Genetic Effect of Chemotherapy Exposure in Children of Testicular Cancer Survivors

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Abstract

Purpose: Cancer survivors express anxiety that chemotherapy exposure may lead to transmissible genetic damage in post-treatment children. Preclinical models suggest that chemotherapy exposure may result in considerable genomic alterations in postexposure progeny. Epidemiologic studies have not demonstrated a significant increase in congenital abnormalities in posttreatment children of cancer survivors, but the inherited genome-wide effect of chemotherapy exposure in humans is unknown.

Experimental Design: Two testicular cancer survivors cured with chemotherapy who had children pre- and postexposure without sperm banking were identified. Familial germline whole genome sequencing (WGS) was performed for these families, and analytic methods were utilized to identify de novo alterations, including mutations, recombinations, and structural rearrangements in the pre- and postexposure offspring.

Results: No increase in de novo germline mutations in postexposure children compared with their preexposure siblings was found. Furthermore, there were no increased short insertion/deletions, recombination frequency, or structural rearrangements in these postexposure children.

Conclusions: In two families of male cancer survivors, there was no transmissible genomic impact of significant mutagenic exposure in postexposure children. This study may provide possible reassuring evidence for patients undergoing chemotherapy who are unable to have pretreatment sperm cryopreservation.

Introduction

For men who present with advanced testicular cancer, combination chemotherapy with bleomycin, etoposide, and cisplatin (BEP) has definitive clinical benefit and is administered with curative intent as the standard of care for over 20 years. However, these treatments may impair future reproductive abilities, including decreased sperm counts, and some cytotoxics are capable of causing secondary malignancies due to their DNA mutagenic properties. As a result of the effect these toxic agents can have on reproduction, patients exposed to chemotherapy express concern about whether these treatments may induce germ cell mutations that lead to transmissible genetic damage in posttreatment offspring.

Multiple preclinical studies have demonstrated that paternal exposure to chemotherapy affects germ cell quality and offspring development. Etoposide exposure induced heritable chromosomal aberrations and aneuploidy in mice, and benzene exposure in mice resulted in increased mutation frequency in spermatogenic cells. Similarly, offspring of rats exposed to radiation have significantly higher germline mutation rates two generations after the exposure when compared with preexposure offspring. Each of the chemotherapies in the BEP regimen has genotoxic effects in model studies.

However, multiple epidemiologic studies of cancer survivors and their children have not demonstrated a significantly increased risk of genetic defects or congenital malformations in the setting of chemotherapy or radiation exposure, even though chemotherapy induces long-term effects on spermatogenesis. Furthermore, no significant increase in large chromosomal abnormalities was observed in offspring of cancer survivors compared with controls.

Given the mutagenic effects of chemotherapy at the base-pair level, epidemiologic studies of even many thousands of cases and controls may not be sufficiently powered to observe significant effects across the entire genome. Similarly, these mutagenic effects may not impact large chromosomal instability. Broader use of whole genome sequencing (WGS) to detect effects of mutagens on patients and their offspring may inform the direct genomic effect of environmental exposures, but such studies have not yet been performed in humans.
We hypothesized that if there were a transmissible genomic effect of chemotherapy exposure in the offspring of cancer survivors, it would manifest through increased de novo mutations across the entire genome. Thus, we identified two testicular cancer survivors who had children before and after significant chemotherapy exposure and performed WGS on the families to determine the effect of chemotherapy on the genomes of posttreatment offspring.

Materials and Methods

Patient enrollment

Eligible patients were defined as those who were exposed to cytotoxic chemotherapy and had children before and after exposure, with confirmation that postexposure children were conceived without cryopreserved tissue. Patients and their families provided informed consent under protocol #13-325 (Dana-Farber Cancer Institute, Boston, MA) to allow genome sequencing of samples for research study.

DNA extraction and sequencing

Samples were obtained using Oragene saliva kits. The chemagic DNA Blood Kit (Perkin Elmer) was used for sample preparation, and the samples were processed for extraction using the chemagic MSM 1 instrument.

Genome sequencing. Libraries were constructed and sequenced on the Illumina HiSeqX with the use of 151-bp paired-end reads for WGS. Output from Illumina software was processed by the Picard data-processing pipeline to yield BAM files containing well-calibrated, aligned reads. All sample information tracking was performed by automated LIMS messaging.

Library construction. Initial genomic DNA input into shearing was reduced from 3 μg to 100 ng in 50 μL of solution. In addition, for adapter ligation, Illumina paired-end adapters were replaced with palindromic forked adapters with unique 8 base index sequences embedded within the adapter. Size selection was performed using Sage Pippin Prep, with a target insert size of 370 bp ± 10%.

Preparation of libraries for cluster amplification and sequencing: Following sample preparation, libraries were quantified using qPCR (kit purchased from KAPA Biosystems) with probes specific to the ends of the adapters. This assay was automated using Agilent Bravo liquid handling platform. On the basis of qPCR quantification, libraries were normalized to 1 nmol/L. Samples were then combined with HiSeq X Cluster Amp Mix 1, 2, and 3 into single wells on a strip tube using the Hamilton Starlet Liquid Handling system.

