Novel next-generation sequencing-based methodologies to characterize the mutational consequences of (prenatal) chemotherapy exposure in non-cancerous tissue.

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Abstract

Purpose of review: Although chemotherapeutics are considered as genotoxins for decades, their exact mutagenic impact on the genome of cancerous and normal cells of cancer patients was unknown for a long time. However, this knowledge is necessary to understand the long-term side effects of chemotherapy. A particular condition represents pregnant cancer patients being treated with chemotherapy. Since certain chemotherapeutics can cross the placenta, concerns exist about possible mutational effects on the fetus' genome with potential long-term health consequences.

Recent findings: Recent advances of next-generation sequencing techniques have opened possibilities to explore the exact mutational footprint of chemotherapies in healthy tissue from treated cancer patients. However, the ultra-low frequency of chemotherapy-induced mutations, introduction of technical artefacts and inaccessibility of normal tissue have posed important limitations. This review discusses five state-of-the-art approaches that were recently designed to overcome these drawbacks.

Summary: Results of the latest investigations give valuable insights into the genome-wide genotoxicity profile of frequently applied chemotherapies, with most of these drugs being associated with a signature of random base substitutions and small indels. Though these findings still might be limited to extrapolate to healthy tissue, they pave the way for studies on the origin of long-term chemotherapy-related adverse health effects.

Keywords: Cancer, Chemotherapy, Non-cancerous tissue, Next-generation sequencing, Mutational signature

Introduction

Despite improved efficacy and enhanced survival rates resulting from modern anticancer treatments, side effects and long-term sequelae of chemotherapy remain a major concern. Chemotherapeutics typically interfere with cell growth- and survival-related processes and have the ability to (in)directly interact with the cancer cell's genetic material (Table 1) [1]. After erroneous DNA replication or repair, chemotherapy-induced DNA lesions might result in the acquisition of *de novo* point mutations (base substitutions, small indels) and large structural variations (SVs, copy number alterations (CNAs), translocations and inversions). Since classical chemotherapeutics have relatively low tumor specificity, they can also interfere with processes active in healthy cells, hence causing numerous severe short-term side effects like hair loss, hematologic toxicity and gastrointestinal and/or excretory system disorders [1–3**]. Additionally, chronic and even long-term effects, like an increased risk of developing secondary malignancies and premature aging, have been associated with chemotherapy exposure [1,3**–5]. While increased cell death, triggered by toxic levels of DNA damage, may underlie some of the long-term effects, somatic mutations resulting from DNA damage across tissues probably also contribute. In fact, recent large-scale cancer genome sequencing projects have revealed that more than half the mutations in cancer genomes arise in healthy cells prior to carcinogenesis [6]. However, the exact link between chemotherapy-mediated mutations in healthy cells and long-term health implications remains unknown [7,8].

This knowledge gap can be partly attributed to the historic lack of techniques to accurately detect chemotherapy-induced DNA damage in normal non-cancerous cells. In the past, the genotoxic ability of chemotherapeutics was only measured via indirect and imprecise *in vitro* cytogenetic tests, either focusing on mutations (in reporter genes) or on chromosome aberrations (via assays like the micronucleus or COMET assay) [9–15]. Although predicting the carcinogenic potential of the tested drug, these tests cannot determine the presence, nor exact genomic location, of genome-wide single and double base substitutions, indels or SVs. The introduction of next-generation sequencing (NGS) enabled sequencing of multiple genomes in parallel. Existing NGS studies on the mutational footprint

of chemotherapeutics have mainly used cancer cells and (cancer) cell lines [16]. Interestingly, these studies demonstrated that chemotherapy-induced mutations occur independently across tissue cells and hence are random and private to each cell [17**]. This is in contrast to cancer tissue, where chemotherapy-induced mutations can expand to high clonality. Consequently, cataloguing such low-frequent DNA aberrations from bulk normal non-cancerous cells *in vivo* is highly challenging [18*], and only starting to be explored. Yet, this knowledge is of crucial importance to explain and predict long-term health effects of chemotherapy in cancer patients. A particularly pertinent condition is that of pregnant cancer patients being treated with chemotherapy [19,20]. Data from *in vivo*, *ex vivo* and clinical studies have indicated that certain chemotherapeutics can pass the placental barrier, suggesting that the fetus is likely exposed to the genotoxic effects of these chemotherapeutics [21–27]. Additionally, mutations introduced earlier in development may be more impactful as they are more likely to be propagated to multiple adult cells as the cell-of-origin proliferates. Because cancer treatment during pregnancy is becoming a standard of care for these patients [28], evaluating this possibility is highly warranted.

