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Short Communication

Alterations in DNA repair gene expression and their possible regulation in rat-liver regeneration

Gai-Ping Wang[1,2] and Cun-Shuan Xu[1,2]

[1]*College of Life Science, Henan Normal University, Xinxiang, Henan Province, China.*
[2]*Key Laboratory for Cell Differentiation Regulation, Xinxiang, Henan Province, China.*

Abstract

Rapidly proliferating tissue may require enhanced DNA repair capacity in order to avoid fixation of promutagenic DNA lesions to mutations. Partial hepatectomy (PH) triggers cell proliferation during liver regeneration (LR). However, little is known on how DNA repair genes change and how they are regulated at the transcriptional level during LR. In the present study, the Rat Genome 230 2.0 array was used to detect the expression profiles of DNA repair genes during LR, and differential expression of selected genes was confirmed by real-time RT-PCR. 69 DNA repair genes were found to be associated with LR, more than half of which distributed in a cluster characterized by a gradual increase at 24-72h and then returning to normal. The expression of base excision repair- and transcription-coupled repair-related genes was enhanced in the early and intermediate phases of LR, whereas the expression of genes related to HR, NHEJ and DNA cross-link repair, as well as DNA polymerases and related accessory factors, and editing or processing nucleases, were mainly enhanced in the intermediate phase. The expression changes of genes in DNA damage response were complicated throughout the whole LR. Our data also suggest that the expression of most DNA repair genes may be regulated by the cell cycle during LR.

Key words: partial hepatectomy, rat genome array, DNA repair genes, liver regeneration.

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The liver has an outstanding capacity for regeneration (Taub, 2004). The process of hepatic cells initiating the cell cycle and proliferating rapidly, in order to compensate for lost liver tissues after rat partial hepatectomy (PH), is called liver regeneration (LR) (Lai *et al.*, 2005; Suzuki and Tsukamoto, 2004). Injured liver cells and cell remnants caused by PH are harmful to the organism, while injured areas therefrom are susceptible to infection by antigens and xenobiotics, all possibly leading to inflammatory and immune responses (Shao *et al.*, 2007; Zhang *et al.*, 2006). Furthermore, carbohydrate, lipid, and protein and amino acid metabolisms are highly active, thereby providing nutrients or energy, especially for active DNA replication in LR (Fausto *et al.*, 2006). As a result, a wider variety of endogenous damage produced by inflammation, normal metabolic byproducts (*i.e.* ROS) or replication errors, may constantly occur in LR.

It is common knowledge that DNA repair processes counteract genetic damage and maintain genome integrity (Wood *et al.*, 2001). Many researchers have discovered that inherited mutations affecting DNA repair genes are strongly associated with high cancer risks (Jass, 2006). Decreased DNA repair capacity may be an important factor predisposing to the development of preneoplastic lesions, neoplastic nodules and malignant tumors (Vielhauer *et al.*, 2001). It is generally believed that rapidly proliferating tissue undergoing DNA synthesis may require enhanced DNA repair capacity, so as to avoid fixation of promutagenic DNA lesions to mutations (Kaufmann *et al.*, 1991; Riis *et al.*, 2002). While hepatic cell proliferation is activated in regenerating liver, diminished rates of DNA repair may contribute to reducing LR capacity (Schmucker, 2005). Therefore, research on how DNA repair operates to prevent the accumulation of damage or mutations, and how to retain the rate of LR, has become a hot topic (Arai *et al.*, 2003). Some researchers have used an LR model to assess expression changes in certain DNA repair enzymes, such as UNG and ATM, as well as their corresponding repair activities. As a result, they found hepatocytes are endowed with increased DNA-repair capacity during the period of highest transformation sensitivity in the cell cycle (Gombar *et al.*, 1981; Lu *et al.*, 2005).

