Multidrug Resistant Protein-Three Gene Regulation

by

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Dedication

This dissertation is dedicated to my father Jerry Mahaffey, without him I would not have believed this possible, to my mother for her love and encouragement, my family (especially my children) for their patience, and my friends for their insight.

Acknowledgements

I would like to thank Dr. Henry Jay Forman for giving me this opportunity (and just enough rope), Dr. Phillip Mack for always having time to help me even when he himself had none, Nichole Farneth for her steady hand and clever mind, and Dr. Paul Gumerlock for giving me an intern position in his laboratory.

Doctoral Dissertation

Christopher M. Mahaffey

Section I

Multidrug Resistant Protein-Three Gene Regulation by the Transcription Factor Nrf2

Section II

Associations between MRP3 levels and *Keap1*, *Nrf2*, and *p53* status in NSCLC cell lines and tumor specimens.

Submitted April, 2010

Abstract

This dissertation is presented in two sections. Section one recounts my investigations of modulating the multidrug-resistant protein-three (MRP3) levels, by either activating or silencing the transcription factor Nrf2, in normal lung cells and *Keap1* wild-type and mutant non-small cell lung carcinoma cell lines (NSCLC). In section two direct interactions between Nrf2 and the MRP3 promoter were evaluated in NSCLC. Additionally, the relationships between MRP3 mRNA levels and *Keap1*, *Nrf2*, and *p53* status were also investigated in cell lines and tumor specimens. Combined, these sections explore the complex regulation of the MRP3 gene.

MRPs are members of the ATP-binding cassette superfamily that facilitate detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. MRP3 is over-expressed in a variety of cancers including NSCLC, and is suspected of playing a role in drug resistance. Chemotherapy, radiation, and other xenobiotic stresses are known to increase levels of select MRPs, although the underlying mechanism remains largely unknown. Analysis of the MRP3 promoter revealed the presence of multiple putative electrophile-responsive elements (EpREs), sequences that suggest possible regulation of this gene by Nrf2, the key transcription factor that binds to EpRE.

Keap1, a key regulator of Nrf2, binds Nrf2 in the cytoplasm, mediating its ubiquitination and degradation. The electrophilic lipid peroxidation product 4-hydroxy-2-nonenal (HNE) has been shown to modify Keap1 allowing its disassociation from Nrf2, allowing Nrf2 to enter the nucleus. We found that HNE up-regulated MRP3 mRNA and protein levels in cell lines with wild-type *Keap1* (the human bronchial epithelial cell line HBE1 and the NSCLC cell line H358), but not in the *Keap1*-mutant NSCLC cell lines

(A549 and H460). Cell lines with mutant *Keap1* had constitutively higher MRP3 that was not increased by HNE treatment. In HBE1 cells, silencing of Nrf2 with siRNA inhibited induction of MRP3 by HNE. Additionally, we found that silencing Nrf2 also increased the toxicity of cisplatin in H358 cells.

ChIP analysis of the MRP3 promoter revealed the presence of Nrf2 binding to the -805bp EpRE sequence distal to the start site after 3 h HNE treatment, demonstrating direct involvement of Nrf2 regulation of MRP3. Next, we examined 5 cell lines and 33 NSCLC pre-treatment patient specimens and found a parallel relationship between Nrf2 protein levels and MRP3 mRNA levels in the cell lines. In addition, a statistically significant correlation between *Keap1*/*Nrf2* mutational status and MRP3 levels was shown.

Studies involving related drug transport proteins have demonstrated both positive and negative regulatory roles for wild-type (wt) and mutant (mt) *p53*; therefore we sought to determine the role of this transcription factor in the regulation of MRP3. Transfection of a *p53* null NSCLC cell line with wt *p53*, empty vector (EV), and mt *p53* plasmids revealed that basal MRP3 levels increased in the absence of functional p53, similar to what we observed in our patient cohort. Conversely, when wt *p53* transfected cells were treated with either HNE or gemcitabine we found an improved response in MRP3 induction compared to the EV group. We also found a significant correlation between harboring both a *Keap1* and a *p53* mutation in our patient cohort. During the course of sequencing *Keap1,* we identified a known coding single-nucleotide polymorphism (cSNP) in exon 4, which had a synonymous codon change of CTC→CTG. Although this is a silent mutation, all patient and cell line samples examined that were homozygous for

this cSNP had levels of MRP3 comparable to *Keap1* or *Nrf2* mutant samples. These results support the hypothesis that MRP3 induction is regulated by both Nrf2 and p53 during normal conditions and during oxidative stress.

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Introduction

MRP3: Roles and Regulation

Overview and Background

Multidrug Resistance Proteins

Development of chemoresistance often occurs during treatment in many types of cancers and is commonly the cause of treatment failure. One cellular component implicated in multifactoral drug resistance in cancer involves the Phase III enzymes known as multidrug resistance proteins (MRPs) [1]. The first member of the MRP transporters was cloned in 1992 from the drug-selected human lung cancer cell line H69AR [2]. MRPs are a branch of the ATP-binding cassette superfamily (*ABC*) of transmembrane proteins [3]. The MRP (*ABCC*) family consists of nine different members [4]. Several MRPs transport a wide range of chemotherapeutic compounds and thus reduce the cellular accumulation of anti-cancer agents [5]. MRP family members are significant contributors to multidrug resistance in cell lines and have been found in numerous classes of cancer types [1, 6, 7]. In addition, certain MRP family-members are expressed 100- to 1,000-fold higher in some drug-resistant lung cancer cell lines [8]. Increased expression of MRPs has been associated with negative clinical outcomes in a variety of cancer types including breast cancer, gastric cancer, neuroblastoma, retinoblastoma, and lung cancer [9-15]. While some cell lines express intrinsically higher levels of MRPs, chemotherapy, radiation, and other xenobiotic stresses are capable of independently increasing levels of select MRPs [16-19]. In either case, the mechanism of regulation remains largely unknown. Therefore, a better understanding of the pathways involved in MRP-mediated chemoresistance may facilitate the development of more

effective therapeutic approaches in treating intrinsic and acquired drug resistance in NSCLC.

MRP3 *(ABCC3)*

Each MRP family member is responsible for the export of various compounds, such as chemotherapeutic drugs. It is therefore crucial to understand both the overlapping and unique genes responsible for the regulation of each MRP in order to develop improved therapies to treat them. The best characterized *ABCC* family member is MRP1, which is capable of conferring resistance to several major families of natural drugs, has been shown to be regulated by Nrf2. MRP3 shares roughly a 58% amino acid homology with MRP1. The human *MRP3* gene is located on chromosome 17, encodes a protein of 1527 amino acids, and is expressed in a variety of tissues including lung, adrenal glands, pancreas, gut, gall bladder, liver, kidney, and prostate [20-25]. MRP3 has an N-terminal region that is comprised of three membrane-spanning domains (MSD): which include five transmembrane helices, an intracellular loop and an extracellular region at the N-terminus [2]. Currently identified substrates of MRP3 include anticancer drugs, glucuronate, sulfate, and glutathione conjugates [26, 27]. One difference between the substrate specificity of MRP3 and those of MRP1 involves its lack of transport of GSH and the reduced ability to transport GSH conjugates [5, 26, 28-31]. Compared to MRP1, MRP3 has a much higher affinity for glucuronidated compounds [26, 29, 32, 33] Analysis of NSCLC cell lines and clinical specimens revealed that MRP3 is expressed at higher levels in NSCLC than SCLC [34]. Young *et al.* found that MRP3 protein levels correlated with decreased sensitivity of lung cancer cell lines to doxorubicin, vincristine, etoposide, and cisplatin [34]. Additionally, MRP3 expression has been associated with

increased resistance to methotrexate and doxorubicin in NSCLC cell lines and patient tumor samples [5, 25]. However, while MRP3 appears to be relevant to chemoresistance in NSCLC, the mechanism underlying its activity and regulation are largely unknown.

Nuclear Factor Erythroid 2-Related Factor 2

Several transcription factors such as AP-1, Nf-KB, and Nuclear factor erythroid 2-related factor 2 (Nrf2) are activated by oxidants [35]. After activation, these transcription factors regulate detoxification genes through cis-acting elements [36, 37]. Among them, the electrophile response element (EpRE) has been shown to be an important regulator of Phase II enzyme expression [38, 39]. A well-established EpRE binding protein, Nrf2, is a member of the basic-leucine zipper NF-E2 family [40]. Upon formation of heterodimers with c-Jun, small Maf, or other proteins, Nrf2 binds to EpRE, which is found in the upstream regulatory region of multiple Phase II genes [41-44]. Nrf2 is negatively regulated by a cysteine-rich cytoplasmic protein known as Kelch-like ECH-associated protein1 (Keap1) [45]. Keap1 is attached to cytoskeletal actin and binds to Nrf2 directly [46]. Once bound, Keap1 has been shown to ubiquitinate Nrf2 for proteasomal degradation. Recent data demonstrates that NSCLC cells which have inactivating mutations in *Keap1* had increased levels of Nrf2 and Nrf2-target genes [8]. Nrf2 RNA-silencing was found to decrease MRP1 and MRP2 expression, suggesting a role for Nrf2 in MRP regulation [8]. An interesting implication comes from the observation that classes of antineoplastic agents including some frontline drugs in the treatment of lung cancers (anthracyclines, most alkylating agents, and platinum compounds) are known to produce significant oxidative stress. Thus, reactive oxygen

species generated by chemotherapy may also serve to activate Nrf2 and contribute to their own transport.

Tumor Suppressor *p53*

p53 is a critical component in the regulation of cell cycle progression, DNA repair, and induction of apoptosis. Following DNA damage, *p53* arrests cell cycle progression to enable the initiation of DNA repair. If the cell is beyond repair, *p53* can initiate the apoptotic process [47]. p53 is frequently dysregulated in human cancers, including lung carcinomas [48]. Functional p53 is capable of repressing Nrf2-regulated expression of several phase II genes, suggesting cross talk between the p53 and Nrf2 pathways [49]. Studies have established a negative link between p53 activation and MRP1 expression, which has been reported to increase with NSCLC cancer stage and invasiveness [50, 51]. The correlation between high levels of MRP1 expression and *p53* mutation is supported by *in vitro* studies of *MRP1* gene regulation that have shown wild-type p53 to be a strong suppressor of *MRP1* transcription [52]. The mechanism by which this occurs could be an example of the indirect effect of transcription factors as described above, as MRP1 does not contain a p53 response element in its promoter. Whether p53 is involved in the regulation of other MRP genes has not yet been determined.

Approximately 45% of NSCLC late stage patients have dysregulation of the *p53* gene. Loss of *p53* is associated with poor prognosis [53]. Base changes resulting in amino acid substitutions that disrupt the *p53* tumor suppressor gene are thought to be important in the development of lung cancer and may additionally indicate etiologic pathways. The majority of mutations found in p53 appear to be missense mutations located within the DNA binding domain (exons 5-8) [54, 55]. The functional significance of these

mutations is often unclear. As demonstrated in 1990 by Hinds *et al.*, different *p53* missense mutations may be pro-oncogenic [56]. Work conducted at UC Davis discovered that *p53* mutants in prostate cancer may have distinct and separate functions [57]. As p53 exists in a tetramer to conduct transcriptional activity, mutant proteins are fully capable of exerting dominant negative effects through formation of hetero-oligomers [55]. Indeed, dominant negative mutations appear to correlate with the frequency of sporadic disease giving rise to mutational hotspots. While these mutations may subvert the wild-type function of p53 resulting in loss-of-function, gain-of-function mutations have also been observed that contribute to cancer proliferation and survival.

Overall Rationale and Objectives

Lung cancer is the leading cause of cancer related death for both men and women in the United States. In 2009 alone, lung cancer was responsible for approximately 160,000 deaths in the United States and currently has only a 2% 5 year survival rate once metastasized. There are multiple reasons for why the survival rate is so low, one of which includes either intrinsic or acquired resistance to standard of care therapies. Understanding the mechanisms behind why certain patients respond to a given therapy could allow the development of personalized medicine for patients. Additionally, understanding processes which may determine acquired resistance could allow for the targeting of these pathways in order to overcome resistance or re-sensitize the cancer to the primary therapy. While this body of work is relevant in drug resistance for cancer, it also has implications in other lung diseases treated with drugs as well. One way cancer can acquire resistance to chemotherapeutic compounds is to actively export the drug out of the cell. This can be accomplished by up-regulating the MRP gene capable of effluxing the specific treatment. MRP3 appears to be involved in the chemoresistance of NSCLC; however, it is currently unknown by what processes it is regulated. Activation of the Nrf2 and p53 signaling pathways in normal cells confer protection against oxidative stress and carcinogens via up-regulation of MRP3. The activation of this same transcriptional program in cancer cells, however, may provide a survival advantage due to lower concentrations of chemotherapeutic drugs. Our examination of *Keap1*, *Nrf2*, and *p53* in cell cycle models should provide a mechanism of regulation for MRP3. Additionally, if our examination of *Keap1, Nrf2,* and *p53* mutational status in lung tissue

samples is predictive for expression of MRP3, it would provide a basis for both prognosis and design of improved personalized therapies for lung cancer treatment.

Hypotheses

Hypothesis A. Induction of MRP3 (*ABCC*3) in response to cytotoxic stress occurs via Nrf2 signaling.

Hypothesis B. p53 modulates induction of MRP3 in response to cytotoxic stress.

Hypothesis C. Cell lines and tissue that lack functional *Keap1* or *Nrf2* will have higher baseline levels of MRP3.

References

1. Cole, S.P., et al., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science, 1992. **258**(5088): p. 1650-4.
2. Deeley, R.G., C. Westlake, and S.P. Cole, Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev, 2006. **86**(3): p. 849-99.
3. Gerk, P.M. and M. Vore, Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. J Pharmacol Exp Ther, 2002. **302**(2): p. 407-15.
4. Borst, P., et al., The multidrug resistance protein family. Biochim Biophys Acta, 1999. **1461**(2): p. 347-57.
5. Kool, M., et al., MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6914-9.
6. Loe, D.W., R.G. Deeley, and S.P. Cole, Biology of the multidrug resistance-associated protein, MRP. Eur J Cancer, 1996. **32A**(6): p. 945-57.
7. Goldstein, L.J., MDR1 gene expression in solid tumours. Eur J Cancer, 1996. **32A**(6): p. 1039-50.
8. Singh, A., et al., Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. PLoS Med, 2006. **3**(10): p. e420.
9. Nies, A.T., et al., Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. Int J Cancer, 2001. **94**(4): p. 492-9.

10. Narasaki, F., et al., Human canalicular multispecific organic anion transporter (cMOAT) is expressed in human lung, gastric, and colorectal cancer cells. Biochem Biophys Res Commun, 1997. **240**(3): p. 606-11.

11. Schaub, T.P., et al., Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. J Am Soc Nephrol, 1999. **10**(6): p. 1159-69.

12. Nooter, K., et al., Multidrug resistance protein in recurrent breast cancer. Lancet, 1997. **349**(9069): p. 1885-6.

13. Nooter, K., H. Burger, and G. Stoter, Multidrug resistance-associated protein (MRP) in haematological malignancies. Leuk Lymphoma, 1996. **20**(5-6): p. 381-7.

14. Chan, H.S., et al., Multidrug resistance protein (MRP) expression in retinoblastoma correlates with the rare failure of chemotherapy despite cyclosporine for reversal of P-glycoprotein. Cancer Res, 1997. **57**(12): p. 2325-30.

15. Ota, E., et al., Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer. Br J Cancer, 1995. **72**(3): p. 550-4.

16. Leslie, E.M., A. Haimeur, and M.P. Waalkes, Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. J Biol Chem, 2004. **279**(31): p. 32700-8.

17. Henness, S., et al., Fractionated irradiation of H69 small-cell lung cancer cells causes stable radiation and drug resistance with increased MRP1, MRP2, and

topoisomerase IIalpha expression. Int J Radiat Oncol Biol Phys, 2002. **54**(3): p. 895-902.

18. Oguri, T., et al., Association between expression of the MRP3 gene and exposure to platinum drugs in lung cancer. Int J Cancer, 2001. **93**(4): p. 584-9.
19. Berger, W., et al., Multidrug resistance markers P-glycoprotein, multidrug resistance protein 1, and lung resistance protein in non-small cell lung cancer: prognostic implications. J Cancer Res Clin Oncol, 2005. **131**(6): p. 355-63.
20. Belinsky, M.G., et al., Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. J Natl Cancer Inst, 1998. **90**(22): p. 1735-41.
21. Ambudkar, S.V., et al., Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol, 1999. **39**: p. 361-98.
22. Konig, J., et al., Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology, 1999. **29**(4): p. 1156-63.
23. Kool, M., et al., Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res, 1997. **57**(16): p. 3537-47.
24. Mottino, A.D., et al., Expression and localization of multidrug resistant protein mrp2 in rat small intestine. J Pharmacol Exp Ther, 2000. **293**(3): p. 717-23.
25. Lockhart, A.C., R.G. Tirona, and R.B. Kim, Pharmacogenetics of ATP-binding cassette transporters in cancer and chemotherapy. Mol Cancer Ther, 2003. **2**(7): p. 685-98.

26. Hirohashi, T., H. Suzuki, and Y. Sugiyama, Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J Biol Chem, 1999. **274**(21): p. 15181-5.

27. Rost, D., et al., Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. Am J Physiol Gastrointest Liver Physiol, 2002. **282**(4): p. G720-6.

28. Atkinson, D.E., et al., Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer. Am J Physiol Cell Physiol, 2003. **285**(3): p. C584-91.

29. Oleschuk, C.J., R.G. Deeley, and S.P. Cole, Substitution of Trp1242 of TM17 alters substrate specificity of human multidrug resistance protein 3. Am J Physiol Gastrointest Liver Physiol, 2003. **284**(2): p. G280-9.

30. Nooter, K., et al., Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. Clin Cancer Res, 1995. **1**(11): p. 1301-10.

31. Zhang, D.W., et al., Characterization of the role of polar amino acid residues within predicted transmembrane helix 17 in determining the substrate specificity of multidrug resistance protein 3. Biochemistry, 2003. **42**(33): p. 9989-10000.

32. Chu, X.Y., et al., Transport of ethinylestradiol glucuronide and ethinylestradiol sulfate by the multidrug resistance proteins MRP1, MRP2, and MRP3. J Pharmacol Exp Ther, 2004. **309**(1): p. 156-64.

33. Zeng, H., et al., Transport of amphipathic anions by human multidrug resistance protein 3. Cancer Res, 2000. **60**(17): p. 4779-84.

34. Young, L.C., et al., Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin Cancer Res, 1999. **5**(3): p. 673-80.

35. Rahman, I. and I.M. Adcock, Oxidative stress and redox regulation of lung inflammation in COPD. Eur Respir J, 2006. **28**(1): p. 219-42.

36. Otterbein, L.E. and A.M. Choi, The saga of leucine zippers continues: in response to oxidative stress. Am J Respir Cell Mol Biol, 2002. **26**(2): p. 161-3.

37. Motohashi, H. and M. Yamamoto, Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med, 2004. **10**(11): p. 549-57.

38. Prestera, T., et al., The electrophile counterattack response: protection against neoplasia and toxicity. Adv Enzyme Regul, 1993. **33**: p. 281-96.