Cluster amplification and sequencing. Cluster amplification of the templates was performed according to the manufacturer’s protocol (Illumina) using the Illumina cBot. Flowcells were sequenced on HiSeqX Sequencing-by-Synthesis Kits, and then analyzed using RTA2.

Analysis

Raw genomic data were aligned to hg19 reference genome using the Picard pipeline. The set of all possible single-nucleotide polymorphisms (SNPs) and short insert/deletion events were identified using HaplotypeCaller (20). Familial de novo mutations and short insertion/deletions were identified using established methodology (21). We considered only high-confidence de novo mutations for our analysis, removing low-coverage calls (i.e., <15 high-quality reads) and variants found in dbSNP. In addition, we filtered out point mutations that were rejected by the MuTect algorithm to account for additional sequencing artifacts (see Supplementary Materials and Methods; ref. 22). Per-child mutation rates were calculated by dividing the number of de novo mutations passing all filters by the number of bases covered to 15× in both parents and child:

\[
\text{de novo mutation rate per Mb} = \frac{\text{de novo count postfiltering/covered bases}}{1,000,000}.
\]

Given the relatively small number of de novo mutations per genome, the range for observed de novo mutation counts was calculated for a Poisson process as \(n \pm 1.96 \times \sqrt{n}\). An additional factor when comparing de novo mutation rates in pre- and postchemoexposure children is a difference in paternal age at conception. Prior studies (23–25) reported estimated increase in de novo substitution rate as 2.5% and 4.29% per year of paternal age. Correspondingly, the range of expected mutation rate after correction for paternal age was calculated as follows:

\[
\text{Expected counts} = (\text{de novo count postfiltering in preexposure child} \times \text{increase in de novo mutations per year based on published literature}) \times (\text{age gap}).
\]

\[
\mu_{\text{lower limit}} = \mu(\text{postfiltering in preexposure child}) \times 1.025^{\text{(age gap)}},
\]

\[
\mu_{\text{upper limit}} = \mu(\text{postfiltering in preexposure child}) \times 1.0429^{\text{(age gap)}}.
\]

Shared coverage was computed using the BEDTools suite (26), with poor mappability regions (27) excluded from consideration. All putative de novo mutations were manually reviewed in the Integrated Genomics Viewer (28).

Rearrangements were detected using dRanger (29) with the requirement of at least 10 reads supporting the rearrangement. To detect paternal recombination events in haplotype data for family 2, we adopted a method previously described (30). First, we identified well-covered (minimum 15×) SNPs which were heterozygous in the father and homozygous in the mother (vice versa for maternal recombination events.) For each pairwise comparison between children, we examined alleles at each informative

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<td>Preclinical models implicate significant effects of chemotherapy exposure on the inherited genome, although epidemiologic studies have not demonstrated this effect in patients. To explore this discrepancy, we performed germline whole genome sequencing on DNA extracted from two patients (and their families) who were exposed to chemotherapy and had children before and after the exposure. We demonstrated the absence of increased de novo genetic events in postexposure children compared with their preexposure siblings. Broadly, this study may have relevance to the cancer survivorship community, and it illustrates the potential for utilizing comprehensive genomic profiling to understand how the environment interacts with the inherited genome in cancer patients and their offspring.</td>
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marker SNP and encoded matches as “0” and mismatches as “1.” We then applied a function to smooth out noise in the data by evaluating the median value within a sliding window along each chromosome. Thus, we define recombination events as genomic regions within which we observe a crossover between concordance and discordance. In the case of three of more children, we determine the child in which the crossover occurs through pairwise comparisons.

**Results**

Two patients and their families took part in this study.

**Family 1.** Patient 1 presented with scrotal discomfort at the age of 35 years. On physical examination, a mass in his left testicle was observed. Serum tumor markers [β-human chorionic gonadotropin (β-hCG) and α-fetal protein (AFP)] were normal. At orchiectomy, the patient had a pT1 seminoma. He was clinical stage I but immediately postorchiectomy he developed a rising β-HCG that did not normalized with testosterone replacement. He received three cycles of full dose BEP chemotherapy [bleomycin 30 units (days 1, 8, 15), etoposide 100 mg/m² (days 1–5), cisplatin 20 mg/m² (days 1–5) with 21-day cycle; refs. 2, 3], and tolerated therapy well. He continued on testosterone replacement and has remained free from testis cancer for 9 years. 3.8 years prior to his testicular cancer diagnosis, the patient had his first child. 2.2 years post-BEP, the patient had a second child that was conceived without the use of banked sperm (Fig. 1A). There were no miscarriages before or after chemotherapy.

