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Research Article

Chemical Exposure Generates DNA Copy Number Variants and Impacts Gene Expression

Samuel M. Peterson[1,2] and Jennifer L. Freeman[1]

[1]*School of Health Sciences, Purdue University, 550 Stadium Mall Drive, HAMP-1263D, West Lafayette, IN 47907, USA*
[2]*Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA*

Correspondence should be addressed to Jennifer L. Freeman; jfreema@purdue.edu

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DNA copy number variation is long associated with highly penetrant genomic disorders, but it was not until recently that the widespread occurrence of copy number variation among phenotypically normal individuals was realized as a considerable source of genetic variation. It is also now appreciated that copy number variants (CNVs) play a role in the onset of complex diseases. Many of the complex diseases in which CNVs are associated are reported to be influenced by yet to be identified environmental factors. It is hypothesized that exposure to environmental chemicals generates CNVs and influences disease onset and pathogenesis. In this study a proof of principle experiment was completed with ethyl methanesulfonate (EMS) and cytosine arabinoside (Ara-C) to investigate the generation of CNVs using array comparative genomic hybridization (CGH) and the zebrafish vertebrate model system. Exposure to both chemicals resulted in CNVs. CNVs were detected in similar genomic regions among multiple exposure concentrations with EMS and five CNVs were common among both chemicals. Furthermore, CNVs were correlated to altered gene expression. This study suggests that chemical exposure generates CNVs with impacts on gene expression warranting further investigation of this phenomenon with environmental chemicals.

1. Introduction

Structural genetic variation in the human genome is present in many forms including single nucleotide polymorphisms (SNPs), variable tandem repeats (e.g., mini- and microsatellites), presence/absence of transposable elements, and structural alterations (e.g., deletions, duplications, and inversions). Until recently, SNPs were thought to be the predominant form of genomic variation and to account for much of the normal phenotypic variation [1]. Recent developments and applications of genome-wide technologies led to the discovery of thousands of copy number variants (CNVs) in the genomes of phenotypically normal humans [2, 3]. CNVs are defined as a duplication or deletion (i.e., a gain or loss of a genomic DNA segment relative to a reference sample) measuring greater than 1 kb in size [4]. Human genomic copy number variation has been studied for over 40 years, but it was assumed that CNVs were few in number, had a relatively limited impact on the total amount of human genetic variation, and were mainly associated with highly penetrant disease phenotypes. In 2004, two studies independently reported the widespread presence of CNVs in the genomes of phenotypically normal individuals [2, 3]. Following these initial studies, additional genome-wide analyses identified and characterized novel human CNVs (e.g., [5]). Widespread copy number variation in the human genome is now well documented with many CNVs spanning genes that are likely to affect gene networks [6]. CNVs result in various phenotypic effects including changes in gene expression levels, disruption of gene dosage and regulatory elements, and loss of regulatory elements [7].

Classical cytogenetics identified a variety of genomic variants that are related to disorders that are caused by a single variant (e.g., a deletion on chromosome 7 in Williams Beuren syndrome). CNVs that did not directly result in early-onset, highly penetrant genomic disorders were initially considered neutral in function, but CNVs are now appreciated to play a role in the onset of complex diseases including autism spectrum disorder (ASD), attention-deficit hyperactivity disorder

(ADHD), and schizophrenia [8, 9]. In addition, CNVs are reported to influence late-onset diseases (e.g., Alzheimer's disease and Parkinson's disease). In addition to genetic factors, these diseases are also implicated to be influenced by environmental factors. The mechanisms by which environmental factors influence onset and pathogenesis of these diseases are not completely understood [10]. Current analysis of functional attributes of CNV regions is revealing enrichment for genes that are relevant to molecular-environmental interactions [3, 5]. Moreover, a study in postmortem brains of individuals with ASD indicates possible involvement of exposure to polychlorinated biphenyls (PCBs) with a duplication event on human chromosome 15 [11]. This study indicates an environmental link with CNVs and influence on complex disease, but it is not known if the copy number alteration was specifically generated by the environmental chemical exposure.

Exposure to environmental chemicals is one environmental factor that may contribute to the formation of CNVs (or copy number aberrations), but the ability of chemical exposure to generate CNVs has not been thoroughly investigated. With the development of genomic technologies including the array comparative genomic hybridization (CGH) technology and NextGen sequencing, copy number alterations are now efficiently detected throughout the genome. Previous assays and techniques applied to investigate the influence of chemical exposure on the genome were limited to detecting single nucleotide mutations or larger chromosomal aberrations (Figure 1). In addition, many of these assays had inefficient integration of structural DNA alterations with the reference genome sequence limiting further studies into the biological and functional significance of these DNA alterations. Thus, this class of DNA alteration was not thoroughly assessed in past genotoxicity studies. Three recent studies began to investigate the generation of CNVs with aphidicolin, hydroxyurea, and ionizing radiation in a cell culture system [12–14], but no other agents have been investigated to date.

The importance of using genomics to identify environmental chemical influence on the human genome is now recognized [15] and the specific influence of CNVs is recognized as an emerging environmental health issue (http://nas-sites.org/emergingscience/meetings/genomic-plasticity/). In this study, a proof of principle experiment using a zebrafish cell line was completed to test the hypothesis that chemical exposure will result in CNVs detectable with the use of array CGH technology to set the stage for future analysis into the influence of environmental chemical exposure in generating CNVs. The zebrafish is a prominent model vertebrate system in a variety of biological disciplines. A finished genome sequence and conserved genetic function between the zebrafish and human genomes permit translation of molecular mechanisms of toxicity observed in the zebrafish model system to humans [16, 17]. Several zebrafish orthologs are reported to play a key role in human disease and large-scale mutant screens demonstrate that mutations in some of these orthologs display phenotypes similar to those present in human diseases [18, 19]. In addition, the zebrafish has been used for many years as a toxicological model [20] and a model for DNA repair mechanisms (e.g., [21]). A CNV map is established for the zebrafish genome and confirms the plasticity of the zebrafish genome permits CNV formation [22] and suitability for application in this study. Two genotoxic chemicals commonly used as reference chemicals, ethyl methanesulfonate (EMS), and cytosine arabinoside (Ara-C) were included in this proof of principle experiment at multiple exposure concentrations to assess dose-response and differences among the two chemicals. In addition, global gene expression analysis was completed to correlate CNVs caused by chemical exposure and alterations in gene expression.

