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Nox1 is over-expressed in human colon cancers and correlates with activating mutations in K-Ras

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Abstract

The NADPH-oxidase 1 (Nox1) is a homolog of gp91phox, the catalytic subunit of the phagocyte superoxide-generating NADPH-oxidase. Nox1 is expressed in normal colon epithelial cells and in colon tumor cell lines, and overexpression in model cells has been implicated in stimulation of mitogenesis and angiogenesis and inhibition of apoptosis. This suggests that aberrant expression of Nox1 could contribute to the development of colorectal cancer. Herein, we examine the expression of Nox1 mRNA in 24 colon tumors of various stages compared with paired adjacent normal tissue from the same patient, and correlate expression with some common mutations associated with colon cancer. Nox1 was overexpressed compared with paired normal tissue in 57% of tumors as early as the adenoma stage, with no correlation of expression level with tumor stage. Overexpression of Nox1 mRNA correlated with Nox1 protein levels assessed by immunofluorescence and immunohistochemistry with an antibody specific for Nox1. There was a strong correlation between Nox1 mRNA level and activating mutations in codons 12 and 13 of K-Ras. Eighty percent (8/10) of tumors with codons 12 and 13 mutations had a 2-fold or more increase in Nox1 mRNA, and 70% (7/10) had a 5-fold or greater increase. Transgenic mice expressing K-RasG12V in the intestinal epithelium also expressed markedly elevated Nox1 in both small and large intestine. There was no correlation between inactivating mutations in the tumor suppressor p53 and Nox1 expression. We conclude that Nox1 mRNA and protein are overexpressed in colon cancer and are strongly correlated with activating mutations in K-Ras.

Keywords

K-Ras; Nox1; reactive oxygen species; colorectal cancer

Introduction

The NADPH-oxidase 1 (Nox1) is a homolog of gp91phox (also known as Nox2), the catalytic subunit of the phagocyte superoxide-generating NADPH-oxidase, and is the original member of the recently described family of Nox/Duox homologs.¹ When activated by protein–protein interactions with its regulatory subunits, gp91phox generates high levels of reactive oxygen species.
of reactive oxygen species (ROS) in neutrophils as a central mechanism of host defense against microbial infection. Many of the other Nox enzymes, including Nox1, generate ROS in lower levels in a regulated manner, such as in response to growth factors and cytokines. Nox1 activity is controlled both by regulation of catalytic activity by the subunits NOXO1, NOXA1 and Rac1 and by regulation of expression of the catalytic and regulatory subunits.

In addition to a role in innate immunity, ROS participate in cellular processes including signal transduction, proliferation, apoptosis and angiogenesis. ROS have also been implicated in pathological processes related to cancer, inflammatory diseases, cardiovascular disease and aging. For example, elevated superoxide (\( O_2^- \)) and hydrogen peroxide (\( H_2O_2 \)) have been reported in cell lines derived from human tumors, and cell lines transformed with oncogenically activated Ras show elevated ROS.

A role for Nox enzymes in normal vascular biology and tumorigenesis has been suggested by several recent studies. Nox1 over-expression is associated with increased mitogenic rate in vascular smooth muscle and with a transformed phenotype in V12-Ras transformed NRK cells. Overexpression of Nox1 and increased ROS generation are seen in highly tumorigenic variants of transformed human keratinocytes and overexpressed Nox1 correlated with increased tumorigenicity in prostate cancer cells. In the latter case, Nox1-derived ROS was associated with induction of VEGF and increased angiogenesis, but little increase in mitogenic rate was seen. Overexpression of Nox1 and Nox5 are associated with increased mitogenic growth and/or tumorigenicity of prostate cancer or cancer-derived cell lines. In addition, 80% of laser-captured human prostate tumor tissue showed increased Nox1 expression compared with normal adjacent epithelium. Similarly, Nox5 has been implicated in Barrett’s esophageal cancer and inhibition of Nox4 in a melanoma cell line resulted in decreased proliferation, consistent with a role for this oxidase in mitogenic signaling. Nox4 has been found to be expressed in a pancreatic cancer cell line, and was associated with cell survival. On the other hand, Nox1 expression in colon cell lines was associated with a more differentiated state and did not correlate with mitogenic state, suggesting a more complex relationship to growth signaling that may depend on the genetic background of the cell, presence of other oncogenes or other unknown factors.

