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Dysregulation of DNA Repair Pathways in a Transforming Growth Factor α/c-myc Transgenic Mouse Model of Accelerated Hepatocarcinogenesis

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SUMMARY: Previous work from our laboratory has implicated oxidative DNA damage and genetic instability in the etiology of transforming growth factor-α (TGFα)/c-myc–associated hepatocarcinogenesis. In contrast, oxidative DNA damage was lower in c-myc single-transgenic mice, consistent with less chromosomal damage and with later and more benign tumor formation. We examined whether defects in the DNA repair pathways contribute to the acceleration of liver cancer in TGFα/c-myc mice. A cDNA expression array containing 140 known genes and multiplex RT-PCR were used to compare the basal levels of expression of DNA repair genes at the dysplastic stage. Thirty-five percent (8/23) and 43% (10/23) of DNA repair genes were constitutively up-regulated in 10-week-old TGFα/c-myc and c-myc transgenic livers, respectively, compared with wild-type controls. The commonly up-regulated genes were OGG1 and NTH1 of base excision repair; ERCC5, RAD23A, and RAD23B of nucleotide excision repair; and RAD50, RAD52, and RAD54 involved in DNA strand break repair. Additional treatment with a peroxisome proliferator, Wy-14,643, known to increase the level of oxidants in the liver, failed to induce a further increase in the expression level of DNA repair enzymes in TGFα/c-myc but not in c-myc or wild-type livers. Moreover, expression of several genes, including Ku80, PMS2, and ATM, was decreased in TGFα/c-myc livers, suggesting a fault or inefficient activation of the DNA repair pathway upon induction of oxidative stress. Together, the results show that DNA damage response is attenuated in TGFα/c-myc mice, creating a condition that may contribute to acceleration of liver cancer in this model. (*Lab Invest 2003, 83:643–654*).

Hepatocellular carcinoma (HCC), the major type of primary liver cancer, is one of the most common cancers worldwide and a leading cause of death in Asia and Africa (Stuver, 1998). Recent studies have revealed that hepatocarcinogenesis occurs in multiple stages, including chronic liver disease, adenomatous hyperplasia, early HCC, and advanced HCC (Sakamoto et al, 1991). Progress has been made in defining the global pattern of genetic alterations in HCC and understanding the relationships between DNA damage, mutagenesis, and cancer (Buendia, 2000; Ozturk, 1999).

It is generally accepted that cancer develops through a progressive accumulation of multiple genetic changes in cells (Hanahan and Weinberg, 2000). There is increasing appreciation that endogenous DNA damage is important in the etiology of many human cancers (Cadet et al, 2000; Frosina, 2000). Oxygen-free radicals, generated continuously during normal cellular metabolism, and their metabolites, collectively called reactive oxygen species (ROS), constitute the major class of endogenous toxic agents in an aerobic organism. At low concentrations, ROS act as intra- and intercellular second messengers modulating various aspects of cellular functions including proliferation, apoptosis, and gene expression (Droge, 2002). When produced in excess, ROS can induce DNA damage through both mutation and recombination (Marnett, 2000). The most frequent types of oxidative DNA damage are modified purine and pyrimidine bases, abasic sites, and DNA strand breaks (Cadet et al, 2000; Floyd, 1990; Otterlei et al, 2000). Alternatively, oxygen radicals can injure other cellular macromolecules such as lipids to generate reactive intermediates that couple to DNA bases (Marnett, 2000). Endogenous DNA damage induced by ROS is primarily repaired by a DNA base excision repair (BER) pathway (Mitra et al, 2001). However, recent evidence indicates that multiple DNA repair pathways, including transcription-coupled repair, nucleotide excision repair (NER), and mismatch repair (MMR) are required to repair oxidative DNA damage in vivo (Le Page et al, 2000; Mitra et al, 2001; Otterlei et al, 2000; Wang et al, 2000). Moreover, coordinated expression of several DNA repair enzymes is needed because the imbalanced overproduction may be harmful rather than beneficial with respect to protection against spontaneous mutagenesis (Glassner et al, 1998; Schar, 2001).

It has been suggested that inactivation of DNA repair pathways may underlie the genomic instability that in turn increases the risk of cancer (Schar, 2001).

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Earlier work from our laboratory has established that a constitutive stimulation of cell proliferation by overexpression of transforming growth factor-α (TGFα) and/or c-myc in the liver accelerates tumor development (Santoni-Rugiu et al, 1996). Significantly, TGFα/c-myc double-transgenic mice displayed a pleiotropic phenotype with much faster development of HCC than in either the TGFα or c-myc parental lines. The trademark features of liver oncogenesis in TGFα/c-myc mice are as follows: (1) high rate of cell proliferation; (2) chronic oxidative stress resulting from increased production of ROS; (3) defective cell cycle checkpoints and suppression of apoptosis; and (4) marked chromosomal instability (Factor et al, 1997, 1998; Santoni-Rugiu et al, 1998; Sargent et al, 1996). The latter was obvious as early as from the dysplastic stage preceding a rapid onset of tumor growth in this model. The chromosomal instability phenotype was characterized by gross chromosomal rearrangements and was observed in TGFα/c-myc but not in c-myc livers. Common chromosomal abnormalities included broken chromosomes and chromatids, fusions, and triradial and quadradial structures, suggesting genetic exchanges between nonhomologous chromosomes (Pfeiffer et al, 2000). The extent of damage was significantly increased in TGFα/c-myc tumors. Spectral karyotyping revealed deletions, amplifications, translocations, and gains or losses of whole chromosomes (Sargent et al, 1999). Extensive random-amplified polymorphic DNA analysis and microsatellite analysis further confirmed that TGFα/c-myc preneoplastic and neoplastic lesions acquire a wide spectrum of genomic damage (Calvisi et al, 2002).

Accumulation of gross chromosomal rearrangements, including double-strand DNA breaks (DSB), in TGFα/c-myc livers may be caused by increased production of oxygen radicals, errors during constitutive DNA replication, or inappropriate DNA repair. In mammalian cells, DSBs are repaired either by homologous recombination, essentially an error-free process, or by the nonhomologous DNA end-joining pathway, which is considered to be an error-prone process (Critchlow and Jackson, 1998; Pfeiffer et al, 2000). Increasing information on DSBs indicates that they originate not only when the appropriate pathway is not functional, but also when the ability to sense and activate damage-responsive functions, including the correct order of DNA repair and cell cycle progression, are impaired (Karran, 2000).

In the present work we have extended our observations to examine whether defects in DNA repair pathways may contribute to a high degree of genomic instability in this model. Because multiple repair pathways are involved in the removal of oxidative DNA lesions, we performed a global analysis of DNA repair gene expression using cDNA microarray technology. This approach allowed us to directly compare expression levels of multiple DNA repair genes both within and between different transgenic lines. Our results suggest that chronic oxidative stress generated in the liver by overexpression of the TGFα/c-myc transgenes leads to less efficient activation of DNA damage-responsive pathways, thereby promoting genomic instability and accelerating development of liver cancer in this transgenic mouse model.