Here, we will review recent efforts undertaken to catalogue the genome-wide mutational footprints of chemotherapeutics in non-cancerous tissue of chemotherapy-exposed (pregnant) patients and describe their advantages and limitations.

Mutational footprints of chemotherapy in healthy tissue of cancer patients

Given the random nature of chemotherapy-induced mutations in non-cancerous tissue, their variant allele frequency (VAF) may fall below the detection limit of standard bulk sequencing [18*]. Hence, only extreme deep bulk sequencing would enable detecting such mutations, yet at extremely high costs and without being able to discern technical noise from signal [29]. Consequently, detailed knowledge about mutagenic effect of chemotherapeutics in somatic cells is still limited today. The following innovative efforts have recently been undertaken to circumvent this issue using either bulk or single-cell-derived approaches (Figure 1).

Deep sequencing of bulk metastatic tumor samples as proxy for healthy tissue

Due to the clonal expansion characteristic of tumor development, low-abundant treatment-induced somatic mutations may become enriched in metastatic tumor cells and hence detectable through bulk whole-genome sequencing ($\sim 106\times$ sequencing depth). Using this strategy, Pich and colleagues mapped the presence of single-base substitutions, double-base substitutions and indels in over 3,500 metastatic tumors originating from different organs of chemotherapy-treated patients (Figure 1A) [17**]. To rule out tumor type specific mutations, both treated and untreated samples were compared per treatment type. Only for three platinum-based drugs (carboplatin, cisplatin and oxaliplatin) and two nucleoside metabolic inhibitors (capecitabine and 5-fluorouracil), treatment-associated mutational signatures could be extracted (Table 1). The investigated chemotherapeutics were found to induce ~ 100 times more mutations than the natural aging process in a similar period [17**]. Pich *et al.* argued that the mutational toxicity identified in these metastatic samples could serve as a proxy for the potential mutational effect of the investigated chemotherapeutics in the patients' healthy tissue. However, differences in rate of cell division and proficiency of DNA repair between cells, as well as pharmacodynamics and drug metabolism, could influence the mutational risk that chemotherapeutics pose for various tissues. Hence, since metastatic tumor samples might react differently to chemotherapeutics than non-cancerous tissues resulting in a differential mutational pattern, results should be interpreted with caution.

Low coverage genome-wide sequencing of bulk primary human cells

Quispe-Tintaya and colleagues developed an algorithm to quantitatively and accurately detect chemotherapy-induced low abundant SVs (large indels, inversions and/or translocations) from ultra-low coverage ($\sim 0.3\times$ sequencing depth) paired-end whole-genome sequencing data [3**,5]. The key features of this algorithm are (i) the use of sequencing libraries free from artificial chimeric fragments, and (ii) a novel non-consensus-based SV-calling algorithm [3**,5]. This algorithm was applied on sequencing data of actively proliferating or quiescent cultures of primary human dermal fibroblasts

that were *in vitro* exposed to bleomycin (Figure 1D). A significant increase in SVs was found, which was highest in quiescent cells (Table 1). This observation was confirmed in post-mitotic mouse liver and heart tissue [3**]. The difference in mutation induction in quiescent versus dividing cells was hypothesized to result from a different employment of DNA repair mechanisms. While DNA double-strand breaks, resulting from bleomycin exposure, are usually repaired by relatively error-free homologous recombination mechanisms in proliferating cells, quiescent cells apply error-prone non-homologous end joining mechanisms, possibly leading to significantly higher frequencies of somatic SVs [3**]. Hence, quiescent cells were suggested to be a reservoir for chemotherapy-induced genome rearrangements. However, *in vitro* chemotherapy exposure of cell cultures might result in differences regarding induced mutations compared to the *in vivo* exposure experienced by patients who receive systemic chemotherapy treatment. Hence, extrapolation to a patient's healthy tissue might be limited.