39. Ishii, T., et al., Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem, 2000. **275**(21): p. 16023-9.

40. Itoh, K., et al., Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. Mol Cell Biol, 1995. **15**(8): p. 4184-93.

41. Itoh, K., et al., An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun, 1997. **236**(2): p. 313-22.

42. Moinova, H.R. and R.T. Mulcahy, Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves binding of Nrf-2 to

an electrophile responsive element. Biochem Biophys Res Commun, 1999. **261**(3): p. 661-8.

43. Wild, A.C., H.R. Moinova, and R.T. Mulcahy, Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J Biol Chem, 1999. **274**(47): p. 33627-36.

44. Alam, J., et al., Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem, 1999. **274**(37): p. 26071-8.

45. Itoh, K., et al., Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev, 1999. **13**(1): p. 76-86.

46. Itoh, K., et al., Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells, 2003. **8**(4): p. 379-91.

47. Sun, S.Y., et al., Implication of p53 in growth arrest and apoptosis induced by the synthetic retinoid CD437 in human lung cancer cells. Cancer Res, 1999. **59**(12): p. 2829-33.

48. Knoll, N., et al., Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione S-transferase A4 expression by butyrate. Toxicol Sci, 2005. **86**(1): p. 27-35.

49. Faraonio, R., et al., p53 suppresses the Nrf2-dependent transcription of antioxidant response genes. J Biol Chem, 2006. **281**(52): p. 39776-84.

50. Sullivan, G.F., et al., The expression of drug resistance gene products during the progression of human prostate cancer. Clin Cancer Res, 1998. **4**(6): p. 1393-403.

51. Sullivan, G.F., et al., Regulation of expression of the multidrug resistance protein MRP1 by p53 in human prostate cancer cells. J Clin Invest, 2000. **105**(9): p. 1261-7.

52. Muredda, M., et al., Cloning and Characterization of the Murine and Rat mrp1 Promoter Regions. Mol Pharmacol, 2003. **64**(5): p. 1259-69.

53. Hashimoto, T., et al., p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. Cancer Res, 1999. **59**(21): p. 5572-7.

54. Greenblatt, M.S., et al., Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res, 1994. **54**(18): p. 4855-78.

55. Petitjean, A., et al., TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene, 2007. **26**(15): p. 2157-65.

56. Hinds, P., C. Finlay, and A.J. Levine, Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol, 1989. **63**(2): p. 739-46.

57. Shi, X.B., et al., Complex functions of mutant p53 alleles from human prostate cancer. Prostate, 2002. **51**(1): p. 59-72.

Section I

Chapter A

Multidrug Resistant Protein-Three Gene Regulation by the Transcription Factor Nrf2 in Human Bronchial Epithelial and Non-Small Cell Lung Carcinoma

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Multidrug Resistant Protein-Three Gene Regulation by the Transcription Factor Nrf2 in Human Bronchial Epithelial and Non-Small Cell Lung Carcinoma

Running Title: Nrf2 Regulates MRP3 in Lung Cancer

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Abbreviations: MRP, multidrug-resistant protein; NSCLC, non-small-cell lung carcinoma; HNE, 4-hydroxynonenal; EpRE, electrophile response element.

Abstract

Multidrug-resistant proteins (MRPs) are members of the ATP-binding cassette superfamily that facilitate detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. Chemotherapy, radiation, and other xenobiotic stresses have been shown to increase levels of select MRPs, although the underlying mechanism remains largely unknown. Additionally, MRP3 is suspected of playing a role in the drug resistance of non-small-cell lung carcinoma (NSCLC). Analysis of the MRP3 promoter revealed the presence of multiple putative electrophile-responsive elements (EpREs), sequences that suggest possible regulation of this gene by Nrf2, the key transcription factor that binds to EpRE. The goal of this investigation was to determine whether MRP3 induction was dependent upon the transcription factor Nrf2. Keap1, a key regulator of Nrf2, sequesters Nrf2 in the cytoplasm, preventing entry into the nucleus. The electrophilic lipid peroxidation product 4-hydroxy-2-nonenal (HNE) has been shown to modify Keap1, allowing Nrf2 to enter the nucleus. We found that HNE up-regulated MRP3 mRNA and protein levels in cell lines with wild-type Keap1 (the human bronchial epithelial cell line HBE1 and the NSCLC cell line H358), but not in the Keap1-mutant NSCLC cell lines (A549 and H460). Cell lines with mutant Keap1 had constitutively higher MRP3 that was not increased by HNE treatment. In HBE1 cells, silencing of Nrf2 with siRNA inhibited induction of MRP3 by HNE. Finally, we found that silencing Nrf2 also increased the toxicity of cisplatin in H358 cells. The combined results therefore support the hypothesis that MRP3 induction by HNE involves Nrf2 activation.

Introduction

Lung cancer is the most common cause of cancer death, accounting for more deaths than colorectal, prostate, and breast cancer combined [1]. One class of lung cancer is non-small-cell lung carcinoma (NSCLC) [2]. NSCLC constitutes 75% of primary lung cancers and comprises large-cell undifferentiated carcinomas, squamous carcinomas, and adenocarcinomas. Unfortunately, most patients present with locally advanced or metastatic disease and are considered incurable. Stagebased therapies may include surgery, radiation, and chemotherapy. In advanced disease current frontline therapies consist of chemotherapeutic doublets (e.g., etoposide with cisplatin). However, as the 5-year survival rate for metastatic NSCLC is a dismal 2%, current treatment options are largely ineffective. One factor that contributes to the poor clinical outcome is that a significant proportion of NSCLCs are intrinsically chemoresistant [3]. Resistance to chemotherapeutic agents in drug-resistant cancers is facilitated, in part, by an increased capacity for detoxification. Like other xenobiotics, chemotherapeutic agents are processed in the body through the detoxification system, which includes Phase II and III enzymes. Phase II genes are a family of enzymes that under increased oxidative stress are up-regulated, eliciting a response that detoxifies the stressors, often through conjugation to glutathione. Exposure to carcinogens and various xenobiotics can increase both Phase II and III enzymes [4–6]. Several transcription factors, such as AP-1, NF-κB, and Nrf2, are known to be activated by oxidants [7]. After activation, these transcription factors can regulate Phase II genes through cis-acting elements [8,9]. Among them, the electrophile response element (EpRE) has been shown to be an important regulator of Phase II enzyme expression [10,11]. A well established EpRE binding protein, Nrf2, is a member

of the basic leucine- zipper NF-E2 family [12]. Upon formation of heterodimers with c-Jun, small Maf, or other proteins, Nrf2 binds to the EpRE domain located in the upstream regulatory region of multiple Phase II genes [13–16]. Nrf2 is negatively regulated by a cysteine-rich cytoplasmic protein known as Kelch-like ECH-associated protein1 (Keap1) [17]. Keap1 is attached to cytoskeletal actin and binds to Nrf2 directly [18]. Once bound, Keap1 has been shown to facilitate ubiquitination of Nrf2 and its proteasomal degradation.

Phase III enzymes, which include multidrug-resistance proteins (MRPs), do not interact with xenobiotics directly but facilitate the excretion of water-soluble compounds (including products of Phase II enzymes) out of the cell. MRPs are a branch of the ATP-binding cassette superfamily (ABC) of transmembrane proteins [19]. The MRP (ABCC) family consists of nine members [20]. Several MRPs are known to transport a wide range of chemotherapeutic compounds and thus reduce the cellular accumulation of anti-cancer agents [21]. MRP family members have been shown to be significant contributors to multidrug resistance in cell lines and have been found in numerous classes of cancer types [22–24]. Increased expression of MRPs has been associated with negative clinical outcomes in a variety of cancer types, including breast cancer, gastric cancer, neuroblastoma, retinoblastoma, and lung cancer [25–31].

The MRP3 (ABCC3) gene is located on chromosome 17, encodes a protein of 1527 amino acids, and is known to be expressed in a variety of tissues including lung, adrenal glands, pancreas, gut, gall bladder, liver, kidney, and prostate [32–36]. MRP3 is the ABCC family member closest in structure to MRP1, sharing roughly 58% amino acid homology [37]. MRP3 has an N-terminal region that comprises three membrane-

spanning domains, which include five transmembrane helices, an intracellular loop, and an extracellular region at the N-terminus [38]. Currently identified substrates of MRP3 include anticancer drugs such as glucuronate, sulfate, or glutathione conjugates [39,40]. Analysis of NSCLC cell lines and clinical specimens revealed that MRP3 is expressed at higher levels in NSCLC than in SCLC [41]. Young *et al.* found that MRP3 protein levels correlated with decreased sensitivity of lung cancer cell lines to frontline chemotherapeutic compounds such as vincristine, etoposide, and cisplatin [41]. Additionally, MRP3 expression has been associated with increased resistance to methotrexate and doxorubicin in NSCLC cell lines and patient tumor samples [21, 37]; however, the mechanisms underlying its activity and regulation are largely unknown.

The α,β-unsaturated aldehyde 4-hydroxynonenal (HNE) is a major lipid peroxidation product formed by the reaction of reactive oxygen or nitrogen species with arachidonic acid in cellular membranes [21]. HNE has been previously established to cause the activation of the Nrf2–EpRE signaling and cytoprotective gene induction in a human bronchial epithelial (HBE1) cell line [42, 43]. The purpose of this study was to examine the involvement of Nrf2 in the up-regulation of MRP3 in lung epithelial cells in response to oxidative stress. Here we demonstrate that activation of Nrf2 by HNE leads to the induction of MRP3 in human epithelial lung and *Keap1* wild-type NSCLC cell lines.

Materials and Methods

Chemicals and Reagents

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO, USA). Antibodies (MRP3: 6D568 mouse monoclonal IgG sc-71605) and small interfering RNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HNE was purchased from Cayman Chemical (Ann Arbor, MI, USA). TRIzol reagent was from Life Technologies (Grand Island, NY, USA). DNA-free reagent was obtained from Ambion (Austin, TX, USA). TaqMan reverse transcription reagent and SYBR green PCR master mix were obtained from Applied Biosystems (Foster City, CA, USA). Luciferase activity assay kit was obtained from Promega (Madison, WI, USA). FuGENE 6 transfection reagent was obtained from Roche (Indianapolis, IN, USA). M-PER mammalian protein extraction reagents were obtained from Pierce (Rockford, IL, USA).

Cell Culture and Treatments

The human bronchial epithelial cell line (HBE1) was cultured in collagen-coated dishes. Cells were grown in serum-free Ham's F-12 medium supplemented with seven additives (5 μg/ml insulin, 3.7 μg/ ml endothelial cell growth supplement, 25 ng/ml epidermal growth factor, 3×10−8 M triiodothyronine, 1×10−6 M hydrocortisone, 5 μg/ ml transferrin) in T-75-cm2 collagen-coated flasks. The NSCLC cell lines H460 (*p53* wild type/*Keap1* mutant), H358 (*p53* null/*Keap1* wild type), and A549 (*p53* wild type/*Keap1* mutant) were obtained from the Mack laboratory at the UC Davis Cancer Center (Sacramento, CA, USA). NSCLC cell lines were maintained in DMEM (Biowhittaker, Walkersville, MD, USA) supplemented with 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA, USA) to which was added MEM Essential Vitamin

Mix, penicillin–streptomycin, and L-glutamine. All cultures were grown at 37°C in 5% CO2 atmosphere. Cells were plated on 10-cm cell culture dishes at a density of 1,000,000 cells/dish. Cells were treated at approximately 85% confluency. The time point studied for both mRNA and protein collection was 24 h. HNE was dissolved in ethanol. HBE1 cells close to confluency were treated with vehicle control (0.05% ethanol) or various concentrations of HNE as indicated under Results.

RT-PCR

The content of MRP3 mRNAwas determined by real-time PCR. RNA samples were treated with DNA-free reagent and reverse transcribed using the TaqMan reverse transcription system. Real-time PCR was carried out using the SYBR GreenER qPCR Supermix Universal (Invitrogen) as specified by the manufacturer. Real-time PCR was performed with a Cepheid 1.2 real-time PCR machine (Cepheid, Sunnyvale, CA, USA) and an iQ5 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). GAPDH and β-actin were used as internal controls. The primers were as follows: MRP3, sense 5′-CAGAGAAGGTGCAGGTGACA-3′, antisense 5′-CTAAAGCAGCATAGACGCCC-3′; GAPDH, sense 5′-TGGGTGTGAACCATGAGAAG-3′, antisense 5′-CCATCACGAC ACAGTTTCC-3′; β-actin, sense 5′-GAGCGCGGCTACAGCTT-3′, antisense 5′-TCCTTAATGTCACGCACGATTT-3′.

Immunofluorescence

HBE1 cells were grown on glass coverslips pretreated with collagen I (20 μg/μl) for 2 h at 20°C in 30-mm plates. NSCLC cells were plated on VRW Scientific glass coverslips in 30-mm plates. Cells were washed twice in PBS and fixed with 95% methanol at 20°C for 5 min. Slides were washed twice in PBS containing 1% BSA,

0.02% saponin, and 0.05% sodium azide. Fixed cells were incubated for 30 min at 37°C with primary antibody at a 1:200 dilution in PBS. Slides were washed and incubated with FITC-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology; sc3699) at a 1:50 dilution for another 30 min. Slides were washed and coverslips were mounted. At least 100 cells were scored for localization and concentration of MRP3 protein under a microscope. Images were obtained using a Nikon Eclipse-TE 2000-U/confocal microscope and an Olympus BX61 confocal system microscope. The images were analyzed for luminosity values and were subsequently exported from the native .ids Nikon file format to .bmp files using the Nikon Ez-C1 viewer software version 3.2, and then Adobe Photoshop was used to construct the figures. Experiments were performed at least in triplicate.

siRNA Transfection: Nrf2

Transfection of Nrf2 siRNA was performed using the target sequence 5′-AAGAGTATGAGCTGGAAAAAC-3′ for human Nrf2 siRNA. Nonspecific siRNA (NS siRNA) was used as a negative control. HBE1 cells were seeded at 1.5×105 cells per well into six-well plates. After 24 h, the cells were transfected with Nrf2 siRNA. Appropriate amounts of Nrf2 siRNA in 250 μl serum-free DMEM/F12 medium and 5 μl transfection reagent in 245 μl serum-free DMEM/F12 medium was prepared in separate centrifuge tubes. After incubation for 5 min, the siRNA and transfection reagent were mixed, incubated for an additional 20 min, and added to each well.

Statistics

The comparative ΔΔCT method was used for relative mRNA quantitation. Comparisons of variants between experimental groups were conducted using one-way

analysis of variance. All data are expressed as the means ± standard deviation. In-Stat software was used for statistical analysis. Statistical significance was accepted when $p<0.05$.

Results

Induction of MRP3 in *Keap1* Wild-Type Cells

RT-PCR analysis was used to determine which MRP family members were present in normal bronchial epithelial cells. We demonstrated the presence of all known MRPs, with the exception of MRP6, in HBE1 cells. Of the detected MRPs, MRP3was a prevalent MRP species (Fig. 1).We then sought to evaluate whether HNE was capable of up-regulating the expression of MRP3 in the selected cell lines. HNE can be toxic, depleting glutathione, the principal antioxidant made by cells. Normal human plasma contains the equivalent of 0.3–0.7 μM HNE (much of it reversibly bound to plasma proteins through Schiff base formation); however, during oxidative stress such as occurs in inflammation and tobacco smoking, plasma HNE concentrations can increase more than 10 times, with affected tissue concentrations in the millimolar range [42,44–47].

Upon exposure to sublethal concentrations of HNE, MRP3 mRNA increased in *Keap1* wild-type cells, but not in *Keap1* mutant cells. We observed an approximate fourfold increase in MRP3 mRNA in the *Keap1* wild-type cell lines compared to untreated controls (Fig. 2). The expression and localization of MRP3 protein was determined by immunofluorescence using monoclonal antibodies. MRP3 protein levels were markedly induced after exposure to HNE in *Keap1* wild-type cell lines. The increase in MRP3 protein by HNE treatment correlated with the observed increase in mRNA levels in these cells. Conversely, we found that MRP3 expression in *Keap1* mutant cell lines was relatively higher than in *Keap1* wild-type cells andwas unaffected by the addition of HNE (Fig. 3). These results were expected as *Keap1* mutants had

higher constitutively active Nrf2 than *Keap1* wild-type cells and therefore were expected to also have high constitutive levels of MRP3.

Nrf2 Silencing of MRP3

Analysis of the promoter sequence of MRP3 located in the 5′-untranslated region revealed the presence of four putative EpRE (TGA(C/T)NNNGC) sites at −434, −628, −805, and −1049 bp from the 5′ UTR of MRP3 (Fig. 4) [48]. To specifically determine Nrf2 involvement in MRP3 induction, we examined the effect of depleting Nrf2, the principal transcription factor involved in EpRE regulation. We have previously demonstrated the effectiveness of Nrf2 siRNA in HBE1 cells [49]. The nonspecific siRNA-treated samples displayed a reduction in cytosolic Nrf2 and an increase in levels of nuclear Nrf2 after exposure to HNE. Transfection with Nrf2 siRNA was shown toreduce cytosolic and nuclear Nrf2 alone or in the presence of HNE (Fig. 5).

We measured the effects of inhibiting the rate of Nrf2 expression with siRNA in cells exposed to HNE. RT-PCR analysis demonstrated that transfection with Nrf2 siRNA 24 h before exposure to HNE inhibits the induction of MRP3 mRNA compared to the nonspecific siRNA treated cells (Fig. 6). Moreover, the levels of MRP3 mRNA in the Nrf2 siRNA HNE treatment group were lower than the basal level of the control group, which was probably due to a dependence upon Nrf2 for basal expression. Fig. 6 shows HBE1 cells that were transiently transfected 24 h before exposure to HNE. The difference in the effectiveness of HNE in Fig. 6 compared to Fig. 2 was probably due to the toxicity of the transfecting agent, FuGENE 6, which alone caused a 25% decrease in viability (data not shown).

Quantitative immunofluorescence analysis of HBE1 cells indicated that MRP3 expression was increased in a dose-dependent manner compared to the control after exposure to HNE (Fig. 7).We found that the differences between the luminosity measurements of the control group were statistically significant ($p > 0.001$) compared to that of either the 10 or the 15 μM HNE groups. Additionally, the luminosity measurements of the 10 and 15 μM HNE groups were statistically significant ($p > 0.001$) compared to those of the Nrf2 siRNA group (Fig. 8). Taken together these observations support the involvement of Nrf2 in MRP3 induction in response to HNE-induced oxidative stress.

Additionally, we sought to measure the change in toxicity to cisplatin (CP) in a NSCLC when Nrf2 expression was knocked down using siRNA. Although not quite statistically significant, we found that in the NSCLC cell line H358 the Nrf2 siRNA → CP group was approximately 25% more sensitive to 48 h of 2.5 μM cisplatin treatment than the nonspecific siRNA → CP group (Fig. 9). Although this was consistent with a role for MRP3, the expected global decrease in activity of other Nrf2-regulated genes had unpredictable consequences.

Discussion

One of the primary treatments for combating NSCLC is the use of chemotherapy. However, the majority of NSCLCs are inherently drug resistant. This presents a major obstacle in successfully treating this disease. One component of this observed multifactorial drug resistance is an increased capacity to efflux chemotherapeutic compounds out of the cell. Studies of clinical specimens and cell lines have shown an increased capacity for drug transport, and subsequent resistance, due to increased levels of Multidrug resistance transporters (MDRs) and MRPs. Given that not all NSCLCs possess this ability it would seem that some additional dysregulation has occurred.

