

Humans and chimpanzees differ in their cellular response to DNA damage and non-coding sequence elements of DNA repair-associated genes

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Abstract. Compared to humans, chimpanzees appear to be less susceptible to many types of cancer. Because DNA repair defects lead to accumulation of gene and chromosomal mutations, species differences in DNA repair are one plausible explanation. Here we analyzed the repair kinetics of human and chimpanzee cells after cisplatin treatment and irradiation. Dot blots for the quantification of single-stranded (ss) DNA repair intermediates revealed a biphasic response of human and chimpanzee lymphoblasts to cisplatin-induced damage. The early phase of DNA repair was identical in both species with a peak of ssDNA intermediates at 1 h after DNA damage induction. However, the late phase differed between species. Human cells showed a second peak of ssDNA intermediates at 6 h, chimpanzee cells at 5 h. One of four analyzed DNA repair-associated genes, *UBE2A*, was differentially expressed in human and chimpanzee cells at 5 h after cisplatin treatment. Immunofluorescent staining of γ H2AX foci demonstrated equally high numbers of DNA strand breaks in human and chimpanzee cells at 30 min after irradiation and equally low numbers at

2 h. However, at 1 h chimpanzee cells had significantly less DNA breaks than human cells. Comparative sequence analyses of approximately 100 DNA repair-associated genes in human and chimpanzee revealed 13% and 32% genes, respectively, with evidence for an accelerated evolution in promoter regions and introns. This is strikingly contrasting to the 3% of DNA repair-associated genes with positive selection in the coding sequence. Compared to the rhesus macaque as an outgroup, chimpanzees have a higher accelerated evolution in non-coding sequences than humans. The TRF1-interacting, ankyrin-related ADP-ribose polymerase (*TNKS*) gene showed an accelerated intraspecific evolution among humans. Our results are consistent with the view that chimpanzee cells repair different types of DNA damage faster than human cells, whereas the overall repair capacity is similar in both species. Genetic differences in non-coding sequence elements may affect gene regulation in the DNA repair network and thus contribute to species differences in DNA repair and cancer susceptibility.

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Our closest extant evolutionary relatives, the chimpanzees, diverged from the human lineage only 4.6–6.2 million years ago (Kumar and Hedges, 1998). The striking species differences, i.e. in morphology and cognitive abilities, are likely due to changes in gene regulation rather than structural changes in the gene products. Chimpanzees have been used as animal models for human diseases, e.g. for HIV (Novembre et al., 1997) or hepatitis A and B infection (Maynard et al., 1975), because in many aspects of physiology and pathology they are more similar to humans than any other model organism. However, despite this close evolutionary

relationship, humans have a characteristic set of disease susceptibilities (Olson and Varki, 2003). This is probably due to genetic variations that occurred during the rapid phenotypic divergence of humans and great apes. Humans living under modern conditions appear to be more susceptible to certain infectious diseases, cardiovascular diseases, carcinomas, obesity, type II diabetes, autoimmune diseases, major psychoses, and neurodegenerative diseases (Gearing et al., 1994; Bertoni et al., 1998; Martin et al., 2005). Much of the biomedical research in economically developed countries is focused on these common human diseases. To the extent of present knowledge, spontaneous neoplasms are rare in nonhuman primates and many investigators have reported negative attempts to induce experimental tumors in these animals (Allen et al., 1970; McClure, 1973; Beniashvili, 1989; Seibold and Wolf, 1997; Waters et al., 1998). In contrast, cancer is a major and growing medical problem in modern human societies. This is particularly true for epithelial tumors, i.e. breast, lung, colon, and prostate carcinomas, that are a major cause (>20%) of human deaths. The different cancer incidences in humans and nonhuman primates may at least be partially explained by exposure to different environments and differences in life expectancy. However, genetic differences are also likely to play an important role. Comparative sequence analyses of more than 300 cancer genes revealed at least some amino acid changes between humans and chimpanzees which appear to be relevant for human cancer (Puente et al., 2006). In this light, human-chimpanzee comparisons may provide new insights into the genetic basis of human health and disease.

Although usually referred to as a single disease entity, cancer represents many different pathologies, which are all characterized by uncontrolled cell growth, invasion of surrounding tissues, and subsequent metastasis (Hahn and Weinberg, 2002). Germline mutations in a number of DNA repair genes are responsible for many hereditary forms of cancer (Futreal et al., 2004; Vogelstein and Kinzler, 2004). Environmental factors including UV and ionizing radiation as well as endogenous factors, i.e. reactive oxygen species derived from oxidative metabolism, continually damage our genome. One mechanism by which these chemical and physical agents exert their effects is by inducing DNA lesions that interfere with replication and transcription. Error-prone translesion synthesis can result in gene mutations and chromosomal aberrations, leading to the development of cancer (Hoeijmakers, 2001; Hanawalt et al., 2003). In dividing cells, additional errors are introduced during DNA replication and mitosis. Therefore, highly efficient DNA repair systems are required for maintaining genome integrity and preventing malignant transformation of cells. Over the last two decades, our knowledge about the DNA repair network with its various main routes, subpathways, and crossroads has dramatically increased (Jeggo, 1998; Christmann et al., 2003; Sancar et al., 2004). Cells use different pathways for the repair of different types of DNA damage. With the exception of direct one-step removal of adducts from the O⁶ position of guanine by methylguanine DNA methyltransferase, most routes are multistep pathways involving the

highly coordinated action of multiple repair enzymes. In general, sites of structurally altered DNA are recognized by specific damage recognition proteins followed by clipping off the damaged bases by glycosylases via base excision repair or by removing a longer patch around the lesion by nucleotide excision repair (NER). The repair-induced gaps are then filled in by DNA polymerases and finally closed by a ligation step. Double strand breaks (DSB) are repaired by nonhomologous end joining and homologous recombination (Osman and Subramani, 1998; Sonoda et al., 2006). Because of the complexity of the different DNA repair machineries little is known about the time courses of complete repair processes from initial damage recognition to the resealing steps.

If humans and chimpanzees differ in their susceptibility to neoplasms, this could be due to modulations in the cellular response(s) to DNA damage. To test this hypothesis, we have compared the DNA repair kinetics of human and chimpanzee lymphoblasts after induction of different types of DNA damage. Completion of the chimpanzee genome sequence (Chimpanzee Sequencing and Analysis Consortium, 2005; Varki and Altheide, 2005) has also made it possible to study whether genetic variations between the human and chimpanzee DNA repair-associated genes might contribute to the observed differences in cancer susceptibility.

