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Transcriptomic Analysis of Cultured Whale Skin Cells Exposed to Hexavalent Chromium [Cr(VI)]

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Abstract

Hexavalent chromium Cr(VI) is known to produce cytotoxic effects in humans and is a highly toxic environmental contaminant. Interestingly, it has been shown that free ranging sperm whales (*Physeter macrocephalus*) may have exceedingly high levels of Cr in their skin. Also, it has been demonstrated that skin cells from whales appear more resistant to both cytotoxicity and clastogenicity upon Cr exposure compared to human cells. However, the molecular genetic mechanisms employed in whale skin cells that might lead to Cr tolerance are unknown.

In an effort to understand the underlying mechanisms of Cr(VI) tolerance and to illuminate global gene expression patterns modulated by Cr, we exposed whale skin cells in culture to varying levels of Cr(VI) (i.e., 0.0, 0.5, 1.0 and 5.0 $\mu\text{g}/\text{cm}^2$) followed by short read (100 bp) next generation RNA sequencing (RNA-seq). RNA-seq reads from all exposures (≈ 280 million reads) were pooled to generate a *de novo* reference transcriptome assembly. The resulting whale reference assembly had 11K contigs and an N50 of 2,954 bp.

Using the reads from each dose (0.0, 0.5, 1.0 and 5.0 $\mu\text{g}/\text{cm}^2$) we performed RNA-seq based gene expression analysis that identified 35 up-regulated genes and 19 down-regulated genes. The experimental results suggest that low dose exposure to Cr (1.0 $\mu\text{g}/\text{cm}^2$) serves to induce up-regulation of oxidative stress response genes, DNA repair genes and cell cycle regulator genes.

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Author contributions: RBW, WW, and JPW Sr principal investigators, conceived the project, arranged for RNA sequencing, provided oversight of data analysis, assisted VP in writing and edited the final manuscript. VP assembled the reference transcriptome, performed bioinformatic mining and data analyses, gene expression analyses and wrote the manuscript. SW, CL, JPW Jr grew the cultured whale fibroblast cells, performed Cr exposures, cell survival data collection and analysis and chromosome damage preparation and aberration analysis. YS, TG, SK processed raw Illumina RNAseq sequence data for use in the study, provided custom bioinformatic scripts for data mining and reference transcriptome assembly, assisted VP in the transcriptome builds and in read mapping for gene expression analysis. MB, AP performed RNA isolations, qRT-PCR primer design and efficiency testing, qRT-PCR analysis of gene expression, and contributed to writing and editing the manuscript. All co-authors have approved the final submitted manuscript.

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However, at higher doses ($5.0 \mu\text{g}/\text{cm}^2$) the DNA repair genes appeared down-regulated while other genes that were induced suggest the initiation of cytotoxicity.

The set of genes identified that show regulatory modulation at different Cr doses provide specific candidates for further studies aimed at determination of how whales exhibit resistance to Cr toxicity and what role(s) reactive oxygen species (ROS) may play in this process.

Keywords

Sperm whale; Chromium; RNA-seq; Illumina HiSeq; ROS (reactive oxygen species); cytotoxicity

1. Introduction

Environmental contamination from heavy metals has been well documented and represents a global concern for both ecosystems and human health (US Department of Health and Human Services, 1993). Among heavy metals, Cr [as Cr(VI)] is considered a human health concern and most of the Cr released into the environment stems from human commercial and industrial activities (e.g., fossil fuel consumption, tanning, textiles, electroplating, etc.) (Cohen. et al., 1993; Salnikow and Zhitkovich, 2008). It is known that Cr(VI) easily enters cells and upon crossing the cell membrane becomes rapidly reduced to Cr(III). The reduced Cr(VI) may bind to DNA and or induce DNA damage and it has been documented that Cr exposure is carcinogenic (O'Brien *et al.*, 2003; Wise et al., 2008). For example, DNA double strand breaks are thought to result from ternary adducts caused by Cr stalling replication forks (Wise et al., 2008). It was found that Cr(VI) exposures decreased clonogenic survival in a dose-dependent manner (from 8-83%) and a strong response of cell cycle arrest, DNA double strand breaks and chromosome damage. In these studies less than 7% of the cells underwent apoptosis, but had increased activation of ATM and SMC1 (Xie et al., 2005).

A recent global assessment of Cr pollution in marine ecosystems measured in biopsies taken from free ranging whales across the globe revealed that many whales had skin Cr levels roughly 28 fold higher than those obtained from human skin samples (Wise et al., 2009). The levels found in whale biopsies correlated with human samples previously observed in occupationally exposed humans with lung cancer (Tsuneta et al., 1980). Complementing the observation of high Cr levels in whales are several reports detailing Cr induced cyto- and genotoxicity using cultured whale and human fibroblast cells exposed to various levels of Cr (Li Chen et al., 2012; Wise, 2012). Cumulatively, these reports indicate that whale cells are able to tolerate higher levels of intercellular Cr, and exhibit lesser cytogenetic damage than human cells. The source and manner in which whales accumulate Cr in the deep ocean is not understood, nor are the genetic mechanisms that allow whale cells to exhibit Cr resistance.