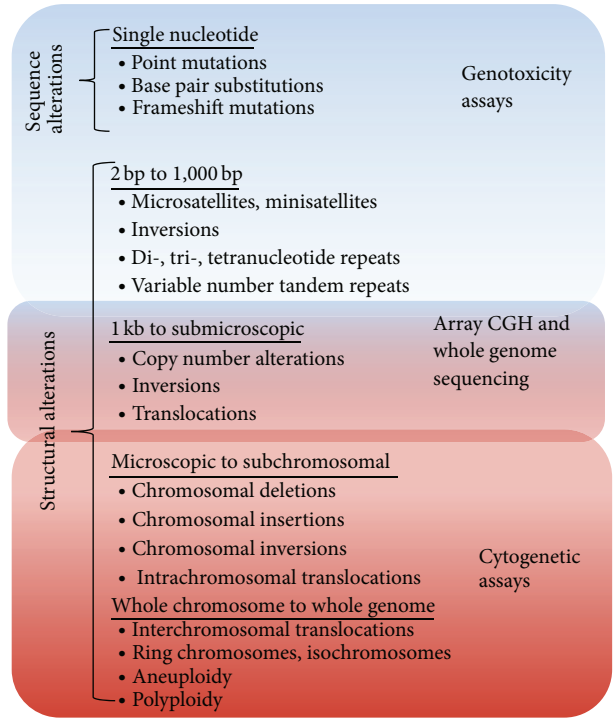

Figure 1: Toxicity assays interrogating DNA sequence alterations by size. Classic cytogenetic methodologies routinely identify whole genome, whole chromosomal, and microscopic structural chromosomal aberrations. At the opposite end of the spectrum, mutation assays are optimized to detect single base pair mutations. Development of genome-wide technologies including array-based assays (e.g., array CGH) and whole genome sequencing now permit efficient detection of DNA structural alterations of an intermediate size including copy number alterations and enable direct integration with a reference genome sequence. As a result, the ability of chemical exposure to induce this type of DNA alteration was not thoroughly investigated in the past and is just now beginning to be addressed.

2. Materials and Methods

2.1. Cell Line and Toxicity Assay. A zebrafish fibroblast cell line established from approximately 100 embryos of the AB zebrafish strain that is described in detail in Freeman et al. [23] was used in this study. This zebrafish cell line was used in this proof of principle study since it is well-characterized, is routinely monitored for cytogenetic changes, and has been used in previous zebrafish cytogenetic studies [23]. In addition, use of this zebrafish cell line will provide ease in moving into *in vivo* studies with *in vivo* zebrafish

in future studies. Two reference chemicals routinely used in genotoxicity assays, ethyl methanesulfonate (EMS; CAS 62-50-0; Sigma, St. Louis, MO) and cytosine arabinoside (Ara-C; CAS 147-94-4; Sigma, St. Louis, MO), were investigated for potential to generate CNVs. A cell confluency assay was first completed to identify the toxicity of EMS and Ara-C in this cell line. This assay is modified from Plewa et al. [24]. Briefly, cells were harvested from cell culture flasks following a standard trypsin protocol and cell concentration determined. The assay was set up in 96-well plates with 7,000 cells per well in an appropriate volume of media and chemical stock to achieve desired chemical test concentrations. Plate set-up included a first column blank and a second column negative control. Each plate contained four subsample wells per chemical concentration. Following set-up, plates were placed in an incubator at 28°C and 5% CO_2. After 72 hours, the cells were fixed in 50% methanol and stained with 1% crystal violet in 50% methanol, and excess crystal violet solution was washed from the plate. The cells were then treated with 1% SDS to bring the crystal violet back into solution. The absorbance of each well was read on a microplate reader at 595 nm and readings from the four subsample wells of each test concentration averaged. A percent negative control value was calculated for each test concentration. This value represents the confluency of the cells grown in the presence of the test compound as compared to the unexposed control cells. Three replicate plates were completed and the average percent negative control values of the three replicate plates for each test concentration calculated, plotted, and fit a sigmoidal curve. The 50% and 75% confluency value of the negative control was calculated for each chemical to determine test concentrations for array CGH analysis. These values were chosen to be able to compare if CNVs would be generated from exposure treatments that ranged from 50% impacts on cell confluency to exposure treatments that did not alter cell confluency in the EMS experiment and to then choose exposure treatments that did not impact cell confluency in the Ara-C experiment.

2.2. Copy Number Analysis. A zebrafish-specific oligonucleotide platform was designed and printed in conjunction with Roche NimbleGen (Madison, WI) for this study to analyze copy number changes. The zebrafish oligonucleotide platform was manufactured using Roche NimbleGen's proprietary Maskless Array Synthesizer technology using photomediated synthesis chemistry. DNA probes were selected using a proprietary probe screening system. T_m-balanced probe selection was coupled with heuristic and Al predictive methods derived from their experimental database. Probe sets were selected to represent the genomic target and to have excellent hybridization characteristics. Specifically for this design, segmental duplications (i.e., regions of the genome with up to 5 close matches) were included as some copy number alterations are reported to be associated with these genomic segments [5] and highly repetitive sequences were excluded. In addition, a number of standards were also included throughout the array. A number of self to self-hybridizations were first conducted with this platform to assess the performance of the array platform and to determine resolution of platform.

Array CGH analysis was performed similarly as described in Peterson and Freeman [25]. Zebrafish cells were exposed to three chemical concentrations of EMS calculated from the cytotoxicity curve to represent (1) a concentration 50% of the negative control value, (2) a concentration 75% of the negative control value, and (3) a concentration where no cytotoxic impacts were observed for a dose-response assessment and a corresponding negative control without chemical exposure. Two exposure concentrations with limited cytotoxicity were included for Ara-C. Cells were harvested from maintenance cultures and cell concentration determined. Appropriate volume of media and chemical stock was added to each petri dish to achieve the desired test concentrations (i.e., 0 mM, 0.5 mM, 2 mM, and 5 mM for EMS and 0 μM, 0.1 μM, and 1 μM for Ara-C). 7.5×10^5 cells were initially seeded into each dish. After set-up, petri dishes were placed in an incubator at 28°C and 5% CO_2 for 72 hours (the equivalent of 1.5 cell cycle lengths). After 72 hours, cells were harvested and genomic DNA was isolated following a standard phenol: chloroform isolation method as described in Freeman et al. [26]. Genomic DNA quantity and quality were determined using a NanoDrop ND-1000 and gel electrophoresis. The negative control was the reference sample for each replicate and was cohybridized with each treatment (i.e., the negative control and the three treatment concentrations) using a two-color hybridization strategy. Genomic DNA samples were labeled and subsequently hybridized upon the zebrafish array CGH platform using the protocol outlined in the Roche NimbleGen User's Guide (Roche NimbleGen, Madison, WI). For each array hybridization, 1 μg of test DNA and 1 μg of reference DNA were fluorescently labeled with dye-labeled 9 mers (i.e., the test DNAs were labeled with Cy3 and the reference DNA was labeled with Cy5). Dye-incorporation and DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer. Cy3-labeled test DNA and Cy5-labeled reference DNA was combined into one tube for each test concentration and injected into a mixer attached to the array CGH chip as described in the NimbleGen Array User's Guide. The chip was placed in a bay of the NimbleGen Hybridization System and DNA hybridized for 16 hours at 42°C. Following hybridization, the arrays were washed in solutions supplied in the Roche NimbleGen wash buffer kit followed by spin drying of the slides in a microfuge slide dryer.