Results

TGFα/c-myc Hepatocytes Show Altered Gene Expression of DNA Repair Enzymes

Radiolabeled cDNA probes synthesized from poly(A)$^+$ RNA isolated from livers of 10- to 12-week-old mice were hybridized to Atlas cDNA expression arrays (Clontech, Palo Alto, California), and the resulting hybridization patterns were compared between TGFα/c-myc transgenic and wild-type (Wt) animals. Expression of 65% of 140 known mouse genes present in the array were clearly detectable, with a threshold intensity at least twice that of the background (Fig. 1). The quantified average signals of nine housekeeping

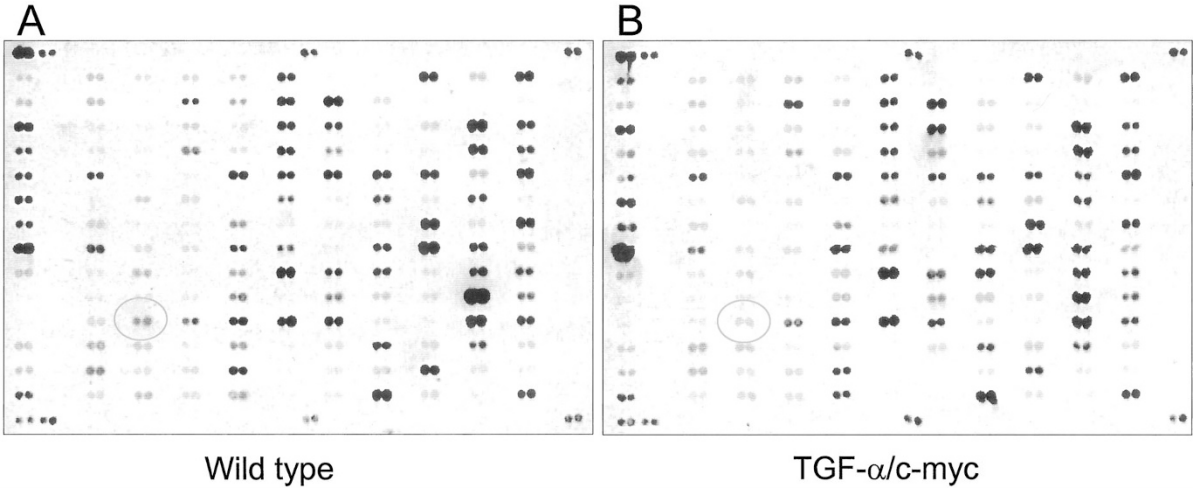

Figure 1.
Representative cDNA array hybridization images showing expression profiles of 140 genes in the livers of wild-type (A) and TGFα/c-myc mice (B). Clontech Atlas Mouse Stress Array membranes were hybridized with the ^{33}P-labeled cDNA probes prepared as described in "Material and Methods." The identity of the gene with *matching circles* is PMS2.

genes present in an array were used to normalize mRNA abundance, whereas negative controls served to confirm hybridization specificity. Genes were only declared differentially expressed if a difference in hybridization intensities between TGFα/c-myc and Wt liver cDNA microarrays was at least 1.5-fold.

We examined three individual mice of each genotype and found the results to be highly reproducible. The data showed consistent differences in mRNA expression profiles at the early stage of TGFα/c-myc–driven hepatocarcinogenesis. Of the 38 DNA repair genes present in the microarray, only 23 (61%) were expressed at detectable levels in both Wt and double-transgenic livers. However, in TGFα/c-myc cells, the steady-state levels of 11 (48%) of these 23 genes were either overexpressed (8 genes) or suppressed (3 genes) compared with Wt controls (Fig. 2).

Among the genes involved in BER—the predominant pathway used for removal of oxidized bases—OGG1 (8-oxo-guanine DNA glycosylase) and NTH1 (thymine glycol DNA glycosylase) were the most differentially expressed. In TGFα/c-myc livers, the steady-state levels of OGG1 and NTH1 mRNAs were increased 1.6- and 1.5-fold, respectively. However, the expression levels of Ungl (uracil-DNA glycosylase), DNL3 (DNA ligase III), and MPG (N-methylpurine-DNA glycosylase) did not change, whereas the level of PARP (poly(ADP-ribose) polymerase) was slightly decreased (1.3-fold). Coincidentally, several NER genes also known to repair oxidative DNA damage were up-regulated, including ERCC5 (excision repair cross-complementing rodent repair deficiency complementation group 5) (1.5-fold), RAD23A (UV excision repair protein homolog A) (1.6-fold), and RAD23B (DNA damage repair and recombination protein 23 homolog B) (1.7-fold), whereas the levels of XPC (xeroderma pigmentosum group C complementing protein) and TFIIH did not change. We also found that of seven genes involved in repair of DNA strand breaks, three were overexpressed, including RAD50 (DNA damage repair and recombination protein 50 homolog) (1.5-fold), RAD52 (DNA damage repair and recombination protein 52 homolog) (2.4-fold), and RAD54 (DNA damage repair and recombination protein 54 homolog) (2-fold), whereas Ku80 (ATP-dependent DNA helicase II 80-kDa subunit) was down-regulated (1.5-fold). Two other suppressed genes were PMS2 (postmeiotic segregation-increased 2 homolog) (1.7-fold), one of the MMR genes playing a role in the maintenance of microsatellite sequences, and ATM (ataxia telangiectasia mutated in human beings homolog; 1.5-fold), involved in sensing DSB and activating numerous damage-induced pathways (Kastan and Lim, 2000). Taken together these results imply that in young TGFα/c-myc livers, DNA