Materials and methods

Cell culture

EBV-transformed lymphoblastoid cell lines from three unrelated humans (*Homo sapiens*, HSA) and three unrelated chimpanzees (*Pan troglodytes*, PTR) were cultured in RPMI 1640 (Gibco) medium supplemented with 10% fetal calf serum (FCS, Biochrom), 0.1 mM MEM non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), and antibiotics. Cells were grown at 37°C in a humidified incubator containing 5% CO₂. There were no differences in growth behavior (population doubling times of 2–3 days) and cell density between human and chimpanzee cell cultures. To induce DNA damage (in particular intrastrand and interstrand crosslinks), 10⁶ cells/ml in the exponential growth phase were incubated for 1 h with 40 μM cisplatin (Neocorb). Pilot experiments had shown that under these experimental conditions 10–20% of cells underwent apoptosis, whereas 80–90% survived and continued to proliferate. Trypan blue staining was used to determine cell viability. Living cells exclude the blue dye, whereas dead cells take it up. After washing the cells were cultured for another 1–12 h to allow repair of the induced DNA damage. To induce DSB, exponentially growing cells were resuspended in icecold PBS in a 75-cm² plastic flask and exposed to a linear accelerator with 15 MV photon beams. A single dose of 4 Gy, as determined by chemical dosimetry, was delivered to the cells. Following irradiation the cells were washed and resuspended in fresh medium.

Dot blot assay for quantification of single-stranded DNA repair intermediates

The induction of ssDNA is a secondary event resulting from cellular response to different types of DNA damage. DNA breaks are first processed by unidirectional 5'- to 3'-exonuclease digestion of one strand of each end to produce rather long 3'-overhanging ssDNA tails. This ssDNA disappears as DNA repair products are formed (Osman and Subramani, 1998; Raderschall et al., 1999). We have developed a sensitive and rapid dot blot method to quantify ssDNA intermediates in genomic DNA from cultured cells. Genomic DNA was isolated from

cell pellets using the QIAamp DNA Mini Kit (Qiagen). Eight aliquots each containing 0.5 μ g genomic DNA of a given sample were dropped onto nylon membrane (GM Health Care), air-dried for 30 min at room temperature, and crosslinked at 80°C for 2 h. The nylon membrane was placed upside down in a Petri dish and all further steps were performed under constant shaking (15 strokes/min) at room temperature. To prevent unspecific antibody binding, the membrane was blocked for 1 h with PBS containing 5% fat-free milk powder (Marvel). Then it was incubated for another hour with human monoclonal antibody against ssDNA, diluted 1:1,000 with blocking solution. The IgM autoantibody MER-1 from an SLE patient recognizes ssDNA, however no cross-reactivity is found with double-stranded DNA and histones (NatuTec, Product Code CTS11152). After washing with PBS, the membrane was incubated for 30 min with horseradish peroxidase-conjugated rabbit anti-human IgG+IgM (Boehringer), diluted 1:2,000 with blocking solution. After washing the membrane 4 \times 10 min with PBS, 0.1% Tween 20 and once with PBS, the secondary antibody signal was detected with the BM Chemiluminescence Western Blotting Kit (Roche). The membrane was incubated for 1 min with substrate solution. The antibody staining intensities of the eight spots per sample were quantified in the Dark Box (Fuji) using AIDA image reader/analyzer software. For quantification of the total amount of DNA in each spot the membrane was stained for 3 min with 0.03% methylene blue, 0.3 M Na acetate (pH 5.2), and then thoroughly washed with water. The methylene blue staining intensities were also measured in the Dark Box. The mean ratio (of the eight spots) between anti-ssDNA antibody signal and methylene blue staining was used as a measure for ssDNA repair intermediates. For the sake of simplicity, we call this ratio the 'R'(repair) value.

Quantification of γ H2AX foci

Because within a few minutes after DSB induction hundreds to thousands of histone H2AX molecules are phosphorylated in a chromatin domain of several megabases around the break site, antibodies against the phosphorylated γ H2AX can be used to directly visualize individual DSB in cell nuclei (Rogakou et al., 1999). Counting of γ H2AX foci has become a widely used method for the quantification of DSB and their repair. Aliquots of 5 \times 10⁵ cells (in 1 ml PBS with 1% FCS) were centrifuged onto clean glass slides at 800 rpm for 3 min using a Shandon Cytospin. After cytocentrifugation, the preparations were fixed in PBS containing 2% formaldehyde for 15 min at room temperature and for 20 min in icecold methanol. Then they were washed 3 \times 5 min in PBS, permeabilized and blocked for 1 h in PBS containing 0.3% Triton X-100 and 5% BSA. The preparations were incubated overnight at room temperature in a moist chamber with mouse antibodies against human γ H2AX (Upstate Biotechnology), diluted 1:1,000 with blocking solution. After three washes with PBS and one wash with PBS containing 0.3% Triton X-100, they were incubated for 2 h with AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen, Molecular Probes), diluted 1:500 with blocking solution. After three further washes with PBS, the preparations were mounted in Vectashield antifade solution (Vector Labs) containing 100 ng/ml DAPI (Sigma). The antibody staining intensity was controlled by eye through an epifluorescence microscope which was equipped with the appropriate filter sets (Vysis). Only preparations of high quality were evaluated further using an automatized system. The fluorescence images were captured with an Axio Imager microscope (Zeiss) and foci were counted with Metafer4 software (Metasystems). γ H2AX foci were scored in at least 200 nuclei of each sample.

Quantitative real-time RT-PCR

Total RNAs were prepared from exponentially growing cell cultures using the Trizol method (Invitrogen). Aliquots of 2.5 μ g RNA each were reversely transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR analyses of *DNAJA3* (QT00076321), *MLH3* (QT00038430), *TNKS* (QT00059920), and *UBE2A* (QT00049273) were performed with pre-designed and optimized Qiagen QuantiTect Primer Assays on an Applied Biosystems 7500 Fast Real-Time PCR system. All reactions were performed in triplicate. Each 30- μ l reaction volume contained 25 ng cDNA template (of a human or chimpanzee cell line), 3 μ l 10 \times Quan-

tiTect Primer Assay, 15 μ l 2 \times QuantiTect SYBR Green I PCR Master Mix, and RNase-free PCR graded water. PCR was performed with one cycle of 95°C for 15 min (first stage) and 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 40 s (second stage). Relative quantification was carried out with the $\Delta\Delta$ CT method (Applied Biosystems 7500 Fast System SDS Software version 1.3), using *TBP* (QT00000721) as endogenous control. When comparing the expression levels of several frequently employed control genes in all analyzed (both treated and untreated) human and chimpanzee cell lines, the TATA-Box binding protein (*TBP*) gene was chosen by the geNORM software as the most reliable endogenous control.