Hybridized arrays were scanned using two-color scanning for Cy3 and Cy5 at 5 microns on a GenePix 4000B (Molecular Devices, Sunnyvale, CA). Scans were optimized for Cy3 and Cy5 signal intensities in the same range and for ~1% of the features saturated. Array image data was extracted using the NimbleScan software program (Roche NimbleGen, Madison, WI). The Cy3 and Cy5 signal intensities were normalized to one another using qspline normalization, a simple and robust nonlinear method of normalization for two-color experiments [27]. Normalized signal intensity files were generated by NimbleScan. Internal control probes and overall variation of signal intensity were used to assess the quality of each array CGH experiment.

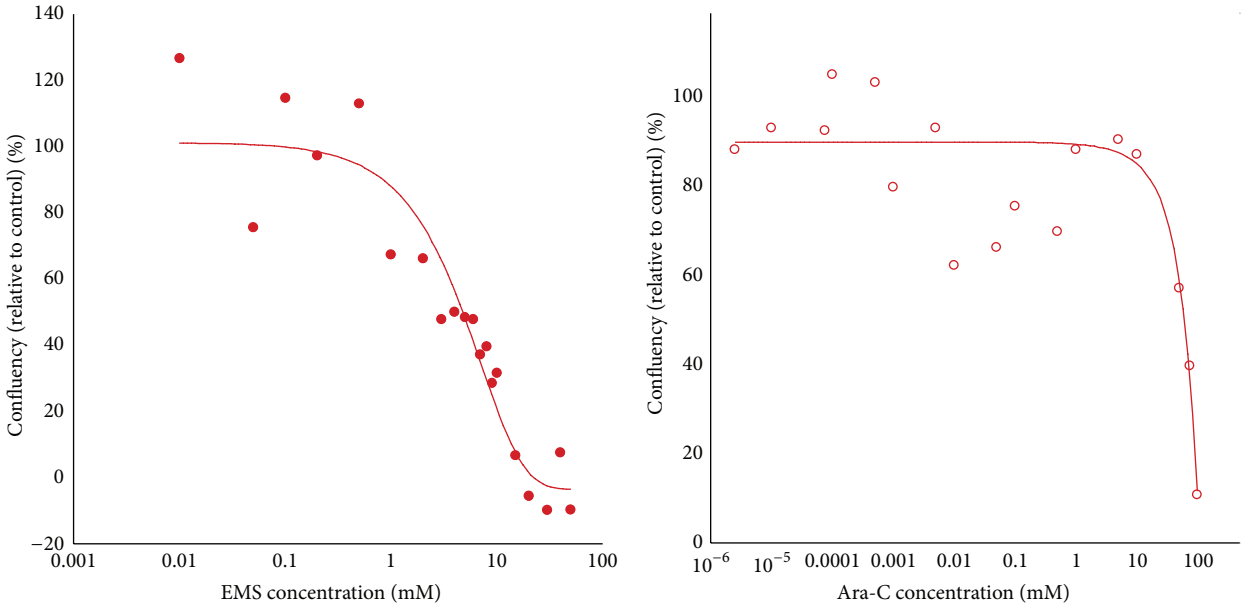

Figure 2: Cell toxicity assay. The toxicity profile of (a) EMS and (b) Ara-C was first completed in the zebrafish cell line to determine impacts on cell confluency in this specific cell line. From this analysis chemical concentrations were determined for array CGH experiments.

The NimbleScan data was then exported into the Nexus Copy Number software to calculate DNA sequence regions that deviated from the expected 1 : 1 molar ratio of the test to reference DNA ($\log_2$ ratio of 0) similar to as reported previously [22]. Called regions represent CNVs as a result of chemical exposure. Genomic locations and magnitude of gain/loss were compared among the experiments and among the chemical treatments. Genomic locations of CNVs were integrated with the zebrafish reference sequence for characterization. Genomic location of CNVs was compared among samples and overlapping segments determined. Two separate experiments were completed for each chemical.

2.3. Global Gene Expression Analysis. To investigate the impacts of CNVs caused by chemical exposure on gene expression, global gene expression analysis was performed with RNA isolated from a 2 mM EMS exposure and a control treatment following similar procedures as described previously [28]. The 2 mM EMS treatment was chosen as an exposure that had a minor effect on confluency of the cells and at which a number of CNVs were detected. Three biological replicates were included that consisted of three separate control samples and three separate samples treated with 2 mM EMS. Microarray analysis was performed similarly to as described in Peterson et al. [28] with the zebrafish 385 K expression platform (Roche NimbleGen, Madison, WI) using the one-color hybridization strategy. As such, six different microarrays were hybridized for this analysis. This platform contains 385,000 60-mer probes interrogating 37,157 targets with up to 12 probes per target. Following hybridization, arrays were washed and scanned at 5 microns using a GenePix 4000B array scanner (Molecular Devices, Sunnyvale, CA). Array image data was extracted using the NimbleScan software program (Roche NimbleGen, Madison, WI). Fluorescence signal intensities were normalized using quantile normalization [29] and gene calls generated using the Robust Multichip Average algorithm [30] following manufacturer recommendations. Further statistical processing of the array data was performed with Array Star (DNASTAR, Inc., Madison, WI) and Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) to identify specific genes altered following EMS exposure. A robust and reproducible list of differentially expressed genes using recommendations from the Microarray Quality Consortium [31, 32] was first determined by genes consistently expressed (Students t-test, $P < 0.05$) and substantially altered with a fold change of ±2.0. Genomic location of genes with altered expression was compared to the genomic location of CNVs and gene ontology analysis and molecular pathway analysis completed using UCSC Genome Browser (http://www.genome.ucsc.edu/) and Ingenuity Pathway Analysis (IPA) software following similar parameters as in previous experiments [28]. All genes were converted and reported as human homologs.

3. Results

3.1. Cell Toxicity. The toxicity of EMS and Ara-C in the zebrafish cell line was first investigated and results were used to determine the exposure concentrations for the array CGH analysis. Test concentrations were calculated at the 50% negative control value, at 75% negative control value, and at a concentration where no impacts on cell confluency were observed for a dose-response assessment in the array CGH analysis for EMS (Figure 2(a)). 5 mM, 2 mM, and 0.5 mM, were chosen as test concentrations for EMS, respectively.