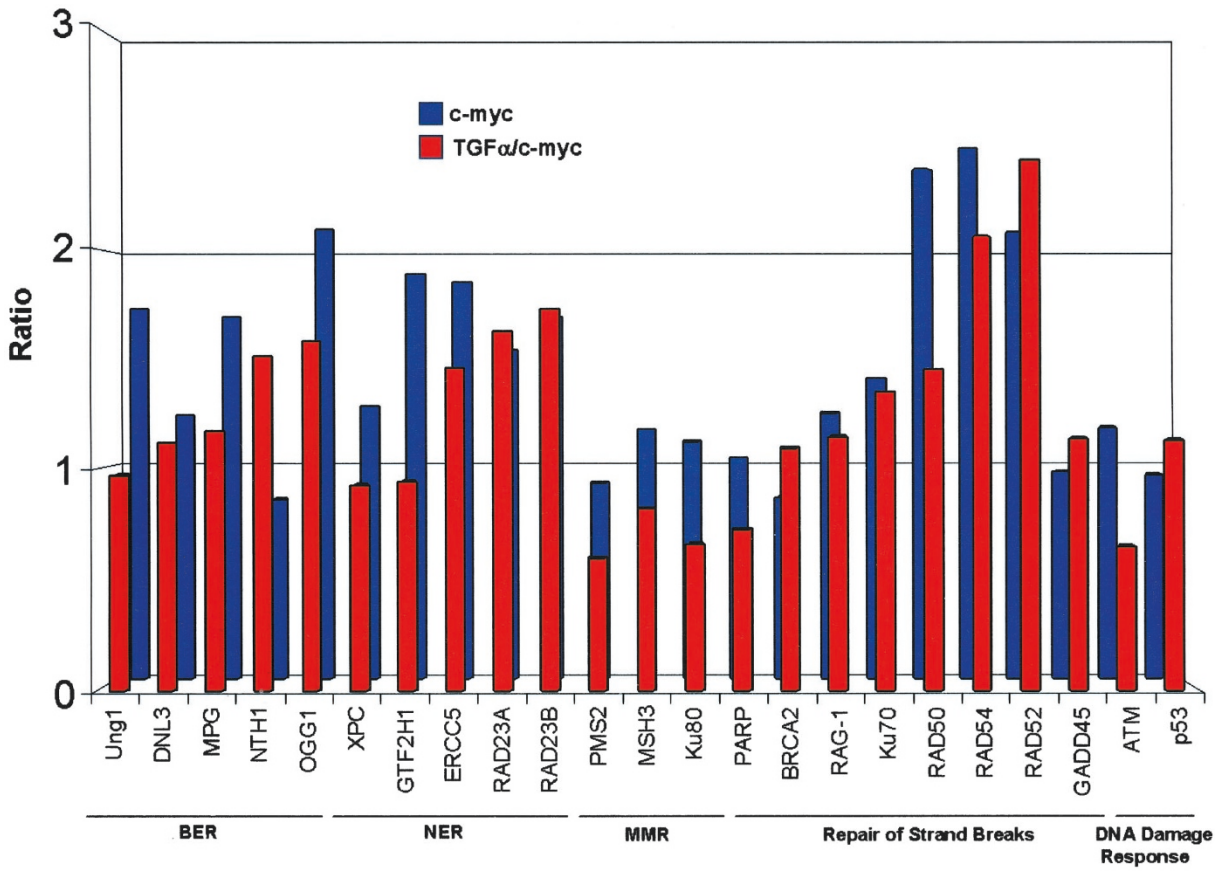

Figure 2.

Differential alterations in DNA repair gene expression profiles in livers of young adult c-myc and TGFα/c-myc mice relative to age-matched wild-type (Wt) controls. The data represent the average of three independent hybridizations expressed as ratio of amount of mRNA for indicated DNA repair genes in each transgenic line relative to corresponding Wt values.

repair enzymes are up-regulated unevenly, creating a state of imbalanced DNA repair.

c-Myc Hepatocytes Manifest Greater Levels of Induction of DNA Repair Genes than TGFα/c-myc Hepatocytes

Surprisingly, c-myc single-transgenic mice, which experience less oxidative load and DNA damage than TGFα/c-myc mice, yielded a similar pattern of up-regulation of DNA repair response when compared with Wt mice (10/23; 43.5%). In both transgenic mouse models, OGG1, RAD50, and RAD54 were the most differentially expressed DNA repair genes. The induction of DNA repair genes in c-myc livers seemed to be greater than in TGFα/c-myc livers (Fig. 2). However, no significant differences were seen in the expression of PMS2, MSH2, Ku80, PARP, and ATM, unlike TGFα/c-myc livers in which these genes were down-regulated when compared with Wt controls (Fig. 2).

Treatment with Wy-14,643 Failed to Induce DNA Repair Gene Response in TGFα/c-myc but Not c-myc Livers

Next we examined whether the capacity to respond to increased oxidative stress is different between transgenic models. For this purpose we performed a 2-week treatment with the peroxisome proliferator Wy-14,643 [4-chloro-6-(2,3-xylidino)-pyrimidynylthioacetic acid], which is known to increase production of oxygen radicals (Yeldandi et al, 2000). Consistent with the results published previously (Akiyama et al, 2001), Wy-14,643 treatment caused approximately a 2-fold liver enlargement in both transgenic mouse lines and Wt controls (Fig. 3). Likewise, RT-PCR analysis demonstrated a similar increase in levels of mRNAs encoding peroxisome proliferator receptor α (PPARα) and acyl-CoA oxidase (ACOX), the rate-limiting enzyme in the peroxisomal β-oxidation pathway (Fig. 4). Thus, Wy-14,643 elicited a comparable efficacy in terms of liver enlargement and induction of peroxisomal enzymes in both transgenic lines and Wt controls.

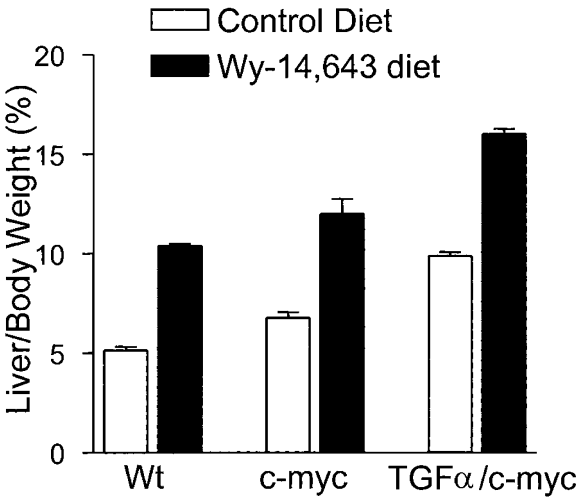

Figure 3.

Effect of Wy-14,643 on liver weight in Wt, c-myc, and TGFα/c-myc mice. Mice with constitutive expression of TGFα and/or c-myc transgenes had greater liver mass than Wt controls (*white bars*). However, Wy-14,643 dietary supplementation (0.1%) for 2 weeks resulted in a similar liver enlargement in mice of all genotypes (*black bars*). Values are expressed as percentage of total body weight ± SE (n = 3–5).

To examine the effects of Wy-14,643 on expression of DNA repair genes, we performed a mouse cDNA microarray analysis independently for three mice of each genotype. In Wt mice, Wy-14,643 resulted in 2.1 ± 0.15-fold (range, 1.5- to 3.8-fold) up-regulation of the majority of DNA repair genes (18/23; 78%) (Fig. 3). Similar induction of DNA repair gene response (13/23, 57%; range, 1.5- to 3.5-fold) was observed in c-myc transgenic mice upon Wy-14,643 treatment. In sharp contrast, TGFα/c-myc mice fed a Wy-14,643–containing diet failed to exhibit further increases in the expression levels of the DNA repair genes (Figs. 3 and 4). Only 2 of 23 DNA repair genes detected by cDNA microarray, including MPG and GADD45 (growth arrest and DNA damage-inducible protein 45), manifested a 1.5- and 1.7-fold elevation in mRNA levels, respectively. Moreover, Wy-14,643 treatment resulted in a 2.3-fold down-regulation of BRCA2 (breast cancer type 2 susceptibility protein), which, in addition to its essential function in suppressing gross chromosomal rearrangements, has the capacity to remove 8-oxoguanine (Kastan et al, 1992) (Fig. 5). In addition, unlike Wt or c-myc livers, the expression levels of five DNA repair genes including PMS2, MSH2, PARP, Ku70 (ATP-dependent DNA helicase II 70-kDa subunit), and ATM were decreased in TGFα/c-myc mice, whereas the majority of genes (14 of 23) exhibited no change in expression pattern upon Wy-14,643 treatment.

To validate and extend this study, we also determined the changes in mRNA levels of selected differentially expressed DNA repair genes by using semiquantitative RT-PCR. Both methods revealed comparable differences in the expression levels of either up-regulated (OGG1, ERCC5, and RAD52) or down-regulated (PMS2, MSH3, and Ku80) DNA repair genes in TGFα/c-myc livers in the presence or absence of Wy-14,643 treatment (Figs. 6 and 7). Taken together, the results show that the response to Wy-14,643 administration was less efficient in TGFα/c-myc mice, whereas c-myc mice showed a predictable increase in the expression of DNA repair genes upon induction of oxidative stress.

