Irradiation-induced p53 expression is attenuated in cells with NQO1 C465T polymorphism

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Introduction

The NAD(P)H:quinone acceptor oxidoreductase (NQO) gene family belongs to the flavoprotein and consists of 2 genes, NQO1 and NQO2, which catalyze the beneficial two-electron reduction of quinones, which results in their detoxification.

NQO1 polymorphism is associated with the increased risk of benzene-induced hematotoxicity as well as the susceptibility to various forms of cancer. More than 93 single nucleotide polymorphisms (SNPs) have been identified in the NQO1 gene. The most widely studied SNP of NQO1 is C609T (rs1800566), also known as NQO1*2. This nucleotide change results in an amino acid change from proline (P) to serine (S) at codon 187 that leads to the loss of enzyme activity due to instability of the protein product. The allele frequency of C609T varies between 16% and 49%. Another genetic polymorphism of NQO1 is a single nucleotide change of C465T (rs4986998), also known as NQO1*3, which changes the amino acid at codon 139 from arginine (R) to tryptophan (W). This SNP also results in alternative mRNA splicing that leads to the deletion of exon 4 and generates a protein lacking the quinone binding site. The allele frequency of the C465T polymorphism ranges between 0% and 5%.

Several studies have reported the association between the NQO1*2 variant allele and infant leukemia with MLL rearrangement, childhood acute lymphoblastic leukemia (ALL), and therapy-related leukemias. p53 is the most commonly mutated tumor suppressor gene in various types of cancers. DNA damage induces p53 accumulation in an Ataxia Telangiectasia Mutated (ATM)-dependent manner. p53 interacts with mouse double minute...
2 (MDM2) protein and undergoes ubiquitination and 26S proteasomal degradation. ATM controls p53 stability by phosphorylation of MDM2 and E3 ligase processivity. Not only 26S proteasomal degradation of p53, 20S proteasomal degradation pathway was also reported. NQO1 and NQO2 interact with p53 and protect p53 against 20S proteasomal degradation.

These findings prompted us to investigate the relationship between DNA damage dependent p53 stabilization pathway and NQO1 polymorphism.

**Materials and Methods**

**Cell lines**

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (EB-LCLs), HCT116 and U2OS cells were maintained as reported elsewhere.

**Plasmid**

NQO1 C465T mutant was generated by in vitro mutagenesis using QuickChange II site-directed mutagenesis kit (Stratagene). NQO1 exon 4 skip variant (isoform c) was generated using PCR method using primers tagged with overlapping homologous sequences; sense primer, CCTTGTGATATTCCAGAGTAAGAGCAGTGCTTTC and antisense primer, CTGCCTTCTTACTCTGGAATATCACAAGGTCTG.

**Reverse transcriptase (RT)-PCR**

RT-PCR for the detection of NQO1 isoform b and/or isoform c was performed using sense primer in exon 3 and antisense primer in exon 6.

**Western blotting**

Western blotting was performed by standard techniques by using the following antibodies: ATM (5C2), p53 (Do-1), MDM2 (SMP14), p21 (C-19), and NQO1 (A180) from Santa Cruz; β-actin (AC-15) from SIGMA; phospho ATM Serine 1981 (10H11.E12) and phospho Chk2 Threonine 68 from Cell Signaling; HA (3F10) from Roche Diagnostics; and tubulin (DM1A) from Calbiochem.

**Expression of NQO1 in the cells with polymorphism**

EB-LCLs were genotyped for NQO1 polymorphism, and 4 EBV-LCLs with different type of polymorphism such as wild type (NQO1 *1*1), heterozygous C609T (NQO1 *1*2), homozygous C609T (NQO1 *2*2), and heterozygous C465T (NQO1 *1*3) were selected for this study. NQO1 expression was undetectable in NQO1 *2*2 cells, as reported previously. Its expression in NQO1 *1*2 cells was significantly less than that in the NQO1 *1*1 cells. Interestingly, NQO1 expression was also undetectable in NQO1 *1*3 cells (Fig. 1A), although it was slightly detected with long exposure (Fig. 1B). Interestingly, Expression of full length mRNA levels (isoform a) and exon 5-deleted splicing variant (isoform b) showed no difference among the LCLs, although expression of exon4-deleted splicing variant (isoform c) was higher in NQO1 *1*3 cells (Fig. 1C) as reported previously, which suggests that undetectable NQO1 in NQO1 *1*3 cells may be post-transcriptionally regulated. Wide variety of chemicals such as benzene metabolites or cellular stimulations are known to induce NQO1 expression. However, in our study, irradiation had no effect on the level of NQO1 expression in any of the LCLs with NQO1 *1*1 (WT), NQO1 *1*3, or NQO1 *2*2 genotype (Fig. 1B).