In an effort to better understand the underlying mechanisms of Cr induced cyto- and genotoxicity, and how whale cells ameliorate Cr induced damage, we performed RNA-seq analysis on sperm whale skin cells after exposure to varying levels of Cr (e.g., 0.0, 0.5, 1.0, $5.0 \mu\text{g}/\text{cm}^2$ Cr(VI)). Herein, we report assembly of a *de novo* sperm whale reference transcriptome based upon next generation sequencing reads (Illumina HiSeq, 100 bp paired-end; PE) from fibroblasts (cell culture). This reference transcriptome was utilized to assess global gene expression and identify genes exhibiting modulated expression upon the exposure to varying levels of Cr.

2. Methods

2.1 Cell Culture and Chromium Exposure

Sperm whale skin fibroblasts were maintained as adherent subconfluent monolayers, fed at least twice a week, and subcultured at least once a week as described previously (Wise et al., 2011). All experiments were conducted on logarithmically growing cells cultured in DMEM/F-12 supplemented with 15% Cosmic calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 mM sodium pyruvate. Cells were grown in a 33°C, humidified incubator with 5% CO₂ and routinely tested for Mycoplasma contamination.

Lead chromate (CAS# 7758-97-6, ACS reagent minimum 98% purity), was used as a representative particulate Cr(VI) compound and was administered as a suspension in water as previously described (Wise et al., 2002). Because lead chromate is an insoluble compound that only partially dissolves, treatments are expressed as weight per surface area (µg/cm²). Lead chromate was chosen because it represents particulate Cr(VI) compounds, which is the more potent carcinogenic form of Cr(VI). Studies show that the genotoxicity of lead chromate is all due to the chromate anion with the lead cation having no role (Wise et al., 1994; Holmes et al, 2005; Wise et al, 2005).

2.2 RNA Isolation and Next generation Sequencing

Logarithmically growing cells were seeded in 100 mm tissue culture plates and treated with 0.0-5.0 µg/cm² lead chromate for 24 h and then their RNA was isolated. RNA samples were sequenced using the Illumina HiSeq platform as 100 bp, PE reads. All short read data was converted to FASTQ files for filtration. The raw sequence reads were filtered using in-house software that utilized the following protocol: (1) adapter sequences from the PE FASTQ reads were removed, (2) raw PE reads with stretches of bad quality scores (score of 2, encoded in FASTQ format as 'B') were then trimmed, being untrustworthy, (3) bases with quality scores below 10 along with positions having a mean quality score of less than 20 for a window of size 3 centered on them were trimmed, (4) only the longest fragment from the remaining reads were then retained, (5) PE reads were ordered and the overlapping reads were merged, (6) lastly the reads that had lost their PE mates were kept as single end reads (Garcia et al., 2012). After the above filtration, we had approximately 52 GB of data representing a total of 250 million reads, paired and single end combined.

A transcriptome assembly was systematically built after using different K-mer values for Velvet assembler (v1.2.03) along with its extension, Oases (v0.2.06) (Zerbino and Birney, 2008). Assemblies were analyzed for K-mer sizes between 63 to 83 bp and N50 values were calculated for each assembly. Based on the N50 plot over the range of K-mer values (Figure 1), we used the assembly constructed from K-mer size of 73 bp, comprised of 193,644 contigs with N50 of 1,961 bp and average length of 915 bp, for further assessment.

To annotate the 73-mer reference assembly, we performed BLASTX comparisons with bovine (*Bos_taurus*.UMD3.1.68.pep.all.fa.gz) and dolphin (*Tursiops_truncatus*.turTru1.68.pep.all.fa.gz) protein sequences downloaded from the Ensemble database (Flicek et al., 2012). These BLASTX searches were performed at an e-value cutoff of e^{-10} (Altschul et al., 1990). Comparison of the 73-mer assembled transcripts versus the bovine protein sequences resulted in ~60% of the whale transcript contigs containing a known peptide motif. Transcripts with the highest coverage in respect to the bovine database were kept (i.e., the transcripts with the longest sequences and highest sequence identity versus bovine protein sequences) and used as the final whale reference assembly. The whale reference assembly so developed is comprised of 11,286 contigs with an N50 value of 2,954 bp.