**Family 2.** At the age of 30 years, patient 2 presented with a right testicular mass. Preoperative tumor markers showed an AFP of 1,035. At orchiectomy, the patient had a pT2 mixed germ cell tumor: 60% yolk sac, 20% teratoma, 15% embryonal, and <5% choriocarcinoma. Ten weeks after orchiectomy, the patient underwent a retroperitoneal lymph node dissection (RPLND), and no tumor was found. Three months post-RPLND, he was found to have a lung mass consistent with recurrent disease and an AFP of 49. The patient received three cycles of BEP chemotherapy as described previously. At completion of BEP, the AFP was normal, and a residual left lung mass was resected; pathology demonstrated mature teratoma. After therapy, he has not required testosterone replacement. The patient has remained without evidence of disease for 10 years. 1.6 years prior to his testicular cancer diagnosis, the patient had his first child. 2.4 years and 5 years post-BEP, the patient had his second and third children, respectively (Fig. 1B). Both postexposure children were conceived without the use of banked sperm. There was no history of miscarriages before or after chemotherapy.

Mean coverage for whole genomes was 50×. The preexposure children had de novo mutation rates of 0.039 and 0.027 mutations per megabase (mutations/Mb) for families 1 and 2, respectively (Fig. 2A and B; Supplementary Tables S1 and S2). The two postexposure children born approximately 2 years after chemotherapy had de novo mutation rates of 0.044 and 0.036 mutations/Mb, respectively. The second family's other postexposure child, born 5 years after exposure, had a de novo mutation rate of 0.038 mutations/Mb. There was no discernible difference between pre- and postexposure children de novo mutation rates in either family (Fig. 2A and B). Furthermore, in all cases, the expected de novo mutation rates based on adjustment for paternal age fell within the 95% confidence intervals (CI) for the observed mutation rates. (Supplementary Table S1; refs. 23–25).

The de novo short insertion/deletion (indel) rate of the postexposure children was also similar to their preexposure counterparts (Fig. 2C and D; Supplementary Table S3). The relative indel rate per Mb was lowest for the two postexposure children born earliest after chemotherapy. There was no enrichment for mutations occurring in a specific site or variant type in postexposure children. The majority of mutations for all children occurred in intergenic regions (Supplementary Table S4), and there were no recurrent de novo events observed at the base-pair level.

No structural rearrangements were detected in either of preexposure or postexposure children. Finally, family 2 had two offspring and thereby afforded an opportunity to determine whether there were different frequencies of meiotic recombination events between preexposure and postexposure children (Fig. 3A; ref. 31). There was no significant difference in the frequency of recombinations, although the total number of recombinations decreased with each child (Fig. 3B). The number of maternal and paternal recombination events we observed was consistent with prior reports in unselected populations (32).

**Discussion**

The use of familial WGS to determine the impact of specific mutagenic exposures on patients and their offspring may provide...
insight into clinical questions related to environmental effects on the human genome. In the cancer survivorship community, a frequent concern raised by patients is whether mutagenic chemotherapy exposure may impact future offspring. Contrary to expectations derived from preclinical models, we did not observe an increase in the de novo mutation rate in postexposure compared with preexposure children within a given family in the context of BEP chemotherapy exposure. These results may allay patient concerns regarding the effect of these exposures on subsequent offspring.

Although this study is limited to two families, it demonstrates an intriguing phenomenon that exposure to mutagenic chemotherapy leading to massive cell death can still leave no 'genomic scars' in offspring. WGS detects genetic alterations in coding and noncoding regions, where vast majority of mutations have no known functional impact and would not likely affect sperm viability. Correspondingly, the observed lack of additional mutational burden in postexposure offspring may reflect its absence in mature sperm, rather than the product of extreme positive selection due to spermatozoa competition. In turn, this observation can be explained by a combination of two factors: presence of germline cells that were not exposed to mutagenic impact and extreme sensitivity leading to senescence or apoptosis of those cells that were.

Larger and more diverse cohorts are necessary to make these findings generalizable, including profiling more families of both male and female cancer survivors exposed to chemotherapy or radiotherapy prior to conception, because it is possible that different environmental exposures in male and female cancer patients may result in different genomic effects (33). In addition,
nongenetic effects, such as epigenetic changes (34, 35) or transcription-based alternative splicing not observable with WGS, may identify transmissible mutagenic effects in humans and warrant further evaluation.

Broadly, this study may provide reassurance for patients undergoing chemotherapy who are unable to have cryopreservation of sperm. Finally, this study illustrates the potential for utilizing clinical genome sequencing to study effects of environmental exposures on the inherited genome.

**Disclosure of Potential Conflicts of Interest**

L. A. Garraway has ownership interests (including patents) at Foundation Medicine, is a consultant/advisory board member for Boehringer Ingelheim, Foundation Medicine, Novartis, and Warp Drive, and reports

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**Figure 3.**
Recombination events in family 2. A representative recombination event occurring in the children from family 2 is seen in panels A to C. We define recombination events as genomic regions within which we observe a crossover between concordant and discordant SNPs. In this example, we see a paternal recombination event in child 2 that is not observed in the pairwise comparison between child 1 and child 3. The overall meiotic recombination frequency events for the children from family 2, where detection of these events are possible, are visualized in D.
receiving commercial research grants from Novartis. E.M. Van Allen has ownership interests (including patents) at Syapse, and is a consultant/advocacy board member for Roche Ventana, Syapse, and Third Rock Ventures. No potential conflicts of interest were disclosed by the other authors.

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References


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