A role for Nox1 in colon neoplasia has been suggested based on its expression in normal colon and in colon carcinoma cell lines and by the finding of marked Nox1 overexpression in virtual Northern blots comparing colon tumors to normal tissues, although a recent study failed to demonstrate a statistically significant overexpression in colon cancers compared with pooled normal colon tissues. Nox1 expression does not correlate with more advanced tumor stage, perhaps indicating an early role for Nox1 at the adenoma stage. Consistent with a role in growth, ROS in colon tumor-derived cells expressing Nox1 are higher in subconfluent/growing cells than in confluent/growth-arrested cells, and low level transfected Nox1 is sometimes associated with increased growth rates, although higher level expression can result in senescence or apoptosis. In addition to regulating cell division, Nox enzymes may contribute to the cancer phenotype by inhibition of apoptosis, stimulation of angiogenesis, effects on integrin signaling, and increased genomic damage, e.g. see Refs. 24 and 29. Thus, by a variety of mechanisms Nox1 overexpression in colon tumors could contribute to the development of colorectal cancer (CRC).

CRC is the second most common cause of cancer deaths in North America, and by age 70, more than 50% of the population develops a precancerous colorectal lesion such as an adenoma, which progresses to malignancy in 10% of cases. In general, sporadic and hereditary CRCs harbor mutations and deletions in genes such as the tumor suppressors...
adenomatous polyposis coli (APC) and p53, or the K-Ras proto-oncogene,\textsuperscript{31–33} as well as germline mutations in mismatch repair genes\textsuperscript{32,34} that can alter their signaling pathways to promote growth, inhibit apoptosis and accelerate further mutations. ROS can also lead to DNA damage and mutations and may be a risk factor for CRC development.\textsuperscript{35–37} In this report, we examined expression levels of Nox1 mRNA and protein in staged colon tumors compared with paired normal colonic tissue from the same patient, and correlated Nox1 levels with oncogenic mutations in K-Ras and with functional mutations in the tumor suppressor p53, both frequently associated with CRC. Herein, we show that Nox1 was overexpressed in a majority of tumors compared with normal tissue (but with no further increase with more advanced tumor stage), and that elevated Nox1 strongly correlated with oncogenic mutations in K-Ras in human colon tumors and in transgenic mice expressing K-Ras\textsuperscript{G12V} in the intestine. These data in the context of earlier studies are consistent with a role for Nox1 in oncogenic Ras-mediated colon tumorigenesis.

**Material and methods**

**Tissue and tissue processing**

Surgically obtained samples of human colon cancer tissue and adjacent normal colon were obtained through Emory University’s Human Tissue Procurement and Banking Center. The procurement of human tissue was approved by Emory’s Institutional Review Board. Tissues were flash frozen within 30 min of resection in liquid nitrogen and stored at −80°C. Tissues were examined histo-logically and tumors were staged by board-certified pathologists according to the CAP-approved AJCC-pTNM method. Samples were coded to maintain anonymity, and blanket informed consent for the experimental use of tissues was obtained during patient hospitalization.

**Mice**

K-Ras\textsuperscript{G12V} transgenic mice in a B62D background were previously shown to develop tumors over time.\textsuperscript{38} The K-Ras\textsuperscript{G12V} gene is under the regulation of the murine villin promoter, which directs expression in intestinal epithelial cells. Transgenic mice or control B62D mice, all 18 weeks of age, were sacrificed by CO\textsubscript{2} inhalation, and intestinal tissue was divided into small intestine (duodenum, jejunum, ileum) and colon (proximal, medial, distal) sections then stored in RNAlater\textsuperscript{™} (Qiagen) until further use. For mRNA quantitation, RNA was extracted from the duodenum (defined as one-third of the small intestine immediately distal to the stomach) and from the distal one-third of the colon.

**Immunofluorescence**

Tissue was embedded in OCT-medium and 6-μm sections were obtained using a Cryostat. Sections were stained with hematoxylin and eosin for histology, or processed for immunofluorescence as follows: sections were fixed in freshly prepared 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and then washed twice in PBS (5 min per wash). Slides were placed in a moist chamber and incubated in PBS containing 1% normal goat serum (or 2% BSA) and 0.1% Triton X-100 for 1 hr. Human tissue sections were incubated 2 hr with a 1:100 dilution of E39.1 monoclonal antibody (generated and characterized by diaDexus, Inc.) directed against the predicted third extracellular loop or with a rabbit polyclonal Nox1 antibody directed against the flavoprotein domain of Nox1. The specificity of the antibodies towards Nox1 using transfected versus control epithelial cells that either do or do not express Nox1 was determined as described previously\textsuperscript{39} and unpublished studies. A Rhodamine Red-conjugated goat anti-mouse antibody was used to visualize Nox1-specific staining. For visualization of the nucleus, the DNA was stained with Sytox (Molecular Probes, Eugene, OR) for 10 min and washed 3 times with PBS. Slides were mounted with Vectashield.
medium (Vector Laboratories, Burlingame, CA) and viewed on a Zeiss laser confocal microscope or standard fluorescence microscope. Nox1 immunofluorescence was quantified by measuring fluorescence intensity per pixel in tumor tissue compared with that in normal epithelium using the following formula: 

$$N = \frac{\sum I_u}{\sum I_t}$$

where \(I_u\) is pixel intensity in the upper half of the intensity range (using the same range in both normal and tumor), \(I_t\) is the total pixel intensity over the entire range, and \(\sum\) represents the summation of values. The ratio \(N/ N_c\) \([N(tumor)/N(control)]\) is reported.