Filtered RNA-seq reads derived from each of the cell cultures representing different Cr exposures were mapped back onto the reference transcriptome assembly. Mapping of the reads was performed using the Bowtie software package (v0.12.7) employing default options, except for the $-M$ 1 (to increase the number of reads mapped), for picking one random best hit for mapping against the assembly (Langmead *et al.*, 2009). Approximately 25% of the filtered Illumina reads from all Cr exposed cell culture datasets were able to map onto the reference assembly. Counting of mapped reads was performed using SAMtools function *Idxstats* (Li. H., et al., 2009), which were further used to assess differential gene expression.

2.3 Gene expression analysis

To identify genes differentially regulated in response to Cr(VI) exposure we employed the DESeq software package [from R, (Anders and Huber, 2010)] which normalizes data to account for differences in the total number of reads per library. Differentially expressed genes were identified by comparison of the reads at each Cr dose to that of a sham treatment (i.e., 0.0 $\mu\text{g}/\text{cm}^2$ dose) with p -value <0.01 . Every permutation of comparison among the four Cr(VI) doses (i.e., 0.0, 0.5, 1.0 and 5.0 ($\mu\text{g}/\text{cm}^2$)) was assessed. An example is shown in Figure 2, for comparison of the 0.0 and 5.0 $\mu\text{g}/\text{cm}^2$ doses. The transcripts with a p -value of <0.01 and $\log_2$ fold change greater than or equal to 2 are significantly differentially regulated and are shown in red. Genes above $\log_2$ fold change (+2 line) represent up regulated genes, while those below the $\log_2$ fold change (-2 line) represent down regulated transcripts relative to the sham treatment. IL8 and PER1 are circled in Figure 2 representing up and down-regulated genes, respectively. These significantly modulated transcripts were further analyzed for dose dependence. Annotation of genes exhibiting modulated expression based on their RNA-seq read counts were confirmed using BLASTX with an e -value cutoff of e^{-10} , versus the NCBI-nr database.

2.4 RT-PCR

To confirm the RNA-seq gene expression analysis, quantitative real time-PCR (qRT-PCR) was performed for a subset of genes that appeared to be differentially expressed in a dose dependent manner, or were of interest due to their suspected cellular function. Total RNA was isolated from the pelleted cultured cells using a pestle to lyse the cells in Trizol (Invitrogen, Carlsbad, CA) and employing an RNAeasy miniRNA isolation kit (Qiagen, Valencia, CA) to purify the RNA. Any residual DNA contamination was eliminated by performing column DNase digestion at 37°C (30 min). The integrity of the RNA was determined by gel electrophoresis (2% agarose in TAE running buffer) and the concentration determined using a spectrophotometer (Nano Drop Technologies, Willmington, DE).

cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Bioscience, Carlsbad, CA). The synthesis was performed in two steps, a DNase treatment with DNase I was performed for 15 min at rt and deactivated with 25 mM EDTA in a 65°C bath for 10 min. This treatment was followed by reverse transcription using 100 mM dNTPs, 10 $\times$ RT Random Primers, RNase Inhibitor, and Multiscribe Reverse Transcriptase in a 20 μL reaction employing a thermocycler using standard conditions (25°C for 10 min, 37°C for 120 min, 85°C for 5 min). Each sample was removed, diluted to a final volume of 500 μL , and analyzed using qRT-PCR. In addition, no RT controls were constructed using the protocol outlined above without adding the Multiscribe Reverse Transcriptase enzyme. This control was tested using qRT-PCR to verify the absence of gDNA in the samples.

Results were verified for selected genes by qRT-PCR analysis and SYBR Green-based detection with an Applied Biosystem's 7500Fast system (Applied Bioscience, Carlsbad,

CA). cDNA was synthesized using the total RNA isolated from each of the four cell cultures representing the various Cr(VI) exposures. Initially, each set of designed primers were tested for efficiency using a dilution series constructed with 0.0 $\mu\text{g}/\text{cm}^2$ Cr(VI) control cDNA (500 ng, 50 ng, 5.0 ng, and 0.5 ng, respectively) to establish a standard curve. Each respective primer and cDNA dilution series was used in a 20 μL reaction consisting of 1.0 μL of cDNA, 0.5 μM of each primer (forward and reverse), and 10 μL SYBR Green ready mix (Applied bioscience, Carlsbad, CA). Each reaction was subjected to 40 cycles each at 95°C for 20 sec, 95°C for 15 sec, and 60°C for 30 sec, before being tested with melt curve analysis. Amplified products were also analyzed for size specificity by agarose gel electrophoresis (2% agarose in TAE running buffer). The β -actin gene was selected as the transcript for normalization of all samples. The mean C_T values from triplicate runs were used to calculate relative expression levels and efficiencies. Once the primer efficiency was established, the relative expression of each of the cDNA samples (0.5 $\mu\text{g}/\text{cm}^2$ Cr(VI), 1.0 $\mu\text{g}/\text{cm}^2$ Cr(VI), and 5.0 $\mu\text{g}/\text{cm}^2$ Cr(VI)) was tested. Each cDNA and primer set was tested in replicates of four and the mean C_T values were used to calculate relative mean expression and fold-change of each respective transcript tested. This analysis was subject to cDNA normalization established by constructing a standard curve from the β -actin gene and co-normalization for each primer set using the 0.0 $\mu\text{g}/\text{cm}^2$ Cr(VI) control cDNA.