Our analysis of the human MRP3 gene (*ABCC3*) revealed multiple EpREs in the tentative promoter region. These findings led us to hypothesize that activation of Nrf2 could contribute to the induction of MRP3. To test this we used HNE, a known Nrf2 activator, in an effort to evaluate the response of MRP3. Our results demonstrate that Nrf2 activation can up-regulate the expression of the endogenous MRP3 gene, producing increases in both mRNA and protein in both human bronchial epithelial and *Keap1* wild-type NSCLC cells. In addition, we demonstrate that selectively inhibiting the expression of Nrf2 has the capacity to abolish induction of MRP3 by HNE. Whereas regulation of MRP3 by Nrf2 has not been described previously, a relationship between Nrf2 and MRP1 and MRP2 expression has been suggested by studies conducted in human cancer specimens, including NSCLC [50].

Recent studies have indicated the potential “negative role” for the Nrf2 pathway, as it is up-regulated in a number of drug-resistant human malignancies, and that inhibiting expression of Nrf2 during chemotherapy could be clinically beneficial [51].

Additionally, Wang *et al.* showed that overexpression of Nrf2 increased resistance to chemotherapeutic drugs and that knocking down Nrf2 decreased resistance to these drugs [52]. Our own experiment was in agreement with the aforementioned study, demonstrating that knocking out Nrf2 produced increased toxicity to the frontline chemotherapeutic drug cisplatin, which is consistent with a decrease in MRP3 expression. Despite that consistency, we cannot definitively state that the increased toxicity was due to the decrease in MRP3 alone as Nrf2 regulates the expression of numerous Phase II genes that could also have contributed.

It has been recently demonstrated by Singh *et al.* that approximately 50% of NSCLC cell lines and 18% of NSCLC clinical specimens examined in their study had inactivating mutations in *Keap1* [50]. These *Keap1* mutant NSCLCs had increased accumulation of Nrf2 and Nrf2-target genes, possibly leading to increased drug resistance [50]. We observed elevated basal levels of MRP3 in *Keap1* mutant cells, which were comparable to the levels observed in the *Keap1* wild-type cells under oxidative stress. Activation of the Keap1/Nrf2 signaling pathway in normal cells confers protection against oxidative stress and carcinogens, whereas deregulation of this transcriptional program in cancer cells may provide a selective survival advantage via the upregulation of MRP3.

In addition to *Keap1* status, other potential mechanisms may lead to aberrant expression of MRP3. One such possibility may involve *p53*. Dysregulation of the *p53* gene (mutant or null) occurs in approximately 45% of NSCLC cases and is associated with drug resistance [53,54]. Additionally, p53 has been demonstrated to repress Nrf2-regulated expression of several Phase II genes (*NQO1*, *X-CT*, and *GST-α1*), suggesting

cross talk between the p53 and the Nrf2 signaling pathways [40]. Studies have demonstrated a negative link between p53 activation and MRP1 expression. The correlation between high levels of MRP1 expression and p53 mutation is supported by in vitro studies of the MRP1 gene regulation in human and murine systems, which have shown that wild-type p53 is a strong suppressor of MRP1 transcription [55]. Additionally, the human papilloma E6 protein initiates the rapid degradation of p53, which subsequently increased MRP1 levels in an HPV16-transformed cell line [56]. Whether p53 is involved in the regulation of the MRP3 gene, as it is with MRP1, has not been determined. Of note, p53 is functionally disrupted in both of the cell lines HBE1 and H358, which we observed to be inducible for MRP3. If p53 acts as a negative regulator of MRP3 as it does for MRP1, it would be expected that these cells would be more responsive to the types of cytotoxic stresses that induce MRP3.

Although some cell lines express higher levels of MRPs intrinsically [19,22], chemotherapy, radiation, and other xenobiotic stresses have been shown to increase levels of select MRPs independently [20,21]. Several classes of antineoplastic agents produce oxidative stress leading to the formation of lipid peroxidation products, such as HNE. An additional factor to consider is that chemo-and radiation therapy will induce lipid peroxidation that generates HNE and could potentially activate Nrf2. This effect of inadvertent activation of the genes involved in drug resistance in “real time” may explain one component of the drug-resistance phenotype common to this malignancy. As this is a normal response to chemotherapy, it suggests that even under conditions under which there is no aberrant signaling in the drug metabolic pathway, tumors may nevertheless naturally develop resistance over time to therapy, in part through an accumulation of

MRP3. Ideally, one solution to resolve this issue may be to determine markers of sensitivity in drug metabolism (e.g., lack of MRP expression) as opposed to resistance.

In conclusion, our results demonstrate that MRP3 belongs to the family of detoxification enzymes whose expression is regulated by Nrf2. This study shows that HNE, at physiologically achievable levels, produces a marked up-regulation of the multidrug-resistance protein transporter, MRP3, in human bronchial epithelial and *Keap1* wild-type NSCLC cells. In addition, we demonstrated that inhibiting Nrf2 significantly attenuates this response. Elucidating the exact mechanisms involved in the up-regulation and/or suppression of MRP3 could lead to the identification of patient subsets that may benefit from different chemotherapy regimens thereby improving upon current therapeutic options for NSCLC.

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References

[1] Soria, J. C.; Lee, H. Y.; Lee, J. I.;Wang, L.; Issa, J. P.; Kemp, B. L.; Liu, D. D.; Kurie, J. M.; Mao, L.; Khuri, F. R. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin. Cancer Res. 8:1178–1184; 2002.

[2] Sanchez-Cespedes, M.; Decker, P. A.; Doffek, K. M.; Esteller, M.; Westra, W. H.; Alawi, E. A.; Herman, J. G.; Demeure, M. J.; Sidransky, D.; Ahrendt, S. A. Increased loss of chromosome 9p21 but not p16 inactivation in primary non-small cell lung cancer from smokers. Cancer Res. 61:2092–2096; 2001.

[3] Young, L. C.; Campling, B. G.; Cole, S. P.; Deeley, R. G.; Gerlach, J. H. Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels. Clin. Cancer Res. 7: 1798–1804; 2001.

[4] Yu, S.; Kong, A. N. Targeting carcinogen metabolism by dietary cancer preventive compounds. Curr. Cancer Drug Targets 7:416–424; 2007.

[5] Itoh, K.; Mochizuki, M.; Ishii, Y.; Ishii, T.; Shibata, T.; Kawamoto, Y.; Kelly, V.; Sekizawa, K.; Uchida, K.; Yamamoto, M. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin J(2). Mol. Cell. Biol. 24:36–45; 2004.

[6] Gong, P.; Stewart, D.; Hu, B.; Li, N.; Cook, J.; Nel, A.; Alam, J. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. Antioxid. Redox Signal. 4:249–257; 2002.

[7] Rahman, I.; Adcock, I. M. Oxidative stress and redox regulation of lung inflammation in COPD. Eur. Respir. J. 28:219–242; 2006.

[8] Otterbein, L. E.; Choi, A. M. The saga of leucine zippers continues: in response to oxidative stress. Am. J. Respir. Cell Mol. Biol. 26:161–163; 2002.

[9] Motohashi, H.; Yamamoto, M. Nrf2–Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10:549–557; 2004.

[10] Prestera, T.; Zhang, Y.; Spencer, S. R.; Wilczak, C. A.; Talalay, P. The electrophile counterattack response: protection against neoplasia and toxicity. Adv. Enzyme Regul. 33:281–296; 1993.

[11] Ishii, T.; Itoh, K.; Takahashi, S.; Sato, H.; Yanagawa, T.; Katoh, Y.; Bannai, S.; Yamamoto, M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J. Biol. Chem. 275:16023–16029; 2000.

[12] Itoh, K.; Igarashi, K.; Hayashi, N.; Nishizawa, M.; Yamamoto, M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. Mol. Cell. Biol. 15:4184–4193; 1995.

[13] Itoh, K.; Chiba, T.; Takahashi, S.; Ishii, T.; Igarashi, K.; Katoh, Y.; Oyake, T.; Hayashi, N.; Satoh, K.; Hatayama, I.; Yamamoto, M.; Nabeshima, Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. 236: 313–322; 1997.

[14] Moinova, H. R.;Mulcahy, R. T. Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves binding of Nrf2 to an electrophile responsive element. Biochem. Biophys. Res. Commun. 261:661–668; 1999.

[15] Wild, A. C.; Moinova, H. R.; Mulcahy, R. T. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J. Biol. Chem. 274:33627–33636; 1999.

[16] Alam, J.; Stewart, D.; Touchard, C.; Boinapally, S.; Choi, A. M.; Cook, J. L. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem. 274:26071–26078; 1999.

[17] Itoh, K.;Wakabayashi, N.; Katoh, Y.; Ishii, T.; Igarashi, K.; Engel, J. D.; Yamamoto, M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 13:76–86; 1999.

[18] Itoh, K.; Wakabayashi, N.; Katoh, Y.; Ishii, T.; O'Connor, T.; Yamamoto, M. Keap1 regulates both cytoplasmic–nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells 8:379–391; 2003.

[19] Gerk, P. M.; Vore, M. Regulation of expression of the multidrug resistanceassociated protein 2 (MRP2) and its role in drug disposition. J. Pharmacol. Exp. Ther. 302:407–415; 2002.

[20] Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. The multidrug resistance protein family. Biochim. Biophys. Acta 1461:347–357; 1999.

[21] Kool, M.; van der Linden, M.; de Haas, M.; Scheffer, G. L.; de Vree, J. M.; Smith, A. J.; Jansen, G.; Peters, G. J.; Ponne, N.; Scheper, R. J.; Elferink, R. P.; Baas, F.; Borst, P. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc. Natl. Acad. Sci. USA 96:6914–6919; 1999.

[22] Cole, S. P.; Bhardwaj, G.; Gerlach, J. H.; Mackie, J. E.; Grant, C. E.; Almquist, K. C.; Stewart, A. J.; Kurz, E. U.; Duncan, A.M.; Deeley, R. G. Overexpression of a

transporter gene in a multidrug-resistant human lung cancer cell line. Science 258:1650–1654; 1992.

[23] Loe, D. W.; Deeley, R. G.; Cole, S. P. Biology of the multidrug resistance-associated protein, MRP. Eur. J. Cancer 32A:945–957; 1996.

[24] Goldstein, L. J.MDR1 gene expression in solid tumours. Eur. J. Cancer 32A:1039–1050;1996.

[25] Nies, A. T.; Konig, J.; Pfannschmidt, M.; Klar, E.; Hofmann, W. J.; Keppler, D. Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. Int. J. Cancer 94:492–499; 2001.

[26] Narasaki, F.; Oka, M.; Nakano, R.; Ikeda, K.; Fukuda, M.; Nakamura, T.; Soda, H.; Nakagawa, M.; Kuwano, M.; Kohno, S. Human canalicular multispecific organic anion transporter (cMOAT) is expressed in human lung, gastric, and colorectal cancer cells. Biochem. Biophys. Res. Commun. 240:606–611; 1997.

[27] Schaub, T. P.; Kartenbeck, J.; Konig, J.; Spring, H.; Dorsam, J.; Staehler, G.; Storkel, S.; Thon, W. F.; Keppler, D. Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. J. Am. Soc. Nephrol. 10:1159–1169; 1999.

[28] Nooter, K.; de la Riviere, G. B.; Klijn, J.; Stoter, G.; Foekens, J. Multidrug resistance protein in recurrent breast cancer. Lancet 349:1885–1886; 1997.

[29] Nooter, K.; Burger, H.; Stoter, G. Multidrug resistance-associated protein (MRP) in haematological malignancies. Leuk. Lymphoma 20:381–387; 1996.

[30] Chan, H. S.; Lu, Y.; Grogan, T. M.; Haddad, G.; Hipfner, D. R.; Cole, S. P.; Deeley, R. G.; Ling, V.; Gallie, B. L. Multidrug resistance protein (MRP) expression in

retinoblastoma correlates with the rare failure of chemotherapy despite cyclosporine for reversal of P-glycoprotein. Cancer Res. 57:2325–2330; 1997.

[31] Ota, E.; Abe, Y.; Oshika, Y.; Ozeki, Y.; Iwasaki, M.; Inoue, H.; Yamazaki, H.; Ueyama, Y.; Takagi, K.; Ogata, T., et al. Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer. Br. J. Cancer 72:550–554; 1995.

[32] Kool, M.; de Haas, M.; Scheffer, G. L.; Scheper, R. J.; van Eijk, M. J.; Juijn, J. A.; Baas, F.; Borst, P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 57:3537–3547; 1997.

[33] Belinsky, M. G.; Bain, L. J.; Balsara, B. B.; Testa, J. R.; Kruh, G. D. Characterization of MOAT-C and MOAT-D, new members of the MRP/cMOAT subfamily of transporter proteins. J. Natl. Cancer Inst. 90:1735–1741; 1998.

[34] Ambudkar, S. V.; Dey, S.; Hrycyna, C. A.; Ramachandra, M.; Pastan, I.; Gottesman, M. M. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu. Rev. Pharmacol. Toxicol. 39:361–398; 1999.

[35] Konig, J.; Rost, D.; Cui, Y.; Keppler, D. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. Hepatology 29:1156–1163; 1999.

[36] Mottino, A. D.; Hoffman, T.; Jennes, L.; Vore, M. Expression and localization of multidrug resistant protein mrp2 in rat small intestine. J. Pharmacol. Exp. Ther. 293:717–723; 2000.

[37] Lockhart, A. C.; Tirona, R. G.; Kim, R. B. Pharmacogenetics of ATP-binding cassette transporters in cancer and chemotherapy. Mol. Cancer Ther. 2:685–698; 2003.

[38] Deeley, R. G.; Westlake, C.; Cole, S. P. Transmembrane transport of endo-and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol. Rev. 86:849–899; 2006.

[39] Hirohashi, T.; Suzuki, H.; Sugiyama, Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). J. Biol. Chem. 274: 15181–15185; 1999.

[40] Rost, D.; Mahner, S.; Sugiyama, Y.; Stremmel,W. Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. Am. J. Physiol. Gastrointest. Liver Physiol. 282:G720–726; 2002.

[41] Young, L. C.; Campling, B. G.; Voskoglou-Nomikos, T.; Cole, S. P.; Deeley, R. G.; Gerlach, J. H. Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin. Cancer Res. 5:673–680; 1999.

[42] Tirumalai, R.; Rajesh Kumar, T.; Mai, K. H.; Biswal, S. Acrolein causes transcriptional induction of phase II genes by activation of Nrf2 in human lung type II epithelial (A549) cells. Toxicol. Lett. 132:27–36; 2002.

[43] Zhang, C. Y.; Feng, Y. X.; Yu, Y.; Sun, W. J.; Bai, J.; Chen, F.; Fu, S. B. The molecular mechanism of resistance to methotrexate in mouse methotrexate-resistant cells by cancer drug resistance and metabolism SuperArray. Basic Clin. Pharmacol. Toxicol. 99:141–145; 2006.

[44] Strohmaier, H.; Hinghofer-Szalkay, H.; Schaur, R. J. Detection of 4-hydroxynonenal (HNE) as a physiological component in human plasma. J. Lipid Mediat. Cell Signaling 11:51–61; 1995.

[45] Uchida, K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog. Lipid Res. 42:318–343; 2003.

[46] Parola, M.; Bellomo, G.; Robino, G.; Barrera, G.; Dianzani, M. U. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. Antioxid. Redox Signal. 1:255–284; 1999.

[47] Zhang, H.; Liu, H.; Iles, K. E.; Liu, R. M.; Postlethwait, E. M.; Laperche, Y.; Forman, H. J. 4-Hydroxynonenal induces rat gamma-glutamyl transpeptidase through mitogen-activated protein kinase-mediated electrophile response element/ nuclear factor erythroid 2-related factor 2 signaling. Am. J. Respir. Cell Mol. Biol. 34:174–181; 2006.

[48] Gradhand, U.; Tegude, H.; Burk, O.; Eichelbaum, M.; Fromm, M. F.; Konig, J. Functional analysis of the polymorphism −211CNT in the regulatory region of the human ABCC3 gene. Life Sci. 80:1490–1494; 2007.

[49] Zhang, H.; Court, N.; Forman, H. J. Submicromolar concentrations of 4-hydroxynonenal induce glutamate cysteine ligase expression in HBE1 cells. Redox Rep. 12:101–106; 2007.

[50] Singh, A.; Misra, V.; Thimmulappa, R. K.; Lee, H.; Ames, S.; Hoque, M. O.; Herman, J. G.; Baylin, S. B.; Sidransky, D.; Gabrielson, E.; Brock, M. V.; Biswal, S. Dysfunctional KEAP1–NRF2 interaction in non-small-cell lung cancer. PLoS Med. 3:e420; 2006.

[51] Lau, A.; Villeneuve, N. F.; Sun, Z.; Wong, P. K.; Zhang, D. D. Dual roles of Nrf2 in cancer. Pharmacol. Res. 58:262–270; 2008.

[52] Wang, X. J.; Sun, Z.; Villeneuve, N. F.; Zhang, S.; Zhao, F.; Li, Y.; Chen, W.; Yi, X.; Zheng, W.; Wondrak, G. T.; Wong, P. K.; Zhang, D. D. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis 29: 1235–1243; 2008.

[53] Hashimoto, T.; Tokuchi, Y.; Hayashi, M.; Kobayashi, Y.; Nishida, K.; Hayashi, S.; Ishikawa, Y.; Tsuchiya, S.; Nakagawa, K.; Hayashi, J.; Tsuchiya, E. p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. Cancer Res. 59:5572–5577; 1999.

[54] Lai, S.; Perng, R.; Hwang, J. p53 gene status modulates the chemosensitivity of non-small cell lung cancer cells. J. Biomed. Sci. 7:64–70; 2000.

[55] Muredda, M.; Nunoya, K.; Burtch-Wright, R. A.; Kurz, E. U.; Cole, S. P.; Deeley, R. G. Cloning and characterization of the murine and rat Mrp1 promoter regions. Mol. Pharmacol. 64:1259–1269; 2003.

[56] Sullivan, G. F.; Yang, J. M.; Vassil, A.; Yang, J.; Bash-Babula, J.; Hait,W. N. Regulation of expression of the multidrug resistance protein MRP1 by p53 in human prostate cancer cells. J. Clin. Invest. 105:1261–1267; 2000.