Finally, to further explore the differences in the DNA repair responses between Wt, c-myc, and TGFα/c-myc mice, we investigated the effect of Wy-14,643 on p53, the tumor suppressor gene that preserves genetic stability by inducing cell cycle checkpoints and enhancing DNA repair (Moller and Wallin, 1998). As expected, exposure to Wy-14,643 induced a marked accumulation of both p53 RNA and protein in Wt livers. In contrast, no increases compared with controls were seen in double-transgenic livers after Wy-14,643 treatment (Figs. 5 and 8).

Discussion

Our results demonstrate that a number of genes involved in different DNA repair pathways were con-

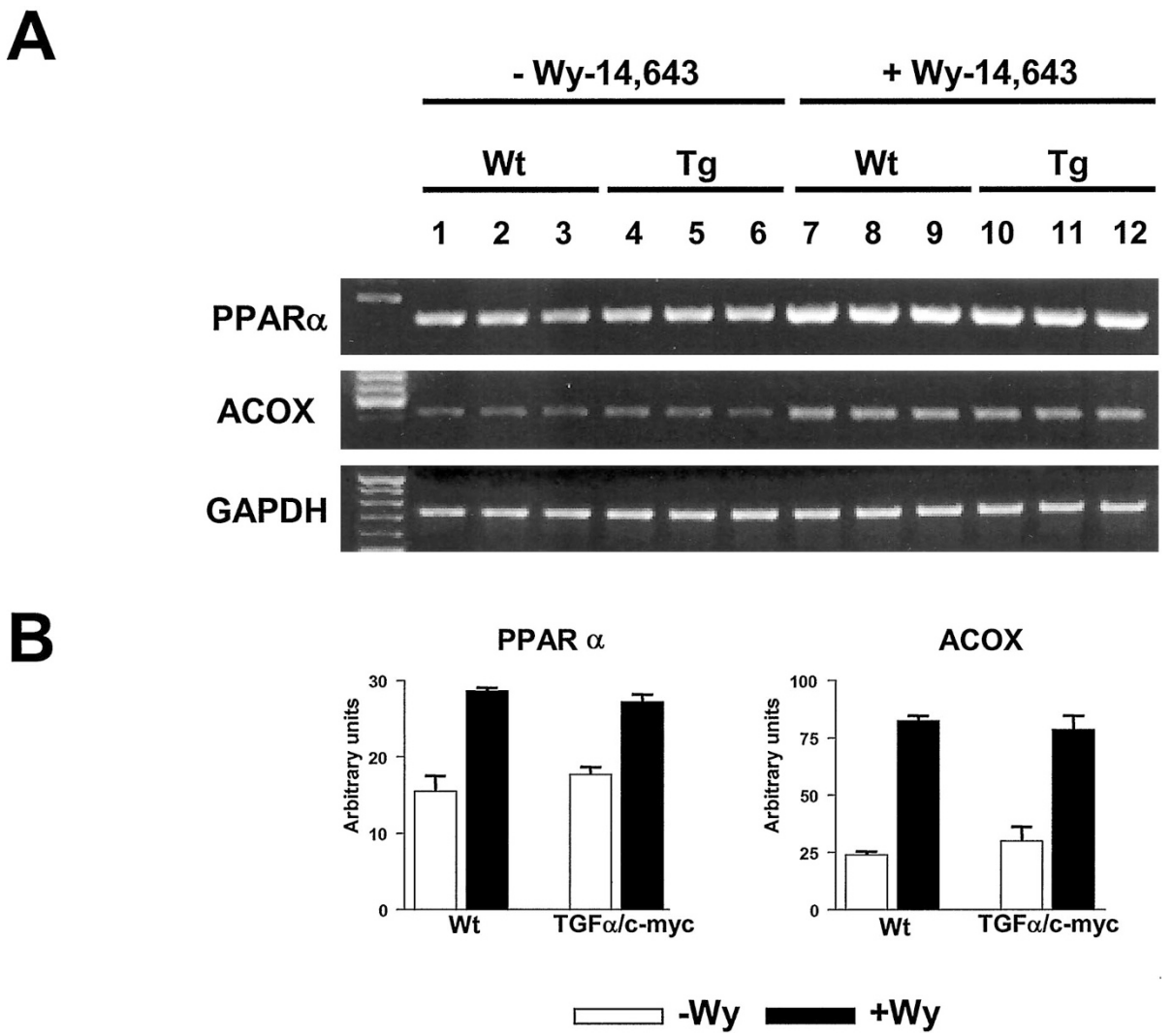

Figure 4.

A, Wy-14,643 treatment induced expression of peroxisome proliferator receptor α (PPARα) and acyl-CoA oxidase (ACOX) both in Wt and transgenic livers as shown by RT-PCR analysis. B, Data from A were quantified by densitometric scanning of ethidium-stained bands of amplified fragments. For each sample, the amount of PPARα and ACOX mRNA was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Each bar represents the mean $\pm$ SE ($n = 3$).

stitutively up-regulated in the dysplastic livers of young c-myc and TGFα/c-myc mice. The largest increases detected by cDNA microarray analysis were seen for the OGG1 and NTH1 of BER, RAD23A, and RAD23B of NER (known to repair oxidative DNA damage) (Cadet et al, 2000; Moller and Wallin, 1998). In addition, three of seven genes involved in repair of DNA strand breaks were also overexpressed, including RAD50, RAD52, and RAD54. The up-regulation of the primary DNA defense system might be replication related to preserve the integrity of the genome in continuously replicating transgenic hepatocytes. This finding is in agreement with observations in which c-myc has been shown to couple DNA synthesis to the induction of genes involved in DNA repair (Menssen and Hermeking, 2002). Increased expression of DNA repair genes may also relate to the fact that overexpression of c-myc and TGFα transgenes creates a state of chronic oxidative stress, consistent with our previous data (Factor et al, 1998). In fact, production of ROS as determined by DCFH, the peroxidation sensitive fluorescent dye, was substantially greater in TGFα/c-myc than in c-myc livers and occurred in parallel with an increase in lipid peroxidation, suggesting that TGFα/c-myc cells are under a greater oxidative load (Factor et al, 1998). Furthermore, TGFα/c-myc hepatocytes accumulated significantly more DNA damage than c-myc cells at any stage of hepatocarcinogenesis (Calvisi et al, 2002; Factor et al, 1998). Importantly, when Wy-14,643 (known to increase the level of oxidants in the liver) (Rusyn et al, 2001) was included in the diet, a clear response was observed only in Wt and c-myc mice. As expected, the genes of BER and NER pathways directly involved in repair of oxidative DNA lesions, including DNL3, MPG, NTH1, OGG1, and RAD23A/B, respectively, were the most differentially expressed, in agreement with previously published data (Rusyn et al, 2000). In addition, expression of GADD45, ATM, and p53, critical for a DNA damage response, was ele-