2.5 Cytotoxicity

Cytotoxicity was determined using a clonogenic assay based on previously published methods (Wise et al., 2002). Briefly, logarithmically growing cells were seeded in a monolayer, treated for 24 h with 0.0-5.0 $\mu\text{g}/\text{cm}^2$ lead chromate. After treatment, cells were harvested and reseeded at colony forming density in 100 mm tissue culture plates with four dishes per treatment group. Colonies were allowed to grow for approximately 14 days then stained and counted. At least 3 independent experiments were performed.

2.6. Genotoxicity

Genotoxicity was determined using the chromosomal aberration assay based on published methods with some minor modifications for whale cell cultures (Wise et al 2002). Briefly, logarithmically growing cells were seeded in 100 mm tissue culture plates and treated with 0.0-5.0 $\mu\text{g}/\text{cm}^2$ lead chromate for 24 h. Five hours before the end of treatment, demecolchicine was added to arrest the cells in metaphase. Cells were harvested, suspended in hypotonic solution for 17 min, fixed, dropped onto slides and stained. One hundred metaphases per treatment were analyzed using standard scoring criteria. At least 3 independent experiments were performed.

3. Results

Among the differentially expressed genes identified by DESeq analysis 35 transcripts showed up-regulation and 19 appeared to be down-regulated (p-value <0.01 and $\log_2$ fold change greater than or equal to ± 2). Of the 35 up-regulated transcripts, 3 transcripts were common in all doses of Cr(VI) exposure and 5, 4 and 20 transcripts were unique to 0.0-0.5, 0.0-1.0 and 0.0-5.0 ($\mu\text{g}/\text{cm}^2$) comparisons, respectively (Figure 3a). Of the 19 down-regulated transcripts, 1 was common for all of Cr(VI) doses and 2, 6 and 9 transcripts were unique for each of the 0.0-0.5, 0.0-1.0 and 0.0-5.0 ($\mu\text{g}/\text{cm}^2$) comparisons, respectively, (Figure 3b).

Gene profiling of these 54 transcripts was performed using BLASTX to search against the NCBI-nr database with e-value cutoff of e^{-10} . For a dose dependent analysis, the data were grouped into three sets, and then compared with the sham (i.e., 0.0-0.5 $\mu\text{g}/\text{cm}^2$, 0.0-1.0 $\mu\text{g}/\text{cm}^2$ and 0.0-5.0 $\mu\text{g}/\text{cm}^2$).

The genes which were significantly up-regulated at the 0.5 $\mu\text{g}/\text{cm}^2$ dose belong to the oxidative stress response and/or anti-inflammatory (immune) response (2 genes), cell-cycle regulation (1 gene), cell adhesion (2 genes) and miscellaneous (2 genes) gene categories. Significantly down-regulated genes were from metabolic (2 genes), cell adhesion (1 gene) and miscellaneous (1 gene) gene categories. A list of differentially expressed genes, their expected functions, and their fold change at 0.5 $\mu\text{g}/\text{cm}^2$ is presented in Table 1.

At the 1.0 $\mu\text{g}/\text{cm}^2$ exposure, we observed oxidative stress response and/or anti-inflammatory (immune) response (4 genes), cell-cycle regulatory (1 gene), signaling (2 genes), DNA repair (2 genes) and transcription regulation (1 gene) genes to be significantly up regulated. Significantly down-regulated genes included metabolic (2 genes), transcription regulation (1 gene) and miscellaneous (5 genes) gene families (See Table 2).

The highest numbers of genes found to have modulated expression within the various exposures occurred at the at 5.0 $\mu\text{g}/\text{cm}^2$ dose. In this category, 25 out of 35 up-regulated, and 12 out of 19 down-regulated, genes were identified. Among the up-regulated genes, oxidative stress response and or inflammatory (immune) response (7 genes), signaling (6 genes), metal-binding (2 genes), cell-cycle regulatory (2 gene), DNA damage or apoptosis related (3 genes), transcription regulation (3 genes) and metabolic (3 genes) were up-regulated. Down-regulated genes included, stress/toxin response (1 gene), signaling (2 gene), cell cycle regulation (1 gene), kinase/apoptosis related (1 genes), transcriptional regulation (3 genes), metabolic (2 genes) and a few miscellaneous (3 genes; Table 3) genes.

In order to visualize the gene expression profiles at each dose, we generated a heatmap using DESeq, where the expression values of the genes was assigned a color based on read counts (Figure 4). As expression moves from higher to lower values, the color changes from red to green (where red represents the higher expressed genes).