Notwithstanding, there has been surprisingly little research on the global changes of mRNA expression in DNA repair-related genes during LR (Riis *et al.*, 2002). In this study, a total of about 180 genes involved in various DNA repair pathways, such as the mismatch, direct, excision, homologous recombination (HR), DNA nonhomologous

Send correspondence to Cunshuan Xu. College of Life Science, Henan Normal University, No. 46, Construction East Road, Xinxiang 453007 Henan, China. E-mail: cellkeylab@126.com; xucs@x263.net.

end-joining (NHEJ), DNA cross-link and translesion repair, were obtained by searching the biological pathway maps at databases, such as RGD, GenMAPP, KEGG, BIOCARTA and Biocompare. They were then reconfirmed through pertinent article retrieval (Arias-Lopez *et al.*, 2006; Wood *et al.*, 2005). Gene expression profiles of the above DNA repair genes were detected using the Rat Genome 230 2.0 array, whereupon their expression changes and possible regulation patterns during LR were primarily analyzed.

A total of 76 healthy male Sprague-Dawley rats, each weighing 200 ± 10 g, were supplied by the Experimental Animal Center of Henan Normal University. They were randomly divided into 19 groups (4 rats in each), viz., 9 PH, 9 sham-operated (SO) and one normal control (NC). The rats in the PH groups underwent 2/3 PH as described by Higgins and Anderson(1931). Four rats a time were ether anesthetized at 0, 2, 6, 12, 24, 30, 36, 72, 120 and 168 h after PH, whereupon their livers were immediately removed and stored at -80 °C for use.

After isolation from the frozen livers, as indicated by the manual of Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA), total RNA was purified, acccording to RNeasy mini protocol (Qiagen, Inc, Valencia, CA, USA). The quality of the final product was assessed by optical density measurement at 260/280 nm, as well as through agarose electrophoresis (180 V, 0.5 h). T7-oligo dT(24) (Keck Foundation, New Haven, CT), SuperScript II RT (Invitrogen Corporation, Carlsbad, CA) and 5 μg of total RNA were used to synthesize the first strand of cDNA, and the Affymetrix cDNA single-stranded cDNA synthesis kit for synthesis of the second. The resultant cDNA products were purified according to manufacturer's cDNA purify protocol. 12 μL of purified cDNA and the reagents in the GeneChip *In Vitro* Transcript Labeling Kit (ENZO Biochemical, New York, USA) were employed for synthesizing biotin-labeled cRNA, which was purified by means of RNeasy Mini Kit columns (Qiagen, Valencia, CA). 15 mL of cRNA (1 μg/μL) were incubated with 6 μL of 5 x fragmentation buffer and 9 μL of RNase free water for 35 min at 94 °C, and then digested into 35-200 bp cRNA fragments. The prehybridized Rat Genome 230 2.0 microarray was placed into a hybridization buffer, and hybridization was allowed to occur in a hybridization oven (Affymetrix) at 45 °C at 60 rpm for 16 h. The hybridized arrays were washed in a wash-buffer, and stained in a GeneChip® Fluidics Station 450 (Affymetrix Inc., Santa Clara, CA, USA). The arrays were then scanned and images captured with a GeneChip® Scanner 3000 (Affymetrix Inc., Santa Clara, CA, USA) (Guo and Xu, 2008).

Images showing gene expression abundance were converted into signal, signal detection (P, A, M) and experiment/control (Ri) values through Affymetrix GCOS 1.2 software (Affymetrix, USA). The data of each array were initially normalized by scaling all signals to a target intensity of 200. P values < 0.05 meant that gene expression is present (P), $p < 0.065$ indicated marginal expression (M), and $p > 0.065$ absence of expression (A). Furthermore, signal values of PH normalized to those of control were used to calculate the relative or ratio values of gene expression abundance. A ratio = 2 meant up-regulated gene expression, = 0.5, significantly down-regulated, and 0.5-2, biologically insignificant expression. To minimize technical errors inherent in microarray analysis, each sample was analyzed at least three times, and the average value was considered reliable.

As a result, 139 of the above-mentioned 180 DNA repair genes were assessed in the Rat Genome 230 2.0 array. Sixty-nine of these yielded meaningful expression changes, at least at one time-point after PH, thereby indicating significant ($0.01 \le p < 0.05$) or extremely significant ($p \le 0.01$) differences between PH and SO groups, thus indicating their involvement in LR. During LR, 55 genes were found to be up-regulated, 8 down-regulated and 6 up/down-regulated. Fold changes for the up-regulated ranged from 2-fold to 34-fold, and in the down-regulated from 2-fold to 5-fold (Supplementary Material, Table S1).