Sequence analysis

Conservation and divergence of necessary gene sequence structures can be considered as a hint for negative and positive selection, respectively. We compared the human and chimpanzee genomic sequences of a set of about 100 representative DNA repair-associated genes, which are responsible for maintaining genome integrity. Nucleotide sequences were taken from Ensembl (<http://www.ensembl.org/index.html>) and the *Homo sapiens* promoter database (HsPD) (<http://rulai.cshl.org/cgi-bin/CSHLmpd2/promExtract.pl?species=Human>) between January 2006 and June 2007. First of all we classified the human and chimpanzee gene sequences in an amino acid coding fraction (open reading frames, ORF) and a non-coding fraction. The latter included 3'- and 5'-untranslated regions (UTR), promoter regions, and introns. Then we compared orthologous sequence parts of human and chimpanzee in terms of selection events. We aligned ORF sequences with ClustalW (BioEdit-version 7.0.5.2.(6/5/05)) and analyzed paired sequences for selection using the modified Nei-Gojobori model with Jukes-Cantor correction for pairwise alignments (MEGA 3.0), which takes transitions/transversions and multiple base substitutions into account. To avoid errors due to the different quality of annotation of the human and chimpanzee reference sequence, we compared only orthologous sequence stretches of the same length (Taudien et al., 2006). Before calculating nucleotide divergence, all sequences were gap-filtered. According to established theories of coding sequence evolution we took the ratio of non-synonymous to synonymous substitutions (dN/dS) >1 as an indicator for positive selection. There is no widely accepted measure of selection for non-coding sequences. This is largely due to the fact that the function(s) of non-coding sequence elements is not well understood. The human and chimpanzee genomes share approximately 96% mean sequence similarity (Chimpanzee Sequencing and Analysis Consortium, 2005; Varki and Altheide, 2005). In this study, we took any nucleotide sequence divergence of 5% or more as a hint for an accelerated evolution. Pair-wise aligned non-coding sequences were analyzed with the sequence identity-tools in BioEdit. Promoter sequences were defined as regions 500 bp downstream and 1,500 bp upstream of the transcription start site in HsPD. Promoter regions, 3'- and 5'-UTR, and introns were taken from Ensembl. Introns were concatenated before analysis.

To find out whether genes with high sequence divergence in their intronic sequence underwent accelerated evolution in the human or the chimpanzee lineage, we used the rhesus macaque (*Macaca mulatta*, MMU) as an outgroup (Rhesus Macaque Genome Sequencing and Analysis Consortium, 2007). MMU genomic sequences of our study genes were retrieved from the UCSC genome browser (<http://genome.ucsc.edu/>). Sequence stretches which fitted in the best way to the human gene sequences were processed further with BioEdit. Alignments were made with ClustalW (BioEdit) and MAFFT version 6 (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/>). For phylogenetic interpretation of multi-species (HSA, PTR, and MMU) alignments, we relied on a maximum likelihood model (Felsenstein and Churchill, 1996). The DNAML maximum likelihood program, version 3.5c (<http://evolution.genetics.washington.edu/phylip/doc/dnaml.html>) was used to create unrooted phylogenetic trees. DNAML accounts for unequal base substitutions and transition and transversion frequencies, allowing different rates of evolution at different sites. The branch lengths are scaled in terms of expected numbers of substitutions, counting both transitions and transversions with an average rate of change, averaged over all sites analyzed, set to 1.0.

Fig. 1. Dot blot assay for the quantification of ssDNA. Membranes with 8 dots of 0.5 μg undigested genomic DNA and *EcoRI*-digested genomic DNA, respectively, were stained with anti-ssDNA antibody and methylene blue. Histograms display the ratio (R) between antibody and methylene blue staining intensities. The R value of the *EcoRI*-digested DNA sample containing single-stranded ends at restriction sites is significantly higher than that of the undigested DNA sample (from the same individual). Standard deviations reflect variation between the 8 dots of each sample.

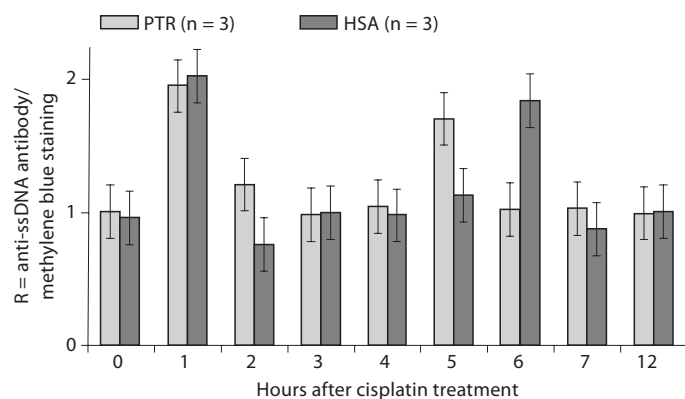
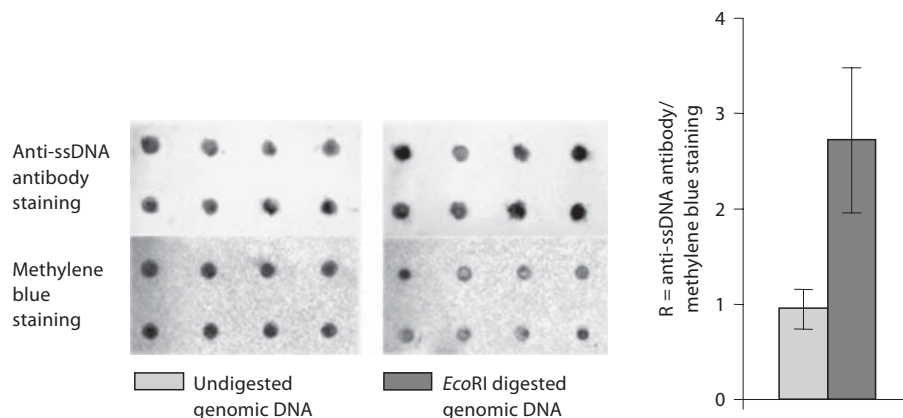


Fig. 2. Quantification of ssDNA repair intermediates in human (HSA) and chimpanzee (PTR) lymphoblast DNAs at different time points after cisplatin treatment. An R value of 1 in our dot blot assay is equivalent to the amount of detectable ssDNA in untreated cells. Standard deviations reflect the variation between the three cell lines analyzed for each species. Both human and chimpanzee cells show a peak of ssDNA at 1 h after DNA damage. Chimpanzees show a second peak at 5 h and humans at 6 h.