Clonal expansion of human induced pluripotent stem cell lines

In their recent work, Kucab *et al.* assessed mutational signatures induced by various known or suspected environmental carcinogens, including five chemotherapeutics (cisplatin, carboplatin, cyclophosphamide, bleomycin and etoposide), using a human induced pluripotent stem cell line (iPSC) obtained upon reprogramming adult skin cells [30**]. After chemotherapy exposure of the iPSC cultures, single cells were isolated and cultured into subclones that were subsequently subjected to whole-genome deep bulk sequencing (~30× sequencing depth) (Figure 1B). Mutation frequencies of *de novo* base substitutions, indels, rearrangements and CNAs were determined by comparison to the genome of the primary iPSC parental clone. Rearrangements and CNAs turned out to be limited and uninformative, suggesting a high intolerance to double stranded breaks in these sensitive stem cells. Bleomycin and etoposide failed to induce a significant number of mutations. However, for cisplatin and carboplatin, mutational signatures for single and double base substitutions were extracted, being similar for both chemotherapeutics (Table 1) [30**]. As most adult human cell types are terminally differentiated and not amenable to growth in culture media, the use of iPSCs has the advantage to

allow for clonal sequencing in otherwise unculturable cell types. However, the process of reprogramming terminally differentiated cells was previously found to be inherently mutagenic, hence precluding reliable extrapolation of mutagenic signatures found in iPSCs upon chemotherapy exposure to those that would be expected in normal human somatic cells [31].

Clonal expansion of human-derived cancer cell lines

Boot *et al.* created clonal cultures of the non-tumorigenic human breast epithelial cell line MCF-10A and the liver cancer cell line HepG2 to determine the cisplatin mutational signature. Single cells from cisplatin-exposed cultures were isolated and expanded for whole-genome sequencing (~33× sequencing depth) and mutational analyses (Figure 1C) [32**]. The detected cisplatin single-base substitution signature did not exactly match that found in iPSCs, as described by Kucab [30**]. Subtle differences could be attributed to the usage of different cell lines. Interestingly, the cisplatin signature of single base substitutions in the breast and liver cancer cell line was the same as found by Pich *et al.* in metastatic tumor cells [17**]. Similarly as in iPSCs [30**], CNAs or SVs were rarely observed in the investigated cancer cell lines, suggesting that cisplatin does not induce detectable genomic instability [32**]. However, since cell lines are artificial systems engineered to divide indefinitely and with unique gene expression patterns not found in any cell type *in vivo*, they might not be representative to study the mutational footprint of chemotherapeutics in normal cells [33].

Organoids derived from primary human cells

Using the ability of singular pluripotent stem cells to form *in vitro* clonal outgrowths, Christensen *et al.* generated organoids of healthy intestinal colon stem cells to model the mutational effect of *in vitro* exposure to 5-fluorouracil (Figure 1E) [34**, 35*, 36]. 5-fluorouracil was found to induce single base substitutions, similar as those reported by Pich *et al.* (Table 1) [17**,34**]. Based on observed *in vivo* mutation rates due to ageing (40 mutations/year) and the estimated mutation rate induced by 5-fluorouracil treatment found in colon tumors (2,000 mutations/year), it was estimated that each 5-fluorouracil treatment (standard therapy of 12 cycles) induces an approximate 50-fold increase in

oncogenic mutations in colon stem cells *in vivo* compared to normal, untreated circumstances [34**,36]. This equals the additive mutation accumulation due to ageing in normal colon stem cells over 20 years. Since organoids mimic the *in vivo* organs from which the stem cells originate, this experimental setup might be ideally suited to characterize the mutational footprints of chemotherapy-exposed organs. Yet, it should be noted that this approach is restricted to stem and progenitor cell types that have the ability to be cultured. Also, selection bias might arise from the selective outgrowth of particular single cells during culturing. Finally, mutations may be induced by the clonal expansion process itself. Yet, this issue can be countered by taking into account differences in VAFs of *in vitro* generated mutations versus those present in the cell-of-origin [16,34**,37].

