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Genetic Evidence for XPC-KRAS Interactions During Lung Cancer Development

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Abstract

Lung cancer causes more deaths than breast, colorectal and prostate cancers combined. Despite major advances in targeted therapy in a subset of lung adenocarcinomas, the overall 5-year survival rate for lung cancer worldwide has not significantly changed for the last few decades. DNA repair deficiency is known to contribute to lung cancer development. In fact, human polymorphisms in DNA repair genes such as xeroderma pigmentosum group C (*XPC*) are highly associated with lung cancer incidence. However, the direct genetic evidence for the role of XPC for lung cancer development is still lacking. Mutations of the Kirsten rat sarcoma viral oncogene homolog (*Kras*) or its downstream effector genes occur in almost all lung cancer cells, and there are a number of mouse models for lung cancer using these mutations. Using activated *Kras*, $Kras^{LA1}$, as a driver for lung cancer development in mice, we showed for the first time that mice with $Kras^{LA1}$ and *Xpc* knockout had worst outcomes in lung cancer development, and this phenotype was associated with accumulated DNA damage. Using cultured cells, we demonstrated that induced expression of oncogenic $KRAS^{G12V}$ led to increased levels of reactive oxygen species (ROS) as well as DNA damage, and both can be suppressed by anti-oxidants. Thus, it appears that XPC may help repair DNA damage caused by KRAS-mediated production of ROS.

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Keywords

lung cancer; XPC; Kras; ROS

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, claiming more than 1.5 million lives every year (which exceeds the combined mortality from breast, prostate and colorectal cancers) (Siegel et al., 2015). Lung cancer is understood to be a largely environmental disease, with cigarette smoking as a major (but not the only) risk factor. Patients with advanced stage of lung cancer, which represents 75% of all new cases, have a median survival time of only 10 months. Thus, understanding the molecular basis of lung cancer has been a major focus in cancer research.

There are a number of genetic alterations in lung cancer, and specific gene mutations are associated with a given subset of tumors (Cooper et al., 2013). For example, mutations of RAS or its upstream/downstream effectors occur almost in every lung cancer cell, and expression of activated KRAS in mice has been a robust model for lung cancer development, particularly non-small cell lung cancer (NSCLC). Furthermore, targeted therapy towards growth factor receptor gene mutations in NSCLC has significantly improved the quality of life in a subset of lung cancer patients (Robert et al., 2015; Thomas et al., 2015; Thress et al., 2015; Tricker et al., 2015; Ugurel et al., 2015; Weber et al., 2015; Yang et al., 2015a, 2015b). In addition, gene mutations in the p53 and RB/p16 pathways are common in lung cancer (Cooper et al., 2013).

Amounting evidence indicate that decreased expression of DNA repair protein XPC, which is responsible for global nucleotide excision repair (NER), is correlated with poor outcomes of lung cancer patients (Wu et al., 2007a). *Xpc* deficient mice also develop several types of cancer, such as lung, liver and skin cancers, after exposure to carcinogens or UV irradiation (Cheo et al., 1996; Berg et al., 1998; Cheo et al., 2000; Friedberg et al., 2000; Hollander et al., 2005). Because most of these mice developed tumors after 15 months, it has been suggested that XPC's effects in tumor development were through regulation of NER system. In human population studies, *Xpc* Lys939Gln polymorphism is highly associated with development of lung cancer (Hu et al., 2005; Lee et al., 2005; Vogel et al., 2005; Matakidou et al., 2006; Jin et al., 2014). Despite the significance of XPC in lung cancer, there is still no direct genetic evidence to demonstrate the significance of XPC for lung cancer development. XPC was originally regarded as nucleotide excision repair molecule, but recent studies have shown that NER factors including XPC contribute to the repair of oxidative DNA lesions including 8-oxo-7,8-dihydroguanine (Menoni et al., 2012; Parlanti et al., 2012). It is not known how XPC is involved in cancer development.

In this study, we use mutant *Kras*-driven lung cancer model to test the effect of XPC deficiency on DNA damage, tumor development and mouse survival in mice. We also tried to identify the underlying molecular mechanisms using inducible expression of activated RAS in cultured cells.

RESULTS

XPC deficiency promotes KRAS-mediated tumor development

To understand the role of XPC in lung cancer development, we examined *Kras*-mediated tumor development in mice with or without *Xpc* expression. In $Kras^{LA1}$ mice, we found all mice developed tumor in the lung, which is similar to a previous report (Johnson et al., 2001). The phenotype became more severe when *Xpc* gene was knocked out (Fig. 1 and 2). Based on the survival time, we found that $Kras^{LA1}$/ $Xpc^{-/-}$mice all died whereas less than 40% of $Xpc^{+/+}$/$Kras^{LA1}$ mice died at 40 weeks. As expected, $Xpc^{-/-}$mice without $Kras^{LA1}$ and the control mice had no mortality within the same period of time (Fig. 1).