Also shown in Figure 4 is a pattern wherein progression from 1.0 $\mu\text{g}/\text{cm}^2$ to 5.0 $\mu\text{g}/\text{cm}^2$ doses, corresponds with a decrease in gene expression related to cell cycle progression and DNA repair. Examples of this include; MUTYH, Ribonucleoside diphosphate reductase subunit M2B (RRM2B) and RECQ4 with -4.7, -2.9, and -6.4 fold down regulation, respectively.

To validate the RNA-seq method we calculated the relative expression levels for each Cr(VI) exposure [0.5, 1.0 and 5.0 ($\mu\text{g}/\text{cm}^2$)] compared to sham using qRT-PCR for six transcripts. In order to compare the two methods, expression values from both analyses were converted to $\log_{10}$ fold change values.

Among the six transcripts selected for qRT-PCR, three genes, CDKN1a, IL8 and HMOX, were up-regulated at all doses compared to the sham treatment based on the RNA-seq analysis. The remaining three; FOSB (transcriptional regulator), RECQ4 (DNA repair) and ZNT-1 (metal transporter), were selected based on their known functional annotations. We were interested in studying the expression patterns for transcriptional regulators, DNA repair genes and metal binding genes to understand their possible role in increased Cr(VI) resistance in whale cells. The expression patterns calculated for all genes using the RNA-seq method had a similar trend at each of the dose comparisons as the qRT-PCR method (Table 4), except for FOSB. The relative expression of FOSB increased at 1.0 $\mu\text{g}/\text{cm}^2$ using the RNA-seq method to analyze FOSB and slightly decreased at the same exposure when analyzed by qRT-PCR. In total, qRT-PCR validated 17 out of the selected 18 dose comparison points in the six selected transcripts.

In order to put the genetic data into context, we treated sperm whale cells with lead chromate and measured the cytotoxic and genotoxic effects. Table 5 summarizes the results.

There was a concentration-dependent decrease in relative survival and an increase in the amount of chromosomal damage with lead chromate treatment.

4. Discussion

Cr(VI) is metabolically reduced to Cr(III) in a step wise process under physiological conditions by reducing agents such as ascorbic acid, glutathione, reductases, etc., (Arslan et al., 1987; Buttner and Beyersmann, 1985). This process generates reactive carbon or oxygen radical species that may induce DNA damage and direct Cr adduct formation within the DNA (Stearns and Wetterhahn, 1994; Stearns et al., 1995).

Cr(VI), itself, has little reactivity with DNA; however, Cr(V), (IV) or (III) may interact with DNA and Cr(III) is considered capable of forming the most stable DNA interactions (Meister, 1994; Stearns et al., 1994; Jacob and Sotoudeh, 2002). Thus, cellular damage induced by Cr may be due to direct Cr-DNA adduct formation or due to ROS produced during reduction. As an outcome of DNA damage, one may expect stimulation of several cellular responses inducing growth factors, cell cycle regulators, DNA repair and select transcription factors.

Induction of anti-oxidant and oxidative response genes such as, HMOX1, GSTO-1, SRXN1, PGHS-2, EGR1 and IL8 along with calcium mediated signaling genes such as, RCAN1, STC1, NKD1, STC1 and EDNRB suggests Cr produces oxidative stress via calcium mediated gene regulation induced from ROS production (Hoyal et al., 1998). These Cr induced calcium signaling genes may be responsible for inducing other pathways such as cell cycle regulatory pathways that precede DNA damage and induction of a DNA repair response. Similar genes have been shown to undergo modulation in their expression patterns upon Cr(VI) exposure in human A549 cells (da Cruz et al., 1995).

One clear difference between the genes expressed at 0.5 $\mu\text{g}/\text{cm}^2$ and 1.0 $\mu\text{g}/\text{cm}^2$ dose is the induction of DNA repair genes (MUTYH, and RecQ4) at the 1.0 $\mu\text{g}/\text{cm}^2$ exposure. This may suggest activation of DNA repair pathways after cellular stress. Such an outcome is consistent with sperm whale cell culture data showing two times more chromosomal damage at 1.0 $\mu\text{g}/\text{cm}^2$ as compared to the damage at 0.5 $\mu\text{g}/\text{cm}^2$ (Wise et al., 2011). For the 1.0 $\mu\text{g}/\text{cm}^2$ exposure we noticed a stronger down-regulation of metabolic gene (KHK), perhaps as an effort to conserve energy for other cellular activities in response to ROS stress or DNA damage.