Figures

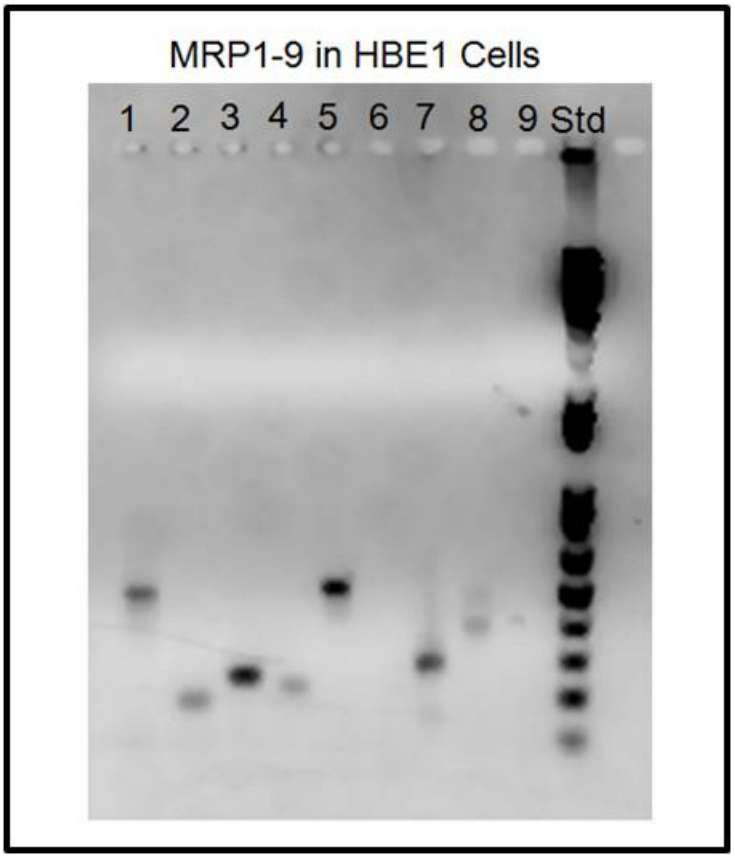

Fig. 1. RT-PCR analysis of MRPs 1–9 in the HBE1 cell line. Total RNA was isolated, reverse transcribed, and amplified by PCR. Products were analyzed on a 1.5% agarose gel. Numbers above the lanes correspond to the MRP member. MRP3 was loaded at 1/10 the initial concentration during PCR amplification.

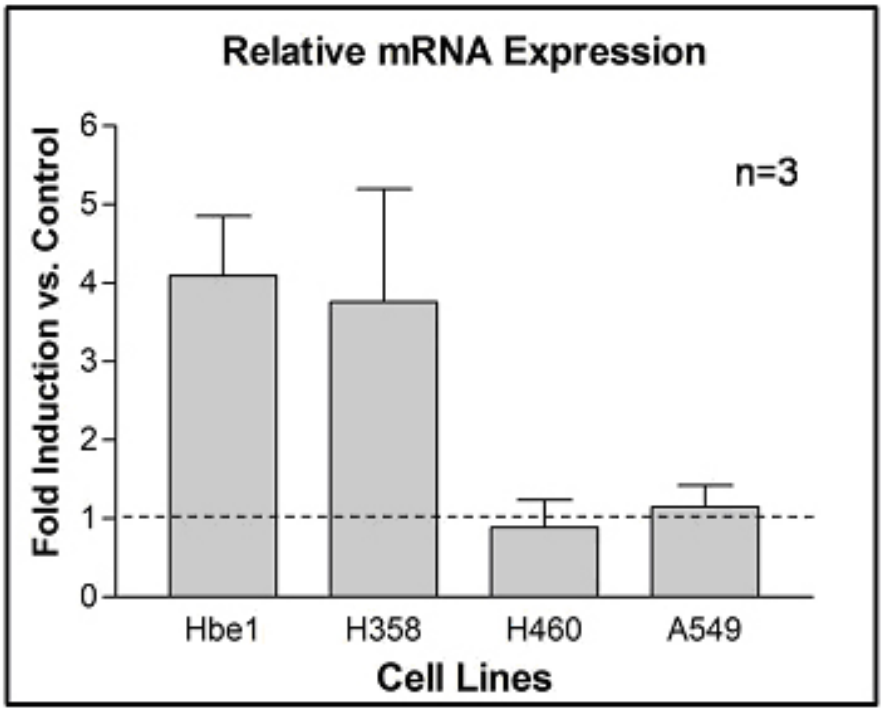

Fig. 2. Relative levels of MRP3 mRNA were determined after 24 h exposure to 15 μM HNE using real-time PCR. GAPDH and β-actin were used as internal controls. Results are expressed as means ± standard deviation (n= 3).

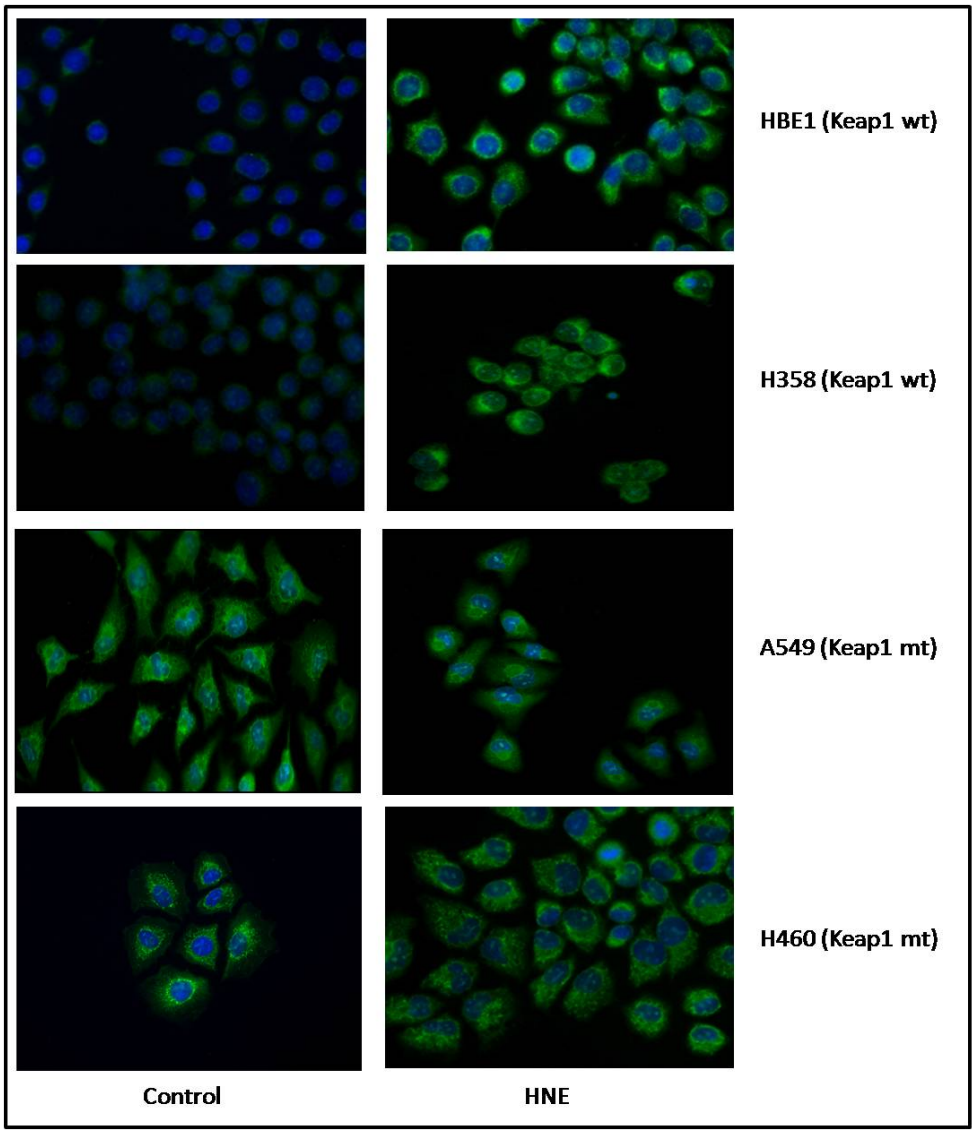

Fig. 3. Immunofluorescence. Selected cell lines were fluorescently labeled for MRP3 protein (green) and counterstained with DAPI (blue) for nuclear detection and examined after 24 h exposure to 15 μM HNE.

Fig. 4. MRP3 (ABCC3) promoter region spans from −11 bp through −1103 bp. Putative EpRE binding sites (blue) are located −434, −628, −805, and −1049 bp upstream of the ABCC3 gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

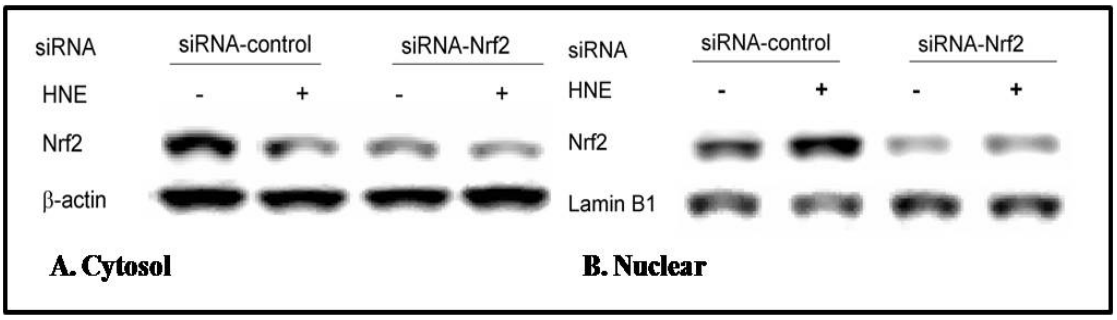

Fig. 5. Effect of Nrf2 siRNA on the activation and constitutive expression of Nrf2 protein. HBE1 cells were transfected with Nrf2 siRNA or nonspecific RNA (NS) 24 h before treatment with 10 μM HNE for 1 h. Cytosolic and nuclear fractions were extracted for Western blot analysis. (A) The NS siRNA samples displayed a reduction in cytosolic Nrf2 after HNE treatment. Transfection with Nrf2 siRNA reduced cytosolic Nrf2 alone or in the presence of HNE. (B) The NS siRNA samples demonstrated increased nuclear Nrf2 after exposure to HNE. Transfection with Nrf2 siRNA reduced nuclear Nrf2 alone or in the presence of HNE.

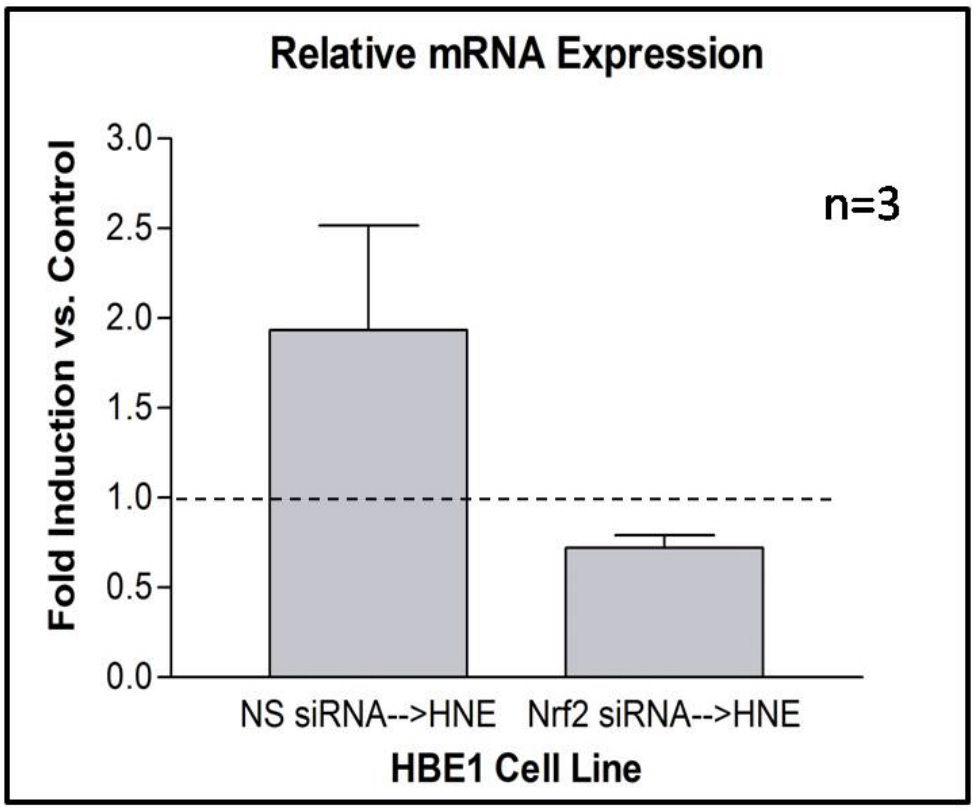

Fig. 6. RT-PCR analysis of Nrf2 siRNA silencing. Transfection with Nrf2 siRNA 24 h before exposure to 15 μM HNE inhibits induction of MRP3 mRNA compared to the nonspecific siRNA group. Results are expressed as means ± standard deviation (n= 3).

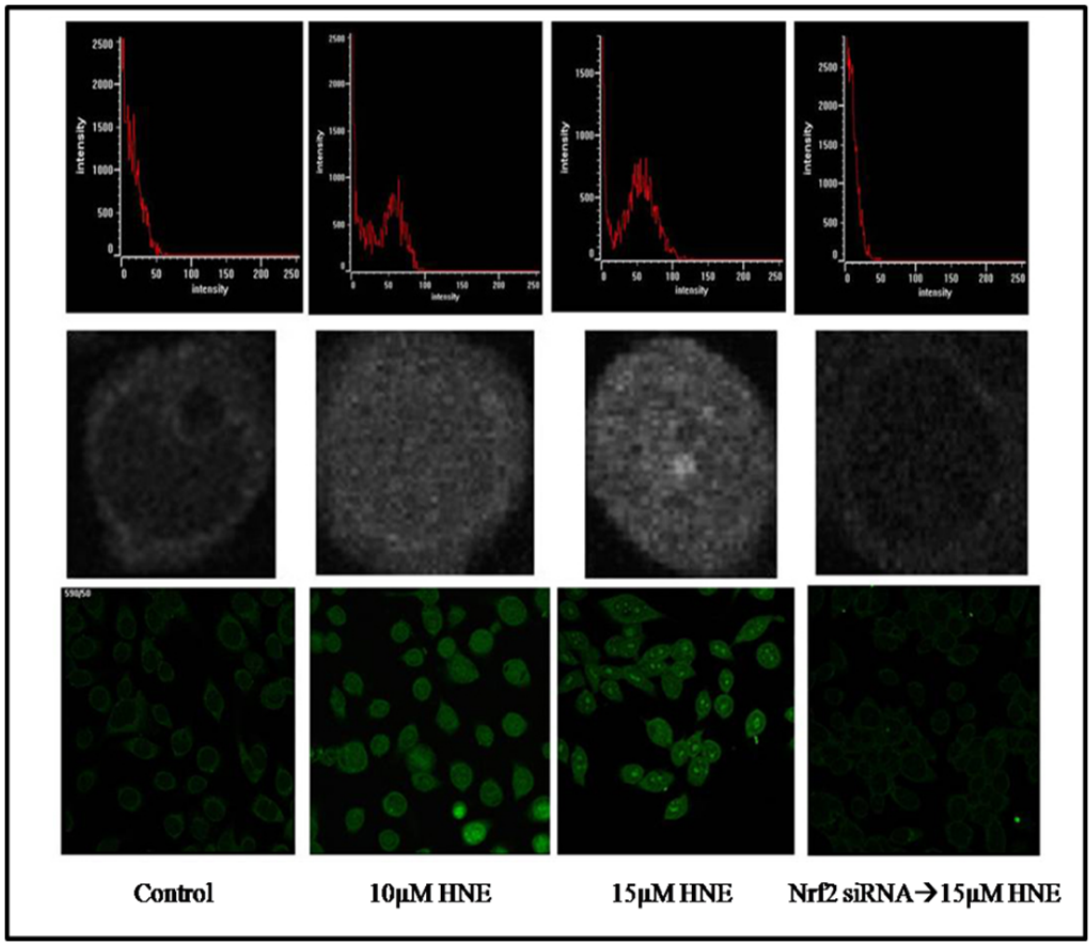

Fig. 7. Fluorescence microscopy and digitized images of representative human bronchial epithelial cells. MRP3 protein levels (green) were analyzed 24 h after exposure to 10 or 15 μM HNE and 24 h after transfection with Nrf2 siRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

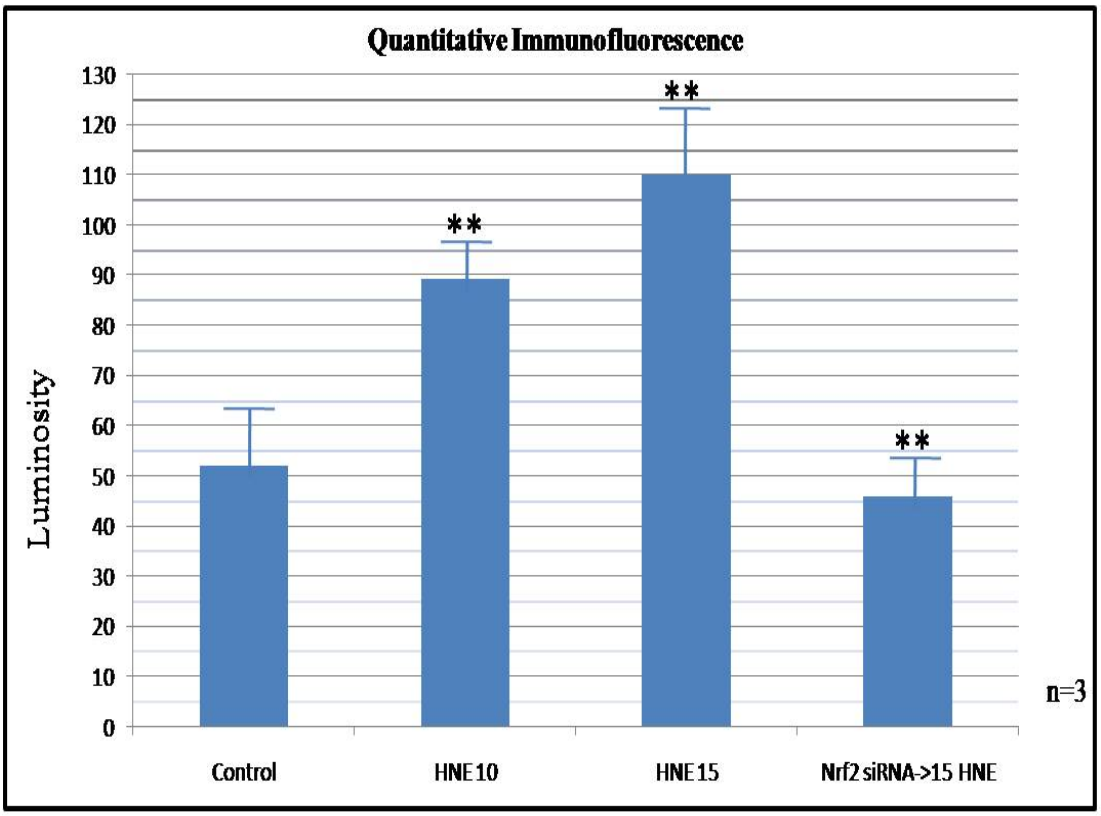

Fig. 8. Quantitative immunofluorescence. Twenty intact HBE1 cells in each treatment group were individually measured for luminosity. Control vs HNE 10 and 15 μM24 h, $p < 0.001$, and HNE 10 and 15 μM 24 h vs Nrf2 siRNA → 15 μM HNE, $p < 0.001$. Results are expressed as means ± standard deviation (n= 3).