**Immunohistochemistry of human tissues**

Six-micrometer thick sections of formalin fixed paraffin-embedded blocks were heated at 45°C, deparaffinized in Histoclear and rehydrated through a series of ethanol and PBS rinses. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 120°C, 15–17 PSI in a decloaking chamber (Biocare, Walnut Creek, CA) for 10 min. Endogenous peroxidase activity was quenched by treating with a 3% hydrogen peroxide solution for 15 min. Slides were incubated with preblocking solution (Immunovision Technologies, Brisbane, CA) and then incubated for 1 hr at room temperature in a DAKO autostainer (Dako, Carpinteria, CA) with either the E39.1 antibody against Nox1 or an isotype matched mouse IgG at 5 \(\mu\)g/ml final concentration. Slides were washed in tris buffered saline (TBS) with 0.5% Tween-20, and then incubated with polymer antimouse IgG conjugated to horse radish peroxidase (HRP) (Immunovision Technologies, Brisbane, CA). After washing in TBS with 0.5% Tween-20, sections were visualized by treatment with 3,3' diaminobenzidine (DAB) chromagen for 2 to 5 min and counterstained with hematoxylin before mounting in Permunt medium after dehydration.

**RNA processing**

Total RNA was prepared using the Qiagen RNAeasy® mini or micro kit (Valencia, CA) according to the manufacturer’s instructions and was treated with RNase-free DNase I to eliminate genomic DNA contamination. The equivalent of 0.2 \(\mu\)g of total RNA was reversed transcribed using the BD advantage RT-for-PCR kit (BD Biosciences, Mountain View, CA).

Real-time PCR was carried out on a BioRad iCycler real-time PCR system using iQ™ SYBR® Green Supermix according to the manufacturer’s directions. Primer pairs designed to detect Nox1 were: human Nox1 5'GGT TTA CCG CTC CCA GCA GAA-3' (forward) and 5'GGG TGC CAT TCC AGG AGA GAG-3' (reverse); mouse Nox1 5'GGT TTA CCG CTC CCA GCA GAA-3' (forward) and 5'GGG TGC CAT TCC AGG AGA GAG-3' (reverse). Primer pairs designed to detect β-actin were: human β-actin 5'CCT AAG GCC AAC CGC GAG AAG-3' (forward) and 5'CCT CGT AGA TGG GCA CAG TGT-3' (reverse); mouse β-actin 5'CCT AAG GCC AAC CGT GAG AAG-3' (forward) and 5'CCT CGT AGA TGG GCA CAG TGT-3' (reverse). PCR conditions included an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The relative concentration of Nox1 mRNA was normalized to the reference gene product β-actin mRNA according to standard methods: the ratio \(R\) of Nox1 mRNA relative to that of actin was calculated semi-quantitatively using the following formula: 

$$R = 2^{[-(Ct_{Nox1} - (Ct_{Actin} - Ct_{Actin average})]$$

where \(Ct\) is the number of cycles required for the fluorescence to cross a threshold value.

The ratio \(R_{T/C}\) of Nox1 mRNA in tumor to control was then calculated by dividing \(R_T\) (the ratio of Nox1 mRNA to actin mRNA in tumor tissue) by \(R_C\) (the ratio of Nox1 mRNA to actin mRNA in control tissue), or \(R_{T/C} = R_T/R_C\). Note that the units are the ratio of Nox1 mRNA in tumor to Nox1 mRNA in control. Samples in which β-actin Ct values in tumor and normal tissues differed by more than 1.4-fold were eliminated due to potential problems with RNA quality.
DNA sequencing of K-Ras

RNA from tissue samples was obtained using the Qiagen RNeasy® DNA Micro Kit (Qiagen). A region encompassing exons 1 and 2 of K-Ras was amplified using the BD Clontech Amplitaq kit (Palo Alto, CA) using forward primer 5′GAC TGA ATA TAA ACT TGT GG-3′ and reverse primer 5′TGG CAA ATA CAC AAA GAA G-3′. PCR conditions were as follows for 35 cycles: denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 73°C for 45 sec. Samples were run on a 1% agarose gel and the PCR fragment was recovered using the Qiagen gel-extraction kit (Valencia, CA). Ras mutations at codons 12, 13 and 61 were determined by nucleotide sequencing through the Emory University DNA sequencing core facility.