To confirm the results of the microarray analysis, some significantly changed genes were chosen for in-depth analysis by real-time quantitative RT-PCR. MGMT is an important direct repair protein which suicidally transfers the methyl moiety from O^6-methylguanine to itself (Pegg *et al.*, 1995), whereas PCNA plays a vital role in BER and the initiation of recombination-associated DNA synthesis (Li *et al.*, 2009), and HMGN1 participates in promoting NER (Birger *et al.*, 2003). Therefore, the above three genes involved in different DNA repair pathways were chosen. Primer sequences were designed by rimer express 2.0 software according to the mRNA sequences of *mgmt*, *hmgn*, *pcna* and that of the internal control gene β-*actin* (GenBank numbersNM_012861, NM_001013184, NM_022381 and NM_031144) (Supplementary Material, Table S2). First-strand cDNA samples underwent quantitative PCR amplification, using SYBR® Green I on a Rotor-Gene 3000A thermocycler (Corbett Robotics, San Francisco, CA). Each was analyzed in triplicate, and standard curves were generated from five repeated ten-fold serial dilutions of cDNA (Wang and Xu, 2010). The absolute values and corresponding relative values of their temporal transcriptional levels in RT-PCR assays appear in Table 1. On a whole, expression trends of the three genes detected by RT-PCR and microarray were generally consistent, thereby indicating that array-check results were reliable (Supplementary Material, Figure S1).

It is common knowledge that hepatocyte DNA replication starts at approximately 12 h after PH and normally reaches a peak at 24 h. In rats, however, there is a second peak at 36-66 h. In this study, the 10 time-points during LR could be allocated to 3 phases, viz., 0-6 h (early phase), in which hepatocytes are activated and G0/G1 transition occurs, 12-72 h (intermediate phase) when cell proliferation

Table 1 - The mRNA quantity of three DNA repair genes detected by real-time RT-PCR during rat liver regeneration.

Verified gene	Recovery time after partial hepatectomy (h)									
	0	2	6	12	24	30	36	72	120	168
mgmt	8.83E+01	1.46E+01	4.16E+00	4.56E+00	4.84E+00	6.41E+00	6.32E+00	1.18E+02	3.46E+01	1.73E+01
	7.59E-03	2.17E-03	2.66E-03	2.84E-03	6.75E-03	3.91E-03	1.03E-02	2.62E-02	6.87E-03	5.96E-03
hmgn1	8.73E-01	3.15E-01	3.62E-01	3.61E-01	5.92E-01	7.53E-01	5.62E-01	6.21E-01	4.91E-01	4.08E-01
	1.74E-05	2.22E-05	1.37E-05	1.55E-05	4.41E-05	3.73E-05	4.47E-05	1.01E-05	1.24E-05	9.26E-06
pcna	1.80E+00	1.38E+00	1.17E+00	1.83E+00	2.99E+00	2.87E+00	1.88E+00	1.71E+00	2.16E+00	1.92E+00
	3.59E-05	9.75E-05	4.44E-05	7.86E-05	2.23E-04	1.42E-04	1.49E-04	2.79E-05	5.45E-05	4.37E-05

Upper panel for each gene: Absolute quantity of mRNA (molecules/ng total RNA); Lower panel for each gene: Relative quantity of mRNA compared to beta-actin. All data were average values of three repeats.

takes place, and 120-168 h (late phase) when regeneration terminates. There was considerable variation in different genes at the time points, as to initial expression and expression persistence during the whole process. As a result, the numbers of initially up and down-regulated DNA repair-related genes were 19 and 4, respectively, in the early phase, 49 and 8 in the intermediate, and 1 and 1 in the late. Total expression frequencies of up- and down-regulated genes in the three phases, were 26 and 5, 159 and 15, 41 and 8, respectively (Figure 1), thereby illustrating that DNA repair-related genes, mainly induced during the early and intermediate stages, played important roles in the different stages.