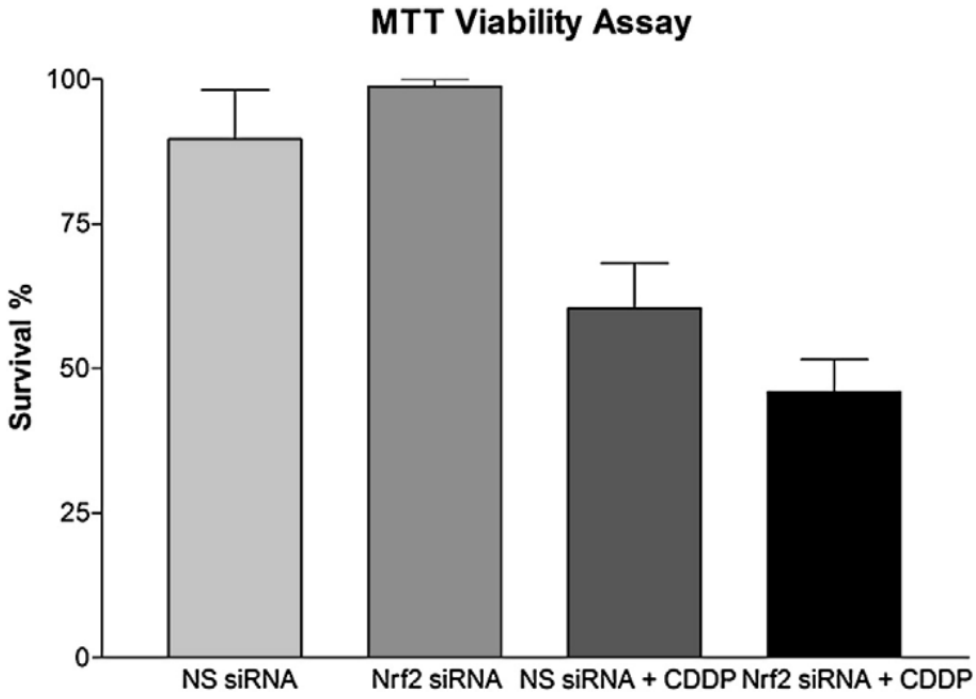

Fig. 9. MTT viability assay. We examined the change in toxicity by Nrf2 silencing by using siRNA in the NSCLC cell line H358 after exposure to 2.5 μM cisplatin for 48 h. Treatment groups included NS siRNA+ CP, NS siRNA – CP, Nrf2 siRNA+ CP, and Nrf2 siRNA – CP. Results are expressed as means ± standard deviation (n= 3).

Section II

Chapter A

Regulation of Multidrug Resistant Protein-Three by the Transcription Factors Nrf2 and p53 in Non-Small Cell Lung Carcinoma

Regulation of Multidrug Resistant Protein-Three by the Transcription Factors Nrf2 and p53 in Non-Small Cell Lung Carcinoma

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Abbreviations: MRP, multidrug-resistant protein; NSCLC, non-small-cell lung carcinoma; HNE, 4-hydroxynonenal; EpRE, electrophile response element.

Abstract

Multidrug Resistant Protein-three (MRP3) is a member of the ATP-binding cassette superfamily that facilitates detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. MRP3 is over-expressed in a variety of cancers including non-small cell lung carcinoma (NSCLC), and is suspected of playing a role in drug resistance. Keap1, the key regulator of the transcription factor Nrf2, binds Nrf2 in the cytoplasm, mediating its ubiquitination and degradation. 4-hydroxynonenal (HNE) is a major lipid peroxidation product which we have previously demonstrated initiates activation of Nrf2 thereby inducing MRP3. Here we show that ChIP analysis of the promoter revealed the presence of Nrf2 binding to the third EpRE sequence distal to the start site after HNE exposure, demonstrating direct involvement of Nrf2 regulation of MRP3. Next we examined 5 cell lines and 33 NSCLC pre-treatment patient specimens and found a parallel relationship between Nrf2 protein levels and MRP3 mRNA levels in the cell lines, and a significant correlation between *Keap1*/*Nrf2* mutational status and MRP3 levels. Additionally, studies involving related drug transport proteins have demonstrated both positive and negative regulatory roles for wild-type (wt) and mutant (mt) p53; therefore we sought to determine the role of this transcription factor in the regulation of MRP3. Transfection of a *p53* null NSCLC cell line with wt *p53*, empty vector (EV), and mt *p53* plasmids revealed that basal MRP3 levels increased in the absence of functional p53, similar to what we observed in our patient cohort. Conversely, when wt *p53* transfected cells were treated with either HNE or gemcitabine we found an improved response in MRP3 induction compared to the EV group. We also found a significant link between harboring a both a *Keap1* and a *p53* mutation in our patient

cohort. These results support the hypothesis that MRP3 induction is regulated by both Nrf2 and p53 during normal conditions and during oxidative stress.

Introduction

Multidrug resistant proteins (MRPs) are a 9-member branch of the ATP-binding cassette superfamily [1]. The role of MRP3 is to facilitate transport toxic compounds out of cells; however this increased transport can contribute to the chemoresistance of cancer cells [2]. Chemotherapy, radiation, and xenobiotic stresses have been shown to increase levels of select MRPs. Three members of the MRP family, MRPs 1-3, have been associated with increased resistance to frontline chemotherapeutic compounds, and are thought to play a role in the drug resistance of non-small cell lung carcinoma (NSCLC) [3, 4]. Increased MRP1 levels have been reported to correlate with both increasing NSCLC cancer stage and invasiveness [5, 6]. MRP3 is the closest structural *ABCC* family member to MRP1 (~58% homology) [3]. MRP3 protein levels have been correlated with decreased sensitivity of lung cancer cell lines to doxorubicin, vincristine, etoposide, and cisplatin [4]. Additionally, MRP3 expression has been associated with increased resistance to methotrexate and doxorubicin in NSCLC cell lines and tumor samples [2, 3].

The lipid peroxidation product, 4-hydroxy-2-nonenal (HNE) is formed from polyunsaturated fatty acids in cell membranes by reaction with reactive oxygen and nitrogen species [7, 8]. Many classes of antineoplastic agents including frontline drugs in the of lung cancer treatment (anthracyclines, most alkylating agents, and platinum compounds) produce significant oxidative stress [9]. The reactive oxygen species generated by chemotherapy subsequently leads to lipid peroxidation resulting in products such as HNE. HNE was used as a surrogate for oxidative stress in this investigation and has been previously established to cause the activation of the Nrf2-EpRE signaling [10,

11]. We have previously demonstrated that HNE induced of MRP3 mRNA and protein levels in a *Keap1* wt NSCLC cell line, but not in *Keap1* mutant NSCLC cell lines. Nonetheless, the failure of HNE to increase MRP3 in the *Keap 1* mutant NSCLC cell line was apparently due to constitutively active Nrf2 leading to high expression of MRP3. Our results also showed that MRP3 induction involves activation of the transcription factor, Nrf2, as MRP3 induction was inhibited using siRNA against Nrf2 [12]. This is consistant with what is known about MRP1 and MRP2 as both have been shown by others to be regulated by Nrf2. In addition, we also treated cells with the DNA damaging drug gemcitabine, a front line NSCLC chemotherapeutic, to investigate the effect on MRP3 levels alone and in cells transfected with a *p53* wt expression vector.

The current accepted model for Nrf2 regulation is one in which two amino-terminal motifs in the Neh2 domain, a weak binding DLG motif and a strong binding ETGE motif, act as binding sites for Keap1 and promote ubiquitination and rapid turnover; also known as the hinge and latch model [13]. We and others have demonstrated that inhibiting Nrf2 decreases resistance to chemotherapeutic agents. Furthermore, Shibata *et al.* found that Nrf2 mutations occurred in approximately 11% of their lung cancer samples, with a striking frequency in the Neh2 domain, and that these patients had a poor prognosis [13]. Keap1, the key functional repressor of Nrf2 is comprised of an N-terminal region (AA 1–60), a BTB domain (AA 61–179), a central IVR (AA 180–314), six Kelch motifs (AA 315–359, 361–410, 412–457, 459–504, 506–551, and 553–598), and a C-terminal domain (AA 599–624) [14]. Singh *et al.* found that *Keap1* loss-of-function mutations occurred in approximately 19% of NSCLC tumors and 50% of NSCLC cell lines examined [14]. In NSCLC cell lines with mutant *Keap1*, Nrf2

was found to be constitutively active and MRP1&2 expression was elevated [14]. In addition to improved drug resistance, *Keap1* mutants have also been demonstrated to possess rapid growth characteristics [15].

p53 is a critical component in the regulation of cell cycle progression, DNA repair, and induction of apoptosis. In response to DNA damage, p53 responds by arresting cell cycle progression to enable the initiation of DNA repair. If the cell is beyond repair, p53 can initiate the apoptotic pathway [16]. Dysregulation of the *p53* gene (mt or null) occurs in approximately 45% of NSCLC cases and is associated with poor prognosis [17]. The majority of mutations found in *p53* appear to be missense mutations located within the DNA binding domain (exons 5-8), some of which can be pro-oncogenic [18, 19].

Functional p53 has been demonstrated to repress Nrf2-regulated expression of several phase II genes, suggesting cross talk between the p53 and Nrf2 pathways [20]. Studies have also demonstrated a negative link between p53 activation and MRP1 expression, which has been reported to increase with NSCLC cancer stage and invasiveness [5, 6]. The correlation between high levels of MRP1 expression and *p53* mutation is supported by *in vitro* studies of *MRP1* gene regulation that have shown wt p53 to be a strong suppressor of *MRP1* transcription [21]. Additionally, the human papilloma E6 protein, which initiates the rapid degradation of p53, subsequently increased MRP1 levels in an HPV16-transformed cell line [6]. The mechanism by which this occurs could be an example of the indirect effect of transcription factors as described above as MRP1 does not contain a p53 response element in its promoter. MDR1, a well characterized drug transporter, but not MRP1 has been shown to be strongly up-regulated

by the addition of gain of function mutant p53 and its interaction with the transcription factor Ets-1 [22]. A more recent study using a *p53* null osteosarcoma model found that a select *p53* mutant caused an increase in baseline MRP1 levels [23]. Whether p53 is involved in the regulation of other MRP genes has not yet been determined.

Materials and Methods

Chemicals and Reagents

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO). DNA-free reagents were obtained from Fermentas (Glen Burnie, MD). TaqMan Reverse Transcription Reagent and SYBR Green PCR Master Mix were obtained from Applied Biosystems (Foster City, CA). FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, IN).

Cell Culture and Treatments

The NSCLC cell line H358 (*p53* null/*Keap1* wt), and H1666 (*p53* wt/*Keap1* wt) were maintained in RPMI 1640 (Biowhittacker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA) to which was added MEM Essential Vitamin Mix, Penicillin-Streptomycin, L-Glutamine. All cultures were grown at 37°C in 5% CO_2 atmosphere. Cells were plated on 10cm cell culture dishes at a density of 1,000,000 cells per dish. Cells were treated at approximately 70% confluence. Agents were diluted to appropriate concentrations in media before use.

RT-PCR

The content of MRP3 mRNA was determined with real-time RT-PCR method. RNA samples were treated with DNA-free RNase reagent and reverse transcribed by using the TaqMan reverse transcription system. Real-time PCR was performed with a BioRad iQ5 iCycler PCR machine (Hercules, CA), with β-actin used as an internal control. The primers are as follows: MRP3, forward 5'-CAGAGAAGGTGCAG

GTGACA-3**',** reverse 5'-CTAAAGCAGCATAGACGCCC-3**';** β-actin**,** forward 5'-TGGGTGTGAACCATGAGAAG-3, reverse 5'-CCATCACGACACAGTTTCC-3.

SSCP PCR

Analysis was performed in 25 μl reactions using 1x AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA), 10 μM of primers, and one μl of genomic DNA were used. The primer sequences for *p53* exons 5-8 are as follows; *p53* exon 5: forward, 5'-TTCAACTCTGTCTCCTTCT-3'; reverse, 5'-CAGCCCTGTCGTCT CTCCAG-3'; *p53* exon 6: forward, 5'-GCCTCTGATTCCTCACTGAT-3'; reverse, 5'-TTAACCCCTCCTCCCAGAGA-3'; *p53* exon 7; forward, 5'-CTTGCCACAGGTC TCCCCAA-3'; reverse, 5'-TGTGCAGGGTGGCAAGTGGC-3'; *p53* exon 8: forward, 5'-TTCCTTACTGCCTCTTGCTT-3'; reverse, 5'-CGCTTCTTGTCCTGCTTGCT-3'. PCR was performed in a BioRad PTC-200 DNA Engine Thermal Cycler (BioRad, Hercules, CA) for 35 cycles at 95^{o}C for 15s, 55^{o}C for 30s, and 68^{o}C for 60s with a final extension at 68 ^{o}C for 10 min. After PCR, 5 μl of PCR products were checked by gel electrophoresis using ethidium bromide. Subsequently, 3 μl of PCR products were mixed with 3 μl of SSCP loading buffer (95% formamide, 5% xylene, and 10 mg bromophenol blue), incubated at 95 °C for 5 minutes, and immediately placed on ice. DNA samples were electrophoresed using the Gene Gel Excel 12.5/24 Kit (Amersham Pharmacia Biotech, Sweden) at 600 volts for 90 minutes. Exons 5, 7, and 8 were run at 18 ^{o}C while exon 6 was run at 10 ^{o}C. The GenePhor Elecotrophoresis Unit (Amersham Pharmacia Biotech, Sweden) was used for all SSCP reactions. DNA samples were visualized by silver stain. Any exon fragments migrating abnormally in SSCP gels were

sequenced to define the base changes. The specific positions and types of nucleotide changes were recorded.

Tumor Specimens

NSCLC tumor tissues were acquired from the IRB-approved UC Davis Cancer Center Specimen Repository (CCSR). Specimens were collected at time of surgery, pathologically reviewed and annotated. Approval for specimen usage for this proposed project was obtained from Dr. Gandour-Edwards, CCSR Leader. Tissue was stored at -80^{o}C until extraction of DNA, RNA, and protein, as described.

Preparation of Frozen Tissue

Genomic DNA was extracted from minced tissue using the QIAamp DNA extraction kit (Qiagen, Valencia, Ca) according to manufacturer's protocol. RNA was extracted using Qiagen RNeasy Kit (Qiagen, Valencia, CA). Quantity and quality of both RNA and DNA were determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Plasmid Cloning

Plasmids were transformed into DH5α competent bacterial cells. Cells grown overnight at 37°C on ampicillin/LB treated plates. Colonies were selected, re-suspended in LB/Amp, and allowed to grow for 8hrs at 37°C while shaking at 270 rpm. Half of the culture was used to prepare a glycerol stock of the *p53* plasmids and half of the culture was used for a mimi-prep to isolate plasmid DNA (Qiagen mini-prep kit). A double restriction digest was performed using EcoRI and BamHI to cut out the *p53* fragment.

The sizes of both plasmids and their respective fragments were confirmed. The *p53* mt plasmids (V143A, R175H, R248W, R273H) were acquired from Addgene (Cambridge, MA), and the wt *p53* plasmid was a kindly gift from Dr. Ruth Vinall (UC Davis Cancer Center).

Isolating plasmid DNA for Transfection

Bacteria from the glycerol stock was streaked out onto fresh ampicillin/LB plates and allowed to grow overnight for colony selection. Colonies were selected and grown in LB/Amp culture for 8 h at 37°C while shaking at 270 rpm. These cultures were spun down and plasmid DNA was isolated using the Qiagen mini-prep kit. Quantity and quality of plasmid DNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Plasmid and siRNA Transfection

Transient transfection of wt *p53* plasmid was performed in H358 cells, at approximately 60-70% confluence. Appropriate amounts of wt *p53*, mt *p53,* or empty vector plasmids (stocks are 10 μM in RNase-free water) were mixed with transfection reagent FuGENE 6 in antibody-free RPMI 1640 medium and incubated for 20 min at room temperature. The mixture was then added to cells and incubated for 24 h and confirmed by Western Blotting. H358 cells were plated in antibody-free RPMI 1640 medium with 10% fetal bovine serum. The cells were transfected using FuGENE6 with 5 μg of *p53* expression vector DNA (or empty pcDNA3.1- DNA). Unless otherwise indicated, the cells were harvested 24 h following the application of plasmid DNA to the cells.

Western blotting

Western blotting was performed as previously reported [24]. Briefly, soluble proteins were extracted with the addition of RIPA lysis solution (RIPA Buffer, Leupeptin, Aprotinin, PMSF, NaVO4 and DNase, Rnase-free). The lysates were cleared by centrifugation at 12,000 rpm at 4°C for 15 minutes, and soluble protein extracts were stored frozen at -80°C. Protein concentrations were quantitated from duplicate readings using a modified Bradford assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates were diluted with RIPA lysis buffer to either 20 or 30 µg/µl to facilitate equal loading of samples, and added to electrophoresis SDS sample buffer and boiled at 95°C for 5 minutes. Total cell extracts were separated by 10 or 15% SDS-PAGE gels using a mini-gel system (Bio-Rad laboratories, Hercules, CA) for 120-150 min. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) overnight at a constant 40 volts. Membranes were then blocked with a powdered milk solution. Mouse p53 antibody at 1:200 (sc-53394), rabbit Nrf2 antibodies at 1/200 (sc-722), and mouse β-actin antibody at 1:10,000 (A5441, Sigma) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were incubated at 4^{o}C overnight with p53 or Nrf2 (1:200) and mouse anti-β-actin antibody (1:10,000). After washing, blots were incubated in horseradish peroxidase-conjugated antimouse IgG or antirabbit IgG secondary antibody (1:2500), (Promega) in TBS-T for 1 h, followed by incubation with chemiluminescent detection reagents (ECL, Fisher Scientific). Membranes were exposed to Kodak XAR film, the film developed and the results interpreted.

ChIP Assay

ChIP assay was performed following the protocol provided with the kit from Millipore (Temecula, CA). Briefly, cells were incubated with formaldehyde (final concentration is 1%) at room temperature for 10 min. Cell pellet was lysed on ice for 10 min and sonicated using a Branson sonifier 150 (Branson, Danbury, CT) under conditions that cause DNA to be broken into 200- to 800-bp fragments (5 sets of 10-second pulses on wet ice with output power at 3). Sonicated cell lysate was precleared with 60 µl of protein G agarose, and the supernatant was used for immunoprecipitation with antibodies to specific transcription factors overnight at 4^{o}C. The protein/DNA complex was collected with 60 ml of protein G agarose and then it was washed and eluted from agarose in elution buffer. Next the crosslink of DNA/protein was reversed by adding 5 M NaCl and incubating the mixture at 65^{o}C for 5 h. The DNA was then extracted with a spin filter. GAPDH was used as the input control. The real time PCR was run for 50 cycles under the conditions: 95^{o}C 25 sec, 61^{o}C 30 sec and 72^{o}C 31 sec. Primers used for PCR in the ChIP assay were as follows; EpRE1 forward, 5'-AGCCAAGGAAGGAAAC ACCT-3'; reverse, 5'-ATCTCTCAATCCCGCTGTTG-3'; EpRE2 forward, 5'-AGCCTTAGGAGGAGGACGAG-3'; reverse, 5'-GAATCTTCCTCCCAGGGTTC-3'; EpRE3 forward, 5'-AGTAGAGGCCCAGAGACGTG-3'; reverse, 5'-GGGTGTGTAC CCGTGAGG-3'; EpRE4 forward, 5'-CCAGTGGTCTCTGGAACCTG-3'; reverse, 5'-GTTCCTCTCCGGAGGTCAGT-3'; GAPDH: forward, 5'-TACTAGCGGTTTTA CGGGCG-3', reverse, 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'.

