2/Neu, has been shown to be effective, both in outcome and cost, in treating advanced stage GA [38–40]. Whether amplifications of *ERBB2IP* would lead to increased expression of Her2/Neu is yet to be determined.

In summary, exome sequencing is becoming increasingly common in clinical practice, especially in the field of oncology and represents a cost-effective approach to comprehensively characterize germline and somatic mutations. This application will lead to the discovery of novel drug targets, driver mutations, as well as known and novel cancer predisposition genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Representative histologic section of a metastatic gastric adenocarcinoma. Section shows malignant epithelial cells arranged in poorly formed cribriform glandular structures infiltrating liver parenchyma. Cytologically the cells exhibit a moderate degree of pleomorphism with high nuclear-to-cytoplasmic ratio and abundant mitotic figures.

Fig. 2.
Family pedigree showing the affected proband with gastric adenocarcinoma and father and paternal great-uncle with colon cancer.

Table 1

Alignment and coverage statistics.

	Total reads	Total mapped (%)	% bases covered at 1×	% bases covered at 10×	% bases covered at 20×	Average coverage
Primary tumor	89,541,912	86,861,333 (97%)	97.1	91.7	87	123×
Patient's germline	108,967,923	106,753,528 (98%)	97	92	88.2	159×
Mother's germline	88,821,743	86,961,114 (98%)	96.9	92.2	88.5	149×

Table II

Single nucleotide variants.

	Total called (Ti/Tv)	Total in dbSNP132 (Ti/Tv)	% in dbSNP132	% dbSNP concordance	Total novel (Ti/Tv)
Primary tumor	23,768 (3.0)	22,370 (3.1)	94.1	99.8	1,398 (2.3)
Patient's germline	23,826 (3.0)	22,408 (3.1)	94.0	99.8	1,418 (2.3)
Mother's germline	23,474 (3.0)	22,093 (3.1)	94.1	99.8	1,381 (2.3)

* Ti/Tv= Transition to transversion ratio

Table III

Small insertions and deletions.

	Total called	Total in dbSNP132	% in dbSNP132	Novel
Primary tumor	1,864	613	32.9	1,251
Patient's germline	1,906	607	31.8	1,299
Mother's germline	1,888	601	31.8	1,287

Table IV

Somatic, protein-damaging variants with Fisher Exact p-values <0.0005.

Position	Ref	Alt	Effect	Prediction	Gene
13:78133970	C	T	Nonsense	.	<i>SCEL</i>
3:160150845 -160150846	-	TCTTCAACCT	Frameshift	.	<i>SMC4</i>
2:170366298	C	T	Nonsense	.	<i>KBTD10</i>
10:91359119 -91359120	-	T	Frameshift	.	<i>PANK1</i>
18:29046573	G	C	NS coding	SIFT=tolerated PolyPhen=probably_damaging	<i>DSG3</i>
18:76755074	G	T	NS coding	SIFT=deleterious PolyPhen=benign	<i>SALL3</i>
21:47334009	G	A	Splice site	.	<i>PCBP3</i>
16:31371662	C	T	NS coding	SIFT=deleterious PolyPhen=probably_damaging	<i>ITGAX</i>
18:6955426	G	-	Frameshift	.	<i>LAMA1</i>
7:1131971	G	A	NS coding	SIFT=tolerated PolyPhen=possibly_damaging	<i>GPER</i>