HAPMAP Phase 2 data and ‘haplotter’ (<http://hg-wen.uchicago.edu/selection/index.html>) were used to analyze DNA-repair-associated genes for recent human intraspecific evolution. The integrated haplotype score (iHS) is a statistic that is based on the different levels of linkage disequilibrium surrounding a positively selected allele compared to the background allele at the same position. An extreme positive iHS score (iHS > 3) was taken as evidence for recent intraspecific selection events among humans (within the last 10,000–15,000 years).

Results

Response of human and chimpanzee lymphoblasts to cisplatin treatment

Cisplatin induces intrastrand and interstrand links in DNA. The major adduct is cisplatin bound to two neighboring guanines. However, binding to two guanines which are separated by one or more bases or to two guanines in opposite DNA strands, also occurs. Cisplatin-induced

DNA damage is mainly repaired by NER. This process is very efficient and usually removes most DNA lesions before the damaged region is replicated. If the replication fork meets unrepaired DNA damage, breaks may occur in one or both strands of the nascent DNA (Jeggio, 1998). To quantify the amount of ssDNA repair intermediates in cultured cells, we have developed a very simple dot blot assay that measures the staining intensity of genomic DNA with an anti-ssDNA antibody versus methylene blue staining. In a test experiment (Fig. 1) we compared *EcoRI*-digested human genomic DNA with undigested DNA from the same cell culture. Because *EcoRI* cuts one strand of the DNA double helix at one point (G’AATTC) and the second strand at a different, complementary point (between the G and the A base), the separated pieces have single-stranded ends. The R value, which is a measure of the amount of ssDNA, was approximately three times higher for *EcoRI*-digested DNA than for undigested DNA, demonstrating the specificity of our assay.

To compare the human and chimpanzee DNA repair kinetics, three exponentially growing lymphoblast cultures of each species were treated for 1 h with 40 μM cisplatin. Cells were harvested at different time points (0, 1, 2, 3, 4, 5, 6, 7, 12, and 24 h) after DNA damage induction. Using our dot blot assay, we observed a biphasic cellular response to cisplatin treatment in both human and chimpanzee (Fig. 2). Human cells showed an R value of approximately 2 at 1 and 6 h after cisplatin treatment, chimpanzee cells at 1 and 5 h, respectively. At all other analyzed time points, the R values were around 1, which corresponds to the normal value of untreated cultures. We conclude that the early response to cisplatin treatment is similar in both species, but the late response is somewhat faster in chimpanzees than in humans. It is noteworthy that this species difference does only concern the repair kinetics but not the repair capacity. The relative amounts of detectable ssDNA repair intermediates at 7, 12, and 24 h after DNA damage were very similar in humans and chimpanzees.

In order to find out whether the different cellular response of human and chimpanzee cells to cisplatin treatment is associated with gene expression differences, we

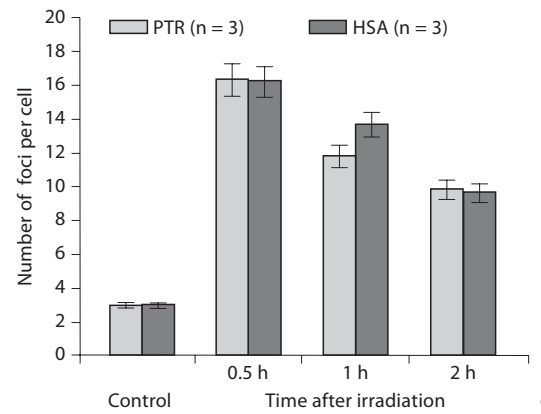
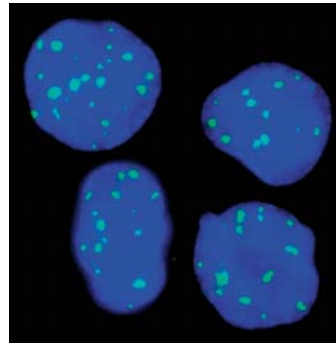
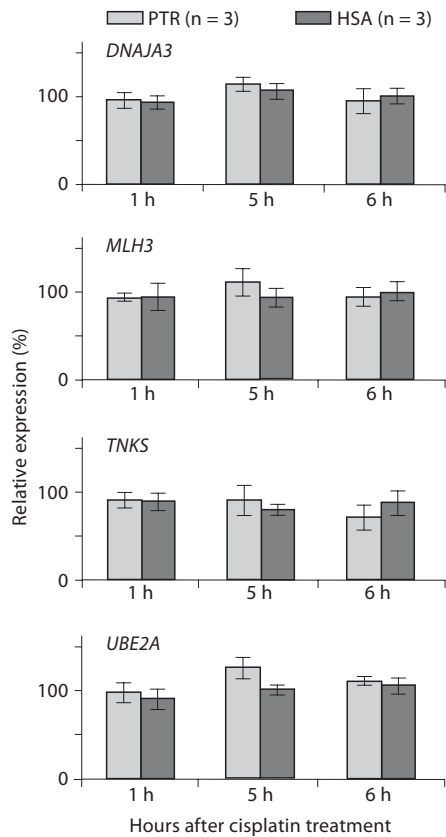


Fig. 3. Relative mRNA expression of *DNAJA3*, *MLH3*, *TNKS*, and *UBE2A* in human (HSA) and chimpanzee (PTR) lymphoblasts at 1, 5, and 6 h after cisplatin treatment, as determined by quantitative real-time RT-PCR. Dark gray bars represent the average expression level of a given gene in humans, light-gray bars in chimpanzees. Standard deviations reflect variation between the three human and the three chimpanzee cell lines analyzed, respectively. The mRNA level in a pool of human lymphoblasts was chosen as a reference (100%). Please note that *UBE2A* shows a higher expression in chimpanzee cells at 5 h after DNA damage.

Fig. 4. Quantification of γ H2AX foci in human (HSA) and chimpanzee (PTR) cells after irradiation with a dose of 4 Gy. Cells were stained at 2 h after DNA damage with green fluorescent anti- γ H2AX antibodies and counterstained in blue with DAPI (left). At least 200 nuclei were analyzed automatically for each cell line and time point. Histograms show the average number of foci in control cells (without DNA damage) as well as at 0.5, 1, and 2 h after irradiation. Bars indicate three standard deviations, reflecting intraspecific variation between the three human and the three chimpanzee cell lines analyzed, respectively.

compared the relative mRNA levels of four representative DNA repair genes in cisplatin-treated human and chimpanzee cells (Fig. 3). Quantitative real-time RT-PCR measurements of *DNAJA3*, *MLH3*, *TNKS*, and *UBE2A* were performed at 1, 5, and 6 h after DNA damage induction. The expression profiles of *DNAJA3*, *MLH3*, and *TNKS* did not differ significantly between species, whereas *UBE2A* was expressed at higher levels in chimpanzees than in humans at 5 h after cisplatin treatment.