DNA sequencing

PCR Primers for *Keap1* sequencing [25] are as follows; *Keap1* exon 2F1, 5'-TGGTGGTGTTGCTTATCTTCTGG-3'; *Keap1* exon 2R1: 5'-TACCCTCAATGGACACCACCTCC-3'; *Keap1* exon 2F2: 5'-AAGGTGGTGCTGGCCTCATCCAG-3'; *Keap1* exon 2R2: 5'-AGACAGTGATGAGCACTCGTCCA-3'; *Keap1* exon 3F1: 5'-GGTGACTGGAGAGTCAGCCCGTC-3'; *Keap1* exon 3R1: 5'-CTCCAGGTAGCTGAGCGACTGTC-3'; *Keap1* exon 3F2: 5'-TGCACAAGCCCACGCAGGTGATG-3'; *Keap1* exon 3R2: 5'-TTGGGACTTGCCAGGAGCAGGAC-3'; *Keap1* exon 4F: 5'-CACGAAGGTCAGCTATAATGGCC-3'; *Keap1* exon 4R: 5'-TCAGTTTCACCCCAGGATGGTAG-3'; *Keap1* exon 5F: 5'-TCTCCCTCAAGGAGGTGATGGCT-3'; *Keap1* exon 5R: 5'-GCAAAAGCAGTCCACAAAAGATG-3'; *Keap1* exon 6F: 5'-GCTCTTGGATGTGGTGTGACAG**G**-3'*; *Keap1* exon 6R: 5'-CAATGATACTCCCCATTGGACTG-3'. Primers for *Nrf2* sequencing [13] are as follows; *Nrf2* exon 2: forward, 5'-ACCATCAACAGTGGCATAATGTG-3'; reverse, 5'-GGCAAAGCTGGAACTCAAATCCAG-3'. All DNA sequencing was performed on an ABI 3730 Capillary Electrophoresis Genetic Analyzer using ABI BigDye Terminator v3.1 Cycle Sequencing chemistry.

Software

Scion Image software was used in this study to quantitate protein levels of western blots, by deriving a ratio between absorbance values.

Statistics

The comparative $\Delta\Delta C_T$ method was used for relative mRNA quantitation. Comparisons of variants between experimental groups were conducted using one-way analysis of variance (ANOVA). All data is expressed as the mean ± standard error of the mean (SEM). In-Stat software was used for statistical analysis. Statistical significance was accepted when $p < 0.05$. The Tukey-Kramer Multiple comparisons test was used for comparison of mRNA levels. Fisher's Exact Test was used to determine nonrandom associations.

Results

ChIP assay detects DNA binding by Nrf2 *in vitro.*

The genomic sequence of the human MRP3 gene (ABCC3) revealed four EpREs sequences in the promoter region from −11 bp through −1103 bp. Specifically located at sites −434, −628, −805, and −1049 bp. These findings led us to hypothesize that activation of Nrf2 could contribute to the induction of MRP3. As an extension of our previous work we sought to further establish the relationship of Nrf2 and MRP3 [12]. The interaction between Nrf2 and MRP3 was elucidated by use of a chromatin immunoprecipitation assay (ChIP) of the Nrf2/DNA complex, and subsequently analyzing the bound DNA using PCR. Nrf2 binding was increased at the -805 bp EpRE after exposure to HNE (**Figure 1**). These results demonstrate a direct interaction of Nrf2 binding in the promoter region of MRP3.

Sequence analysis of cell line and patient specimens for *Keap1*, *Nrf2*, and *p53* mutations reveals a correlation between *Keap1* and *p53* mutational status in patient samples.

To determine whether mutations in *Keap1*, *Nrf2*, or *p53* were present in our samples we amplified and sequenced the five protein coding exons of the *Keap1* gene, exon 2 (Neh2) of the *Nrf2* gene. In the patient specimens, *p53* mutational status was determined using Single-Strand Conformation Polymorphism (SSCP) of exons 5-8, with positive samples confirmed by sequencing (in patients with lung cancer, *p53* mutations are most common in exon 5-8) (**Figure 2A and 2B**). Sequencing of *Keap1* in these cell lines confirmed the homozygous mutation at amino acid 333 found by Singh *et al.* in

A549, while all other cell lines evaluated were wild type for both *Keap1* and *Nrf2*. In our patient cohort we identified four homozygous and one heterozygous *Keap1* mutants. All mutations were missense mutations: three homozygous mutants were G→T transversions, one homozygous mutant was a G→A transition, and the hetrozygous mutant was a C→T transition. Singh *et al.* found 18.5% of their patient samples had a mutation in *Keap1*, which is similar to our findings of 15%.

Only one *Nrf2* exon 2 mutant was found (~3%), which was a heterozygous G→A transition, resulting in a missence mutation (**Table 1**). Shibata *et al.* found approximately 11% of their patients analyzed were *Nrf2* mutant; a possible explanation for the observed discrepancy may be that our sample size was lower, and/or that mutation frequencies may differ between different ethnic populations as their patient samples were Japanese. Additionally, they found a higher frequency of mutations in cancers of squamous cell histology, of which we only had seven. Finally, we found that eleven of our patient samples were mutant for *p53* (33%), which were cross checked with the IARC TP53 data base (*www-p53.iarc.fr*), and are defined in **Table 2**. We found that in all five of our patient samples that were mutant for *Keap1* were also mutant for *p53*. Using Fisher's Exact Test we found a very significant ($p<0.0019$) correlation of this not being a random event (**Figure 2C**).

MRP3 levels trend higher in mutant *Keap1*/*Nrf2* cell line and NSCLC patient samples.

Using quantitative RT-PCR we evaluated levels of MRP3 mRNA in four NSCLC cell lines, and one prostate cancer cell line, and thirty-three pre-treatment surgical specimens with varying genetic status for *Keap1*, *Nrf2,* and *p53* (**Figure 3A**). We found that H1650 (*Keap1* wt, *Nrf2* wt, *p53* wt) and H358 (*Keap1* wt, *Nrf2* wt, *p53* null) expressed relatively low levels of MRP3 mRNA, and that A549 (*Keap1* mt, *Nrf2* wt, *p53* wt) had high levels of MRP3 mRNA. However, we found that H1666 (*Keap1* wt, *Nrf2* wt, *p53* wt) also had high levels of MRP3 despite being wild-type for all three genes, we then evaluated the prostate cancer cell line DU-145 (*Keap1* wt, *p53* mt), as it has recently been demonstrated to produce low levels of Keap1 due to promoter methylation and differentially spliced Keap1 mRNA [26]. These cell lines were evaluated further by measuring their total Nrf2 protein levels via western blotting. Low levels of the predicted size Nrf2 was observed in H1650 and H358 cells, and higher levels of the Nrf2 were found in A549. While we found elevated levels of Nrf2 protein in H1666, it was the 100 kD variant, which was also detected in DU-145 (**Figure 3B**). Relative levels of total Nrf2 protein levels of our cell line model were quantified using Scion Image software. We found that Nrf2 protein levels (H1650 had insufficient levels of Nrf2 by western blot to accurately measure) in our cell line model had very similar ratios to their MRP3 mRNA levels (**Figure 3C**). When we compared MRP3 mRNA levels in *Keap1/Nrf2* wt against *Keap1*/*Nrf2* mt patient samples (only homozygous *Keap1* mt samples were included in our evaluation) we found a statistically significant correlation between the *Keap1*/*Nrf2* wt

(Mean ~2.6 fold vs. H358) and *Keap1*/*Nrf2* mt (Mean ~5.6 fold vs. H358) patient samples (**Figure 4**).

Mutant p53 expression in cell lines and patient samples correlates with higher basal MRP3 mRNA levels.

In an effort to determine the effect of having either null, wt, or mt *p53* on basal MRP3 levels we transiently transfected the NSCLC cell line H358 (*p53* null) with either empty vector, wt *p53*, or one of four different mt *p53* plasmids (V143A, R175H, R248W, R273H) (**Figure 5A and 5B**). We evaluated MRP3 mRNA levels 24 h post transfection, and found cells containing wt *p53* had approximately 33% lower levels of MRP3 mRNA than when compared to the average level of the four *p53* mt groups (**Figure 5C**). We then compared the *p53* wt/*Keap1* wt vs. *p53* mt/*Keap1* wt patients, and found that while not statistically significant; the *p53* wt group had 32% less MRP3 mRNA, which is consistent with our cell line data (**Figure 5D**).

We then sought to evaluate MRP3 levels after exposure to cytotoxic stress caused by either HNE or gemcitabine, with or without the presence of wt *p53*. Cells were treated 24 h after transfection with either 15 μM HNE or 25 μM of gemcitabine for an additional 24 h and relative MRP3 mRNA levels were determined by RT-PCR (**Figure 6A**). In *p53* wt cells, HNE and gemcitabine significantly increased the induction of MRP3 expression when compared to the EV. Tukey's Multiple Comparison Test demonstrated a significant difference between the *p53* with versus *p53* with HNE or the gemcitabine treated groups ($p < 0.05$), while the EV versus EV treated with HNE or gemcitabine groups were not significantly different. To evaluate what effects HNE and gemcitabine were having on

p53 protein levels we conducted a western blot, and found that p53 levels increased in both treatment groups **(Figure 6B)**.

Discussion

The primary treatment for combating NSCLC often involves surgery, radiation, and the use of chemotherapy. Unfortunately, the majority of NSCLCs either acquire, or are inherently drug resistant. Multifactorial drug resistance is one contributing factor to the abysmal 5 year survival rate for metastatic NSCLC, which is just 2%. Chemoresistance is thought to be derived by two principle mechanisms: the first involves an improved capacity for DNA repair; the second involves up-regulation of the cellular detoxification pathways such as the super family of P-glycoproteins. Studies of clinical specimens and cell lines have shown an increased capacity for drug transport, and subsequent resistance, due to increased levels of the Multidrug resistance transporters (MDRs) and MRPs. The MRP (*ABCC*) family consists of nine different members [27]. Several MRPs are known to transport a wide range of chemotherapeutic compounds and thus reduce the cellular accumulation of anti-cancer agents [2]. MRP family members have been shown to be significant contributors to multidrug resistance in drug-resistant cell lines and have been found in numerous classes of cancer types [28, 29].

MRP3 is a member of the ATP-binding cassette superfamily that facilitates detoxification by transporting toxic compounds, including chemotherapeutic drugs, out of cells. MRP3 is over-expressed in a variety of cancers including non-small cell lung carcinoma (NSCLC), and is suspected of playing a role in drug resistance. Given that not all NSCLCs express high levels of MRPs, such as MRP3, it would seem that some additional dysregulation must occur to acquire this trait. Our analysis of the human MRP3 gene revealed four putative Nrf2 binding sites (EpREs) in the tentative promoter region. This finding led us to hypothesize that activation of Nrf2 could contribute to the

induction of MRP3. ChIP analysis demonstrated increased Nrf2 binding to the -805bp EpRE after treatment with the Nrf2 activator, HNE. This data, in conjunction with our previous work involving Nrf2 and MRP3 regulation, would strongly implicate direct involvement of Nrf2 in the upregulation of MRP3 [12].

We evaluated our cell line model for total basal levels of MRP3 mRNA and Nrf2 protein, and found that knowing the mutational status of *Keap1*, *Nrf2*, and *p53* alone was insufficient to predict MRP3 levels. Although the cell lines H1650, H358, and A549 had MRP3 levels that corresponded to our hypothesis based on their respective mutational status, the NSCLC cell line H1666 (*Keap1* wt, *Nrf2* wt, *p53* wt) had levels of MRP3 that exceeded those of the *Keap1* mt A549. Recent data from Johns Hopkins University demonstrated that hypermethylation of the promoter and aberrant splicing of Keap1 mRNA occurred in the *Keap1* wt cell line DU-145, causing low levels of Keap1, increased growth rates, and upregulation of Nrf2 controlled genes [26]. We examined this cell line and found modest induction of MRP3. Additionally, a roughly 100 kD band of Nrf2 was present in only DU-145 and H1666, this high weight variant of Nrf2 has been previously reported, and has been shown to be functionally active. What precisely is bound to Nrf2 is of much debate; some evidence points to polyubiquitination or actin [30]. When we quantitated both bands of Nrf2 protein as a ratio against β-actin using Scion Image software we discovered an almost identical pattern to the cell lines MRP3 mRNA levels. The splicing errors in DU-145 occur in the DGR domain (kelch 1-6), which is the portion of Keap1 that interacts with actin [31]. It is tempting to speculate that cells which have atypical splicing of Keap1 may also have high levels of 100 kD Nrf2. In light of this information it is possible that H1666 has a similar dysregulation in

Keap1 transcriptional and posttranscriptional processing. How prevalent hypermethylation of the promoter and aberrant splicing of Keap1 is in patient populations could be of clinical relevance in order to determine the appropriate populations for chemoresistance and proliferation.

Sequencing of *Keap1* and the Neh2 domain of *Nrf2* in 33 primary tumors from lung cancer patients revealed missense mutations in a total of six tumors. All but one of these patient specimens, our only sample of neuroendocrine histology, expressed high levels of MRP3 when compared to the *Keap1*/*Nrf2* homozygous wt cell line H358. All of the *Keap1* mutations were found to occur in the kelch domains, and interestingly they were all mutant for *p53*. It has been demonstrated that human lymphoblastoid cells that have mt *p53* acquire new mutations at a higher frequency [32]. It would seem reasonable to expect that patients who have mt *p53* will consequently have reduced ability to preserve genomic integrity; as such one would expect these patients to acquire new mutations at an increased rate over their *p53* wt counterparts. Additionally, loss of *Keap1* has been demonstrated to increase growth rate in cell line models, and having an increased rate of growth would potentially be a selectable trait. Although not significant due to sample size, we had two metastatic patient samples in our study, both of which had *Keap1* mutations. When taking into account our data demonstrating that cells lacking wt p53 do not upregulate MRP3 as effectively, losing Keap1 function would re-establish higher levels of MRP3 (**Figure 5D**). However, as the patients in our study had not yet received treatment, acquiring a mutation in *Keap1* due to selective pressure from chemotherapy would seem an inadequate explanation.

While our data demonstrates a role for p53 in suppression of baseline MRP3 levels, it does not provide evidence by which p53 is accomplishing these observed effects. One possible mechanism of how p53 may be acting to suppress MRP3 may include direct binding of p53 to the MRP3 promoter to suppress its upregulation. Analysis of the MRP3 promoter sequence revealed the presence of a putative p53 binding site (RRRCA/T T/AGYYY) –285 bp from the transcriptional start site. In wt *p53* transfected cells, treatment with the electrophilic compound HNE and gemcitabine, caused a statistically significant increase in MRP3 mRNA and an increase in p53 protein levels when compared to the untreated and treated empty vector control groups. Cytotoxic compounds, such as high dose HNE or the chemotherapeutic drug gemcitabine, can initiate induction of phase II detoxification genes. These genes include the Nrf2 targets quinone oxidoreductase 2 (*NQO2*) and NAD(P)H quinone oxidoreductase 1 (*NQO1*). Subsequently, both NQO1 and NQO2 act to stabilize p53 against 20S proteasomal degradation [33]. A potential model of MRP3 regulation after cytotoxic stress may involve p53, p21 WAF, and c-myc. The consequence of increased levels of p53 would be the upregulation of p21 [34]. Recent data has shown that p21, which is directly upregulated by p53 and is known to be involved in an antioxidant capacity, and that it has the capacity to binds to Nrf2 directly interfering with Keap1 mediated degradation [35]. We recently demonstrated that the oncogenic transcription factor c-myc has the ability to negatively regulate Nrf2 driven genes by two distinct mechanisms; it directly competes with Nrf2 for binding to EpRE sites, and through increased rate of Nrf2 degradation [36]. In addition to being able to reduce Nrf2 related induction, c-myc has been well established to suppress p21 [37]. Cytotoxic induced

stabilization of p53 could act to increase levels of MRP3, as it has been demonstrated to repress c-myc production through histone deacetylation (**Figure 7**) [38].

Cells exposed to cytotoxic compounds will attempt to survive, in part by effluxing the causative agent. We propose a system of regulation for the transport protein, MRP3, in which either through mutation or cytotoxic insult, will facilitate the release and activation of the transcription factor Nrf2 from its repressor Keap1 causing the subsequent up-regulation Nrf2 regulated genes such as MRP3. Furthermore cells with wt *p53* are able to achieve higher levels of MRP3 after cytotoxic stress than those lacking functional *p53*. Therefore, better understanding of the interplay between the mutational status of *Nrf2* and *p53* in MRP3 regulation could lead to improved therapeutic decision-making for NSCLC.

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References

1. Gerk, P.M. and M. Vore, Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. J Pharmacol Exp Ther, 2002. **302**(2): p. 407-15.
2. Kool, M., et al., MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6914-9.
3. Lockhart, A.C., R.G. Tirona, and R.B. Kim, Pharmacogenetics of ATP-binding cassette transporters in cancer and chemotherapy. Mol Cancer Ther, 2003. **2**(7): p. 685-98.
4. Young, L.C., et al., Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. Clin Cancer Res, 1999. **5**(3): p. 673-80.
5. Sullivan, G.F., et al., The expression of drug resistance gene products during the progression of human prostate cancer. Clin Cancer Res, 1998. **4**(6): p. 1393-403.
6. Sullivan, G.F., et al., Regulation of expression of the multidrug resistance protein MRP1 by p53 in human prostate cancer cells. J Clin Invest, 2000. **105**(9): p. 1261-7.
7. Parola, M., et al., 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. Antioxid Redox Signal, 1999. **1**(3): p. 255-84.
8. Uchida, K., 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res, 2003. **42**(4): p. 318-43.
9. Kim, H.J., et al., Roles of NADPH oxidases in Cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci. **30**(11): p. 3933-46.

10. Tirumalai, R., et al., Acrolein causes transcriptional induction of phase II genes by activation of Nrf2 in human lung type II epithelial (A549) cells. Toxicol Lett, 2002. **132**(1): p. 27-36.

11. Zhang, H., N. Court, and H.J. Forman, Submicromolar concentrations of 4-hydroxynonenal induce glutamate cysteine ligase expression in HBE1 cells. Redox Rep, 2007. **12**(1): p. 101-6.

12. Mahaffey, C.M., et al., Multidrug-resistant protein-3 gene regulation by the transcription factor Nrf2 in human bronchial epithelial and non-small-cell lung carcinoma. Free Radic Biol Med, 2009. **46**(12): p. 1650-7.

13. Shibata, T., et al., Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci U S A, 2008. **105**(36): p. 13568-73.

14. Singh, A., et al., Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. PLoS Med, 2006. **3**(10): p. e420.

15. Ohta, T., et al., Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. Cancer Res, 2008. **68**(5): p. 1303-9.

16. Sun, S.Y., et al., Implication of p53 in growth arrest and apoptosis induced by the synthetic retinoid CD437 in human lung cancer cells. Cancer Res, 1999. **59**(12): p. 2829-33.

17. Hashimoto, T., et al., p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. Cancer Res, 1999. **59**(21): p. 5572-7.

18. Greenblatt, M.S., et al., Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res, 1994. **54**(18): p. 4855-78.

19. Petitjean, A., et al., TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene, 2007. **26**(15): p. 2157-65.

20. Faraonio, R., et al., p53 suppresses the Nrf2-dependent transcription of antioxidant response genes. J Biol Chem, 2006. **281**(52): p. 39776-84.