Table 1. The Primer Sequences and PCR Strategy Used in This Study

Gene	Primer sequences (5′-3′)		Annealing temperature (°C)	Product size (bp)	Ratio (GAPDH: target gene) for multiplex RT-PCR	PCR cycles
	Sense	Antisense				
PMS2	GCGTGTTATCCTCTTATCCTTC	GGTGCTGTAACTGCTTCATTCG	56	537	0.75:6	35
MSH3	AAGCAGCAGCATAAAGACGCAG	GAAGCAGTCAAACACAACCTCG	60	498	0.15:1	35
MLH1	TTTGGTGTTCTGAGGTTATCGG	TGTGAAATGCTTCGGAGGTAGG	58	495	—	35
MSH2	GATGAACTTTGAGTCTTTCGTG	GTCGGCTCTTTCTTTCTGTG	58	448	—	35
MSH6	CGTTACCAGTTAGAGATTCCAG	CTTCTTCCTCGCAGCCTATTAG	58	439	—	35
ERCC1	AGGAGCAAAGTCTAATAGCATC	GTCGGTCTTGTTCACAGATTTC	58	466	0.25:1	30
ERCC2	GGAGTATGTCAAGTGGCGTCTG	GGCAAAGTTAGCAAGGAGTGTG	62	202	—	35
ERCC3	GAAGAGGAAGAGGAAGACGAAG	CTTGAAGAAGATGCTGGATAAC	58	282	0.25:1	30
ERCC4	TGAGAGCAACAGAGAAGGTG	ATAAAGTCGCAGCAGGAAGG	62	371	—	35
ERCC5	GCCCTCTCACCAGCATTCATAG	CCCACCTTCTGTTTCATCGTCAC	60	217	0.3:1	35
XPA	CCTTGCCTGGTGATGGAGTAG	TCTTGCTTCGCTTCTGCTTGG	57	525	—	35
XPC	GGAGAGGGAGAAGAAGAAAGAC	CAGTTACTGGATGGGAATAGCC	58	212	0.2:1	30
OGG1	ATTCCAAGGTGTGAGACTGC	TTAGGATGCCAGCCGTAGTC	56	474	0.4:1	30
MPG	CAGATTCAGAGCAGCAGCAGAC	CCAGCCACACAGCATCATCTTG	64	579	—	35
DNA-PKcs	CGTACGGTGTTGGCTACTGC	ATCAGAAGGTCTAAGGCTGGAAT	60	375	—	28
Ku70	TTCCTCCCTTATGCCGATGAC	TTACCCAGTGTGCCCTTACG	58	419	—	30
Ku80	GCTGTTGTGCTGTGTGTGGAC	TTGAGGGAAGGAGGGTTTGAG	60	558	0.2:1	35
RAD50	AAGAAGAAAGTGCTAAAGTGTGCC	TTCCAATGCTGTCTCCAAAGGG	58	419	—	33
RAD51	TGTAGCATATGCGCGAGGGT	CCAATGGGTTTTTTGGGATC	56	301	—	33
RAD52	CTGAGACAGAGACTGGGTCC	TCATGTGGGGGGCTTGATCT	56	574	0.125:1	33
RAD54	TCCAGCCAATGATGAACAAG	CAAACAACTACTCCAGACCAG	56	485	—	33
AOX	AGGTTCAGTCGGGGAAGCTGG	ATCTGAGCCCCTGTGATGATG	60	430	—	40
PPARα	GCGGCCCCATACAGGAGAGCAG	CTAACCTTGGGCCACACCCTGACT	60	409	—	36
GAPDH	CCATGTTTGTGATGGGTGTGAACC	TGTGAGGGAGATGCTCAGTGTTGG	56–60	712	NA	NA

NA, not available.

vated after a 2-week treatment with Wy-14,643 in both c-myc and Wt mice. In contrast, Wy-14,643 failed to induce a further increase in the expression level of DNA repair enzymes in TGFα/c-myc mice. Moreover, the expression of 3 of 23 DNA repair genes, including ATM, Ku80, and PMS2, was decreased in TGFα/c-myc dysplastic livers, suggesting a fault or inefficient activation of DNA repair pathways that may lead to the bypass of DNA damage checkpoints, accumulation of mutation, and acceleration of tumorigenesis observed in these mice.

Thus ATM, one of the down-regulated genes in double-transgenic livers, encodes a multifunctional protein involved in the maintenance of cellular homeostasis in response to oxidative damage (Gatei et al, 2001). Cells deficient in ATM fail to induce a DNA damage response and show increased frequency of chromosomal aberrations after exposure to ionizing radiation (Cornforth and Bedford, 1985; Morgan et al, 1997). Similarly, cells deficient in Ku80 display impaired nonhomologous DNA end-joining repair and increased sensitivity to DNA damaging agents inducing DSBs (Nussenzweig et al, 1996; Zhu et al, 1996). ATM $-/-$ cells also display an impaired ability to correctly regulate an apoptotic pathway (Humar et al, 1997; Meyn et al, 1994) as well as a defective or delayed increase in the p53 protein after ionizing radiation (Khanna and Lavin, 1993). In normal cells, the amount of p53 protein is increased after DNA damage through a posttranscriptional stabilization mechanism that includes both phosphorylation and suppression of ubiquitination, leading either to damage repair coupled with arrest of the cell cycle, predominantly in the G1 phase, or to apoptosis (Banin et al, 1998; Maki and Howley, 1997).

It has been suggested that the ATM protein functions by sensing DNA damage and signaling events that lead to activation of p53 through a phosphorylation process (Hartley et al, 1995; Morgan and Kastan, 1997). In this context, it seems likely that down-regulation of ATM expression might affect the activation of the p53 protein in TGFα/c-myc mice. The basal levels of p53 expression were not elevated in TGFα/c-myc livers despite the presence of intrinsic DNA damage, including DNA single- and double-strand breaks (Khanna and Jackson, 2001). Given that p53 is a redox-sensitive protein (Hainaut and Milner, 1993; Meplan et al, 2000), these data raise the possibility that p53 activity may be defective in TGFα/c-myc hepatocytes that undergo excessive exposure to endogenously generated ROS. In support of this hypothesis, we have recently found that expression of several genes involved in reactive oxygen metabolism and transcriptionally regulated by p53, is dramatically altered in TGFα/c-myc mice. Thus, both mRNA and protein levels of an inducible form of nitric oxide synthase (NOS2), which is repressed by p53 (Ambs et al, 1998a, 1998b; Forrester et al, 1996), were increased along with a progression of hepatic lesions. We also found up-regulation of nitrotyrosine levels in