Table 1: Genome coverage of the zebrafish array CGH platform.

Seq. ID	Number of probes	Mean interval (bp)	Median interval (bp)	Coverage (bp)
Chr1: 1–56204684	16932	3204	3208	956774
Chr2: 1–54366722	16410	3204	3208	925430
Chr3: 1–62931207	18916	3204	3209	1066623
Chr4: 1–42602441	12659	3204	3209	711800
Chr5: 1–70371393	21149	3204	3209	1192946
Chr6: 1–59200669	17707	3204	3209	1000358
Chr7: 1–70262009	21110	3204	3208	1193208
Chr8: 1–56456705	16885	3204	3209	953452
Chr9: 1–51490918	15560	3204	3208	879929
Chr10: 1–42379582	12784	3204	3208	722041
Chr11: 1–44616367	13512	3204	3209	763782
Chr12: 1–47523734	14322	3204	3209	808732
Chr13: 1–53547397	16177	3204	3208	913627
Chr14: 1–56522864	16923	3205	3211	951969
Chr15: 1–46629432	14027	3204	3209	790586
Chr16: 1–53070661	16002	3204	3208	902893
Chr17: 1–52310423	15830	3204	3208	893908
Chr18: 1–49281368	14898	3204	3209	841550
Chr19: 1–46181231	13923	3204	3209	786221
Chr20: 1–56528676	16867	3204	3210	951071
Chr21: 1–46057314	13802	3205	3210	778363
Chr22: 1–38981829	11629	3204	3210	653771
Chr23: 1–46388020	14049	3204	3208	792579
Chr24: 1–40293347	12198	3204	3208	688239
Chr25: 1–32876240	9860	3204	3209	554616
Summary	**384131**	**3204**	**3209**	**21674468**

In addition, two concentrations with limited impacts to cell confluency were included for Ara-C (0.1 μM and 1 μM; Figure 2(b)).

3.2. CNVs following Chemical Exposure. The zebrafish oligonucleotide array CGH platform contains 385,000 probes, approximately 50 to 75 nucleotides in length, tiling the zebrafish genome with a median spacing of ~3.2 kb (Table 1). Four self to self-hybridization experiments were first conducted to assess the performance of the platform and to determine the resolution of the platform. No calls were found to present in these self to self-hybridization experiments greater than 5 consecutive probes in length (~12.8 kb) and the resolution of the platform was estimated at 16 kb (6 consecutive probes in length). All oligonucleotide array-based platforms have some degree of background noise, which varies among each specific platform. As a result there is generally a lack of confidence in single probe calls. Evaluating a series of self to self-hybridizations (in which no copy number alterations should be observed) and confirmatory experiments for calls observed on this platform, we determined that calls containing at least

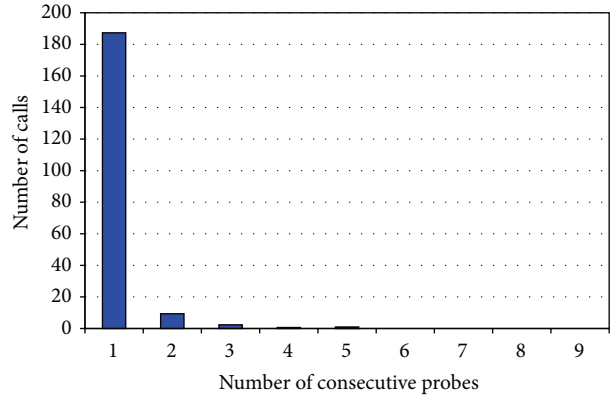

Figure 3: Self to self-hybridization assessment. A series of four self to self-hybridizations were conducted to assess the performance of the array CGH platform to determine the number of consecutive probes that are needed to have high confidence in a true call. From these experiments, it was determined that high confidence is attained in calls in which at least six consecutive probes significantly deviate from the expected 1 : 1 molar ratio. As a result, resolution of this platform is approximately 16 kb.

6 consecutive probes have a high degree of confidence (Figure 3). Using these calling parameters, the number of false positive calls was significantly decreased. In addition, calls had an average segmentation mean ±0.075 or greater in magnitude.

For assessment of EMS, two separate experiments were performed using the two-color hybridization strategy. Genomic DNA from each chemical treatment was cohybridized with the respective negative control as the reference sample. In the first experiment, 5, 17, and 1 CNVs were called in the 0.5, 2, and 5 mM treatments, respectively (Table 2). In the second experiment, 10, 0, and 11 CNVs were called in the 0.5, 2, and 5 mM treatments, respectively (Table 2). In total 44 CNVs were called with a loss in copy number in 28 regions and a gain in copy number in 16 regions. CNVs ranged from 19.8 to 7,069.8 kb in size. The number of CNVs did not increase with dose, but there were 11 CNVs with overlapping genomic locations among the EMS chemical treatments (Table 3). All overlapping CNVs agreed in their respective loss or gain in copy number furthering confidence in these calls. In addition, magnitude of change was also similar among the overlapping CNVs. Overall 8 overlapping CNVs had a loss in copy number, while 3 overlapping CNVs had a gain in copy number. The overlapping genomic segments were refined regions with a size ranging from 33.5 to 1,283.8 kb. Three of the overlapping CNVs were in three different samples including a loss on chromosome 4, a loss on chromosome 5, and a gain on chromosome 14 (Figure 4), while the remaining were present in two samples (Table 3). Considering overlapping CNVs, a total of 29 different copy number variable regions were present among the EMS treatments (Figure 5(a)). When CNVs generated by EMS exposure were compared to CNVs found in the AB strain of zebrafish (the strain from which the cell line was derived) 39% of the CNVs were found to overlap (Table 2).

Table 2: CNVs generated by EMS exposure.