Further examination indicated that all dead mice had lung cancer and aberrant growth in intestine and skin epithelium. For lung cancer, we observed different stages of tumor, including adenomas, adenocarcinomas as well as mixed tumor types (Fig. 2). The tumors were mostly adenocarcinomas in 2-month-old $Xpc^{-/-}$/$Kras^{LA1}$ mice (Fig. 2D) whereas the same aged mice with $Kras^{LA1}$ had mostly adenomas (Fig. 2C). For $Xpc^{-/-}$mice, we did not observe any tumors even after one year, which is consistent with a previous report that $Xpc^{-/-}$mice only develop spontaneous lung tumors at an old age (> 15 months) after exposure to carcinogens (Cheo et al., 1997, 2000; Hollander et al., 2005). These data suggest that unrepaired oncogenic gene mutations resulting from the defective DNA repair system in $Xpc^{-/-}$ mice may be responsible for tumor development. Based on BLISS independence analysis, we observed a more than additive effect (synergy) between *Xpc* loss and oncogenic *Kras* gene expression in development in Aberrant crypt foci (ACF; see Fig. S1 for a typical ACF image). We found that $Xpc^{-/-}Kras^{LA1}$ mice, on average, had >15 ACF whereas $Kras^{LA1}$mice had only 5 ACF on average (Fig. 3). No ACF lesions were found in $Xpc^{-/-}$or the control mice within the same period of time. Taken together, these data indicate that *Xpc* deficiency accelerates $Kras^{LA1}$-mediated tumor development.

Effects of *Xpc* on production of ROS and genomic DNA damage

Next we tried to determine the molecular basis by which XPC deficiency promotes *Kras*-mediated tumor development. COMET assay is a reliable way to measure DNA damage (Dusinska and Collins, 2008). Using COMET assay, we found that *Xpc* loss results in elevated levels of DNA damage, including single and double strand breaks in both the tumor-prone lung tissue (Fig. 4) and lymphocytes (data not shown). We further confirmed elevated frequency of *Hprt* gene mutation in cells with XPC knockout cells, suggesting that an increased DNA damage is associated with an elevated gene mutation rate (Fig. S2). The effect of *Xpc* loss on DNA damages is consistent with the role of XPC in DNA repair in previous studies (Friedberg et al., 2000).

To determine the effect of *Kras* expression on DNA damage in lung tissues, we performed COMET assay, and found that $Kras^{LA1}$ positive mice had an elevated level of DNA damage in the lung tissue (Fig. 4). More interestingly, $Xpc^{-/-}Kras^{LA1}$ mice had much more damaged DNAs in the lung tissues than either $Xpc^{-/-}$or $Kras^{LA1}$ mice (Fig. 4). Based on BLISS analysis (Gu et al., 2013), we found that the DNA damage in the $Xpc^{-/-}Kras^{LA1}$ mice were greater than the additive value of DNA damage from both $Xpc^{-/-}$mice and $Kras^{LA1}$ mice.

These results suggest that XPC deficiency synergizes with $Kras^{LA1}$ in inducing DNA damage, possibly through loss of DNA repair regulation in lung cancer development.

Induced expression of activated RAS increases ROS and DNA damage

The DNA damage detected in our studies likely came from internal insults within the cell because no DNA damage was observed in wild type tissues (Fig. 4). Previous studies from other groups have shown that oncogenic KRAS increases the level of ROS that can lead to DNA damage (Weinberg et al., 2010). Production of 8-deoxogunine and 4-hydroxynonenal (4-HNE) are important markers for oxidative stress and generation of ROS (Yang et al., 2003; Logan et al., 2014; Zhong and Yin, 2015). We examined the level of 8-deoxogunine and 4-HNE in lung tissues in all four groups of mice (wild type, $Xpc^{-/-}$, $Kras^{LA1}$, and $Xpc^{-/-}Kras^{LA1}$), and found that $Xpc^{-/-}Kras^{LA1}$ mice had the highest level of 8-deoxogunine and 4-HNE (Fig. 5). The close association of ROS with DNA damage supports our hypothesis that elevated production of ROS may be responsible for $Kras^{LA1}$-mediated DNA damage during lung cancer development.

To determine whether induced expression of oncogenic RAS can induce ROS, we used lung epithelial BEAS-2B1 cells with inducible expression of $KRAS^{G12V}$. Following induction of KRASG12V, cells were loaded with H_2DCF-DA to assess changes in the ROS level. Fig. 6A show that ROS levels were increased by 100% at 8 hours and by 200% by 24 hours after $KRAS^{G12V}$ induction. When anti-oxidant N-acetyl cysteine (NAC, 10 mmol/L) was added, no elevation of H2DCF-DA fluorescence was detected (Fig. 6A), confirming that induced expression of RAS^{G12V} did induce ROS production. We further detected DNA damage in these cells by the COMET assay, and found that DNA damage was significantly elevated following $KRAS^{G12V}$ expression. DNA damage was observed after induction of $KRAS^{G12V}$, suggesting that ROS production occurred before DNA damage occurrence (Fig. 6B). Confirming this mechanism, we found that NAC significantly reduced the level of DNA damage in $KRAS^{G12V}$-expressing cells. These results suggest that activated KRAS is sufficient to induce the cellular ROS level and subsequently to increase genomic DNA damage.