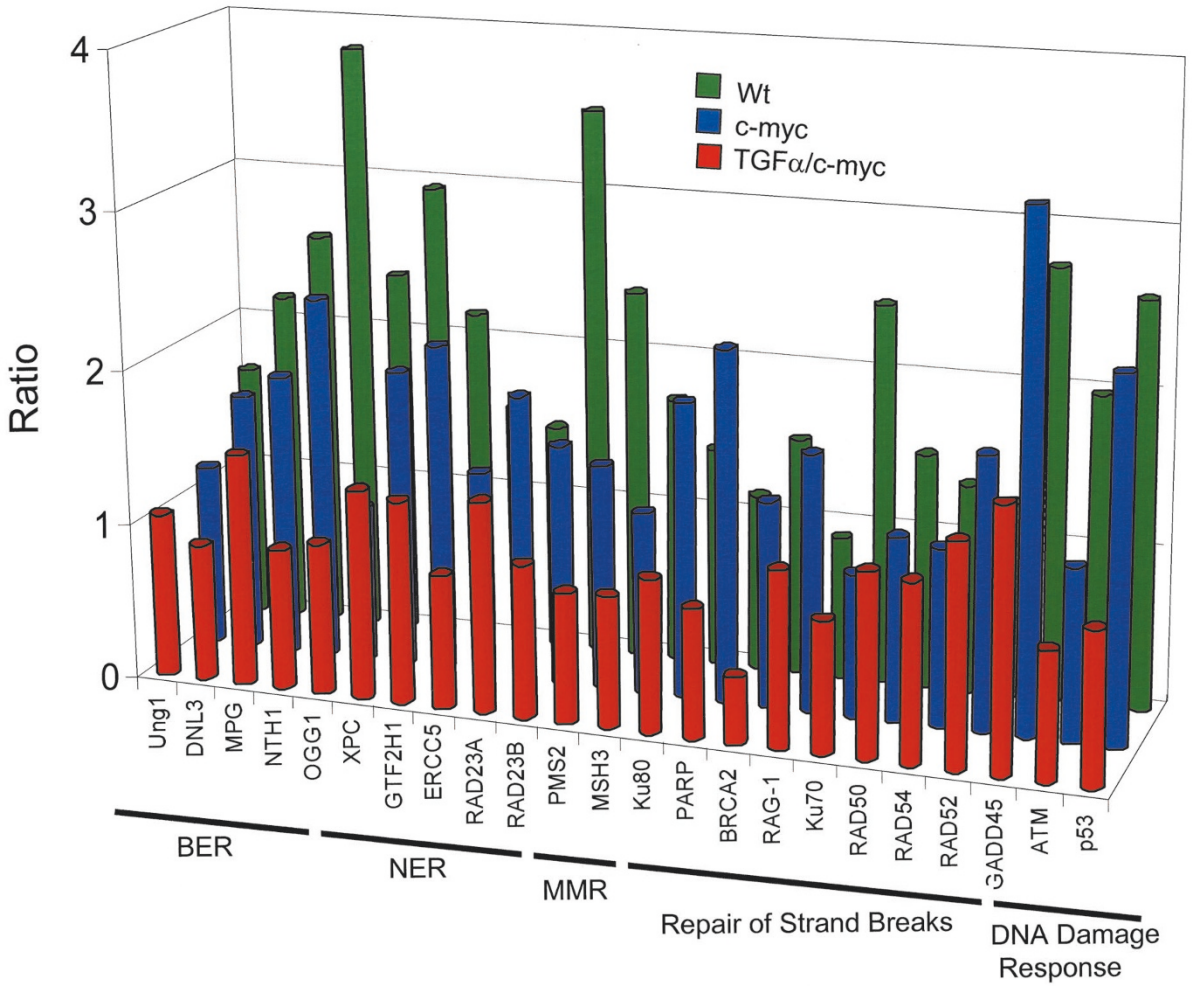

Figure 5.
Comparison of DNA repair responses in Wt, c-myc, and TGFα/c-myc mice treated with Wy-14,643. The data are presented as ratios calculated by dividing the amount of RNAs for each indicated DNA repair gene in Wy-14,643–treated mice by the corresponding value obtained in untreated mice of the same genotype.

livers of young adult TGFα/c-myc mice, which indicates NO-mediated oxidative damage of proteins (DF Calvisi et al, unpublished observations).

Similarly, the activity of a primary antioxidant enzyme glutathione peroxidase, another gene transcriptionally activated by p53 (Tan et al, 1999), was decreased at the dysplastic stage and almost totally absent in TGFα/c-myc HCC (Factor et al, 1998). Although the role of p53 during TGFα/c-myc–associated hepatocarcinogenesis requires further experimental evaluation, it seems plausible that activation of the damage-response pathway might be less efficient in TGFα/c-myc livers. The decreased ability of cells to adequately respond to increased DNA damage might permit cellular proliferation under genotoxic stress conditions and account for genomic instability and cancer predisposition in this model. In contrast, cells with chromosomal abnormalities do not accumulate in c-myc transgenic livers (Sargent et al, 1999), suggesting either more efficient DNA damage repair or apoptosis. In agreement with this, only TGFα/c-myc livers exhibited significant levels of net proliferation (mitosis minus apoptosis), whereas c-myc mice displayed a higher rate of apoptotic cell death at all stages of hepatocarcinogenesis (Santoni-Rugiu et al, 1996). The more effective DNA damage response pathways in c-myc mice accompanied by less oxidative stress might account for delayed tumor development and for a lower frequency and malignancy of c-myc tumors (Santoni-Rugiu et al, 1996).

PMS2, another DNA repair gene that was downregulated in the TGFα/c-myc dysplastic liver, functions in MMR. It was the only MMR gene that was detectable on the cDNA array. However, we found no differences in the expression of MSH2, MSH6, and MLH1 between Wt and TGFα/c-myc mice by RT-PCR (data not shown). MMR genes maintain genome stability by correcting mismatches and small insertion or deletion loops introduced by DNA polymerases (Buermeyer et al, 1999; Kolodner and Marsischky, 1999; Modrich and Lahue, 1996). In addition, evidence is emerging that the MMR genes may participate in various other cellular functions, such as transcription-coupled repair (Mellon et al, 1996), recombination (Worth et al, 1994), and cell cycle regulation (Hawn et al, 1995). The importance of MMR in preventing ac-