The 5.0 $\mu\text{g}/\text{cm}^2$ results exhibits more genes significantly up- or down-regulated than the other two Cr doses analyzed. At this dose, there is an increase in the number of induced genes related to apoptotic pathways including genes such as NR4A1, NR4A2, ESM1, and DUSP6. None of the DNA repair genes observed to be induced at the lower doses are significantly up regulated at the 5.0 $\mu\text{g}/\text{cm}^2$ dose. Among the cell cycle regulators, CDKN1A was induced slightly more at the 1.0 $\mu\text{g}/\text{cm}^2$ dose, whereas a positive regulator of cell proliferation (TNS3) was down-regulated. This is consistent with a shift from cellular repair to apoptosis. It is also consistent with sperm whale cell culture data showing twice as much clonogenic cell death at 5.0 $\mu\text{g}/\text{cm}^2$ compared to the lower doses (Wise et al., 2011).

The comparison of relative expressions using qRT-PCR and RNA-seq methods reflected similar patterns for all six selected transcripts at all dose comparisons relative to the sham treatment. CDKN1a expression remained constant for 0.5 and 1.0 $\mu\text{g}/\text{cm}^2$ dose exposures and increased at 5.0 $\mu\text{g}/\text{cm}^2$ Cr(VI) exposure for both RNA-seq and qRT-PCR analysis (Table 4). For both IL-8 and HMOX-1, the expression values increased as Cr(VI) exposure increased for both methods. The DNA repair transcript, RECQ4, had an initial increase in expression at 1.0 $\mu\text{g}/\text{cm}^2$ and then decreased as the Cr(VI) exposure increased from 1.0 $\mu\text{g}/$

cm² to 5.0 µg/cm². This pattern indicates a gradual decrease in DNA repair mechanisms at higher levels of Cr exposure. In addition, results obtained from qRT-PCR for the ZNT-1 gene aligned with the RNA-seq analysis at each exposure tested, further validating the RNA-seq method.

A minor deviation was observed during the analysis of the FOSB transcript at 1.0 µg/cm² using qRT-PCR. A decrease in expression was noted from the qRT-PCR experiment from 0.5 to 1.0 µg/cm², while an increased expression value was observed in the RNA-seq method. This deviation was observed for one Cr(VI) exposure on a single transcript out of the 18 dose comparisons that were analyzed using qRT-PCR.

5. Conclusions

In the current analysis, we constructed a *de novo* reference transcriptome assembly from cultured whale cells that had been exposed to different levels of Cr(VI). We were able to identify genes that showed modulated expression and also validated the observed expression pattern using qRT-PCR.

Our results suggest that as the levels of Cr exposure increase, there is a shift in gene expression patterns from cellular repair mechanisms to apoptosis. Comparisons of gene expression profiles support this shift in expression patterns and show that 5.0 µg/cm² may produce sufficient DNA damage to result in terminal growth arrest for the majority of cells, as suggested previously (Li Chen et al., 2012).

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A *de novo* reference transcriptome assembly has been produced.

RNseq gene expression analyses from whale cells exposed to various Cr(VI) levels was performed.

Genes exhibiting modulated expression were identified and validated using qRT-PCR.

As levels of Cr increase there is a shift in expression patterns from cellular repair to apoptosis.

Results suggest why whale cells are more resistant to Cr than human cells.

Figure 1.

Plot showing K-mer sizes and N50 values for assemblies generated using Velvet-Oases assembler. Note: the highest N50 value is associated with K-mer size of 73 bp.

Figure 2.

DESeq plot showing $\log_2$ fold change expression against the base mean expression for 5.0 $\mu\text{g}/\text{cm}^2$ dose as compared to sham (0.0 $\mu\text{g}/\text{cm}^2$). Red dots represent statistically significant altered regulation for genes at a p-value < 0.01 and $\pm 2 \log_2$ fold change in expression. For example, we label IL8 and PER1 genes which exhibit a $\log_2$ fold change of 7.11 and -3.34 fold, respectively. There are 26 significantly up-regulated and 11 down regulated genes represented by the red dots.

Figure 3. Venn diagram showing genes identified with up or down modulated expression (p-value <0.01) for each of the doses (0.5, 1.0 and 5.0 $\mu\text{g}/\text{cm}^2$) and sham, (0.0 $\mu\text{g}/\text{cm}^2$). (A) Up-regulated genes; 3 genes are common for all dose comparisons, (B) Down-regulated genes; 1 gene is common for all three dose comparisons.

Figure 4.

Heat map representing expression patterns of significantly expressed genes with red being up-regulated and green down-regulated. Comparing the pattern from 1.0 to 5.0 $\mu\text{g}/\text{cm}^2$ we observe a decrease in RECQ4, RRM2B, MUTYH (compared to their expression from 0.5 to 1.0 $\mu\text{g}/\text{cm}^2$).

Table 1

Genes up-regulated (≥ 2 fold) and down-regulated (≤ -2 fold) at p-value <0.01 in whale cultured cells exposed to $0.5 \mu\text{g}/\text{cm}^2$ of Cr (VI).