Taken all the data together, we have provided genetic evidence to support the critical role of XPC in maintenance of genomic stability in oncogenic *Kras*-expressing lung tissues. Our data demonstrate a synergy between *Xpc* loss and oncogenic *Kras* expression in promoting lung tumor development. We have evidence to suggest that these two genes act through regulation of ROS-mediated DNA damage.

DISCUSSION

Our studies have provided genetic evidence for XPC-KRAS interactions in the development of lung cancer. In this report, we have evidence to indicate that activated KRAS can stimulate the production of ROS, leading to an increase in DNA damage. Our genetic study supports that *Xpc* loss increases oxidatively damaged DNA and promotes KRAS-mediated lung cancer development. In our studies using cultured cells, we demonstrated that activated RAS increases DNA damage levels *via* ROS production within the cancer cell because antioxidant effective in decreasing both ROS and DNA damage (Fig. 6). Based on these

data, we propose that in addition to downstream signaling such as phosphoinositide-3-kinase (PI3K), protein kinase B (a serine/threonine-specific protein kinase; AKT), and mitogen-activated protein kinase (MAPK), activated KRAS has significant effects on ROS production and DNA damage changed in lung cancer. Based on this model, we predict that strategies to enhance XPC functions may be effective in prevention of KRAS-mediated lung cancer development, and enhancing XPC functions may also help cancer treatment.

It is known that expression of XPC can be suppressed by p53 through transcriptional regulation of the XPC promoter (Adimoolam and Ford, 2002; Sengupta and Harris, 2005; Wu et al., 2007b; Hastak et al., 2012). In addition, the mutual interactions between XPC and p53 have been reported (Krzeszinski et al., 2014). Based on these data, we predict a role of XPC in p53-associated tumors, such as head and neck cancers and lung squamous cell carcinomas. Whether XPC knockout indeed plays a critical role in other cancer models await further experimental evidence.

It is not clear how XPC suppression promotes KRAS-driven tumor development. It is known that XPC is an important DNA repair gene for nucleotide excision repair pathway (NER), particularly global genomic DNA repair (Friedberg et al., 2000). Our results suggest that it is likely that the DNA damage in KRAS-mediated tumors were caused by ROS production. Oxidatively damaged DNA is primarily repaired by base excision repair processes (BER). How does XPC deficiency affect ROS-mediated DNA repair? In the last few years, increasing evidence indicate that XPC may be involved in repairing of oxidative DNA damages, either through NER or BER (Melis et al., 2011). NER is known to recognize and repair bulky DNA adducts and helix-distorting structures. It is known that several oxidative DNA lesions have structures similar to helix-distorting structures. It is thus feasible to speculate that NER DNA repair pathway can also repair oxidative DNA damages. Based on the fact that xeroderma pigmentosum group A (XPA) null mice do not have the similar phenotypes as the XPC null mice (Melis et al., 2008), XPC may have functions outside of the NER pathway. Indeed, XPC-hHR23B complex is shown to function as a co-factor for BER machinery to repair 8-hydroxyguanosine-related lesions (D'Errico et al., 2006; Melis et al., 2013). The exact molecular links between XPC and the BER machinery remains to be established.

At present, it is still not clear how activated RAS induces ROS production. A previous study indicates that activated RAS alters mitochondrial metabolism by supporting the pentose phosphate pathway and production of glycolytic ATP and ROS induction (Weinberg et al., 2010). Other studies indicate that ROS production is dependent on NADPH Oxidase 1/4 and Ras-Related C3 Botulinum Toxin Substrate 1 (RAC1) (Trachootham et al., 2009; Lu et al., 2012; Ogrunc et al., 2014; Park et al., 2014; Wang et al., 2015). It is still not very clear whether ROS production in *Kras*^{LA1}-expressing cells occurs all in mitochondria. In addition to DNA damage, ROS is also known to induce cell proliferation through activation of several cell proliferation pathways, such as MAP extracellular signal-regulated kinases (ERK) (Melis et al., 2011). The exact role of *Kras*^{LA1} in ROS production, DNA damage and the consequent tumor development remains to be delineated.