Response of human and chimpanzee lymphoblasts to irradiation

High-energy photon beams induce DNA single- and double-strand breaks. Single-strand breaks are efficiently repaired by NER (Cleaver, 2000) and do not represent a major threat for cell survival, whereas DSB are potentially lethal. Mammalian cells are presumed to repair most DSB by nonhomologous end joining, but homologous recombination also plays an essential role (Jackson, 2002; Sonoda et al., 2006). To compare the baseline frequency of DNA breaks in human and chimpanzee cells, we counted the number of γ H2AX foci in 200 nuclei each from three unrelated individuals. The average number of foci (equivalent to the number of DNA breaks) in the rapidly dividing immortalized cells from non-irradiated lymphoblast cultures was 3.0 ± 0.1 in both humans and chimpanzees. To study the response to irradiation-induced DNA damage, we exposed human and chimpanzee lymphoblasts to a single 4 Gy dose and

counted the number of γ H2AX foci in 200 cell nuclei each at 30 min, 1 h, and 2 h after irradiation. Cells from three unrelated humans displayed 16.2 ± 0.3 foci at 30 min, 13.6 ± 0.3 foci at 1 h, and 9.6 ± 0.3 foci at 2 h after irradiation. Chimpanzee cells were endowed with 16.3 ± 0.3 foci at 30 min, 11.8 ± 0.1 foci at 1 h, and 9.8 ± 0.1 foci at 2 h after irradiation (Fig. 4). The identical number of foci (16.2 and 16.3, respectively) in human and chimpanzee cells at 30 min indicates that irradiation induced similar DNA damages in both species. However, at 1 h after irradiation chimpanzee cells displayed significantly fewer foci than human cells (11.8 versus 13.6; χ^2 test, $P < 0.05$), indicating faster DSB repair in chimpanzee. At 2 h after irradiation, the number of foci had decreased to 9.6 in humans and 9.8 in chimpanzees. This implies that over time DNA breaks are repaired with approximately the same efficiency in both species.

Comparative pairwise sequence analyses of human and chimpanzee DNA repair-associated genes

Our working hypothesis is that species differences in the regulation of DNA repair genes modulate the cellular response to DNA damage. Regulation of gene expression can be achieved by a large number of mechanisms, including changes in the amino acid-coding sequences and/or in non-coding regulatory DNA elements. Therefore, we compared the genomic sequences of approximately 100 representative DNA repair-associated genes representing the entire DNA repair network between humans and chimpanzees (Ta-

Table 1. Sequence evolution of DNA repair genes

Gene	Human intraspecific evolution	Comparative pairwise sequence analyses of human and chimpanzee				
	iHS	dN/dS ratio	Sequence divergence (%) of non-coding elements ^a			
		ORF	3'-UTR	5'-UTR	Promoter	Introns
<i>ABL1</i>	<2	0.5	0.8	0.9	1.0	3.2
<i>ADPRT</i>	<2	0.3	1.9	0.9	3.9	1.9
<i>ADPRTL2</i>	<2	0.0	2.5	1.2	1.6	9.2
<i>AHSG</i>	<2	1.3	0.5	0.0	3.0	1.5
<i>APAF1</i>	<2	0.4	0.9	1.0	1.1	6.1
<i>APE1</i>	<2	0.1	0.8	0.6	1.1	1.2
<i>ATM</i>	<2	0.4	n.a.	n.a.	n.a.	n.a.
<i>BCKDHA</i>	<2	0.8	0.8	0.0	1.8	4.2
<i>CCNH</i>	<2	0.0	1.7	0.0	2.5	1.1
<i>CDK7</i>	<2	0.0	0.4	0.0	1.2	5.2
<i>CDKN1A</i>	<2	0.0	0.6	2.1	0.9	1.5
<i>CETN2</i>	<2	0.0	0.7	0.0	6.8	4.7
<i>CTSL</i>	<2	0.0	3.5	1.7	1.6	0.3
<i>DDB1</i>	<2	0.9	0.0	0.0	1.0	8.4
<i>DDB2</i>	<2	0.0	1.4	0.0	2.0	7.8
<i>DMAPI1</i>	<2	0.0	0.0	1.2	1.2	0.9
<i>DNAJA3</i>	<2	0.0	1.6	3.3	10.4	2.1
<i>ERCC4</i>	<2	0.2	n.a.	n.a.	0.7	1.6
<i>ERCC8</i>	<2	0.0	1.3	0.0	8.3	2.0
<i>FANCC</i>	<2	0.4	3.1	3.8	3.5	4.6
<i>FANCF</i>	<2	0.9	0.0	n.a.	1.2	1.6
<i>FANCG</i>	<2	0.3	1.0	1.4	2.9	1.6
<i>FEN1</i>	<2	0.0	1.4	1.3	1.2	0.8
<i>FKBPL</i>	<2	0.3	0.0	1.0	4.0	0.4
<i>GADD45A</i>	<2	0.0	1.0	4.2	1.8	1.0
<i>GADD45B</i>	<2	0.0	2.0	0.9	1.0	0.9
<i>GRB2</i>	<2	0.0	1.4	3.9	3.2	7.1
<i>GTF2H3</i>	<2	0.3	2.9	0.0	16.4	2.1
<i>HUS1</i>	<2	0.2	1.2	0.0	1.0	1.6
<i>ILF1</i>	<2	0.0	7.2	n.a.	1.5	6.5
<i>ILF2</i>	<2	0.0	1.0	1.2	2.1	3.5
<i>ITGB2</i>	<2	0.1	1.1	2.1	2.6	4.7
<i>LIG4</i>	<2	0.2	3.1	2.2	1.0	1.1
<i>MAPK14</i>	<2	0.0	0.5	2.2	1.2	3.8
<i>MBD4</i>	<2	0.2	0.9	1.7	1.0	0.9
<i>MDM2</i>	<2	1.0	1.0	1.4	3.0	2.8
<i>MGMT</i>	<2	1.1	2.0	1.2	2.9	2.3
<i>MLH3</i>	<2	0.6	1.0	1.7	1.6	2.1
<i>MNAT1</i>	<2	0.0	1.0	0.0	0.8	0.8
<i>MSH6</i>	<2	0.2	4.7	3.5	11.2	4.4
<i>NBS1</i>	<2	0.0	1.2	1.8	1.8	2.5
<i>NTPBP</i>	<2	0.6	0.9	0.0	n.a.	0.5
<i>NUDT1</i>	<2	0.2	2.1	n.a.	4.8	2.0
<i>OGG1</i>	<2	0.7	1.6	0.9	0.8	7.5
<i>PCNA</i>	<2	0.0	0.6	0.9	3.2	0.9
<i>PMS1</i>	<2	0.7	1.0	2.6	3.6	2.3
<i>PMS2</i>	<2	0.3	1.9	0.0	2.6	4.8
<i>PMS2L4</i>	<2	0.0	n.a.	n.a.	3.8	9.7
<i>PMS2L5</i>	<2	2.1	3.0	n.a.	7.8	3.0
<i>POLH</i>	<2	0.3	3.3	2.0	3.5	7.5
<i>POLI</i>	<2	0.4	1.2	4.5	1.5	2.0
<i>POLM</i>	<2	0.3	1.2	0.0	8.2	1.4
<i>POLR2H</i>	<2	0.0	n.a.	n.a.	11.0	1.7
<i>PVALB</i>	<2	0.5	0.5	0.0	1.9	3.2
<i>RAD18</i>	<2	0.5	0.9	2.6	0.8	4.6
<i>RAD23A</i>	<2	0.0	3.8	n.a.	1.9	1.7
<i>RAD23B</i>	<2	0.3	0.5	n.a.	7.6	8.7
<i>RAD51C</i>	<2	0.7	4.7	2.7	4.0	6.0
<i>RAD51L3</i>	<2	0.2	2.0	0.0	1.0	5.3
<i>RAD52</i>	<2	0.8	1.3	1.9	5.0	6.7
<i>RAD9A</i>	<2	0.0	1.4	n.a.	9.3	7.2