No.	Genes	Function	Fold Change Up	Fold Change Down
<i>Stress response</i>				
1	Heme Oxygenase (HMOX1)	Oxidative stress response	2.33	
2	Inter-Leukin 8 (IL8)	Inflammatory response	4.19	
<i>Cell adhesion</i>				
3	Cell adhesion molecule 1 (L1CAM)	Cell Adhesion		-3.18
4	Claudin-18 (CLDN18)	Ca ²⁺ independent cell adhesion activity	2.20	
5	Patched domain containing protein1 (PTCHD1)	TNF precursor	2.38	
<i>Cell cycle regulator</i>				
6	Cyclin dependent kinase inhibitor-1A (CDKN1a)	p21, G2/S checkpoint	2.00	
<i>Metabolic</i>				
7	Carbohydrate sulfotransferase C4ST1(CHST11)	11 Carbohydrate metabolism		-2.89
8	Ketohexokinase (KHK)	Glucose/Fruktose kinase		-3.59
<i>Miscellaneous</i>				
9	Protein HIDE1	Uncharacterized	4.71	
10	SERPINB5	Endopeptidase inhibitor	2.18	
11	WD repeat domain 54 (WDR5)	4) Uncharacterized	4.37	

Table 2

Genes up-regulated (≥ 2 fold) and down-regulated (≤ -2 fold) at a p-value <0.01 in cultured whale cells exposed to $1.0 \mu\text{g}/\text{cm}^2$ of Cr (VI).

No.	Genes	Function	Fold Change Up	Fold Change Down
<i>Stress response</i>				
1	Brain natriuretic peptide (BNP)	Immune response	4.25	
2	Glyceraldehyde-3-phosphate dehydrogenase (B3GAT2)	Oxidoreductase	4.27	
3	HMOX1	Oxidative stress response	2.80	
4	IL8	Inflammatory response	5.49	
<i>Signaling</i>				
5	Chemokine-6 (CXCL6)	Cell signaling/immune response	2.41	
6	Endothelin receptor type-B (EDNRB)	Calcium mediated secondary messenger	2.11	
<i>Cell cycle regulator</i>				
7	CDKN1a	p21, G2/S checkpoint	2.03	
<i>DNA repair</i>				
8	Adenine DNA glycosylase (MUTYH)	DN(base excision repair gene) A adenine glycosylase	7.02	
9	DNA helicase Q4 (RECQ4)ATP dependent	Helicase	8.58	
<i>Transcription regulator</i>				
10	Prospero homeobox protein 1 (PROX1)	Transcription factor activity		-2.06
11	Zinc finger protein (ZNF398)	Transcriptional activator	4.94	
<i>Metabolic</i>				
12	KHK	Glucose/Fructose kinase		-6.06
13	Lactase phlorizin hydrolase (LCT)	Carbohydrate metabolism		-2.97
<i>Miscellaneous</i>				
14	NCK5	Endopeptidase inhibitor		-2.59
15	ProteinCLEC16A (CLEC16A)	Uncharacterized		-2.73
<i>Miscellaneous</i>				
16	SET-binding protein (SETBP1)	Uncharacterized		-2.44
17	Tetraco peptide 28 (TTC28)	Uncharacterized		-3.79
18	UPF0705 protein C11orf49 homolog (C11orf49)	Uncharacterized		-2.35

Table 3

Genes up-regulated (≥ 2 fold) and down-regulated (≤ -2 fold) (p-value <0.01) fold change in whale cell cultures exposed to $5.0 \mu\text{g}/\text{cm}^2$ of Cr (VI).

No.	Genes	Function	Fold Change Up	Fold Change Down
<i>Stress response</i>				
1	5 Hydroxy tryptamine receptor 1-D (5HTR1D)	Toxin response		-2.05
2	BNP	Immune response	3.94	
3	Early growth response 1 (EGR1)	Stress response	2.66	
4	Glutathione S-transferase omega-1 (GSTO1)	Anti-oxidant	2.04	
5	HMOX1	Oxidative stress response	3.79	
6	IL8	Inflammatory response	7.11	
7	Prostaglandin G/H synthase-2 (PGHS-2)	Anti-oxidant, peroxidase	3.04	
8	Sufiredoxin-1 (SRXN1)	Oxidative stress response	2.28	
<i>Signaling</i>				
9	Bestrophin 1 (BEST1)	Ca ²⁺ mediated chloride channel	2.41	
10	Calciopressin-1 (RCAN1)	Activating NFAT in response to calcium mobilization	3.02	
11	CXCL6	Cell signaling/immune response	2.33	
12	EDNRB	Calcium mediated secondary messenger	2.60	
13	Follistatin (FST)	Inhibit follicle stimulating hormone	2.68	
14	Naked cuticle homolog (NKD1)	Ca ²⁺ binding control Wnt signaling		-2.30
15	cGMP dependent protein kinase (PRKG1)	Serine/threonine-protein kinase		-2.11
16	Stanniocalcin-1 (STC1)	TNF precursor	2.00	
<i>Metal binding genes</i>				
17	Transferrin receptor TFRC	Maintains Iron homeostasis	2.10	
18	Zinc transporter (ZNT1)	Ion transport	2.10	
<i>Cell cycle regulator</i>				
19	CDKN1a	p21, G2/S checkpoint	2.67	
20	Endothelial cell specific molecule-1 (ESM1)	Angiogenesis	3.35	
21	Tensin 3 (TNS3)	Actin remodeling (Cell proliferation)		-2.08
<i>DNA damage</i>				
22	Dual specificity protein phosphatase 6 (DUSP6)	Inactivate MAPkinase	2.23	
23	Nuclear receptor subfamily group 4 member 1 (NR4A1)	Helicase	2.37	
24	Nuclear receptor subfamily group 4 member 2 (NR4A2)	DNA adenine glycosylase (base excision repair gene)	2.60	
<i>Transcription regulator</i>				