MATERIALS AND METHODS

Animal Studies

All animal studies were approved by the Institutional Animal Care and Use Committees in University of Texas Medical Branch (Galveston, TX) and Indiana University (Indianapolis, IN). *Kras*^{LA1} mice were generously provided by the Emice Program in National Cancer Institute (Jackson et al., 2001), and *Xpc*$^{-/-}$C57BL/6 mice were generously provided by Dr. Errol Friedberg (Cheo et al., 1997). To obtain *Xpc*$^{-/-}$*Kras*^{LA1} mice, *Xpc*$^{-/-}$mice were first mated with *Kras*^{LA1} mice, and resulting *Xpc*$^{+/-}$*Kras*^{LA1} mice mated each other to generate mice with the following four genotypes: wild type, *Xpc*$^{-/-}$, *Kras*^{LA1}, and *Xpc*$^{-/-}$*Kras*^{LA1}. Mice were provided with normal food and water, and a 12 h light–dark cycle. All genotyping of mice was performed by PCR with specific primers using previously reported procedures (Fan et al., 2014). For survival analysis, 8 mice each from wild type, *Xpc*$^{-/-}$, and *Kras*^{LA1}, and 13 mice from *Xpc*$^{-/-}$*Kras*$^{\mathrm{LA1}}$ were used to monitor animal viability for 60 weeks. The low gastrointestinal part of mouse were stained with 0.1% methylene blue and counted under a dissecting microscope (40×). Aberrant crypt foci (ACF) were identified by elevated appearance from the surrounding mucosa and categorized as small (1–3 crypts per focus) and large ACF (4 or more crypts per focus). Total number of ACF was counted as the sum of the small and large number of ACF per group. Five mice were included in each group for ACF study.

Histology and Immunohistostaining

Histology was performed according to a previously published procedure (Fan et al., 2014) after paraffin embedding and hematoxylin & eosin staining. 4-HNE antibodies (Cat# HNE-13-M) were purchased from Alpha Diagnostic International Inc., and anti-8-oxo-dG (Clone 2E2) antibodies were purchased from Trevigen (Gaithersburg, MD, USA). Tissues were processed according to the procedures provided by the vendor. The primary antibodies were diluted 1:200 in 10% fetal bovine serum (FBS)/PBS at 4°C overnight for immunofluorescent staining. Before antibody staining, tissue sections were processed and blocked with 10% FBS for 3 hours to prevent non-specific staining. The secondary antibodies (goat anti-mouse IgG proteins labeled with Alexa Fluor 488 and 596 from Molecular probes) were incubated for 1h at room temperature (1:300 dilution in 10% FBS/PBS), and the nucleus was stained with 4′,6-diamidino-2-phenylindole (2 μg/mL) before being viewed under microscope.

The Single Cell Gel Electrophoresis assay

The Single Cell Gel Electrophoresis assay (SCGE, also known as COMET assay) was performed using the Trevigen™ COMET assay kit (Trevigen, Gaithersburg, MD). Changes in DNA strand breaks in the freshly harvested tissues or cells were determined using neutral (double-strand breaks, DSBs) and alkaline (SSBs) electrophoretic conditions (Wickliffe et al., 2003). DNA was stained with SYBER Green and >200 cells were analyzed for each data point, using the COMET Assay IV v4.2 system (Perceptive Instruments, Suffolk, UK). Tail moment (TM) of exfoliated lung epithelial cells or lymphocytes from each group (n = 5 for experimental groups and n = 3 for the control group) was quantitatively measured for evidence of DNA damage.

Hprt gene mutation analysis

The mutation analysis was performed according to a previous procedure using the *Hprt* cloning assay as described in Meng et al. (Meng et al., 1998). In brief, same number of cells from each group was grown in the presence of increasing concentrations of 6-thioguanine for 7–10 days. The number of surviving colonies was counted to score the mutation efficiency.

Cell culture

Lung epithelial BEAS-2B1 cells were generously provided by Dr. Cutis Harris (Reddel et al., 1993), and were cultured in DMEM with 10% fetal bovine serum (FBS). Cells with inducible expression of KRAS were generated with LacSwitch eukaryotic expression system (Strategene, La Jolla, CA), and maintained in 150 μg/mL hydromycin B. Isopropyl-1-thio-b-D-galactopyranoside (IPTG, Life Technologies, Inc.) at 5 mmol/L was used to induce $KRAS^{G12V}$ expression in the cells. N-acetyl cysteine (NAC) at 5 or 10 mmol/L was used to suppress the production of reactive oxygen species (ROS).

Cellular ROS analysis

2′–7′-dihydro-dichlorofluorescein diacetate (H_2DCF-DA, Invitrogen Co., Carlsbad, CA, USA) was used to determine cellular ROS levels according to previous publications (Boldogh et al., 2003a, 2003b, 2005). In short, cells on microscope cover slips were placed in a thermo- chamber and loaded with 10 μmol/L (final concentration) H_2DCF-DA for 15 min. After induction of KRAS expression for 8, 24 and 28 hours, cells were washed with medium pre-warmed to 37°C and fluorescent images captured using a Photometrix CoolSNAP Fx digital camera mounted on a NIKON Eclipse TE 200 UV microscope. Fluorescence intensities of > 200 cells were determined by Metamorph™ software (Universal Imaging Corporation). To confirm the results in cultured cells, cells were grown to 70% confluence in 24 well plates and loaded with 50 μmol/L H_2DCF-DA at 37°C for 30 minutes. DCF fluorescence was recorded in an FLx800 (Bio-Tek Instruments Inc., Winooski, VT, USA) microplate reader (with 485 nm excitation and 528 nm emission).