Table 1 (continued)

Gene	Human intraspecific evolution	Comparative pairwise sequence analyses of human and chimpanzee				
	iHS	dN/dS ratio	Sequence divergence (%) of non-coding elements ^a			
		ORF	3'-UTR	5'-UTR	Promoter	Introns
<i>RAG2</i>	<2	0.0	2.3	0.6	1.7	1.2
<i>RAP1</i>	<2	0.1	0.7	1.5	1.5	3.6
<i>RBBP5</i>	<2	0.0	n.a.	0.7	1.5	0.1
<i>REV1</i>	<2	0.0	0.3	2.4	3.4	4.8
<i>RFC2</i>	<2	0.0	1.3	0.0	1.2	6.5
<i>RFC3</i>	<2	0.3	2.5	4.4	1.4	1.6
<i>RFC4</i>	<2	0.3	4.1	0.5	6.0	1.2
<i>RFC5</i>	<2	0.3	0.9	3.2	1.4	6.7
<i>RPA3</i>	<2	0.8	0.0	0.0	2.9	0.1
<i>SCYL1</i>	<2	0.2	2.1	n.a.	1.0	8.5
<i>SIRT1</i>	<2	0.1	1.3	0.0	1.6	5.7
<i>SIRT3</i>	<2	0.9	0.7	n.a.	1.3	6.6
<i>SIRT5</i>	<2	0.4	2.1	2.3	1.6	7.1
<i>SIRT6</i>	<2	0.2	0.8	1.7	5.1	4.1
<i>SIRT7</i>	<2	0.1	1.9	n.a.	1.5	1.1
<i>SMUG1</i>	<2	0.3	1.9	1.6	1.9	1.2
<i>TDG</i>	<2	0.5	1.0	1.5	2.3	4.4
<i>TERF2</i>	<2	0.8	0.7	0.0	1.4	8.2
<i>TERT</i>	<2	0.0	3.9	0.0	1.4	15.7
<i>TINF2</i>	<2	0.4	n.a.	1.2	3.7	1.9
<i>TNKS</i>	3.5 (<i>P</i> = 0.000358)	0.2	1.0	n.a.	1.6	5.0
<i>TNKS2</i>	<2	0.0	1.0	1.3	1.6	5.9
<i>TOP1</i>	<2	0.0	0.5	1.2	1.2	3.9
<i>TOP3B</i>	<2	0.1	0.0	1.1	3.4	3.4
<i>TP53</i>	<2	0.0	1.1	0.0	0.8	5.2
<i>TP53BP1</i>	<2	0.7	0.0	2.7	0.9	9.2
<i>TREX1</i>	<2	0.5	1.0	n.a.	3.6	5.2
<i>TRF4-2</i>	<2	0.0	1.0	n.a.	0.9	3.7
<i>UBE2A</i>	<2	0.0	2.4	1.1	2.6	1.2
<i>UBE2B</i>	<2	0.0	0.5	2.4	1.9	5.4
<i>UBE2N</i>	<2	0.0	1.5	2.3	3.5	6.1
<i>UBE2V1</i>	<2	0.4	1.7	6.0	1.7	4.9
<i>UBL3</i>	<2	0.0	2.3	0.3	1.3	1.2
<i>UNG2</i>	<2	0.0	0.5	0.6	2.7	0.8
<i>XRCC2</i>	<2	0.8	n.a.	n.a.	2.1	6.7
<i>XRCC3</i>	<2	0.1	2.0	11.3	3.1	4.1
<i>XRCC4</i>	<2	0.6	1.2	2.3	1.2	3.1
Number and percentage of						
genes with accelerated evolution	1 (1%)	3 (3%)	1 (1%)	2 (2.5%)	12 (13%)	31 (32%)
conserved genes	96	94	89	78	83	66
analyzed genes	97	97	90	80	95	97

^a n.a.: Not analyzed (the quality of the available chimpanzee sequence did not allow comparison with the human sequence).

ble 1). First the proportion of functional changes was estimated by computing the dN/dS values for the open reading frames of 'master' transcripts. From this we estimate that approximately 3% of DNA repair-associated genes show positive selection in the coding sequence. This more or less corresponds to the average number of positively selected genes in the human genome (Hellmann et al., 2003). We then compared non-coding sequences, namely 3'- and 5'-UTR, promoters, and introns, of the same gene set. When considering a nucleotide sequence divergence of 5% (gap- and length-filtered) as conspicuous, 1% of the analyzed 3'-

UTR, 2.5% of 5'-UTR, 13% of promoter regions, and 32% of introns showed evidence for an accelerated evolution. The sequence divergence of introns is even higher if one takes length differences of homologous DNA segments into account. So far this is difficult because of the different qualities of annotation of the human and chimpanzee reference sequence. The 13% and 32% of DNA repair-associated genes showing an accelerated evolution in non-coding promoter and intron sequences, respectively, are in striking contrast to the 3% with positive selection in the coding sequence.