DNA damage-induced induction of p53 expression in NQO1 C609T homozygous variant and C465T heterozygous variant

The induction of p53 expression is lowered in NQO1 knock-out cells after benzo(a)pyrene treatment or irradiation. p53 induction is attenuated in cells treated with Dicoumarol (NQO1 inhibitor) after irradiation. These results prompted us to investigate whether p53 induction is attenuated in NQO1 *2*2 cells after irradiation. Unexpectedly, the induction of p53 expression in NQO1 *2*2 cells was similar to that in NQO1 *1*1 cells after exposure to irradiation (Fig. 2A). These findings were consistent with those reported by Fagerholm et al. who also observed similar p53 expression between NQO1 *1*1, NQO1 *1*2, and NQO1 *2*2 cells after epirubicin treatment. On the basis of our results and the findings of previous reports, it was concluded that the NQO1 *2*2 status has no effect on p53 accumulation after DNA damage in EB-LCLs. Next, induction of p53 expression in heterozygous NQO1 C465T (NQO1 *1*3) variants was assessed. It was noted that the induction of p53 expression after irradiation was attenuated in NQO1 *1*3 cells (Fig. 2A, B and C). The expression of p53 downstream protein p21, in
NQO1 *1*3 cells was also attenuated (Fig. 2B and D). However, ATM and its downstream protein Chk2 were phosphorylated to similar extents in NQO1 *1*3 and NQO1 *1*1 cells, and MDM2 kinetics were indistinguishable between them (Fig. 2B). This finding suggests that the defective p53 and p21 induction in NQO1 *1*3 cells is not due to a failure of ATM activation. These observations prompted the elucidation of the biochemical mechanism underlying defective p53 induction after DNA damage in NQO1 *1*3 cells. HCT116 colon cancer cell line is known to carry C465T polymorphisms in the heterozygous state. Hemagglutinin (HA)-tagged wild-type NQO1 was transiently transfected to HCT116 cells, and p53 induction was investigated after irradiation. Induction of p53 expression after irradiation was shown to be indistinguishable between mock transfected and wild-type NQO1 transfected cells (Fig. 3). Interestingly, the expression level of endogenous NQO1 was restored by exogenous transfection of wild-type HA-tagged NQO1 cells.

C465T NQO1 does not play dominant negative effect on p53 induction

The transduction of wild-type NQO1 does not contribute to DNA damage-dependent p53 induction, and the question was raised whether NQO1 isoform c or C465T NQO1 has a dominant negative effect against wild-type NQO1 with regard to DNA damage-dependent p53 induction. To evaluate this possibility, NQO1 isoform c or C465T NQO1 expression vector were transfected into NQO1 wild-type (NQO1 *1*1) U2OS cells. However, no dominant negative effect on NQO1 protein stability or the induction of p53 expression was observed in any of these cells (Fig. 4A and B).

Discussion

Individuals with polymorphic variants of NQO1 are at an increased risk of cancer development, however, this association has not yet been confirmed. Since NQO1 plays an important role in the detoxification of benzene-derived quinones, it is reasonable to speculate that a defect in NQO1 activity is associated with the increased risk of cancer development, though the biological role of NQO1 in cancer susceptibility is not known yet. Several recent studies have revealed that NQO1 performs a novel function of stabilization of p53 expression. p53 is a tumor suppressor protein and plays a critical role in defense mechanisms of cells against cancer. The expression of p53 is mainly regulated by MDM2 and ubiquitin ligase E3, and 26S proteasome is involved in the degradation of p53. NQO1 is reported to inhibit the degradation of p53 by...
Figure 2: (A) Western blot analysis for the detection of p53 induction, NQO1 and β-actin protein expression in cells with NQO1 *1*1, NQO1 *2*2 and NQO1 *1*3 genotypes. The cells were irradiated with X-rays (5 Gy) and harvested at the indicated time point.
(B) Monitoring of ATM-dependent signal pathway and the expression of p53 and p21 in NQO1 *1*1 and NQO1 *1*3 cells.
(C), (D) Relative amount of p53 and p21, respectively, densitometrically obtained at indicated time point after 5Gy irradiation. Mean ± SE of 3 experiments are shown.
the 20S proteasome pathway but not by the 26S proteasome pathway. This newly identified NQO1-p53 pathway was expected to uncover a missing link between epidemiological data and the biochemical role of NQO1.

In this study, we determined whether the loss of function polymorphism of NQO1 affects the induction of tumor suppressor protein p53 expression. The results of our study suggest that p53 was induced in NQO1 *2*2 cells that showed almost no expression of NQO1. In contrast, NQO1 *1*3 cells that showed very low level of NQO1 expression demonstrated attenuated induction of p53 and p21 expression after irradiation. It is not known why p53 induction in NQO1 *2*2 cells is normal in human LCL cells but abnormal in knock-out mouse cells. The expression of NQO1 in NQO1 *1*3 cells was almost undetectable and less than that in NQO1 *1*2 cells. To our knowledge, this finding is novel and corresponds to the loss of function of NQO1 in NQO1 *1*3 variants. This observation suggested the C465T allele might exert a dominant negative effect against wild type NQO1 stabilization. However, we could not demonstrate the dominant negative effect of C465T allele by overexpression of C465T NQO1 or NQO1 isoform c in U2OS cells. Taken together, NQO1 expression was decreased and irradiation-induced p53
and p21 expression was attenuated in NQO1 *1*3 cells, which might affirm the hypothesis that individuals with NQO1 *1*3 polymorphism have a higher risk of cancer development such as childhood leukemia.

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References


