Title: Genetic effect of chemotherapy exposure in children of testicular cancer survivors

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ABSTRACT

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

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

Results: No increase in de novo germline mutations in post-exposure children compared to their pre-exposure siblings. Furthermore, there was no increased short insertion/deletions, recombination frequency or structural rearrangements in these post-exposure children.

Conclusions: In two families of male cancer survivors, there was no transmissible genomic impact of significant mutagenic exposure in post-exposure children. This study may provide possible reassuring evidence for patients undergoing chemotherapy who are unable to have pre-treatment sperm cryopreservation. Expanded cohorts that utilize WGS to identify environmental exposure effects on the inherited genome may inform the generalizability of these results.

STATEMENT OF TRANSLATIONAL RELEVANCE

Preclinical models implicate significant effects of chemotherapy exposure on the inherited genome, although epidemiological studies have not demonstrated this effect in

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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 post-exposure children compared to their pre-exposure 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.
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 twenty years(1-3). 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 post-treatment offspring(4-6).

Multiple preclinical studies have demonstrated that paternal exposure to chemotherapy impact germ cell quality(7) and offspring development(8, 9). Etoposide exposure induced heritable chromosomal aberrations and aneuploidy in mice(10), and benzene exposure in mice resulted in increased mutation frequency in spermatogenic cells(11). Similarly, offspring of rats exposed to radiation have significantly higher germline mutation rates two generations after the exposure when compared to pre-exposure offspring(12). Each of the chemotherapies in the BEP regimen has genotoxic effects in model studies(13).

However, multiple epidemiological 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(4, 5, 14, 15), even though chemotherapy induces long term effects on spermatogenesis(16, 17). Furthermore, no significant increase in large chromosomal abnormalities was observed in offspring of cancer survivors compared to controls(18).
Given the mutagenic effects of chemotherapy at the base pair level, epidemiological 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. Broadly, the use of whole genome sequencing to detect effects of mutagens on patients and their offspring may inform the direct genomic effect of environmental exposures(19), 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 whole genome sequencing on the families to determine the effect of chemotherapy on the genomes of post-treatment 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 post-exposure children were conceived without cryopreserved tissue. Patients and their families provided informed consent under protocol #13-325 (Dana-Farber Cancer Institute) 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 I instrument.

Genome Sequencing: Libraries were constructed and sequenced on the Illumina HiSeqX with the use of 151-bp paired-end reads for whole-genome sequencing. 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 3µg to 100ng in 50µL 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’s Pippin Prep, with a target insert size of 370bp +/- 10%.

Preparation of libraries for cluster amplification and sequencing: Following sample preparation, libraries were quantified using quantitative PCR (kit purchased from KAPA biosystems) with probes specific to the ends of the adapters. This assay was automated using Agilent’s Bravo liquid handling platform. Based on qPCR quantification, libraries were normalized to 1nM. 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, then analyzed using RTA2.

Analysis

Raw genomic data was aligned to hg19 reference genome using the Picard pipeline. The set of all possible single nucleotide polymorphisms 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., less than 15 high-quality reads) and variants found in dbSNP. In addition, we filtered out point mutations which were rejected by the MuTect algorithm to account for additional sequencing artifacts (see Supplementary Methods)(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 15X in both parents and child:

\[
\text{de novo mutation rate per mb} = \left( \frac{\text{de novo count post-filtering}}{\text{covered bases}} \right) \times 1000000
\]

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 \( n \pm 1.96 \sqrt{n} \). An additional factor when comparing de novo mutation rates in pre- and post- chemoexposure 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:
Expected counts = (de novo count post-filtering in preexposure child) * 
(increase in de novo mutations per year based on published literature) ^ (age
gap).

\[ \text{mulower}\_\text{limit} = \text{mu}(\text{post-filtering in preexposure child})^{1.025}(\text{age gap}) \]

\[ \text{muupper}\_\text{limit}=\text{mu}(\text{post-filtering in preexposure child})^{1.0429}(\text{age gap}) \]

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 15X) single nucleotide polymorphisms (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 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 age 35. On physical examination, a mass in his left testicle was observed. Serum tumor markers (beta-
human chorionic gonadotropin [beta-hCG] and alpha fetal protein [AFP]) were normal. At orchiectomy, the patient had a pT1 seminoma. He was clinical stage 1 but immediately post-orchiectomy he developed a rising beta-HCG that did not normalize with testosterone replacement. He received three cycles of full dose BEP chemotherapy (bleomycin 30 units (days 1, 8, 15), etoposide 100mg/M² (days 1-5), cisplatin 20 mg/M² (days 1-5) with 21 day cycle)(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, 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, <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 above. At completion of BEP the AFP was normal and a residual left lung mass was resected; pathology demonstrated mature teratoma. Post 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 post-exposure 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 50X. The pre-exposure children had de novo mutation rates of 0.039 and 0.027 mutations per megabase (mutations/Mb) for families one and two, respectively (Fig. 2A-B, Table S1-2). The two post-exposure children born approximately two years after chemotherapy had de novo mutation rates of 0.044 and 0.036 mutations/Mb, respectively. The second family's other post-exposure child, born five years after exposure, had a de novo mutation rate of 0.038 mutations/Mb. There was no discernable difference between pre- and post-exposure children de novo mutation rates in either family (Fig. 2A-B). Furthermore, in all cases, the expected de novo mutation rates based on adjustment for paternal age fell within the 95% confidence intervals for the observed mutation rates. (Table S1)(23-25).

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

No structural rearrangements were detected in either of pre- or post- exposure children. Finally, family two had three offspring and thereby afforded an opportunity to determine whether there were different frequencies of meiotic recombination events between pre- and post-exposure children (Fig. 3A)(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 whole genome sequencing 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 post-exposure compared to pre-exposure 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.

While 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. Whole-genome sequencing detects genetic alterations in coding and non-coding 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 post-exposure 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 radiation therapy prior to conception, since it is possible that different
environmental exposures in male and female cancer patients may result in different genomic effects\(^{33}\). In addition, non-genetic effects, such as epigenetic changes\(^{34, 35}\) or transcription-based alternative splicing not observable with whole genome sequencing, 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.

**AUTHOR CONTRIBUTIONS**


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FIGURE LEGENDS

Figure 1. Pedigrees for chemoexposure families. Pedigrees of Family 1 and Family 2 are displayed in Panels A and B, respectively. Additional information denoting birth of offspring relative to the timing of chemotherapy exposure is showed. Post-exposure offspring are denoted as red.

Figure 2. de novo alteration rates in pre- and post-exposure children. Point mutation rates for each child in family one and two are shown in Panels A and B, respectively. The dashed horizontal lines represent the predicted de novo mutation rate prediction based on published correction estimates(23, 24), and the solid bars give the 95% confidence intervals for these estimated rates. Short insertion/deletion rates for the children from families one and two are in Panel C and D, respectively.

Figure 3. Recombination events in family 2. A representative recombination event occurring in the children from family two is seen in Panel A-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 Panel D.
REFERENCES


Figure 1

A

Testicular

3.8 years pre-exposure

2.2 years post-exposure

B

Testicular

1.6 years pre-exposure

2.4 years post-exposure

5.0 years post-exposure
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