No.	Genes	Function	Fold Change Up	Fold Change Down
<i>Stress response</i>				
25	3-phosphoinositide-dependent protein kinase 1 (PDPK1)	Transcription regulation		-2.80
26	Early growth response -2 (EGR2)	Transcriptional factor	3.47	
27	Fbj murine associate viral oncogene protein homolog B (FOSB)	Jun promoter	4.64	
28	Mastermind like domain containing protein-1 (MAMLD1)	Promoter of HES3 which inhibit protein with bHLH domain	2.68	
29	Nuclear factor 1A type (NFIA)	DNA dependent transcription regulation		-2.80
30	Period circadian protein homolog-1 (PER1)	DNA dependent transcription regulation		-3.34
<i>Metabolic</i>				
31	Abhydrolase domain containing 5 (ABHD5)	Hydrolase		-3.34
32	KHK	Gluco/Frukto kinase	3.47	
33	Pyruvate dehydrogenase kinase isozyme 4 (PDK4)	Carbohydrate metabolism	2.68	
<i>Miscellaneous</i>				
34	Leucin rich repeats and immunoglobulin protein 16 (LRIG16)	Uncharacterized		-2.04
35	Leucin rich repeats and immunoglobulin protein 3 (LRIG3)	Uncharacterized		-2.80
36	NCK5	Uncharacterized		-2.47
37	Transmembrane protein (TM200a)	Uncharacterized		-2.57

Table 4
Relative expression using RNA-seq analysis and qRT-PCR as compared to sham 0.0 µg/cm²

Transcript	RNAseq expression values (log ₁₀)			qRT-PCR expression value (log ₁₀)			
	Dose	0.5 (µg/cm ²)	1 (µg/cm ²)	5 (µg/cm ²)	0.5 (µg/cm ²)	1 (µg/cm ²)	5 (µg/cm ²)
CDKN1a		0.57	0.61	0.80	1.19 ± (8.96 e ⁻³)	1.26 ± (8.59 e ⁻³)	1.43 ± (7.48 e ⁻³)
IL8		1.26	1.65	2.14	1.44 ± (9.67 e ⁻³)	1.81 ± (2.08 e ⁻²)	2.14 ± (1.49 e ⁻²)
HMOX1		0.70	0.84	1.14	1.63 ± (2.39 e ⁻³)	2.08 ± (7.58 e ⁻³)	2.41 ± (6.58 e ⁻³)
RECQ4		0.81	2.58	0.63	0.72 ± (3.14 e ⁻³)	1.32 ± (7.26 e ⁻³)	0.81 ± (1.62 e ⁻³)
ZNT1		0.33	1.47	0.51	1.17± (1.17 e ⁻²)	1.71 ± (7.20 e ⁻³)	1.32 ± (5.35 e ⁻³)
FOSB		0.75	1.20	1.38	1.04 ± (1.79 e ⁻²)	0.79 ± (9.84 e ⁻³)	1.51 ± (7.08 e ⁻³)

Table 5
Lead Chromate Induced Cytotoxicity and Genotoxicity

Lead Chromate Concentration ($\mu\text{g}/\text{cm}^2$)	Relative Survival (Percent of Control)	Chromosome Damage	
		Percent of Metaphases with Damage	Total Damage in 100 Metaphases
0.0	<i>a</i>	2 ± 0.5	2 ± 0.5
0.05	84.5 ± 9.5	ND ^b	ND
0.1	85.5 ± 0.5	ND	ND
0.5	63 ± 9.0	6 ± 0.8	7 ± 1.1
1.0	56 ± 15.0	12 ± 1.3	13 ± 1.7
5.0	25.6 ± 13.9	18 ± 5.0	19 ± 5.3

^a control is the reference value so by default it is 100%

^b ND = not done