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Whole Exome Sequencing of Pediatric Gastric Adenocarcinoma: A Germline and Somatic Mutation Analysis

A thesis submitted in partial satisfaction

of the requirements for the degree of Master of Science

in Bioinformatics

by

Vivian Y Chang

2012

ABSTRACT OF THE THESIS

Whole Exome Sequencing of Pediatric Gastric Adenocarcinoma:

A Germline and Somatic Mutation Analysis

by

Vivian Y. Chang

Master of Science in Bioinformatics

University of California, Los Angeles, 2012

Professor Stanley F. Nelson, Chair

Professor John Peter Novembre

Professor Matteo Pellegrini

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 *FAP* and *APC1* were negative. Whole exome sequencing was used 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 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 120x. A rare non-synonymous coding SNV in *TP53* was identified in the germline. There were 10 somatic cancer protein-damaging variants. 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.

The thesis of Vivian Y. Chang is approved.

John Peter Novembre

Matteo Pellegrini

Stanley F. Nelson, Committee Chair

University of California, Los Angeles

2012

This thesis is dedicated to the patient and his family described herein, and to all the patients I have had the honor and privilege of taking care of. You have each made me a better and more compassionate physician; you are the motivation behind all my scientific pursuits.

TABLE OF CONTENTS

Introduction……..1-2

Methods……...2-5

Results…….5-8

Discussion……...8-10

References……...11-14

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We would like to thank our patient and family for their gracious participation. We also thank Traci Toy for her technical assistance with sequencing.

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]. Lastly, 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. 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 [11-15]. About 85% of known disease-causing mutations occur within the 1% of the genome encoding for proteins [16]. 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 16x6x14 centimeters. He underwent a liver biopsy which showed a poorly differentiated adenocarcinoma of unknown primary (**Figure I**). He then underwent a colonoscopy and upper endoscopy which confirmed metastatic gastric adenocarcinoma. The patient was started on chemotherapy with docetaxel, cisplatinum, and oral capecitabine with near complete response by RECIST criteria and PET SUV metabolic uptake [17].

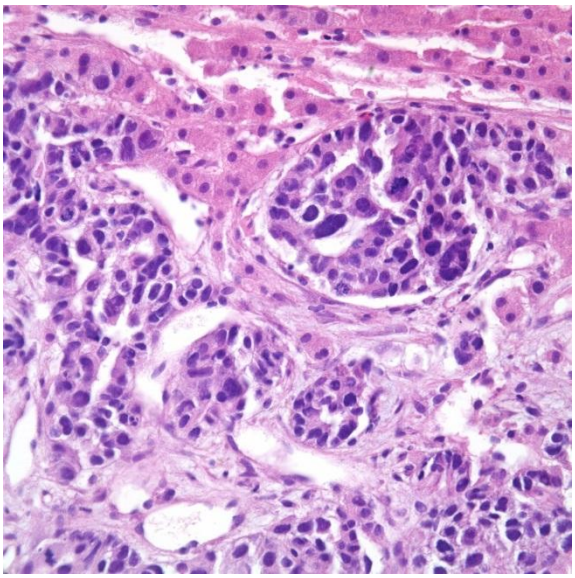


Figure I. Representative histologic section of a metastatic gastric adenocarcinoma. Section shows malignant epithelial cells arranged in poorly formed cribriforming glandular structures infiltrating liver parenchyma. Cytologically the cells exhibit a moderate degree of pleomorphism with high nuclear-to-cytoplasmic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

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. Clinical PCR-based genetic testing for *CDH1* and *APC* mutations were negative.

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 [18, 19]. 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 VariantQualityScoreRealibrator (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 [20].

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 [21]. This is a statistical tool that uses coverage and alternative allele frequencies to estimate copy number variation and loss of heterozygosity.

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 20x or more. Average overall coverage was 123x reads for the tumor, 159x reads for patient's germline, and 149x reads for mother's germline (**Table I**).

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

Table I. Alignment and coverage statistics. Reads were aligned to the NCBI human genome reference build 37 using Novoalign, which outputted these alignment statistics, including the total number of reads for each sample and the total number of reads mapped. The Genome Analysis Toolkit depth of coverage tool was used to calculate coverage statistics.

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**).

	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)

Table II. Single nucleotide variants. The Genome Analysis Toolkit's UnifiedGenotyper were used to call SNVs. The percentage of variants that were in the same position as a known polymorphism in dbSNP132 was calculated by GATK's Variant Evaluation tool. If the variant allele matched the polymorphism in dbSNP132, the variant was calculated as concordant. Novel variants were those that were not in dbSNP132. Transition to transversion ratios were calculated as a quality check.

	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 III. Small insertions and deletions. The Genome Analysis Toolkit's UnifiedGenotyper were used to call indels. The percentage of variants that were in the same position as a known polymorphism in dbSNP132 was calculated by GATK's Variant Evaluation tool. Novel variants were those that were not in dbSNP132.

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) [20]. There were no other mutated genes in the patient's germline, 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 [22, 23], 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 present only in the tumor and not present in the patient's germline or his 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, which highlight their potential role as driver mutations [24].