Experiment	Concentration (mM)	Chromosome	Start (bp)	End (bp)	Length (kb)	Segmentation mean	Overlap with CNVEs[a]
1	0.5	4	34804051	35101102	297.1	0.092	No
1	0.5	8	12592572	13822847	1230.3	−0.088	Yes
1	0.5	9	50187567	50303563	116.0	0.248	No
1	0.5	14	16605821	16732619	126.8	0.276	Yes
1	0.5	24	35425802	35674998	249.2	−0.173	No
1	2	4	22583174	22620167	37.0	−0.792	No
1	2	5	32488684	32820694	332.0	−0.222	No
1	2	5	64239559	64293196	53.6	−0.391	Yes
1	2	6	49556168	50808094	1251.9	−0.199	No
1	2	7	48895436	48948526	53.1	0.583	Yes
1	2	8	32921651	33404812	483.2	−0.321	No
1	2	9	50190782	50306820	116.0	0.445	No
1	2	13	14841216	14864686	23.5	0.705	No
1	2	13	40036069	40492860	456.8	0.151	Yes
1	2	14	16612394	16785422	173.0	0.361	Yes
1	2	16	17135247	18061907	926.7	0.157	No
1	2	18	31915173	33340460	1425.3	−0.235	Yes
1	2	20	8220598	8240372	19.8	0.799	No
1	2	20	16618414	16980789	362.4	0.196	No
1	2	21	8967733	9185499	217.8	0.244	Yes
1	2	21	21053248	21398259	345.0	−0.228	No
1	2	25	31027622	31064013	36.4	−0.579	No
1	5	14	16609009	16769181	160.2	0.282	Yes
2	0.5	4	22586693	22620167	33.5	−0.739	No
2	0.5	5	32662667	32817496	154.8	−0.441	No
2	0.5	7	87	418056	418.0	−0.182	Yes
2	0.5	8	12566432	13625424	1059.0	−0.138	No
2	0.5	13	51315349	51444199	128.9	0.363	No
2	0.5	14	27806152	34875942	7069.8	−0.079	Yes
2	0.5	18	31506143	31908755	402.6	0.117	No
2	0.5	18	43478909	43530599	51.7	−0.521	No
2	0.5	19	8399336	10004277	1604.9	−0.109	No
2	0.5	24	35472028	35709297	237.3	−0.231	No
2	5	4	22586693	22624474	37.8	−0.905	No
2	5	5	32481902	32817496	335.6	−0.398	No
2	5	5	37581072	38022638	441.6	−0.226	No
2	5	5	64165549	65694964	1529.4	−0.139	Yes
2	5	6	49600941	50828098	1227.2	−0.268	No
2	5	7	87	355049	355.0	−0.285	Yes
2	5	8	22262700	23108696	846.0	−0.227	Yes
2	5	13	51328104	51486986	158.9	0.482	No
2	5	14	31449275	33739196	2289.9	−0.159	Yes
2	5	18	27977948	31396054	3418.1	−0.161	Yes
2	5	18	31911993	33198929	1286.9	−0.260	Yes

[a]CNVEs as called in AB strain zebrafish in [22].

Table 3: Common copy number variable regions among EMS treatments.

Genomic region of overlap	Size (kb)	Concentration (mM)	CNV genomic region	Length (kb)	Segmentation mean
Chr4: 22586693–22620167	33.5	0.5	Chr4: 22586693–22620167	33.5	−0.739
		2	Chr4: 22583174–22620167	37.0	−0.792
		5	Chr4: 22586693–22624474	37.8	−0.905
Chr5: 32662667–32817496	154.8	0.5	Chr5: 32662667–32817496	154.8	−0.441
		2	Chr5: 32488684–32820694	332.0	−0.222
		5	Chr5: 32481902–32817496	335.6	−0.398
Chr5: 64239559–64293196	53.6	2	Chr5: 64239559–64293196	53.6	−0.391
		5	Chr5: 64165549–65694964	1529.4	−0.139
Chr6: 49600941–50808094	1207.2	2	Chr6: 49556168–50808094	1251.9	−0.199
		5	Chr6: 49600941–50828098	1227.2	−0.268
Chr7: 87–355049	355.0	0.5	Chr7: 87–418056	418.0	−0.182
		5	Chr7: 87–355049	355.0	−0.285
Chr8: 12592572–13625424	1032.9	0.5	Chr8: 12566432–13625424	1059.0	−0.138
		0.5	Chr8: 12592572–13822847	1230.3	−0.088
Chr9: 50190782–50303563	112.8	0.5	Chr9: 50187567–50303563	116.0	0.248
		2	Chr9: 50190782–50306820	116.0	0.445
Chr13: 51328104–51444199	116.1	0.5	Chr13: 51315349–51444199	128.9	0.363
		5	Chr13: 51328104–51486986	158.9	0.482
Chr14: 16612394–16732619	120.2	0.5	Chr14: 16605821–16732619	126.8	0.276
		2	Chr14: 16612394–16785422	173.0	0.361
		5	Chr14: 16609009–16769181	160.2	0.282
Chr18: 31915173–33198929	1283.8	2	Chr18: 31915173–33340460	1425.3	−0.235
		5	Chr18: 31911993–33198929	1286.9	−0.260
Chr24: 35472028–35674998	203.0	0.5	Chr24: 35425802–35674998	249.2	−0.173
		0.5	Chr24: 35472028–35709297	237.3	−0.231

Similar to the EMS experiments, two separate experiments were completed for Ara-C. In the first experiment, 1 CNV was called in the 0.1 μM treatment, while 2 CNVs were called in the 1 μM treatment. In the second experiment no CNVs were called in the 0.1 μM treatment and 15 CNVs were called in the 1 μM treatment (Table 4). Of the 18 total CNVs, 5 were losses and 13 were gains in copy number and ranged from 28.9 to 1,505.2 kb in size (Figure 5(b)). When CNVs generated by Ara-C exposure were compared to CNVs found in the AB strain of zebrafish 44% of the CNVs were found to overlap (Table 4). There was no overlapping CNVs among the two concentrations or among the two experiments, but 5 CNVs did overlap with CNVs in the EMS experiment (Table 5). The length of these CNVs in the Ara-C and EMS treatments was similar. Two CNV regions on chromosomes 9 and 14 had consistent gains in both chemical treatments. Two CNV regions on chromosomes 5 and 6 had a loss in the EMS treatment and a gain in copy number following Ara-C treatment, while the final CNV region on chromosome 21 had a gain in the EMS treatment and a loss in the Ara-C treatment.

3.3. Comparative Gene Expression Analysis. To elucidate impacts of CNVs on gene expression, global gene expression analysis was completed with the 2 mM EMS treatment. After removal of redundant probes and accounting for gene orthology a total of 1,146 genes were mapped with altered expression. 979 genes were downregulated and 167 genes were upregulated (see Supplementary Table 1 available in supplementary material online at http://dx.doi.org/10.1155/2014/984319). Gene ontology and pathway analysis with IPA indicated enrichment with genes associated with diseases and disorders, molecular and cellular functions, and physiological system development and function (Table 6).

59% of CNV regions (10/17 regions) resulted in a direct impact on gene expression for genes mapping within the CNVs. Five of the ten regions contained genes orthologous to human genes (Table 7). Three CNVs were correlated with a single gene, while two CNVs were correlated with two genes. There were 86% positive associations (a copy number gain associated with increased expression or a copy number loss with decreased expression) and 14% negative associations (a gain associated with decreased expression or a loss associated with increased expression).