21. Muredda, M., et al., Cloning and Characterization of the Murine and Rat mrp1 Promoter Regions. Mol Pharmacol, 2003. **64**(5): p. 1259-69.

22. Sampath, J., et al., Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. J Biol Chem, 2001. **276**(42): p. 39359-67.

23. Tsang, W.P., et al., Modulation of multidrug resistance-associated protein 1 (MRP1) by p53 mutant in Saos-2 cells. Cancer Chemother Pharmacol, 2003. **51**(2): p. 161-6.

24. Mahaffey, C.M., et al., Schedule-dependent apoptosis in K-ras mutant non-small-cell lung cancer cell lines treated with docetaxel and erlotinib: rationale for pharmacodynamic separation. Clin Lung Cancer, 2007. **8**(9): p. 548-53.

25. Shibata, T., et al., Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. Gastroenterology, 2008. **135**(4): p. 1358-1368, 1368 e1-4.

26. Zhang, P., et al., Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. Mol Cancer Ther. **9**(2): p. 336-46.

27. Borst, P., et al., The multidrug resistance protein family. Biochim Biophys Acta, 1999. **1461**(2): p. 347-57.

28. Cole, S.P., et al., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science, 1992. **258**(5088): p. 1650-4.

29. Loe, D.W., R.G. Deeley, and S.P. Cole, Biology of the multidrug resistance-associated protein, MRP. Eur J Cancer, 1996. **32A**(6): p. 945-57.

30. Kang, K.W., et al., Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. Mol Pharmacol, 2002. **62**(5): p. 1001-10.

31. Kang, M.I., et al., Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. Proc Natl Acad Sci U S A, 2004. **101**(7): p. 2046-51.

32. Morris, S.M., A role for p53 in the frequency and mechanism of mutation. Mutat Res, 2002. **511**(1): p. 45-62.

33. Gong, X., et al., NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20s proteasomal degradation leading to stabilization and activation of p53. Cancer Res, 2007. **67**(11): p. 5380-8.

34. el-Deiry, W.S., et al., WAF1, a potential mediator of p53 tumor suppression. Cell, 1993. **75**(4): p. 817-25.

35. Chen, W., et al., Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. Mol Cell, 2009. **34**(6): p. 663-73.

36. Levy, S. and H.J. Forman, C-Myc is a Nrf2-interacting protein that negatively regulates phase II genes through their electrophile responsive elements. IUBMB Life. **62**(3): p. 237-46.

37. Mitchell, K.O. and W.S. El-Deiry, Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. Cell Growth Differ, 1999. **10**(4): p. 223-30.

38. Ho, J.S., et al., p53-Dependent transcriptional repression of c-myc is required for G1 cell cycle arrest. Mol Cell Biol, 2005. **25**(17): p. 7423-31.

Tables

Cell Line/Sample	Mutation	Base Change	Amino Acid Change	Domain	Tumor Histology	Smoking Status
A549	Keap1/Homo	G→T	G333C=non-polar to polar	First Kelch Domain	Adenocarcinoma	Unk
PT377	Keap1/Homo	G→T	G333V=non-polar to non-polar	First Kelch Domain	Metastatic	Never
PT146	Keap1/Homo	G→A	D422N=acidic to polar	Third Kelch Domain	Squamous	Current
PT135	Keap1/Homo	G→T	W497C=non-polar to polar	Forth Kelch Domain	Neuroendocrine	Former
PT423	Keap1/Homo	G→T	A548S=non-polar to polar	Fifth Kelch Domain	Metastatic	Former
PT373	Keap1/Hetro	C→T	T598I=polar to non-polar	Sixth Kelch Domain	Adenocarcinoma	Current
PT367	Nrf2/Hetro	G→A	R43Q=basic to polar	Neh2	Adenocarcinoma	Never

Table 1. *Keap1* and *Nrf2* mutational status in cell line and patient samples.

Sample	p53 Mutation
PT377	E298X/E298X
PT373	Deletion-Codons 244-246
PT126	P151S
PT421	D281E
PT140	Deletion-Codons 158-161
PT423	G154V
PT369	C141G
PT135	R248Q
PT297	Insertion-Codon 201
PT130	P190L
PT146	D259Y

Table 2. *p53* mutational status in patient samples.

Figures

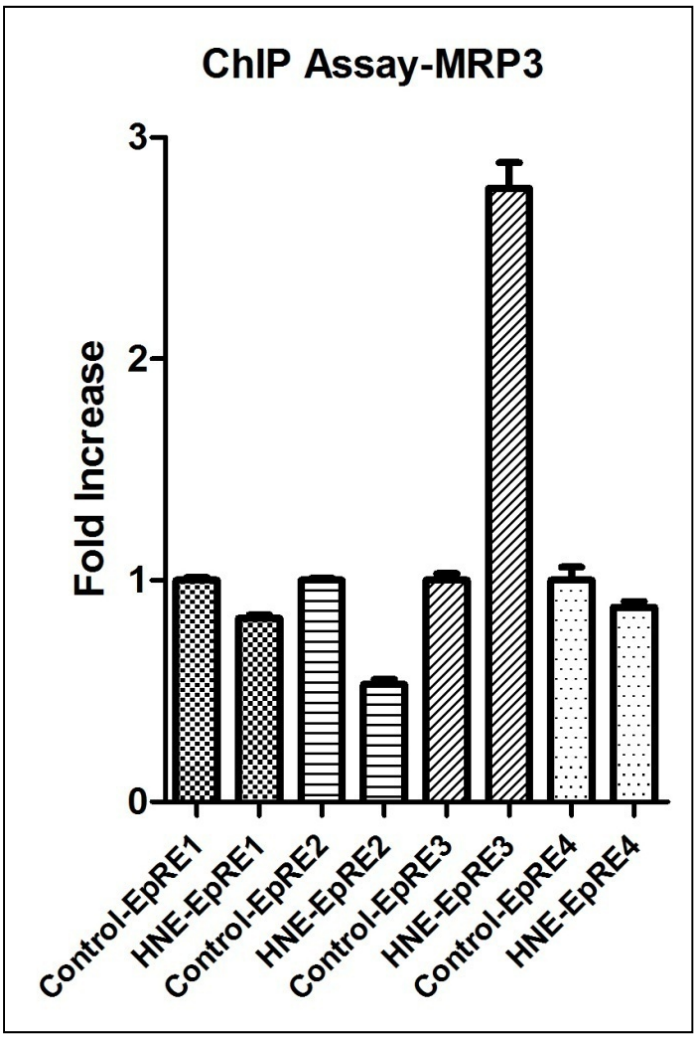

Figure 1. EpRE binding sites are located −434, −628, −805, and −1049 bp. Nrf2 binding was increased ~2.8 fold vs. control at the -805 bp EpRE after 3 h exposure to 15μM HNE.

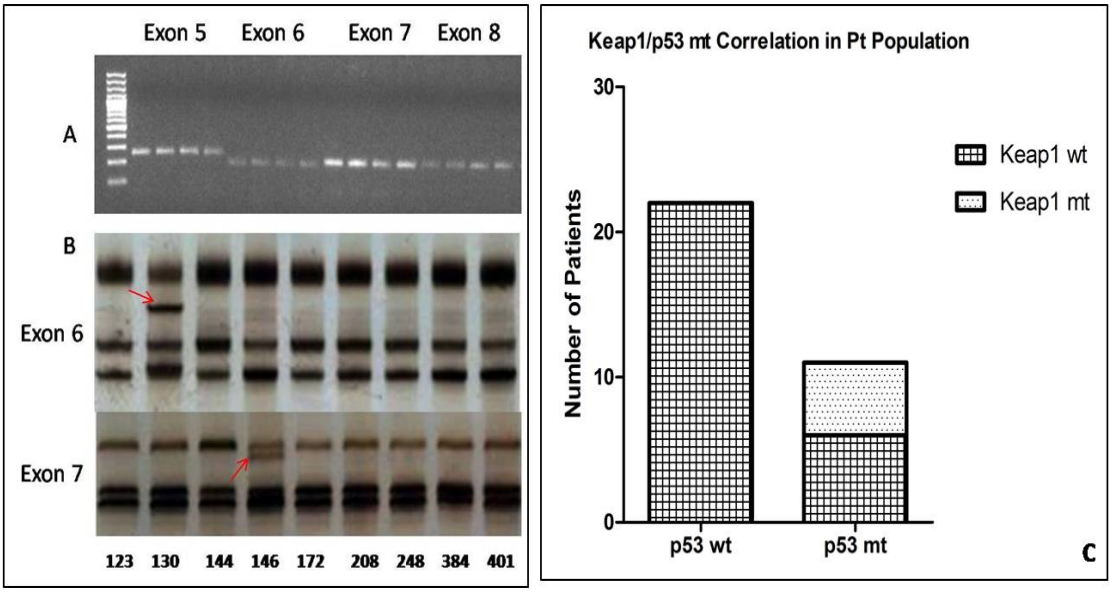

Figure 2. A, PCR amplification of p53. Exons 5-8 were successfully amplified in patient samples. **B,** Single-Strand Conformation Polymorphism (SSCP). Mutations were found in eleven of thirty-three samples, and confirmed by sequencing. **C,** The left column represents the number of *p53* wt patient samples in our cohort (22/33), the right column is the number of *p53 mt* patient samples (11/33). All five *Keap1* mutations occurred in conjunction with a *p53* mutation; Fisher's Exact Test= two-sided P value is 0.0019**.**

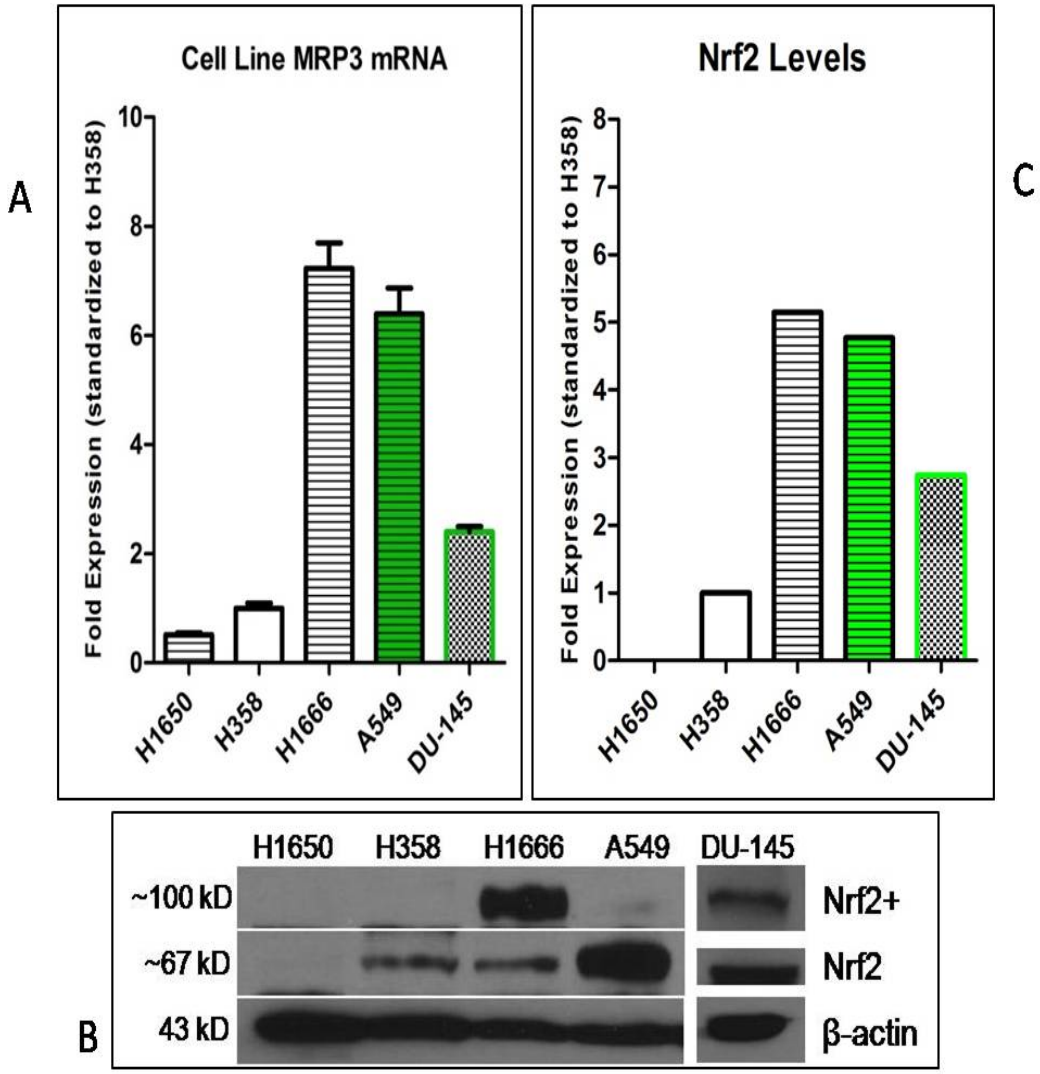

Figure 3. A, Cell lines H1650, H358, H1666, A549, and DU-145. Levels of MRP3 mRNA of our cell line model are compared. **B,** Western blot of standard and ~100 kD Nrf2 in our cell line model**. C,** Scion Image absorbance measurements. Levels of total standard and ~100 kD Nrf2 as a ratio to respective β-actin levels. **Legend**: Horizontal lines= *p53* wt, Grid= *p53* mt, Empty= *p53* null, Green= *Keap1* mt, Green border= splicing errors. Results are expressed as means ± SEM (n= 3).

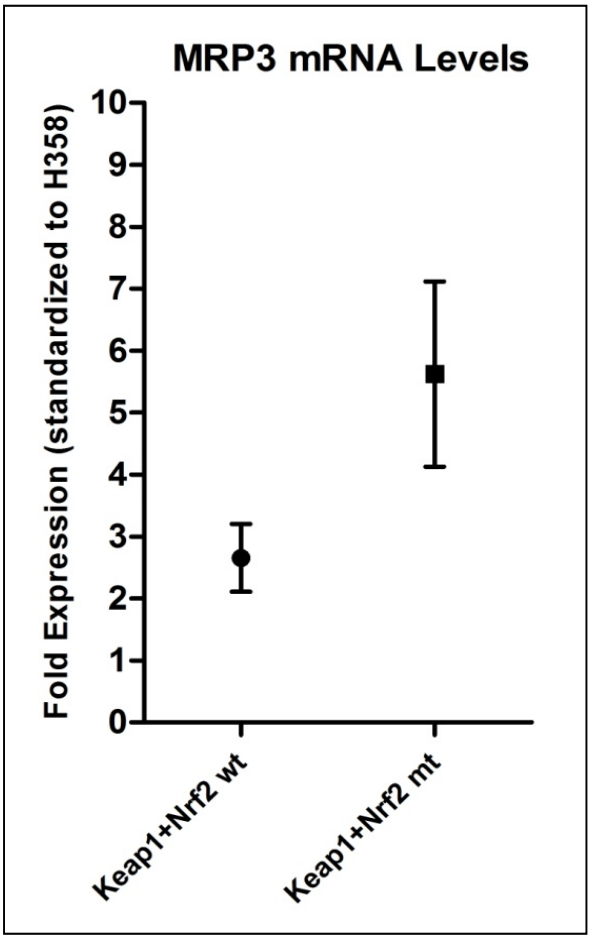

Figure 4. MRP3 mRNA levels were normalized to H358. *Keap1*/*Nrf2* wt (Mean ~2.6 fold) and *Keap1*/*Nrf2* mt (Mean ~5.6 fold) were significantly higher in patient samples ($p<0.05$). Results are expressed as means ± SEM.

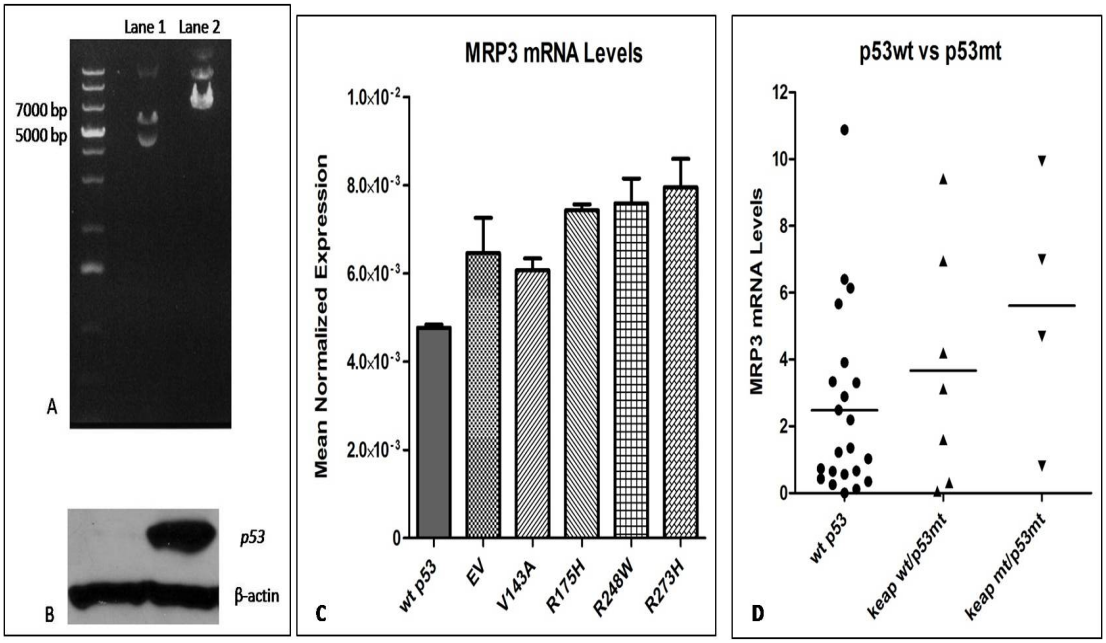

Figure 5. A, Lane 1 contains empty pcDNA3.1- (5427 bp), lane 2 contains pcDNA3.1+ (5428bp) with the wild-type *p53* insert (~1800 bp). **B,** Transient transfection of the *p53* null NSCLC cell line H358 (lane 1) with the pcDNA3.1+ *p53* wt insert (lane 2), and subsequent p53 expression (24 h) was confirmed using western blot analysis. **C,** Cells transfected with wt *p53* had an approximately 33% lower average level of MRP3 mRNA than when compared to the four *p53* mt groups. *p53* wt was significantly lower ($p<0.05$) than the R248W and R273H mutants, results are expressed as means ± SEM (n= 3). **D,** Scatter plot of patient MRP3 mRNA levels, bar represents the mean.