Position	Ref	Alt	Effect	Prediction	Gene
13:78133970	C	T	Nonsense	.	*SCEL*
3:160150845-160150846	-	TCTTCAACCT	Frameshift	.	*SMC4*
2:170366298	C	T	Nonsense	.	*KBTBD10*
10:91359119-91359120	-	T	Frameshift	.	*PANK1*
18:29046573	G	C	NS coding	SIFT=tolerated PolyPhen=probably_damaging	*DSG3*
18:76755074	G	T	NS coding	SIFT=deleterious PolyPhen=benign	*SALL3*
21:47334009	G	A	Splice site	.	*PCBP3*
16:31371662	C	T	NS coding	SIFT=deleterious PolyPhen=probably_damaging	*ITGAX*
18:6955426	G	-	Frameshift	.	*LAMA1*
7:1131971	G	A	NS coding	SIFT=tolerated PolyPhen=possibly_damaging	*GPER*

Table IV. Somatic mutations. 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.

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 (**Figure II**). 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 [25]. There were also novel regions of abnormal copy number in our sample such as amplifications of 12q, 17p, 18p, and 22p.

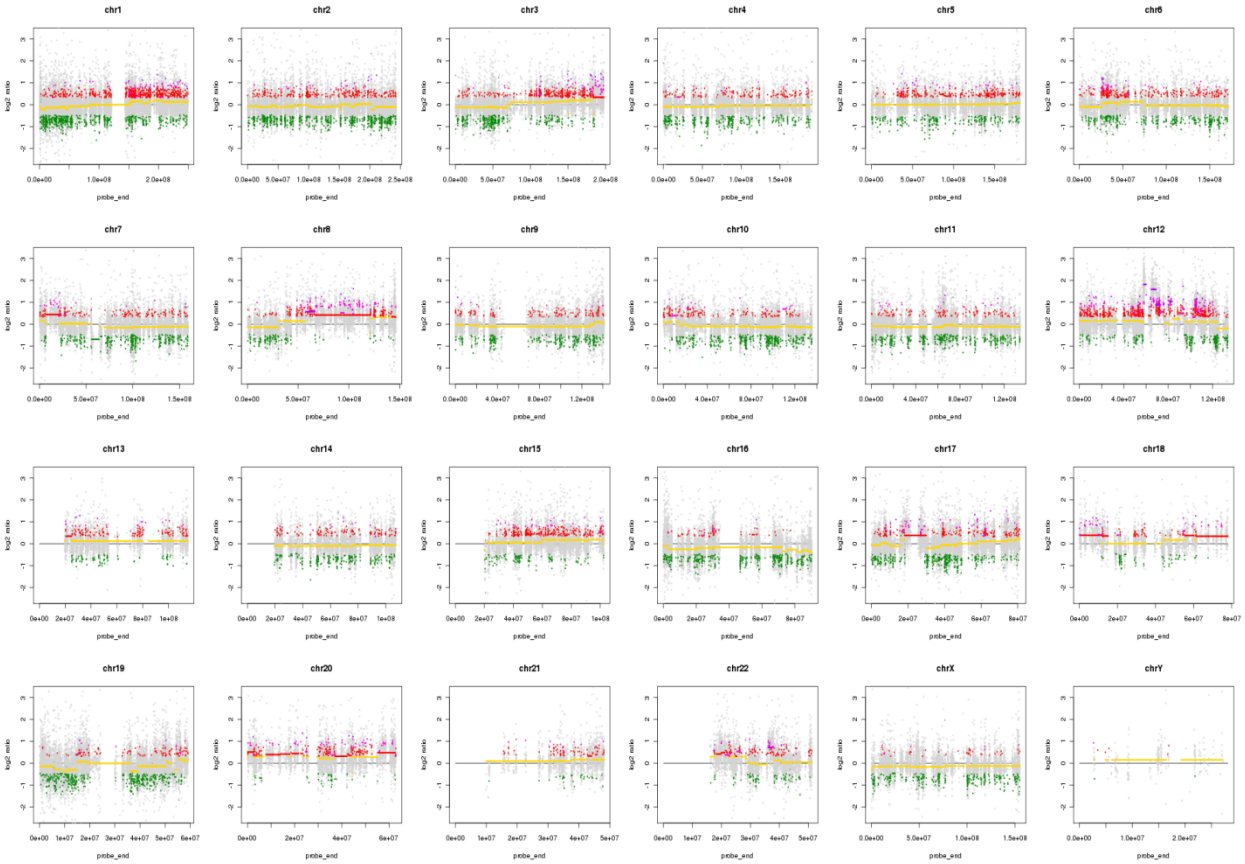


Figure II. ExomeCNV results plotting chromosome position on the x-axis and log2 ratio of the depth-of-coverage on the y-axis. (Yellow = copy-neutral, red = amplification, green = deletion, gray= exons with insufficient coverage to call copy number)

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. There was no tissue available for further testing from the affected father who was deceased.

Germline *TP53* mutations are associated with Li-Fraumeni Syndrome (LFS) [26]. 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 [27, 28], it is not a common cancer associated with LFS, and thus was not considered for high priority sequencing. 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. 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 [26, 28]. 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. 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 [29].

The somatic mutation analysis revealed 10 damaging somatic variants, three of which were previously described [28]. 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 [30].

ERBB2IP is an adaptor for the Her2/Neu receptor and is involved in the location and signaling function of Her2/Neu [31]. 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 [32-34]. 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.

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