Genotoxicity in fetal DNA upon prenatal exposure to chemotherapy

Pregnant cancer patients are a particular vulnerable population, as maternal treatment options must be balanced with possible fetal risks [19,20]. Accumulating evidence indicates that certain anti-cancer therapies are relatively safe if given during the second or third trimester of pregnancy, without major adverse effects on short-term pediatric outcome [38–41]. Yet, a relationship was found between prenatal chemotherapy exposure and incidence of small for gestational age (SGA) babies, the effect being largest for treatment with platinum-derivatives [41]. SGA can be caused by an inherent small constitution or by improper growth in the uterus due to fetal growth restriction conditions, the latter being associated with higher risks of cardiovascular and metabolic diseases later in life [42]. Data from our group suggest that the latter possibility may be true, since prenatal chemotherapy exposure affects both birth weight and placenta [43]. Furthermore, our pharmacokinetic studies in mice and baboons models demonstrated that chemotherapeutics can cross the placental barrier with passage rates ranging from 0.8-2.4% for taxanes, over 1-10.8% for alkylating agents and anthracyclines to 43-56% for platinum-based compounds [21–23,27]. *Ex vivo* and clinical studies, though obtained in small cohorts of pregnant cancer patients, support these findings [24–26]. Consequently, it might be questioned whether prenatal chemotherapy administration has a genotoxic impact on the fetus' genome and

whether this could be associated to some of the observed outcome effects. So far, in humans, toxic effects of prenatal chemotherapy exposure have only been identified based on observable congenital anomalies in infants and miscarriages [44,45*, own unpublished data]. Over the last decade, only five rodent studies addressed this topic, thereby focusing on cyclophosphamide and etoposide [46–50]. Using basic cytogenetic assays, higher levels of apoptosis, DNA fragmentation and/or gross chromosomal aberrations were found in organs and/or blood from *in utero*-exposed newborn pups (Table 2). However, important species-related differences in DNA repair mechanisms and genome maintenance render these models ill-suited to study the genetic damage caused by prenatal chemotherapy exposure without prior knowledge of what to expect [51]. Based on the high placental transfer rate of alkylating agents, in particular of platinum-based drugs, and on the observed mutational signatures of these drugs in cancer patients' healthy tissue (cfr above), it might be hypothesized that prenatal exposure to alkylating agents, particularly platinum-based drugs, might confer the highest risk for the fetus' genome. This needs urgent investigation in a clinically relevant setup. As it is difficult to non-invasively obtain fetal primary tissue, the approach used by Christensen *et al.* [34**], in which stem cells are clonally expanded into organoid structures, might offer a feasible direction to study potential effects of prenatal chemotherapy exposure on fetal DNA derived from stem cells isolated from cord blood or other excrements of the neonate [52].

Conclusions and relevance

Recent technical revolutions using high-throughput NGS approaches have initiated the accrual of genome-wide somatic mutational loads and spectra in non-cancerous human cells arising upon chemotherapy exposure [16,37]. Alkylating agents are mostly investigated and were found to induce mutations in a random pattern. However, the use of artificial cell lines and metastatic tumor samples as surrogates for healthy tissues complicates the interpretation of obtained results. Also, most presented studies rely on the evaluation of cells and tissues that were *in vitro* exposed to chemotherapy. In fact, none of the above described studies investigated the mutational footprint of

chemotherapy in *in vivo* exposed primary healthy tissue from chemotherapy-treated patients. Therefore, information obtained here must be complemented with carefully planned prospective studies of healthy cell samples from treated patients and survivors [17**]. We anticipate that future advances in true single-cell sequencing technologies will provide means for high-throughput profiling of low-frequent somatic mutations in single cells across the human body [16,37]. Furthermore, as studying specific cell types that are difficult to access, may require highly invasive procedures, the evaluation of blood-derived cell-free DNA could offer an alternative [16].