Statistical analyses

Kaplan-Meier curves were generated using the SPSS program. Hprt MFs were analyzed by univariate ANOVA followed by post hoc mean comparisons (Bonferroni-corrected), using the SPSS program (SPSS, Chicago, IL). A P value < 0.05 was used to determine statistical significance. Comparison of two groups was done using Student's t test (two-tail analysis), with a P value < 0.05 as statistically significant. BLISS independence analysis was performed to evaluate the synergistic effect between *Xpc* loss and $Kras^{LA1}$ expression in DNA damage in lung tissues using a previously reported method (Berenbaum, 1978; Gu et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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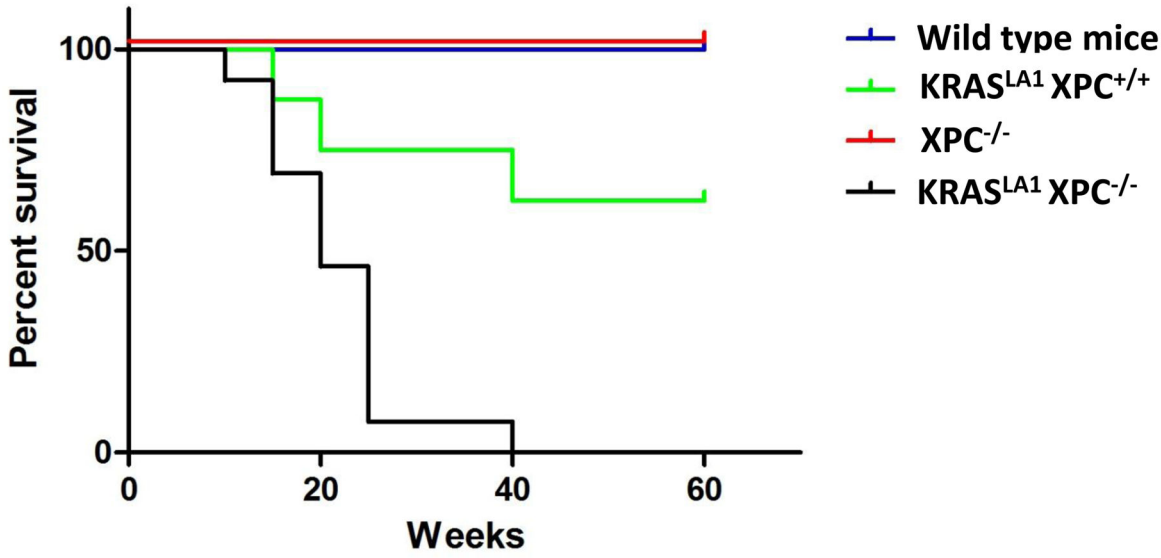

Fig. 1. Kaplan-Meier curves of different groups of mice

Mice from four groups (8 mice each for the control, *Xpc*$^{-/-}$, *Kras*^{LA1} groups and 13 mice for *Xpc*$^{-/-}$*Kras*^{LA1} group) were monitored for 60 weeks, and their surviving time was recorded. The data were analyzed using Kaplan-Meier analysis.