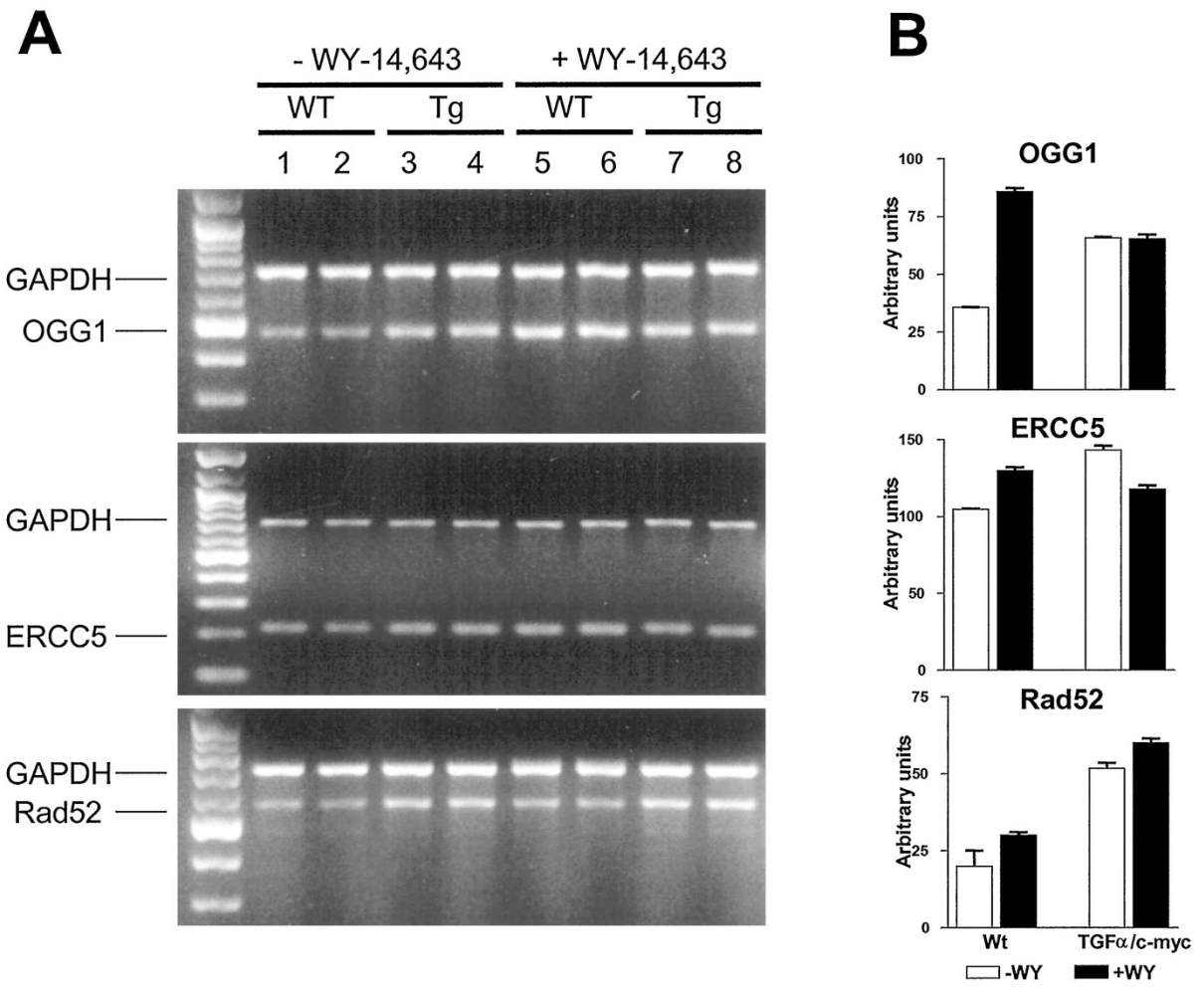

Figure 6.

RT-PCR analysis of up-regulated genes identified by Atlas cDNA expression array. A, *Lanes 1 and 2*, Wt mice; *lanes 3 and 4*, TGFα/c-myc mice; *lanes 5 and 6*, Wt mice treated with WY-14,643; *lanes 7 and 8*, TGFα/c-myc mice treated with WY-14,643. B, Levels of mRNA expression of OGG1, ERRC5, and RAD52 were quantified by densitometric scanning of ethidium-stained bands of amplified fragments after normalization with GAPDH. Each bar is the mean $\pm$ SE.

cumulation of mutations in cancer-related genes has been demonstrated during the multistage process of tumorigenesis (Vogelstein and Kinzler, 1993). In particular, transgenic and knockout mice proved to be very useful for studying the relationship between individual MMR proteins and cancer development (Baker et al, 1995; Prolla et al, 1998). Mice deficient in any of the MMR proteins, except PMS1, have been shown to develop a high frequency of tumors after 2 months of age (Prolla et al, 1998).

In TGFα/c-myc mice, down-regulation of PMS2, which is located in chromosome 5G2, might be associated with a common balanced translocation (5:6)(G2:G2) (Sargent et al, 1999). In addition, nonrandom chromosomal aberrations during TGFα/c-myc–driven hepatocarcinogenesis include a frequent loss of whole chromosomes 7 and 9, where ERCC1 and ATM are located. According to our data, the mRNA levels of ERCC1 in HCCs in TGFα/c-myc mice were barely detectable (data not shown).

In conclusion, our results imply that DNA repair responses are altered in young adult TGFα/c-myc mice. The overall difference in the expression profile of multiple genes, often small relative to control, may undermine the efficiency of DNA repair and constitute a risk factor in this model of liver cancer.

Materials and Methods

Mice

c-Myc single transgenic, TGFα/c-myc double-transgenic, and Wt male mice were generated as described previously (Factor et al, 1997; Jhappan et al, 1990; Murakami et al, 1993). The animal study protocols were conducted according to the National Institutes of Health guidelines for animal care.

Wy-14,643 Treatment

WY-14,643 was obtained from Chemsyn Science Laboratories (Lenexa, Kansas). Pelleted mouse chow containing 0.1% of WY-14,643 was prepared by Bio-Serve (Frenchtown, New Jersey). Three groups of mice (3–4 animals each) including c-myc, TGFα/c-myc, and Wt mice were fed a WY-14,643–containing diet for 14 days starting from 8 to 10

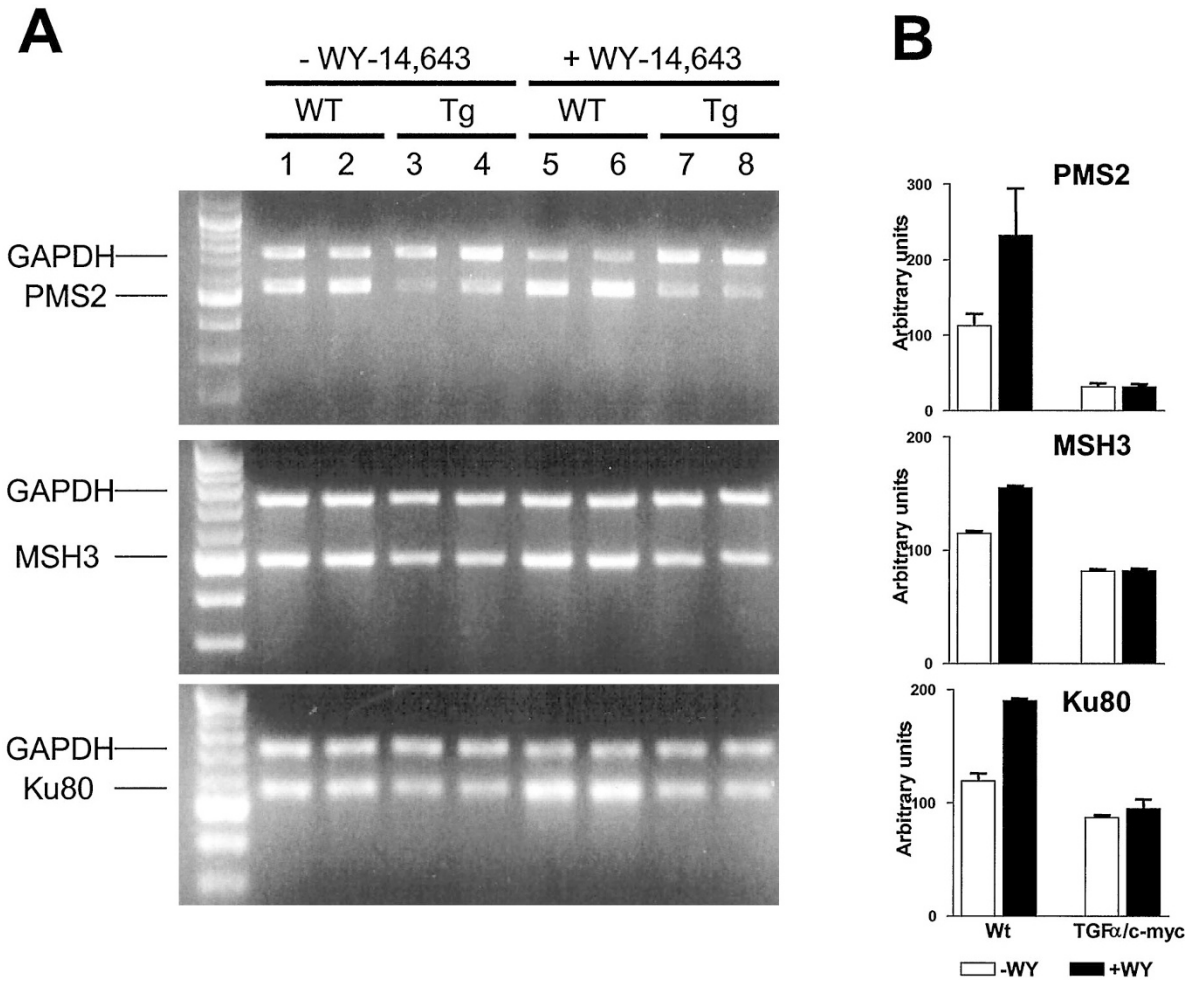

Figure 7.
RT-PCR analysis of down-regulated genes identified by Atlas cDNA expression array. A, *Lanes 1 and 2*, Wt mice; *lanes 3 and 4*, TGFα/c-myc mice; *lanes 5 and 6*, Wt mice treated with WY-14,643; *lanes 7 and 8*, TGFα/c-myc mice treated with WY-14,643. B, Levels of mRNA expression of PMS2, MSH3, and Ku80 were quantified by densitometric scanning of ethidium-stained bands of amplified fragments after normalization with GAPDH. Each bar is the mean ± SE.