4. Discussion

Current knowledge on the role of chemical exposure in the generation of CNVs is limited. In this study, we applied a zebrafish array CGH platform to investigate this phenomenon. CNV identification in the zebrafish genome was recently completed and confirmed the plasticity of the zebrafish genome permits CNV formation [22]. In this proof of principle experiment a zebrafish cell line was initially used to investigate CNV generation associated with chemical

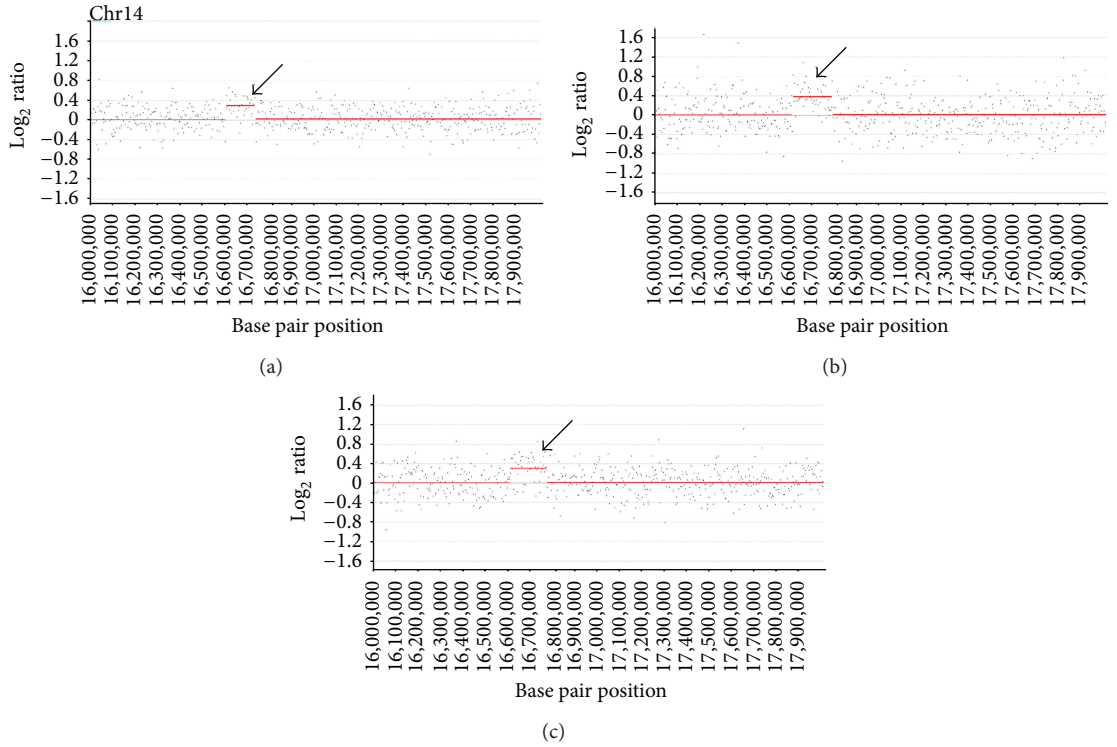

Figure 4: CNVs in common genomic locations among EMS treatments. Multiple CNVs mapped to common genomic locations following the EMS treatments including a gain in copy number in all three concentrations on chromosome 14. This overlapping genomic segment was 120.2 kb in size and mapped to base pair region 16,612,394–16,732,619 on chromosome 14. (a) 0.5 mM, (b) 2 mM, and (c) 5 mM EMS treatments.

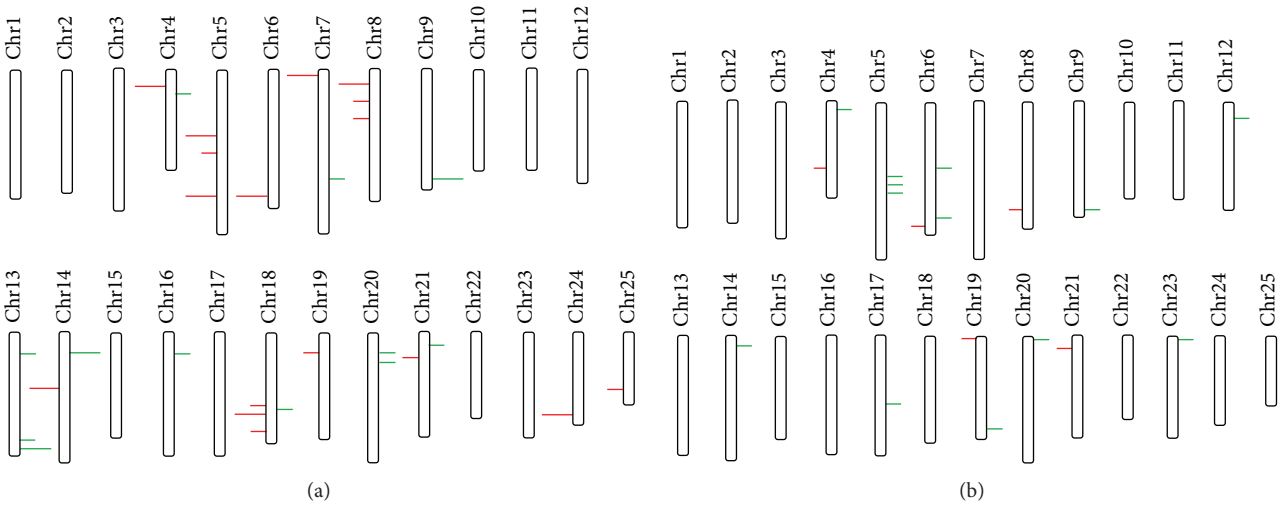

Figure 5: CNVs generated by chemical exposure. (a) Exposure to EMS resulted in 44 CNVs among all treatments and consisted of 11 CNVs with overlapping genomic locations. Considering genomic location overlap, 29 different copy number variable regions were identified and included both gains (12; green bars) and losses (17; red bars). Length of bar indicates frequency. (b) Ara-C exposure resulted in 18 CNVs among the treatments including 13 gains (green bars) and 5 losses (red bars). There were no overlapping CNVs among the Ara-C treatments.

exposure with the intention to translate these findings in future *in vivo* studies using this model.

Two genotoxic chemicals, EMS and Ara-C, were tested for their ability to generate CNVs. EMS is routinely used as reference chemical in genotoxicity assays that is reported to directly produce random point mutations in genetic material by direct alkylation and is often used as a chemical mutagen in studies with model organisms (e.g., [33]). Although alkylating agents are thought to primarily generate point mutations, EMS is also reported to cause other genetic alterations

Table 4: CNVs generated by Ara-C exposure.