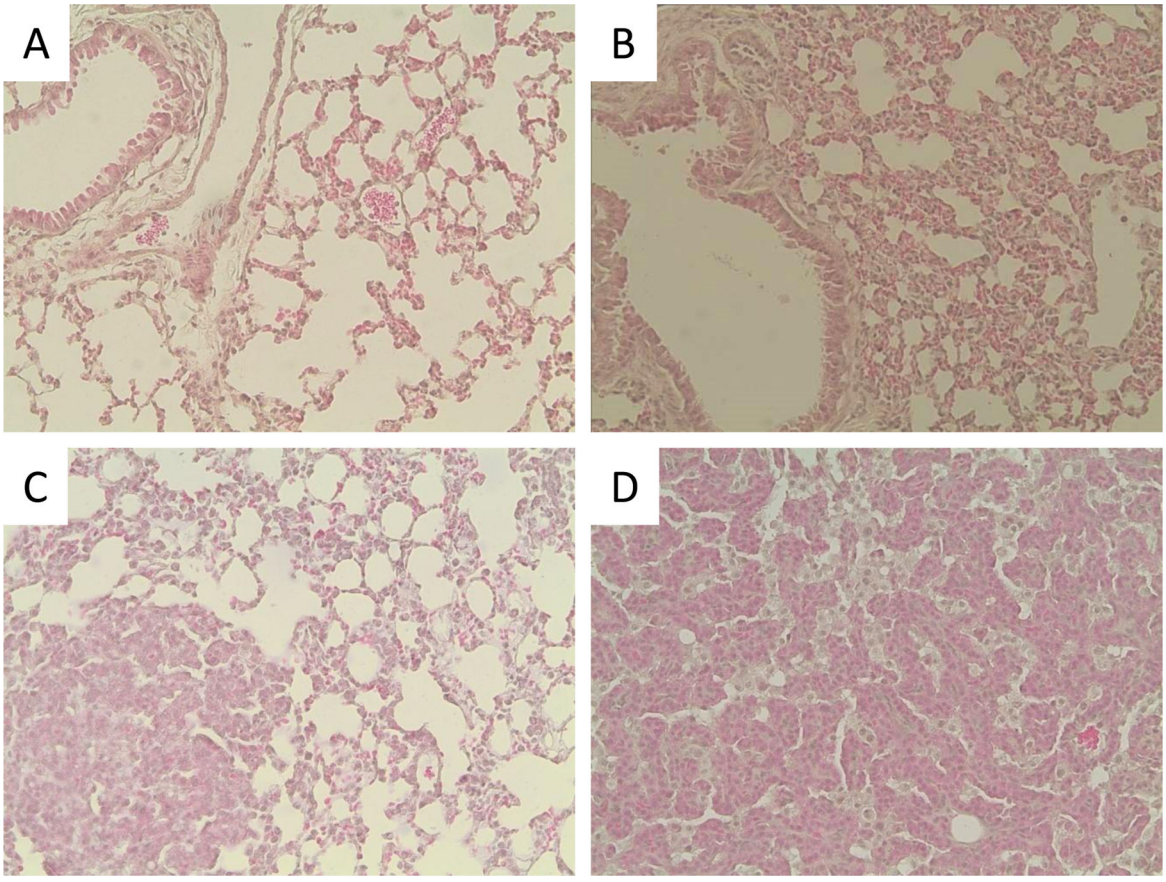

Fig. 2. Lung tissue morphology in different groups of mice

Lungs were sectioned and processed for Hematoxylin and eosin staining and photographs were taken at 100× magnification. **A** shows a normal lung morphology from the control group; **B** shows lung morphology from *Xpc* knockout mice; **C** shows adenoma morphology from *Kras*^{LA1} mice; and **D** shows adenocarcinoma morphology from *Xpc*$^{-/-}$*Kras*^{LA1} mice.

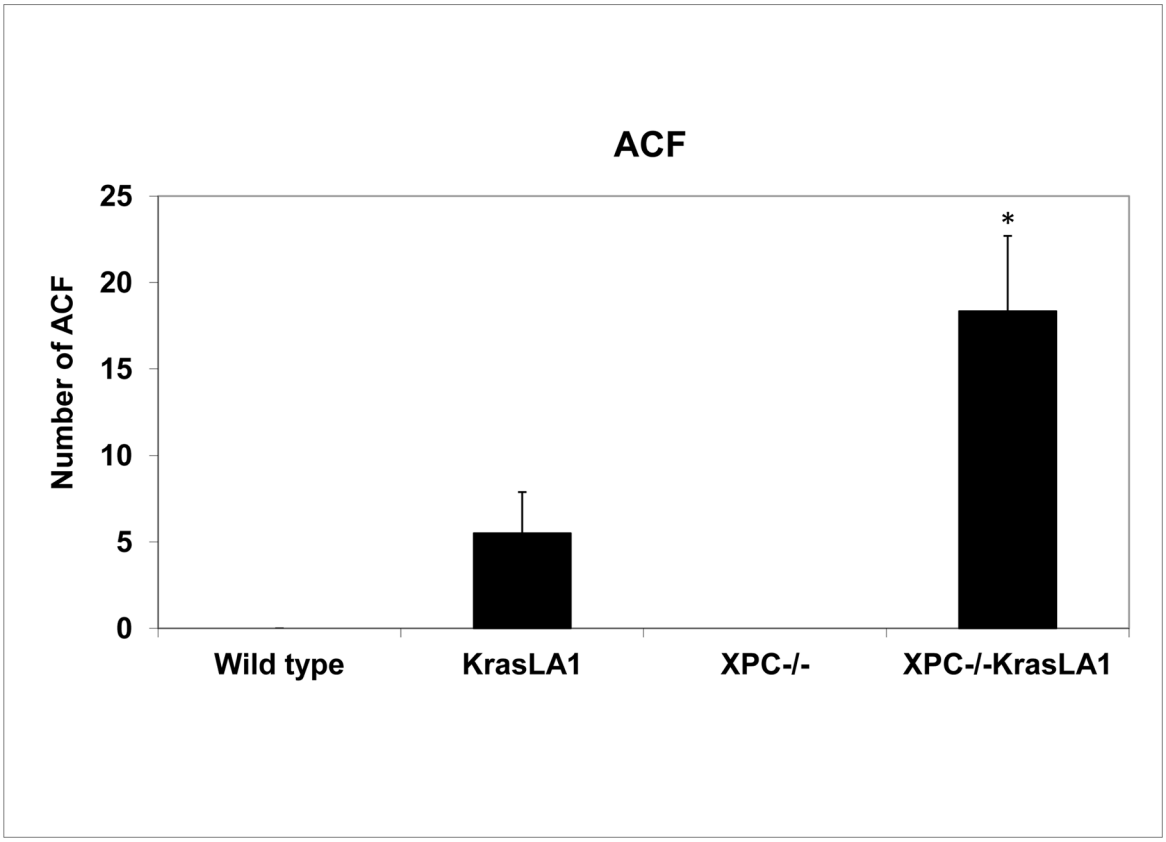

Fig. 3. Development of ACF in mice with different genetic background

The number of aberrant crypt foci (ACF) was visualized after special staining (see methods for details). No ACFs were observed in $Xpc^{-/-}$and the normal control mice, while $Xpc^{-/-}Kras^{LA1}$ mice had a significantly higher number of ACF than those from $Xpc^{+/+}Kras^{LA1}$ mice (P value < 0.05).