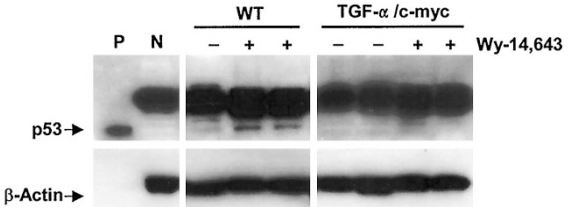

Figure 8.
Representative Western blotting of p53 protein in whole cell lysates prepared from Wt and TGFα/c-myc livers before and after Wy-14,643 treatment. *P* = positive control (Cos7); *N* = negative control (p53 −/− liver).

weeks of age. Mice were killed by cervical dislocation. Livers were then fixed in 10% formalin and frozen in liquid nitrogen with subsequent storage at −80° C.

Mouse cDNA Expression Array

The Altas cDNA mouse array was purchased from Clontech Laboratories. A list of the genes spotted on the array is available at Clontech's web site (http://www.clontech.com).

cDNA Synthesis and Microarray Hybridization

^{33}P-labeled cDNA probes were prepared using liver poly(A)$^+$ RNA isolated from Wt and transgenic livers as described previously (Ye et al, 1999). Hybridizations were performed as recommended by the manufacturer (Clontech). The arrays were exposed to phosphor screens overnight and scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, California). The results were quantified using the ImageQuant software (Version 3.3; Molecular Dynamics). Background signal was defined as the average of the hybridization signals produced by the negative controls on the array. A gene was considered to be expressed when the hybridization signal extended at least 2-fold over a background signal. The average intensity of all nine housekeeping genes was used to normalize the expression level of each gene. The genes with more than 1.5-fold differences in the

integrated density values were considered to be differentially expressed. According to the manufacturer, the radioactive cDNA signal is linear for RNAs present at levels of 0.01% to 3% of the total population.

RT-Procedure

Reverse transcription of poly(A)$^+$ RNA with oligo(dT) primers was performed using the SuperScript Preamplification System for First Strand cDNA Synthesis (Invitrogen, Carlsbad, California) according to the manufacturer's instruction. Briefly, the denaturation of RNA was performed at 65° C for 5 minutes in a reaction mixture consisting of 0.1 μg poly(A) RNA, 500 ng oligo(dT)$_{12–18}$ primer, and 1 mM each dNTP in a volume of 10 μl. After quick chilling on ice, 0.1 M dithiothreitol, RNase inhibitor, and 5$\times$ buffer were added to the reaction mixture. Then, the primer extension was performed at 42° C for 50 minutes in the presence of 0.1 μg poly(A)$^+$ RNA, 500 ng oligo(dT)$_{12–18}$ primer, 500 μM each dNTP, 1$\times$ RT buffer, 10 mM dithiothreitol, 40 U of RNase inhibitor, and 50 U of SuperScript II reverse transcriptase in a final volume of 20 μl. Subsequently, reaction mixtures were heated to 70° C for 10 minutes to inactivate the reverse transcriptase; then 2 U of RNase H were added to each tube, incubated for 20 minutes at 37° C, and stored at −70° C.

To check for the presence of contaminating genomic DNA, RT-PCR was performed using poly(A)$^+$ RNA without avian myeloblastosis virus-reverse transcriptase.

Multiplex-PCR Analysis

The PCR primers were designed based on the published sequences from GenBank. The optimal primers were selected with GeneRunner Software (Version 3.05; Hastings Software, Inc., Allen, Texas). The primer sequences used in this study, the expected size of each RT-PCR amplicon, and the annealing temperature for each primer set are listed in Table 1. Both glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping control gene, and a target gene were amplified in a single reaction. To ensure that the analysis was performed at the linear range of the amplification assay, a range of ratios and cycle numbers were used and the quantification was conducted at a cycle number within a linear range. The ratios of primer sets between a target gene and GAPDH are shown in Table 1. Each PCR reaction contained 1 μl of cDNA synthesis reaction, indicated concentrations of primers for each target gene and GAPDH, 200 μM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 22 U of PLATINUM *Taq*DNA polymerase and PLATINUM *Taq* antibody (Invitrogen) in a final volume of 50 μl. Thermal cycling was performed by using the following amplification profile: initial denaturation at 94° C for 4 minutes, various cycles of denaturation at 94° C for 30 seconds, annealing at various temperatures (as shown in Table 1) for 30 seconds, extension at 72° C for 1 minute, and a final elongation step at 72° C for 10 minutes.

PCR Analysis

Normal PCR reactions were performed for all genes shown in Table 1. The primer sequences, the expected size of each RT-PCR amplicon, and the annealing temperature for each primer set are shown in Table 1. Briefly, each PCR reaction contained 1 μl of cDNA synthesis reaction, indicated concentrations of primers of each target gene, 200 μM each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), and 22 U of PLATINUM *Taq*DNA polymerase and PLATINUM *Taq* antibody (Invitrogen). Amplification reactions were conducted in a 50 μl volume with an initial step at 94° C for 2 minutes. This was followed by 25 to 35 cycles of amplification depending on the gene abundance in liver tissue. Each cycle was 30 seconds at 94° C, 30 seconds at various temperatures (shown in Table 1), and 1 minute at 72° C, with a final extension of 10 minutes at 72° C.

Quantitation

To quantify relative levels of gene expression, the PCR products were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide, and photographed. The bands on the photos were scanned, and the densities of the peaks were calculated in arbitrary units by ImageQuant Software (Version 3.3; Molecular Dynamics). To evaluate the relative levels of expression of the target genes in multiplex RT-PCRs, the value of the GAPDH was used as the baseline gene expression in that sample, and the relative values were calculated for each of the target genes amplified in that reaction.

Western Blot Analysis

Hepatic tissue samples were homogenized by glass dounce homogenizer in lysis buffer (30 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, and 2 mM EDTA) containing the Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals, Indianapolis, Indiana). Protein concentrations were determined by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, California) using BSA as standard. Proteins were separated by SDS-PAGE in 10% acrylamide gels and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed after incubating the membrane in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 hour and then sequentially probing with p53 mAb diluted 1:1000 (Novus Biologicals, Inc., Littleton, Colorado) and β-actin mAb diluted 1:300 (Chemicon International, Temecula, CA). Each primary antibody was followed by incubation with mouse IgG-horseradish peroxidase-linked whole antibody (1:10,000, 1 hour) and ECL+PLUS detection reagents (Amersham International, Arlington Heights, Illinois).

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