From a clinical point of view, mutational signature analyses of chemotherapeutics will be valuable for assessing the mutational risk of different chemotherapies or to develop prevention strategies [32**,34**]. Since the fetus is at risk of being exposed to chemotherapeutics administered during pregnancy, these analyses could also define which chemotherapeutics are safe for the unborn child. Furthermore, long-term follow-up of the defined mutational signatures in treated cancer patients and prenatally exposed children could provide insight into the mechanisms by which long-term side effects of chemotherapy develop. When the exact link between such signatures and the manifestation of diseases later in life is established, they could serve as prognostic biomarkers.

Key points

- Since chemotherapy-induced mutations are often random and private to each cell, several difficulties exist in identifying these ultra-low frequent mutations.
- Revolutionary advances conferring NGS-based methodologies have made it possible to extract mutational signatures from human-derived cell lines, metastatic tumor samples and induced pluripotent and adult stem cells exposed to chemotherapeutic drugs.
- Results of these studies provide valuable insights into potential genotoxicity in healthy, non-cancerous tissues of chemotherapy-treated cancer patients, as well as the prenatally exposed children of chemotherapy-treated pregnant cancer patients.
- Assessing the mutational profiles of chemotherapeutics in healthy, non-cancerous tissues might open perspectives to mechanistic/epidemiologic studies on the long-term side effects of chemotherapy exposure.

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Conflicts of interest

None

Figure 1

Figure Legends

Figure 1: Schematic overview of novel Next Generation Sequencing methodologies that are currently applied to characterize the mutational footprint of different chemotherapeutics in surrogates of healthy tissues. A, B and C describe approaches using either cancer cells or immortalized cell lines. A, Bulk sequencing of metastatic tumor cells of chemotherapy-treated patients as a surrogate for healthy normal tissue. This approach was applied by Pich *et al.* [17**] B, Use of induced pluripotent stem cell (iPSC) cultures that are *in vitro* exposed to chemotherapy. Next, single cells are expanded into clones for subsequent bulk sequencing [30**]. C, Use of cultured cancer cell lines that are *in vitro* exposed to chemotherapy, after which single cells are expanded into clones for subsequent bulk sequencing, as applied by Boot *et al.* [32**] D and E represent approaches in which primary healthy cells are evaluated. D, Bulk sequencing of cultures of primary human cells that were *in vitro* exposed to

chemotherapy. Quispe-Tintaya *et al.* developed a novel bioinformatics algorithm to define low-abundant chemotherapy-induced SVs [3**,5]. E, Chemotherapy exposure of adult stem cell cultures after which single cells are expanded into clones for subsequent bulk sequencing [34**, 35*, 36].

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Table 1 : Different classes of chemotherapeutic agents with their mode of action, specific effect on DNA and known mutational signature induced in surrogate normal tissue					
Class of agent	Examples	Reactivity class	Effect on DNA molecule	Mode of action	Mutational signature in surrogate normal tissue/cells
Alkylating agents	Nitrogen mustards (Cyclophosphamide, ifosfamide, chlorambucil), platinum-based agents (cisplatin, carboplatin), dacarbazine	DNA-reactive	Covalent DNA-adducts and DNA-crosslinks	Inhibit DNA replication process	<p>SBS cisplatin:</p> <ul style="list-style-type: none"> - C>T & C>G [17**,32**] - C>T & G>A [30**] <p>DBS cisplatin:</p> <ul style="list-style-type: none"> - CT>NN & TC>NN [17**] - AG>TT & GA>TT [30**] - Mostly in CC, CT, TC & TG sequences [32**] <p>SBS carboplatin:</p> <ul style="list-style-type: none"> - C>A, C>G, C>T & C>G [17**] - C>T & G>A [30**] <p>DBS carboplatin:</p> <ul style="list-style-type: none"> - CT>NN & TC>NN [17**] - AG>TT [30**] <p>SBS oxaliplatin:</p> <ul style="list-style-type: none"> - C>A, C>T & C>G [17**] <p>DBS oxaliplatin:</p> <ul style="list-style-type: none"> - CT>NN & TC>NN [17**] <p>SBS Cyclophosphamide:</p> <ul style="list-style-type: none"> - C>A [30**] <p>Others:</p> <ul style="list-style-type: none"> - No NGS data available
Topoisomerase inhibitors	Anthracyclines (Doxorubicin, epirubicin), etoposide	DNA-reactive	Covalent DNA-adducts, Single and double DNA strand breaks	Prevent religation of DNA molecules after topoisomerase activity or enhance topoisomerase activity	<p>Etoposide:</p> <ul style="list-style-type: none"> - No detectable signature found [30**] <p>Anthracyclines:</p> <ul style="list-style-type: none"> - No NGS data available
Microtubule interacting agents	Taxanes (Paclitaxel, docetaxel), Vinca alkaloids (vinblastine, vincristine)	DNA non-reactive	Interference with intracellular trafficking of DNA-repair proteins	Polymerisation and/or stabilization of microtubules or depolymerisation and/or destabilization of microtubules	<p>Taxanes:</p> <ul style="list-style-type: none"> - No NGS data available <p>Vinca alkaloids:</p> <ul style="list-style-type: none"> - No NGS data available