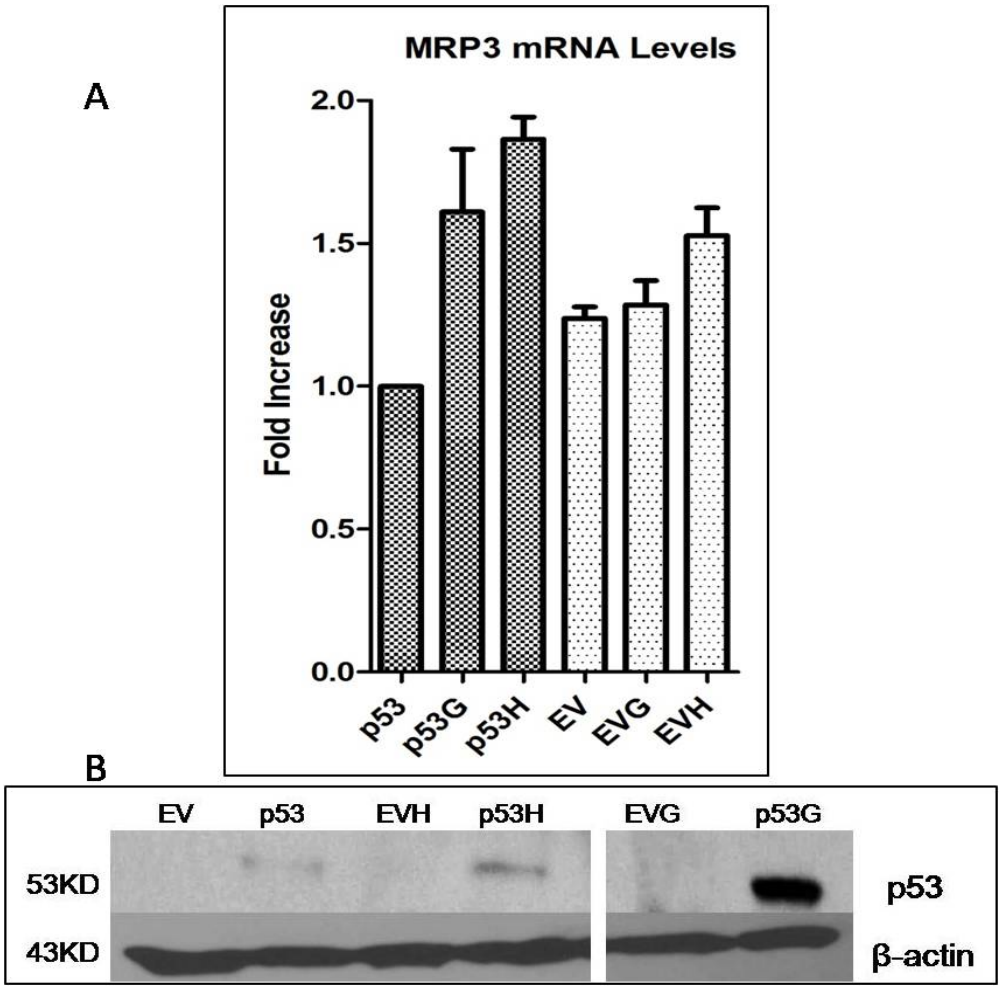

Figure 6. A, Tukey's Multiple Comparison Test demonstrated a significant difference between the *p53* wt vs. *p53* with HNE or the gemcitabine treated groups ($p < 0.05$), while the EV vs. EV treated with HNE or gemcitabine groups were not significantly different. Results are expressed as means ± SEM (n= 3).

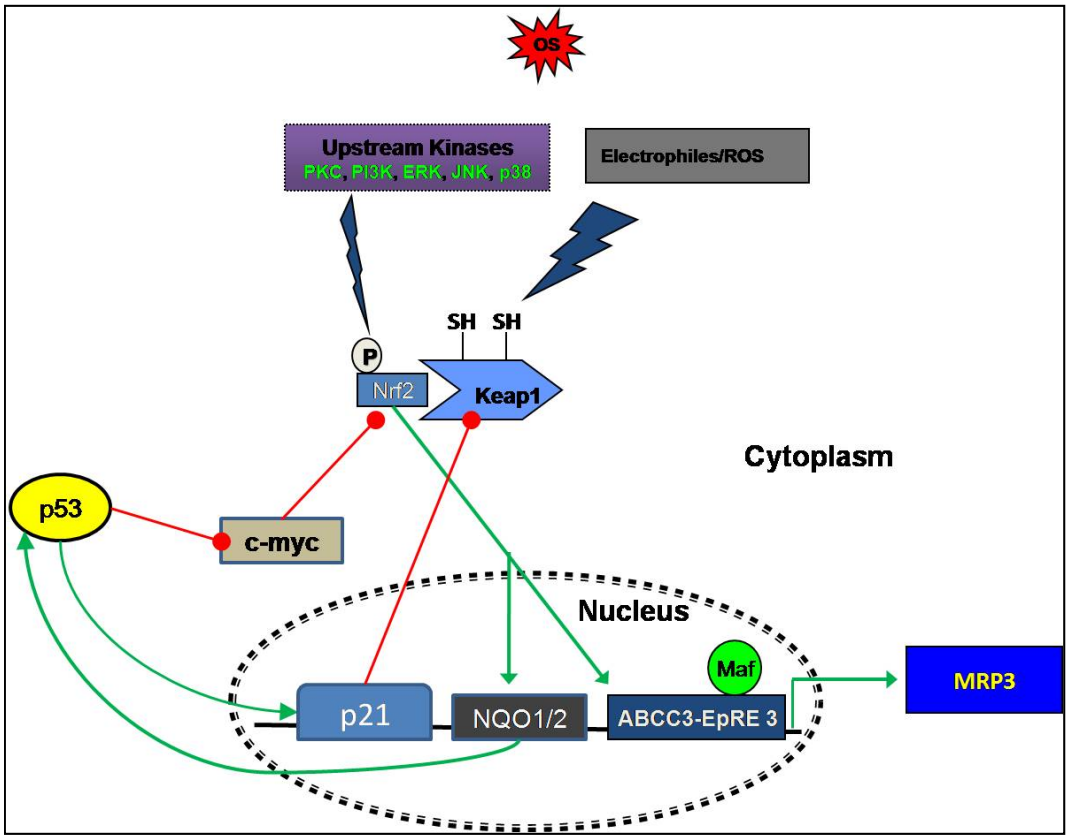

Figure 7. Green lines=activation/stabilization; Red lines=Inhibition

Section II

Chapter B

Keap1 Exon 4 cSNP Associated with Elevated MRP3 Levels in *Keap1* wt NSCLC Cell Lines and Tumor Specimens

Introduction

It is well known that point mutations in the coding regions of genes can disrupt normal protein function by changing the native amino acid. However, what is becoming evident is that translationally silent coding-region mutations can alter normal protein formation by affecting exon splicing sites, resulting in mRNA splicing errors [1]. One such class of sites is known as exonic splicing enhancers (ESEs). ESEs are short oligonucleotide sequences that enhance exon recognition by the splicing machinery [2]. ESEs interact with members of the SR protein family, which bind to ESEs and recruit members of the core splicing machinery [3]. Several diseases have now been associated with splicing errors affecting ESEs, as of 2002 there were at least twenty-three known silent mutations which caused exon skipping [1]. For example, Colapietro *et al*. demonstrated that exon skipping was occurring in the NF1 (neurofibromatosis) gene, in part due to reduced binding efficiency of the SR protein SC35 to an ESEs due to a silent mutation, contributing to the production of a truncated protein [4].

Results

While the presence of a *Keap1* or *Nrf2* mutation was shown to be significantly associated with increased levels of MRP3 mRNA in our patient specimens, we found instances where MRP3 levels were high when wild-type for these genes. During the course of sequencing *Keap1,* a known coding single-nucleotide polymorphism (cSNP) in exon 4 (NCBI; rs1048290), which has a synonymous codon change of CTC→CTG (L471L) was identified in multiple high expressers of MRP3. This is a prevalent cSNP in the human genome with a homozygous frequency ranging from approximately 7% in Europeans, to 60% in Sub-Saharan African populations (HapMap). In our patient cohort, whose ethnic composition is unknown to us, we found a homozygous frequency of approximately 12% (**Figure 1**). Pupasuite 3 (http://pupasuite.bioinfo.cipf.es/), an online database cataloging and characterizing functional properties of SNPs, predicted this cSNP as a potential ESEs site. Members of the SR protein family that were predicted to interact with this site include SC35 and SRp40. Using the ESEs analysis program ESEfinder we found that this cSNP reduce the binding efficiency score of SC35 at this site from 3.31 to 2.41 [5, 6].

Using methods described in the previous chapter of this dissertation we examined *Keap1* exon 4 and found the homozygous cSNP in the NSCLC cell line H1666 (*Keap1* wt) and the prostate cell line DU-145 (*Keap1* wt). Recently, DU-145 was shown to have splicing errors in Keap1 mRNA, low levels of functional Keap1, and high levels of Nrf2 related genes including MRP1 and MRP2 [7]. Both H1666 and DU-145 had high levels of MRP3 when compared to *Keap1*/cSNP wt cell lines (**Figure 2**). When we compared

MRP3 levels of our patient specimens with regard to *Keap1* mt and cSNP status, we found that wild-type and *Keap1*/cSNP heterozygous mutants were not significantly different, and that *Keap1*/cSNP homozygous mutants were significantly higher than either the wt group ($p<0.01$) or the heterozygous mt group ($p<0.05$) (**Figure 3**).

Discussion

Constitutive activation of Nrf2 has been demonstrated to be involved in the upregulation of a number of genes associated with cellular detoxification, including the drug transporter MRP3. Further studies are needed to determine the prevalence of *Keap1* inactivation in the patient population due to transcriptional and posttranscriptional mechanisms such as splicing errors. Future directions of this work could lead to: a) identification and development of tools for screening of tumor profiles that place NSCLC patients at increased risk for chemoresistance and metastasis, making this a prognostic as well as a predictive marker; b) understanding the molecular mechanisms that lead to clinically significant lung cancer could lead to improved therapies. For example, NSCLC patients harboring a loss of either wt *Keap1* or *Nrf2* could have improved benefit from chemotherapy or radiotherapy when given in combination with compounds designed to inhibit the Nrf2 pathway. This research could lead to improved understanding of tumor genetic interactions on NSCLC treatment efficacy, and further, provide the proof-of-principle required for expanded basic, translation and clinical research that will have direct impact on patient care.

References

1. Cartegni, L., S.L. Chew, and A.R. Krainer, Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet, 2002. **3**(4): p. 285-98.
2. Fairbrother, W.G., et al., Single nucleotide polymorphism-based validation of exonic splicing enhancers. PLoS Biol, 2004. **2**(9): p. E268.
3. Graveley, B.R., Sorting out the complexity of SR protein functions. RNA, 2000. **6**(9): p. 1197-211.
4. Colapietro, P., et al., NF1 exon 7 skipping and sequence alterations in exonic splice enhancers (ESEs) in a neurofibromatosis 1 patient. Hum Genet, 2003. **113**(6): p. 551-4.
5. Smith, P.J., et al., An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Hum Mol Genet, 2006. **15**(16): p. 2490-508.
6. Cartegni, L., et al., ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res, 2003. **31**(13): p. 3568-71.
7. Zhang, P., et al., Loss of Kelch-like ECH-associated protein 1 function in prostate cancer cells causes chemoresistance and radioresistance and promotes tumor growth. Mol Cancer Ther. **9**(2): p. 336-46.

Figures

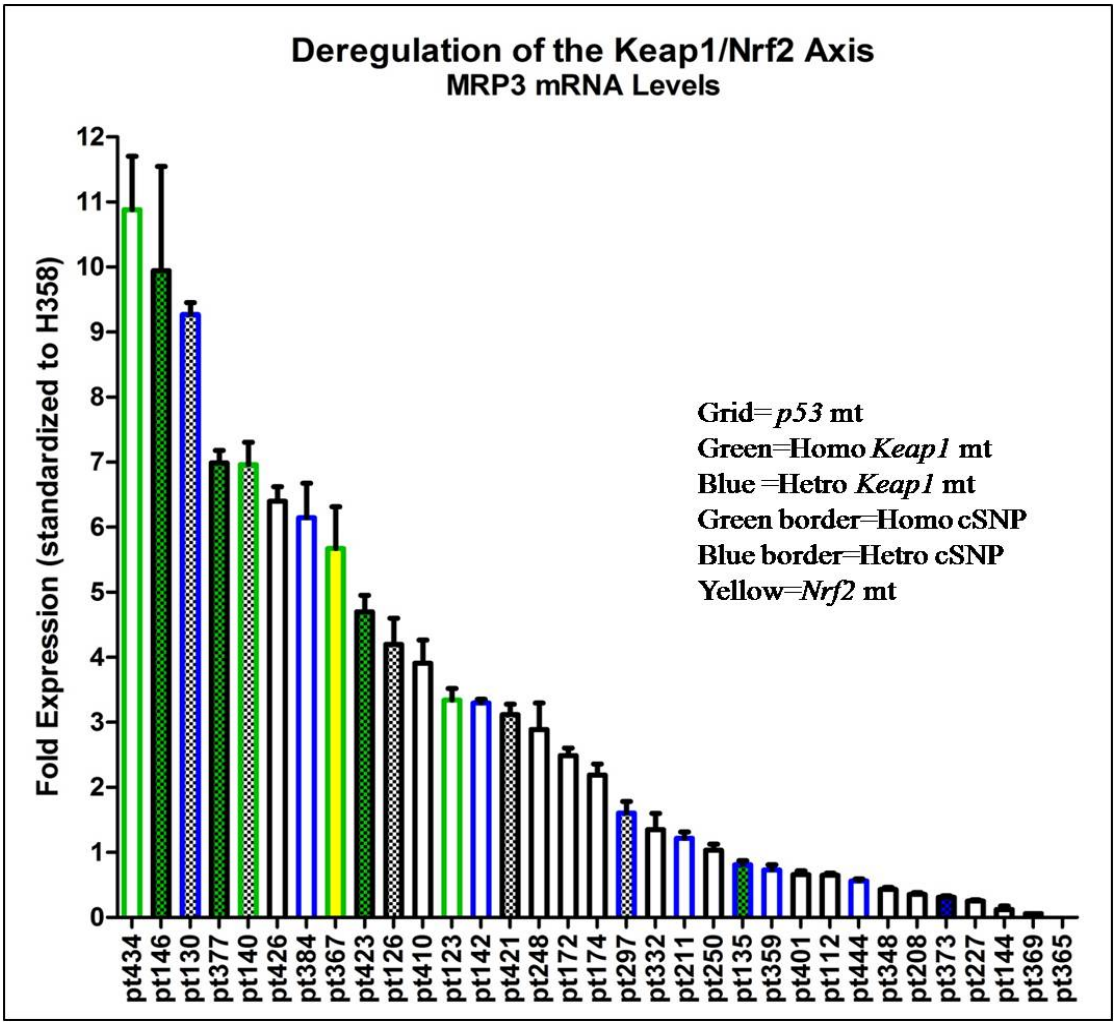

Figure 1. MRP3 mRNA levels of patient specimens.

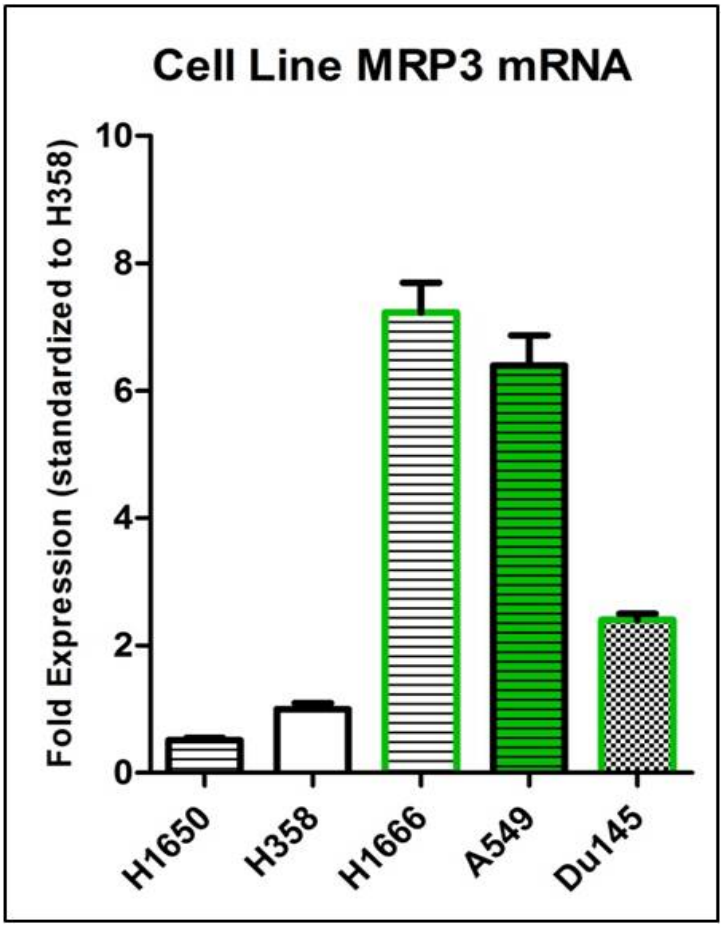

Figure 2. Cell lines H1650, H358, H1666, A549, and DU-145. Levels of MRP3 mRNA of our cell line model are compared. **Legend**: Horizontal lines= *p53* wt, Grid= *p53* mt, Empty= *p53* null, Green= *Keap1* mt, Green border= Homozygous cSNP. Results are expressed as Mean ± SEM (n= 3).

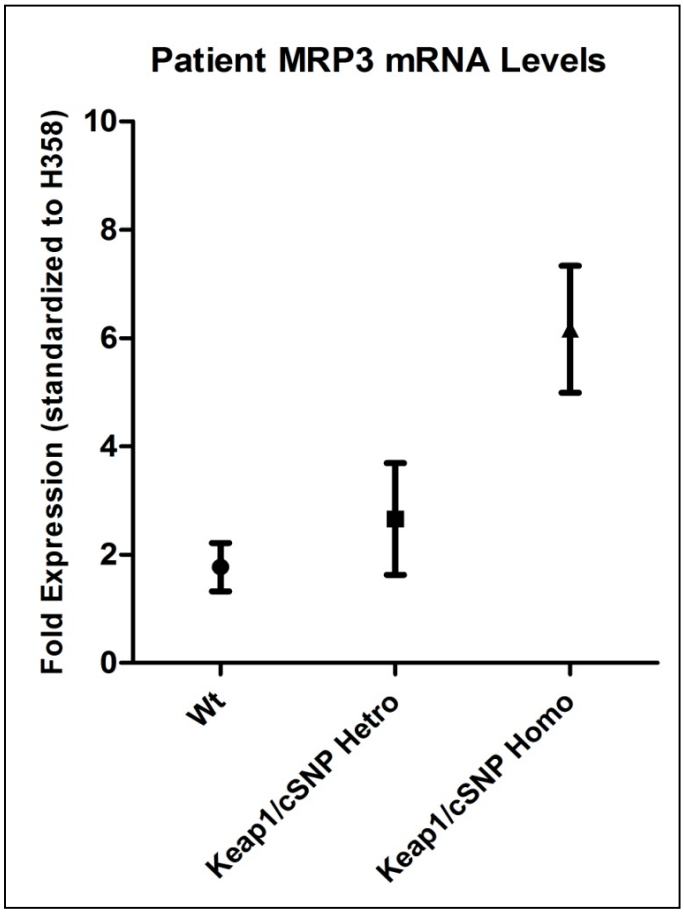

Figure 3. Patient levels were normalized to the *Keap1*/cSNP wt cell line H358. *Keap1*/*cSNP* wt (Mean ~1.8 fold), *Keap1*/*cSNP* heterozygous mt (Mean ~2.7 fold), and *Keap1*/*cSNP* homozygous mt (Mean ~6.2 fold). Homozygous mt patient samples were significantly higher than wild-type patient samples ($p<0.01$), and heterozygous mt patient samples for MRP3 mRNA levels ($p<0.05$). Results are expressed as Mean ± SEM.