To facilitate the visualization and interpretation of gene expression profiles, 69 DNA repair genes, with 2-fold-plus variation in intensity, at least at one time-point after PH, were hierarchically clustered, according to expression similarities (Figure 2A). The result, in compact graphical format, showed their arrangement into three groups (Fiure. 2B). Cluster C1 contained 22 genes which were rapidly up-regulated, 2-30 h after PH and persisted so, whereas the expression of the 38 genes in cluster C2, gradually increased at 24-72 h and then returned to normal, and the 9 genes in Cluster C3, were rapidly down-regulated at 6-12 h and continued to be so. Furthermore, more than half of the DNA repair genes in cluster C2 were up-regulated, mainly at 24-72 h after PH.

Four base excision repair-related genes were mainly present in the C1 cluster, this including *mbd4*, *tdg,* and

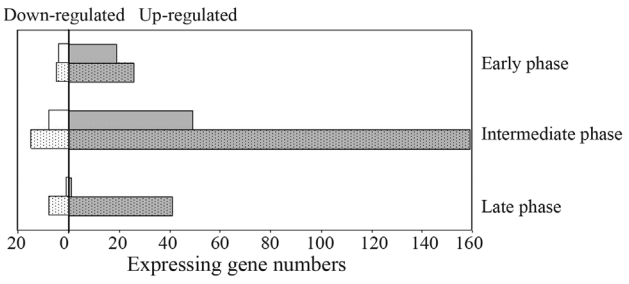

Figure 1 - Initial and total expression profiles of 69 identified DNA repair genes at three phases of liver regeneration**.** Blank bars, initial gene expression; dotted bars, total gene expression.

apex1. However, Gombar *et al.* (1981) found that the specific enzyme activities of UNG reached maximal levels between 18-24 h after PH, and then rebounded by 48 h. Furthermore, Riis *et al.* (2002) found that *ogg1* expression increased 5-fold by 24 h after PH. In the present study, the mRNA levels of UNG and OGG1 were not found to be significantly enhanced after PH, although the expression of two other glycosylases, MBD4 and TDG, was dramatically so.

The genes related to HR, NHEJ, DNA cross-link repair, DNA polymerases and related accessory factors, as well as editing and processing nucleases, were mainly contained in cluster C2. Amongst those associated with HR, *brca1, mus81*, *blm*, *mre11a* and *brca2* were enhanced, with two expression peaks, one between 24-30 h and the other 36-72 h. Since most DNA synthesis in hepatocytes occurs between 12-24 h, with non-parenchyma cells proliferating later (Koniaris *et al.*, 2003; Khan and Mudan, 2007), it was supposed they may play a key role in DNA replication and repair during LR. In this study, the genes involved in HR were all extremely enhanced, with 34-fold peak expression above control at 24 h, thus quite consistent with the results of Thyagarajan *et al.*, (1996) that homologous DNA recombination activity in regenerating liver closely mirrors the first wave of DNA synthesis, reaching a peak 24h after regenerative stimulus. The genes specifically associated with NHEJ, DNA cross-link repair, editing and processing nucleases, and DNA polymerases and related accessory factors which operate in distinct DNA repair pathways or bypass specific classes of adducts in DNA (Wood *et al.*, 2005), were enhanced between 24-36 h with one peak at 24 h after PH. The above results give us to understand that expression of most DNA repair genes is closely related to progression through the cell division cycle during liver regeneration.

The genes involved in both mismatch repair and nucleotide excision repair (NER), and DNA damage response, were distributed among all the three clusters, with most in cluster C1. Among the DNA lesion recognition genes of interest in NER, *cetn2* and *xpc*, both involved in global genome repair (GGR), were down-regulated between 6-24 h and 36-72 h, respectively, whereas *ercc8*,