DNA strand breaking agents	Bleomycin	DNA-reactive	Single and double DNA strand breaks	Induction of DNA strand breaks	Bleomycin: <ul style="list-style-type: none"> - Increased fraction of interchromosomal SVs and reduced fraction of intrachromosomal SVs in proliferating & quiescent primary human fibroblast [3**] - No detectable signature found [30**]
Nucleoside analogs and antimetabolites	Cytosine arabinoside, 5-fluorouracil, capecitabine	DNA-reactive	Incorporation into DNA molecule	Impairing DNA or RNA synthesis, blocking nucleotide metabolism	SBS 5-fluorouracil: <ul style="list-style-type: none"> - T>G [17**] - T>G & T>C [34**] SBS capecitabine: <ul style="list-style-type: none"> - T>G [17**] Cytosine arabinoside: <ul style="list-style-type: none"> - No NGS data available
SBS: Single-base substitution; DBS: Double-base substitution; NGS: Next generation sequencing; SV: Structural variants					

Table 2: Animal studies investigating genotoxic effects of prenatal exposure to chemotherapy using *in vitro* cytogenetic testing.

Class	Compound	Test species	Test tissue	GA at administration (days)	GA at tissue sampling	Test	Outcome	Reference
Alkylating agent	Cyclophosphamide	Rabbit	Peripheral tail blood from the newborn	25-30 (end of organogenesis)	Birth	Micronucleus assay	Significant increase in micronucleus frequency indicates increase of chromosomal loss or breakage. No significant difference in PCE values indicates no observable cytotoxicity of cyclophosphamide to the cells	[50]
Alkylating agent	Cyclophosphamide	Mouse	Newborn lymphocytes, bone marrow, liver & kidney cells	16 - 20 (late gestational period)	Birth	Alkaline COMET assay & micronucleus assay	Significant increase in micronucleus frequency indicates increase of chromosomal loss or breakage; COMET assay results indicate significant increase of DNA strand breaks	[48]
Alkylating agent	Cyclophosphamide	Mouse	Fetal thymus	11	19 days	DNA fragmentation & MTT cell viability assay	Significant increase of DNA fragmentation & significant inhibition in thymocyte proliferation	[47]
Alkylating agent	Cyclophosphamide	Rat	Amniotic fluid cells	14-18	19 days	Micronucleus assay	Significant increase in micronucleus frequency indicates increase of chromosomal loss or breakage	[46]
Topoisomerase inhibitor	Etoposide	Mouse	Fetal brain	12	1, 2, 4, 8, 12, 24 & 48h after treatment	cell cycle analysis, DNA microarray & RT-PCR	S-phase accumulation, G2/M arrest & apoptosis of neural progenitor cells in fetal telencephalon	[49]

GA, gestational age; PCE: polychromatic erythrocytes; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; RT-PCR: Real-time polymerase chain reaction