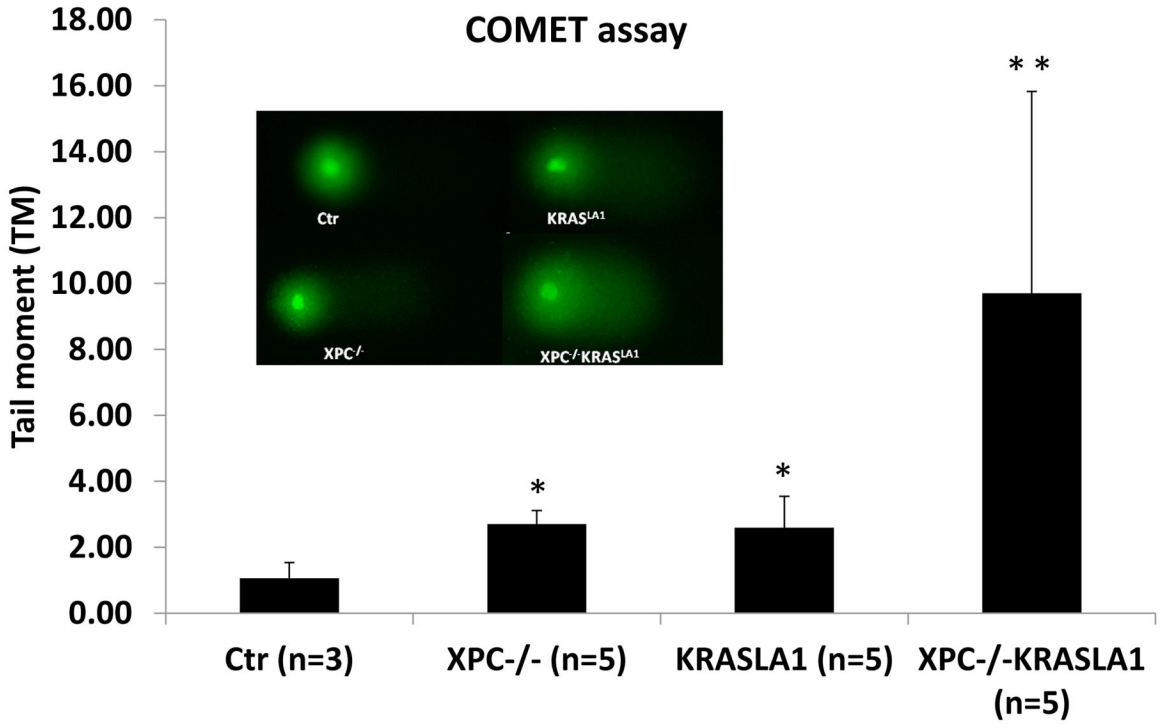

Fig. 4. DNA damage analysis in lung tissues from 2-months old mice with different genetic alterations

A: Fresh lung tissues from the control, *Xpc*$^{-/-}$, *Kras*^{LA1} and *Xpc*$^{-/-}$*Kras*^{LA1} mice were processed to measure DNA damage using the COMET assay. Bronchial epithelial cells were isolated and subjected to COMET assay. For each sample, 200 independent cells were evaluated. The difference between the control mice and the other three groups (*Xpc*$^{-/-}$, *Kras*^{LA1} or *Xpc*$^{-/-}$*Kras*^{LA1}) were significant (with a *P* value <0.05; as indicated by *). Data from the *Xpc*$^{-/-}$*Kras*^{LA1} mice were significantly higher than mice with a single gene mutation (*Xpc*$^{-/-}$or *Kras*^{LA1}) (with a *P* value < 0.05, indicated by **) or the sum from two single mutant mice. According to BLISS independence analysis, *Xpc* loss and *Kras*^{LA1} expression had a more than additive effect (synergy) on induction of DNA damage.