Experiment	Concentration (μM)	Chromosome	Start (bp)	End (bp)	Length (kb)	Segmentation mean	Overlap with CNVEs[a]
1	0.1	6	51918902	51957898	39.0	−0.443	No
1	1	4	41297060	41404074	107.0	−0.298	Yes
1	1	19	3146476	3308498	162.0	−0.206	Yes
2	1	4	6479244	7984418	1505.2	0.104	Yes
2	1	5	28310743	28556303	245.6	0.158	No
2	1	5	32149703	32834420	684.7	0.144	Yes
2	1	5	37513893	38141429	627.5	0.117	No
2	1	6	33587936	33807346	219.4	0.196	No
2	1	6	49575981	50700812	1124.8	0.126	No
2	1	8	46328539	46502625	174.1	−0.237	Yes
2	1	9	50187567	50306820	119.3	0.460	No
2	1	12	11689110	12180785	491.7	0.173	Yes
2	1	14	16605821	16765982	160.2	0.473	Yes
2	1	17	39893636	39923656	30.0	0.455	No
2	1	19	37183601	37212491	28.9	0.559	No
2	1	20	3298313	3486787	188.5	0.226	No
2	1	21	8953651	9198587	244.9	−0.194	Yes
2	1	23	3153298	3185448	32.2	0.374	No

[a]CNVEs as called in AB strain zebrafish in [22].

Table 5: Common copy number variable regions in EMS and Ara-C treatments.

Chemical	Concentration	Chromosome	Start (bp)	End (bp)	Length (kb)	Segmentation mean
EMS	5 mM	5	37581072	38022638	441.6	−0.226
Ara-C	1 μM	5	37513893	38141429	627.5	0.117
EMS	2 mM	6	49556168	50808094	1251.9	−0.199
EMS	5 mM	6	49600941	50828098	1227.2	−0.268
Ara-C	1 μM	6	49575981	50700812	1124.8	0.126
EMS	0.5 mM	9	50187567	50303563	116.0	0.248
EMS	2 mM	9	50190782	50306820	116.0	0.445
Ara-C	1 μM	9	50187567	50306820	119.3	0.460
EMS	0.5 mM	14	16605821	16732619	126.8	0.276
EMS	2 mM	14	16612394	16785422	173.0	0.361
EMS	5 mM	14	16609009	16769181	160.2	0.282
Ara-C	1 μM	14	16605821	16765982	160.2	0.473
EMS	2 mM	21	8967733	9185499	217.8	0.244
Ara-C	1 μM	21	8953651	9198587	244.9	−0.194

including DNA strand breaks [34, 35]. A range of EMS chemical treatments from those that resulted in a 50% decrease in cell confluency to no impacts on cell confluency were included in this experiment. An increase in the number of CNVs was not observed with increasing dose, but CNVs were detected in similar genomic regions among the multiple test concentrations of EMS indicating a potential hotspot of genomic instability and a nonrandom genotoxic mechanism for this chemical. Moreover, 39% of the CNVs generated by EMS exposure overlapped with known CNVs in the genome of the AB strain of zebrafish [22] indicating these regions may be more susceptible to genomic rearrangements than other regions. Each of these experiments was started from the same batch of cells with similar cytogenetic structure to alleviate detection of background CNVs and thus support CNVs observed in this study were due to the chemical exposure.

Ara-C was included as a comparative chemical to assess if CNVs are chemical-specific and to further assess CNVs at concentrations with limited alterations on cell confluency. Ara-C is also often used as a reference chemical in genotoxicity assays, is a chemotherapy agent, interferes with DNA synthesis, and results in chromosomal aberrations [36, 37].

Table 6: Gene ontology analysis for enrichment of biological function with 2 mM EMS treatment.

Biological function	P value[a]	Number of genes[b]
Diseases and disorders		
Developmental disorder	$1.66E-08 - 1.24E-02$	177
Skeletal and muscular disorders	$2.35E-05 - 1.02E-02$	66
Infectious disease	$9.31E-05 - 1.04E-02$	170
Connective tissue disorder	$1.03E-04 - 6.50E-03$	40
Cardiovascular disease	$1.27E-04 - 7.41E-03$	43
Molecular and cellular functions		
Cellular movement	$7.52E-07 - 1.34E-02$	204
Amino acid metabolism	$3.36E-06 - 1.14E-02$	78
Small molecule biochemistry	$3.36E-06 - 1.39E-02$	183
Cellular assembly and organization	$5.52E-06 - 1.39E-02$	179
Cellular function and maintenance	$5.52E-06 - 1.39E-02$	189
Physiological system development and function		
Tissue morphology	$3.17E-09 - 1.34E-02$	209
Organismal survival	$4.22E-08 - 9.58E-03$	187
Embryonic development	$2.67E-07 - 1.34E-02$	221
Organismal development	$3.24E-06 - 1.35E-02$	284
Organ morphology	$1.29E-05 - 1.17E-02$	143

[a]Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone. A maximum false discovery rate of 5% was accepted in this analysis.

[b]Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

In the Ara-C treatments, a higher number of CNVs were generated at the higher exposure treatment and no overlapping CNVs were detected between the two treatments, but 44% of the CNVs generated by Ara-C exposure overlapped with CNVs in the genome of the AB strain of zebrafish [22]. While a number of chemical-specific CNVs were called, 5 CNVs were generated in similar genomic regions in both the EMS and Ara-C treatments indicating these regions may be more susceptible to genomic alterations in a nonchemical-specific manner. Two of the five regions were consistent in gain and/or loss in copy number in the specific genomic region. These findings are similar to the patterns of CNVs generated by exposure to aphidicolin and hydroxyurea in a study where CNVs were distributed among the genome with some hotspots of formation [13].

Overall a dose-response was observed with the two treatments of Ara-C, but not among the EMS treatments. It is hypothesized that this difference is due to the effects on cell confluency among the two ranges of test concentrations in the EMS versus the Ara-C experiments (i.e., the EMS exposure treatments ranged from those that resulted in 50% cell confluency to no impact on cell confluency, while both of the Ara-C treatments did not impact cell confluency).