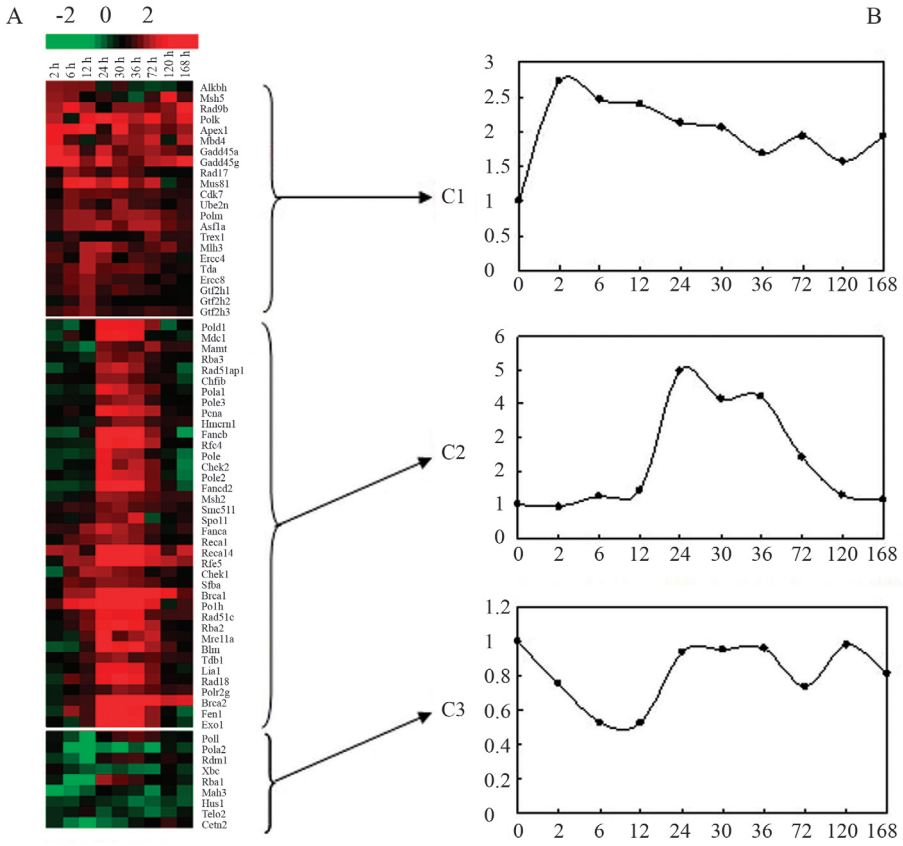

Figure 2 - Expression profiles of DNA repair genes during rat liver regeneration. A**.** Hierarchical clustering of the 69 genes involved in DNA repair pathways. The heat map was colored, using red for up-regulation, green for down-regulation and black for no significant change in expression. The degree of color saturation reflects expression-ratio magnitude, as indicated by the color scale at the top. The 69 genes were gouped into three clusters, **C1**, **C2** and **C3**. B. Expression pattern of the genes in each cluster. The general gene expression trend of each cluster is shown in a line graph.

hmgn1 and *polr2g,*, involved in transcription coupled repair (TCR), were up-regulated after PH, and GTF2H1, GTF2H2, and GTF2H3, the three main subunits of the general transcription factor TFIIH (Tian *et al.*, 2004), were simultaneously enhanced at the mRNA level, after 12 h. Due to cell proliferation triggered by PH, there was an increase in RNA polymerase II-dependent transcription (Dong and Xu, 2008), and the above-mentioned NER factors or TFIIH were more essential to transcription than to their normal roles in DNA repair. Thus, during LR, their expression changes could lead to changes in transcriptional activity rather than in DNA repair. The true relationship between NER factors or TFIIH and DNA repair activity requires further study. As a component of the trimeric Cdk7-cyclin H-Mat1 complex, which functions as a cyclin-dependent kinase-activating kinase (Rossi *et al.*, 2001), *cdk7* was up-regulated at 6 h, while the expression of *cyclin h* did not change significantly, as alike in a previous report (Albrecht *et al.*, 1999). LIG1, which catalyzes DNA joining in the final step of NER (Gariboldi *et al.*, 1995), was expressed in the liver undergoing active cell proliferation. In this study, the expression of *lig1* was increased at 24-36 h, thus correlated with enhanced cell proliferation activity in this phase.