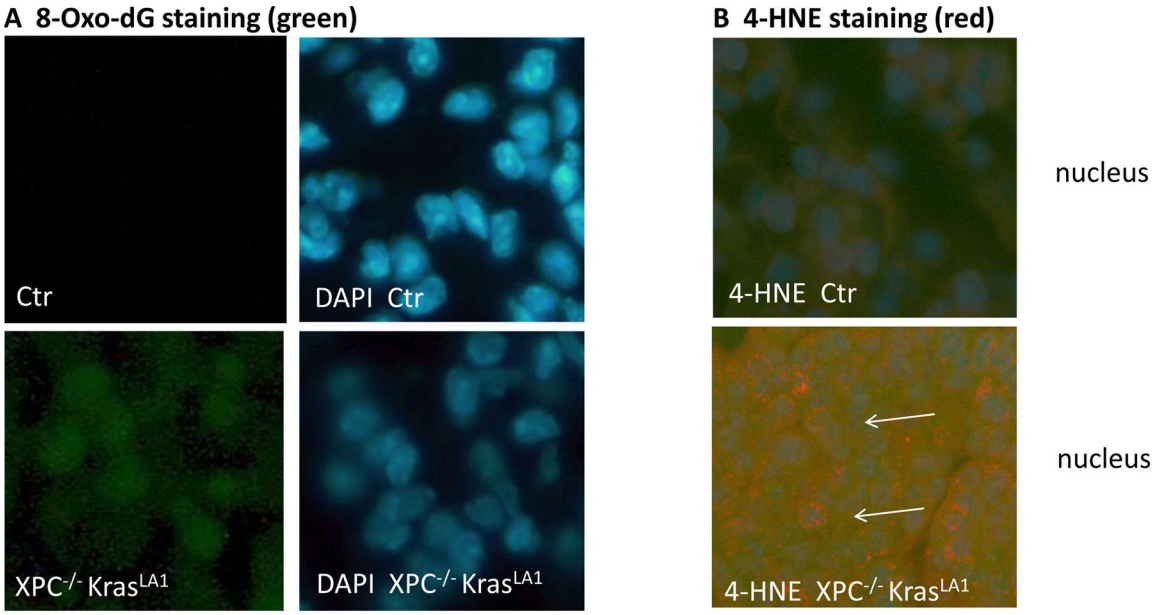

Fig. 5. Detection of relative ROS in lung tissues using the amount of 4-HNE and 8-deoxogunine as markers

Specific antibodies to 8-deoxogunine and 4-HNE were used to detect the relative level of ROS in lung tissues by immunofluorescent staining (**A** shows 8-deoxogunine, and **B** shows 4-HNE). Lung tissues from the $Xpc^{-/-}Kras^{LA1}$ mice had the highest levels of 8-deoxogunine and 4-HNE than other groups. The control mice were shown in upper **A** and **B**. Representative images from $Xpc^{-/-}Kras^{LA1}$ and the control mice were shown.

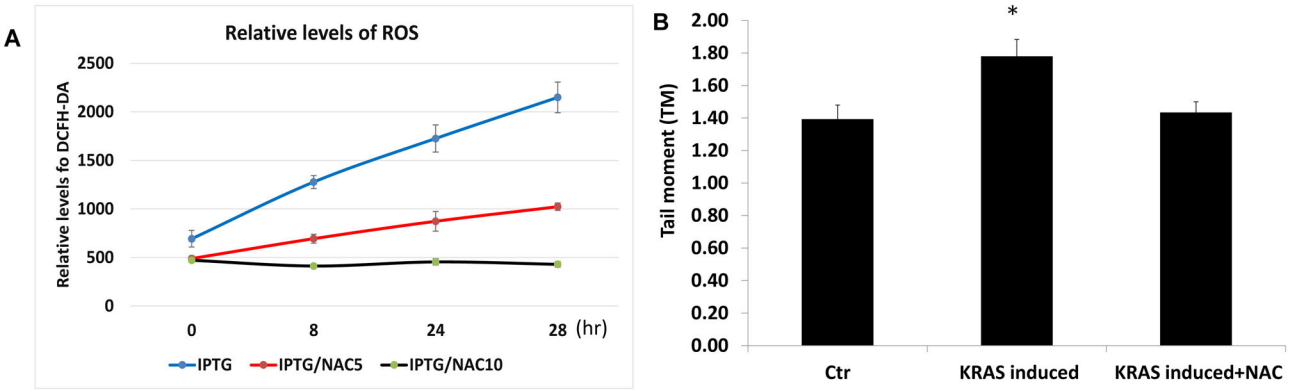

Fig. 6. The effect of induced expression of KRAS^{G12V} on ROS and DNA damage in lung epithelial cells

Lung epithelial BEAS-2B1 cells were engineered to express KRAS^{G12V} under the control of IPTG. We monitored the relative ROS level by H2DCF-DA after induced expression of KRAS^{G12V} for 24 hours. NAC (N-acetyl-L-cysteine, 10 mmol/L) was used to decrease the ROS level. **A:** Changes in the levels of H2DCF-DA in cultured cells under different conditions. **B:** The levels of DNA damage as shown by COMET assay. Tail moment was used to express level of DNA damage. In the presence of NAC, KRAS^{G12V} failed to induce tail moment, suggesting that KRAS^{G12V} induces DNA damage through ROS production. * indicates statistical significance from other groups (P value < 0.05).