To further assess the influence of CNVs generated by chemical exposure, global gene expression analysis was conducted for the 2 mM EMS treatment. Overall gene expression alterations were identified to be associated with developmental disorders, skeletal muscular disorders, infectious diseases, connective tissue disorder, and cardiovascular disease. In addition, alterations were associated with genes involved in cellular movement, amino acid metabolism, small molecular biochemistry, cellular assembly and organization, and cellular function and maintenance. Expression alterations were also enriched for genes associated with tissue morphology, organismal survival, embryonic development, organismal development, and organ morphology. Furthermore, a direct comparison of genomic regions harboring CNVs with the genomic location of genes with altered expression in the 2 mM EMS treatment indicates that CNVs generated by chemical exposure impact gene expression. This analysis identified both direct associations and negative associations. The negative association may be regulatory in nature. Genes with altered expression associated with CNVs include genes involved in SNAP receptor activity (*STX16*), the initiation of transcription (*MED12*), the initiation of protein synthesis (*EIF2S*), acetyl-CoA transport (*SLC33A1*), the *de novo* synthesis of purine nucleotides (*GMPS*), DNA binding (*ARID5B*), and signal transduction (*CAPN5*). Genes are also associated with various diseases including a deletion in *STX16* with autosomal dominant pseudohypothyroidism [38], a decrease in expression of *EIF2S* with uveal melanoma [39], a polymorphism in *ARID5B* with an increased risk of MLL rearrangements in early childhood leukemia [40], and mutations in *CAPN5* with autosomal dominant neovascular inflammatory vitreoretinopathy [41]. In addition, *ARID5B* is essential for adipogenesis and liver development, while *CAPN5* plays an important role in developmental processes [42]. Moreover, it is likely the CNVs are also linked to altered expression of other genes as CNVs are reported to have global influence on the transcriptome [43]. It should also be recognized that exposure to the genotoxic chemicals can result in single nucleotide mutations. Thus, some of the detected gene expression changes may be due to single nucleotide mutations and/or other DNA alterations, but overall the data indicates that the CNVs generated by the chemical treatments are likely to contribute to gene expression changes and support further studies in the functional effects of the CNVs generated by the chemical exposures.

The zebrafish genome is reported to have gone through two rounds of whole genome duplication during the course of evolution with a third event occurring before the last teleost radiation [44]. The duplicated genome did lead to some difficulty in mapping of the zebrafish genome compared to the rodent and human genomes, but a finished reference sequence is now available [17]. These duplication events may influence the presence of segmental duplications and is suggested to lead to an increase in CNVs in the zebrafish genome [22]. As such, the genome duplication may also influence the frequency at which CNVs generated by chemical exposures may be observed. Additional studies will be needed with

Table 7: Genes with altered expression mapping within CNVs in the 2 mM EMS treatment.

CNV region			Gene expression array data					
Chromosome	Genomic region	Gain/loss	Chromosome	Genomic region	Sequence ID	Fold change	Human gene	Gene symbol
6	49556168–50808094	Loss	6	49674955–49688887	ENSDART00000002693	−5.037	Syntaxin 16	*STX16*
			6	49947447–49963592	BC066706.1	−2.129	Eukaryotic translation initiation factor 2, subunit 2 beta	*EIF2S*
8	32921651–33404812	Loss	8	32982721–32982807	NM_001039461	−6.194	Mediator complex subunit 12	*MED12*
18	31915173–33340460	Loss	18	32385597–32398337	NM_201108	−5.699	Solute carrier family 33 (acetyl-CoA transporter), member 1	*SLC33A1*
			18	32408894–32442474	NM_200587	−13.404	Guanine monophosphate synthetase	*GMPS*
21	8967733–9185499	Gain	21	9178136–9675872	ENSDART00000054092	−9.299	AT rich interactive domain 5B (MRF1-like)	*ARID5B*
21	21053248–21398259	Loss	21	21135672–21166560	NM_001080007	−3.236	Calpain 5	*CAPN5*

other model systems to investigate and further understand the influence of chemical exposure on generating CNVs.

5. Conclusions

Overall this study indicates that chemical exposure results in CNVs that alter gene expression. This study is setting the stage for future investigations into the specific mechanism of formation and expansion to assess environmental chemicals, additional structural genomic alterations using sequencing technologies, and inclusion of *in vivo* systems to study the biological and functional significance of the CNVs and their influence on disease pathways.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

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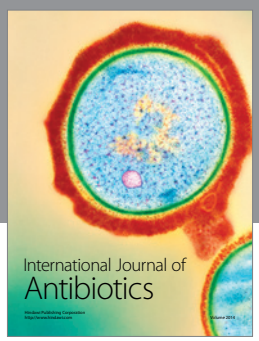

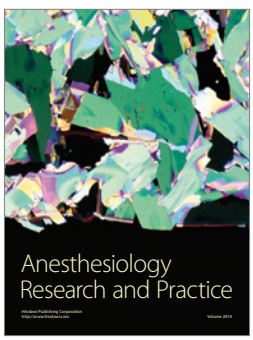

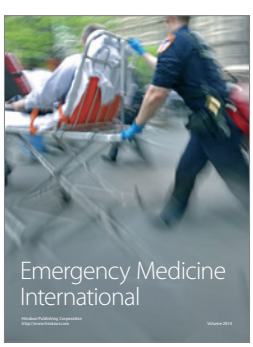

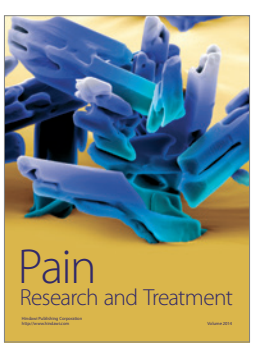

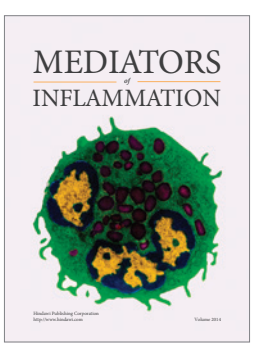

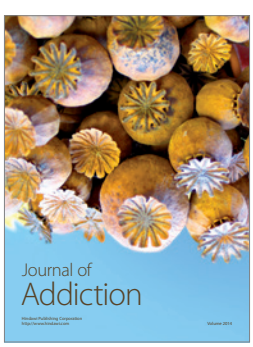

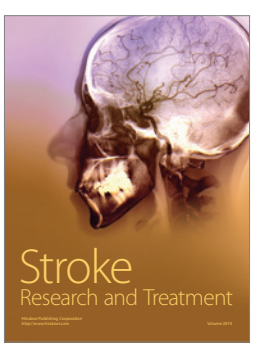

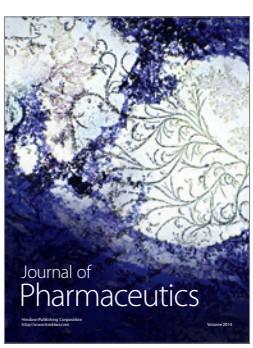

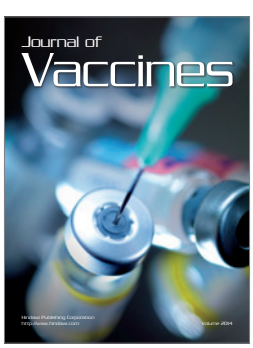

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