Table 2. Sequence divergence and phylogenetic analysis of human (HSA), chimpanzee (PTR) and rhesus macaque (MMU) introns

Gene	Intronic sequence divergence (%)			Branch length ranking ^a			Branch length ^b		
	HSA/PTR	PTR/MMU	HSA/MMU	HSA	PTR	MMU	HSA	PTR	MMU
<i>ADPRTL2</i>	9.2	10.4	10.5	1	2	3	0.00517	0.00507	0.06159
<i>APAF1</i>	6.1	10.2	9.9	2	1	3	0.00446	0.00511	0.06894
<i>CDK7</i>	5.2	10.8	14.6	1	2	3	0.01463	0.00423	0.06509
<i>DDB1</i>	8.4	11.9	11.0	2	1	3	0.00564	0.01018	0.06298
<i>DDB2</i>	7.8	9.3	9.1	2	1	3	0.00612	0.00956	0.06852
<i>GRB2</i>	7.1	12.3	12.2	2	1	3	0.00638	0.00727	0.11177
<i>ILF1</i>	6.5	15.3	16.2	2	1	3	0.01027	0.01162	0.05734
<i>OGG1</i>	7.5	10.0	8.7	2	1	3	0.00531	0.01519	0.07378
<i>POLH</i>	7.5	12.4	11.0	2	1	3	0.00454	0.01028	0.06560
<i>RAD23B</i>	8.7	10.7	10.5	2	1	3	0.00465	0.00739	0.07130
<i>RAD51C</i>	6.0	10.7	10.1	2	1	3	0.00466	0.00948	0.07383
<i>RAD51L3</i>	5.3	9.2	10.2	2	1	3	0.00550	0.00596	0.12297
<i>RAD52</i>	6.7	20.3	17.9	2	1	3	0.00869	0.01604	0.06751
<i>RAD9A</i>	7.2	9.8	9.2	2	1	3	0.00497	0.00625	0.08151
<i>RFC2</i>	6.5	12.7	11.6	2	1	3	0.00666	0.01079	0.05970
<i>RFC5</i>	6.7	9.0	8.6	2	1	3	0.00613	0.00626	0.06513
<i>SCYL1</i>	8.5	7.9	8.7	1	2	3	0.00520	0.00307	0.07443
<i>SIRT1</i>	5.7	14.1	12.9	2	1	3	0.00615	0.01225	0.07727
<i>SIRT3</i>	6.6	12.1	11.0	2	1	3	0.00695	0.00925	0.06230
<i>SIRT5</i>	7.1	9.3	9.4	2	1	3	0.00717	0.00721	0.07355
<i>TERF2</i>	8.2	15.4	12.1	2	1	3	0.00631	0.02739	0.09082
<i>TERT</i>	15.7	14.4	14.0	2	1	3	0.01335	0.01342	0.05850
<i>TNKS</i>	5.0	9.2	9.2	2	1	3	0.00946	0.00956	0.06027
<i>TNKS2</i>	5.9	9.2	9.2	2	1	3	0.00389	0.00462	0.07066
<i>TP53</i>	5.2	10.3	10.5	2	1	3	0.00530	0.00687	0.05998
<i>TP53BP1</i>	9.2	9.4	9.2	2	1	3	0.00453	0.00532	0.09151
<i>TREX1</i>	5.2	13.1	13.5	1	2	3	0.00640	0.00456	0.07173
<i>UBE2B</i>	5.4	10.9	10.7	2	1	3	0.00407	0.00745	0.06587
<i>UBE2N</i>	6.1	12.1	12.2	2	1	3	0.00657	0.00724	0.06201
<i>XRCC2</i>	6.7	8.9	8.6	2	1	3	0.00471	0.00581	0.06201
Rank 1				13%	87%	0%			
Rank 2				87%	13%	0%			
Rank 3				0%	0%	100%			

^a Relative distances; rank 1 is more distant to rank 3 than rank 2 to rank 3; $P < 0.05$.

^b Average rate of change, averaged over all sites analyzed, is set to 1.0; $P < 0.05$.

A subset of 30 DNA repair-associated genes which showed an intronic sequence divergence of at least 5% between human and chimpanzee was analyzed further using the rhesus macaque as an outgroup (Table 2). When comparing homologous intronic sequence stretches, nine genes exhibited more sequence divergence between human and rhesus macaque than between chimpanzee and rhesus macaque, whereas 19 genes showed more sequence divergence between chimpanzee and rhesus macaque than between human and rhesus macaque. However, for most (27 of 30) analyzed genes the PTR-MMU and HSA-MMU sequence divergences differed by less than 1.5%. *CDK7* displayed 3.8% more sequence divergence between human and macaque, *RAD52* and *TERF2* showed 2.4% and 3.3% more sequence divergence, respectively, between chimpanzee and macaque. We then applied the DNaml maximum likeli-

hood program to calculate branch lengths (Felsenstein and Churchill, 1996). Four of the 30 analyzed genes (13%) showed slightly shorter branch lengths in chimpanzee than in human ($P < 0.05$), compared to macaque; 26 genes (87%) showed longer branch lengths ($P < 0.05$) in chimpanzee than in human (Table 2). This is consistent with the view that more genes underwent accelerated evolution of intronic sequences in the chimpanzee lineage than in the human lineage.

In addition, we used HAPMAP data to look for human intraspecific sequence evolution. Only one gene of the analyzed set, *TNKS*, had an $iHS > 3$ (Table 1) and thus may be under selection among humans. Interestingly, the coding sequence of *TNKS* was conserved between humans and chimpanzees, whereas the non-coding introns also showed a slightly accelerated interspecific evolution.