The protein kinases ATM and ATR are emerging as core sensors of DNA damage, capable of activating the downstream effector kinases CHK2/CHK1, as well as many other protein factors, through phosphorylation. The efficient transduction of DNA damage signals initiated by ATM/ATR, is not only CHK2/CHK1-dependent, but also requires a class of checkpoint mediators (Liu *et al.*, 2006). In the ATM signalling pathway, MDC1 assists other mediators in accumulating at sites of damaged DNA (Lukas *et al.*, 2004), whereas in the ATR, the RAD17-RFC2-5 and HUS1-RAD9-RAD1 complexes are possibly capable of recognizing and binding to DNA damage sites in substitution of RFC and PCNA (Ellison and Stillman, 2001). TELO2, essential for the mammalian S-phase checkpoint, impacts on CHK1 stability (Collis *et al.*, 2007), and p53, one of the targets of ATM, ATR and Chk1/Chk2, contributes to G1/s arrest (Canman *et al.*, 1998; Yang *et al.*, 2004). GADD45, dependent on p53, also participates in activating G2/m checkpoints, DNA repair and apoptosis (Vairapandi *et al.*, 2002). The interactions among these factors, and their expression changes during LR, are shown in Figure 3. The general view was that abundance of ATM proteins was invariable in the different cell-cycle phases. However, Lu *et al.* (2005) have shown that their expression levels, be-

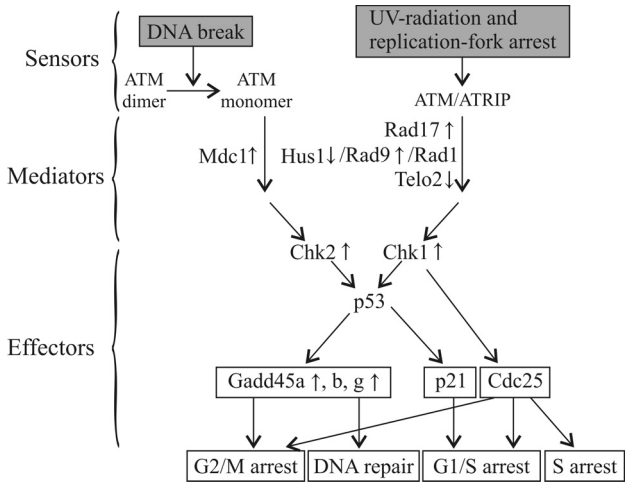

Figure 3 - Scheme of the signal transduction pathway in response to DNA damage. The genes with ↑ were up-regulated during LR, whereas those with ↓ were down-regulated.

sides being increased, were correlated with the onset of DNA replication during LR. Nonetheless, in the present study, there appeared to be no significant change, suggesting that their role in LR may be played through their downstream targets. Among the genes of interest shown in Figure 3, most of the other mediators and effectors, apart from weakly down-regulated *hus1* and *telo2*, were dramatically enhanced between 6-36 h. Furthermore, *gadd45a* and *gadd45g* in cluster C1, which immediately reached their peak levels at 2 h, were up-regulated almost throughout LR, this indicating their possibly significant role in signal transduction of DNA damage during the process.

In conclusion, the expression of BER- and TCR-related genes was enhanced at the transcriptional level in the early and intermediate phases of LR, whereas the expression of genes related to HR, NHEJ and DNA cross-link repair, as well as DNA polymerases and related accessory factors, and editing or processing nucleases, were mainly enhanced in the intermediate phase. Furthermore, gene expression in response to DNA damage was rather complicated throughout the whole LR process. It was also proposed that the expression of most DNA repair genes may be regulated by, or play a role in, progression through the cell division cycle during LR. However, the whole process (genem → RNA → protein) is influenced by many factors, including protein interactions. More important, it remains to be demonstrated that these changes at mRNA levels result in changes both at protein levels and in DNA repair capacity. Therefore, western blot, protein chip and RNA interference assays are required for further analysis of DNA repair genes and their regulation in regenerating liver.

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Supplementary Material

The following online material is available for this article:

Table S1 - Expression abundance of 69 DNA repair genes during rat liver regeneration.

Table S2 - Primer sequences used in real-time quantitative RT-PCR.

Figure S1 - Comparison of relative mRNA levels in regenerating liver detected by real-time RT-PCR and Affymetrix Rat Genome 230 2.0 microarray.

This material is made available as part of the online article from http://www.scielo.br.gmb.

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