Discussion

The role of DNA repair defects for cancer development is well established. In fact, cancer can be seen as a result of incorrectly repaired DNA lesions (Hoeijmakers, 2001; Hanawalt et al., 2003). Because variations in DNA repair mechanisms for maintaining genome integrity are one plausible explanation for the differential cancer susceptibility between humans and chimpanzees, we were interested to find possible species differences in the cellular response to DNA damage. In this light, we compared the repair kinetics of different types of DNA lesions between human and chimpanzee cells. The number of radiation-induced DNA breaks was quantified in three independent cell lines of each species by immunofluorescence staining. Although shortly after irradiation cells of both species displayed comparable numbers of γ H2AX foci, chimpanzee cells repaired faster and had significantly less foci at 1 h than human cells. Dot blots for the quantification of ssDNA repair intermediates revealed that the early response to cisplatin-induced damage was identical in humans and chimpanzees with a peak of ssDNA intermediates at 1 h after DNA damage. However, human cells showed a second peak of ssDNA intermediates at 6 h, chimpanzee cells at 5 h. One of four tested DNA repair genes, *UBE2A*, which is required for post-replicative DNA damage repair (Koken et al., 1991), showed a higher expression level after cisplatin treatment in chimpanzee cells, compared to human cells. Thus, several lines of evidence suggest a species-specific cellular response to DNA damage. The early repair phase of radiation- and cisplatin-induced DNA damage in our study is consistent with papers on the repair kinetics of UVC-induced single- and double-strand breaks. The comet assay showed a maximum tail length at 60–90 min after exposure to UVC, however unfortunately most studies did not include time points longer than 240 min (Zheng et al., 2005; Cipollini et al., 2006). The biphasic repair kinetics of cisplatin-treated cultures in our study is consistent with radiobiological data on DSB repair (Lange et al., 1997; Pinto et al., 2005). The bulk of radiation-induced DSB is repaired most likely by nonhomologous end-joining within the early phase (within 1–2 h). Cells responding with the slow system which may involve homologous recombination are repaired within 5–6 h. It has been noted previously that mammalian species may differ in their sensitivity to the induction of chromosome breakage (Brewen and Preston, 1974; Yunis and Soreng, 1984).

The molecular basis of the observed DNA repair differences between humans and chimpanzees remains to be elucidated. Comparative sequence analysis of approximately 100 representative DNA repair-associated genes revealed that all examined human genes are present in chimpanzee, showing a high degree of conservation in the amino acid-coding sequence. Only 3% of the analyzed genes had been subjected to positive selection in the coding sequence. Similar to cancer genes (Thomas et al., 2003; Puente et al., 2006), the limited interspecies variations in the coding sequence of DNA repair-associated genes may reflect their essential cellular functions. Like the ORF, the 3'- and 5'-UTR of DNA

repair-associated genes displayed only limited sequence variations between humans and chimpanzees. In contrast, 13% and 32% of the analyzed genes showed evidence for an accelerated evolution in the promoter regions and introns, respectively. Because the coding regions of DNA repair-associated genes may be under strong conservative pressure of selection, it seems plausible to assume that phenotypic diversification within and between closely related species is driven by the evolution of non-coding sequence elements with regulatory functions.

It has been shown previously (Bakewell et al., 2007) that more genes underwent positive selection of coding sequences in chimpanzee evolution than in human evolution. Here we used the rhesus macaque as an outgroup to analyze the evolutionary status of 30 DNA repair-associated genes that exhibited a high ($\geq 5\%$) intronic sequence divergence between humans and chimpanzees. Most analyzed genes (87%) showed evidence for higher accelerated evolution in the chimpanzee than in the human lineage, implying that human introns display a more ancestral state. Although the observed differences are weak and may be to some extent flawed by low chimpanzee and/or macaque sequence quality, these results are not unexpected. Because of their smaller long-term effective population size, the efficacy of natural selection is reduced in humans (Bakewell et al., 2007).

One of the genes, which displayed an accelerated evolution of non-coding elements between humans and chimpanzees, also showed an accelerated intraspecific evolution among humans. *TNKS* encodes a poly(ADP-ribose) polymerase (PARP) with multiple functions for maintaining genome integrity. It interacts with the telomere DNA binding protein TRF1 and plays a role in telomere length control. Interestingly, *TNKS* overexpression promoted telomere elongation in human but not in mouse cells, suggesting a human (or primate) specific elaboration (Donigan and de Lange, 2007). *TNKS* is also important for sister telomere (chromatid) resolution before anaphase and proper mitotic progression (Dynek and Smith, 2004). In addition, *TNKS* is known to prevent oxidative and alkylating genotoxins from activating PARP1, thus protecting cells from genotoxin-induced apoptosis (Yeh et al., 2005). Interaction of *TNKS* with the myeloid cell leukemia proteins Mcl-1L and Mcl-1S appears to be another mechanism to modulate apoptosis (Bae et al., 2003). In our expression analyses *TNKS* was not differentially regulated in human and chimpanzee cells. This may be explained by the fact that *TNKS* is not important for the repair of cisplatin-induced DNA damage. On the other hand, *UBE2A* showed expression differences between species, but was not subject to an accelerated sequence evolution. However, gene expression can be regulated by many other mechanisms.

Consistent with our hypothesis that sequence variations in non-coding elements may affect gene regulation and represent a diversifying force of evolution, it has been shown previously that sequence variations in the promoter region of the human and chimpanzee apolipoprotein A gene are responsible for higher transcriptional activity of the chimpanzee gene and elevated Lp(a) plasma levels in chimpan-

zees (Huby et al., 2001). Intron polymorphisms in the *MCM6* gene stimulate promoter activity of the lactase gene and cause lactase persistence (high expression of the lactase gene) into adulthood (Olds and Sibley, 2003). Although there is still no consensus on the origin and role of introns, several lines of evidence suggest that they may serve as regulatory elements at the gene and/or genome level. Because transcription and splicing of long introns requires energy, highly expressed genes are selected for shorter introns (Castillo-Davis et al., 2002). Both the non-coding introns and the coding part are longer in tissue-specific genes than in housekeeping genes. In addition, there is a tight correlation between average intron length and genome size in widely different organisms (Vinogradov, 2006). Because introns can contain several types of non-coding but functional RNA molecules, sequence changes may also be a mechanism to create species-specific non-coding RNAs (Fedorova and Fedorov, 2005).

Collectively, our results suggest a faster response of chimpanzee cells to different types of DNA damage. This may be advantageous for rapidly dividing cells with short G₁ phase, i.e. certain bone marrow and epithelial cell types, the malignant transformation of which is responsible for

many human cancers. To test this hypothesis, it will be necessary to study the DNA damage response in more cell types and tissues of humans and chimpanzees. Interestingly, chimpanzees have an accelerated evolution in intronic sequences of DNA repair-associated genes, compared to humans. Genes that are under selective pressure among humans may exhibit an even more accelerated evolution between species. Whether the observed sequence divergence in non-coding elements affects gene regulation and thus contributes to phenotypic differences in DNA repair and tumor susceptibility between humans and chimpanzees remains to be shown. While it is difficult to tease out functional perspectives of our present results, they form the foundation of follow-up studies. It will be particularly challenging to identify the biomedically and functionally important sequence changes both within and between species.

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