Evaluation of p53 mutations

Loss of function of the p53 tumor suppressor was determined using a p53 functional assay.41,42 RNA was purified and reverse transcribed as above. The p53 open reading frame was amplified using phosphorothioate (S)-linked primers 5′ATT TGA TGC TGT CCCC CGG ACG ATA TTG AA(S)C-3′ and ACC CTT TTT GGA CTT CAG GTG GCT GGA GT(S)G-3′. PCR conditions were as follows for 30–35 cycles: Denaturation at 94°C for 30 sec, annealing at 65°C for 60 sec, and extension at 78°C for 80 sec. 150 ng of p53 cDNA was cotransformed with 75 ng of linearized vector pLS7642 and carrier DNA into Ade2− yeast strain yIG397 by the lithium acetate procedure. Transformed cells were grown for 3–5 days at 35°C in complete medium supplemented with 5.0 μg/ml adenine. The pLS76 and prD112 yeast-expression vectors, which respectively contain wild-type and mutant p53, were used as controls. (Both the vectors and the yeast strain yIG397 were kind gifts of Dr. Richard Iggo.)

Statistical tests

Unpaired data in Figure 3 with a nonparametric distribution were analyzed by the Mann-Whitney test. Paired data were compared using Student’s t-test.

Results

Nox1 mRNA and protein expression in human normal and colon tumor tissues

We first evaluated Nox1 mRNA expression in samples from different regions of 23 normal colorectal tissues. As shown in Table I, in the normal tissue, there was a generally lower expression of Nox1 mRNA (expressed relative to β-actin mRNA) in ascending colon [0.5 ± 0.1 (S.E.M., n = 10)] compared with that in transverse/descending/sigmoid colon [2.4 ± 0.6 (S.E.M., n = 13)] (p < 0.0004). This is in agreement with an earlier report.25 We also find a general increase in Nox1 levels in mouse gastrointestinal track in going from small intestine through the regions of the colon (see below). This increase in Nox1 expression in more distal regions of the normal colon, together with some variability of Nox1 mRNA levels in samples obtained from the same region from different patients (Table I), made it necessary to evaluate Nox1 mRNA expression in tumors by comparing with paired tissue from the same patient and from the region adjacent to the tumor. Tumor mRNA levels are also shown in Table I, and show elevated levels compared with control paired tissue in more than half of the tumors.

To more easily visualize the relative increase in Nox1 and to control for variations due to anatomic region and individual patient-specific variations, data are also reported in Table I as the ratio of mRNA in tumor compared with that in the adjacent histologically normal tissue. Results show that Nox1 mRNA was elevated 2-fold or more in 57% of the tumors. About a third (8/23) of the tumors showed 5-fold or more elevation of Nox1 mRNA.
compared with histologically normal adjacent tissue. An increase was apparent as early as
the adenoma stage, and elevated Nox1 mRNA was seen in stages I, II and III tumors (an
insufficient number of stage IV tumors did not allow conclusions about this stage). There
was no correlation between Nox1 expression and either the stage of tumor or the age of the
patient (Table I).

Nox1 protein expression was evaluated by immunofluorescence in a subset of 11 tumor/
normal pairs. These were selected randomly based upon availability of sufficient tissue for
histologic analysis, and samples used for immunofluorescence are indicated in Table I.
Immunofluorescence was specific to epithelial cells, where it was strongest towards the
apical pole of the cell (Figs. 1c and 1d). In multiple samples, immunofluorescence was
similar throughout the crypt. While immunofluorescence was seen in both normal and tumor
tissue, it appeared to be considerably stronger in many of the tumor samples (Fig. 1).
Fluorescence was Nox1 specific because omission of the primary monoclonal antibody
eliminated the signal (not shown). We confirmed the immunofluorescence results by
immunohistochemistry performed on a subset of the same samples. Figure 2 shows strong
apically oriented staining of Nox1 protein in colon cancer epithelial cells within the tumor
tissue. The tumor staining was similar to that obtained with a CEA antibody although less
broadly distributed. In contrast, the staining of Nox1 protein in the matched normal adjacent
tissue was very weak, in agreement with the immunofluorescence and mRNA data.

To determine whether Nox1 immunofluorescence correlated with mRNA levels,
immunofluorescence in 11 tumor-normal pairs was quantified. The ratio of
immunofluorescence in the tumor compared with that in normal tissue was determined, and
mRNA ratios were compared with protein immunofluorescence ratios, stratified according
to mRNA levels that were either greater than or less than 2-fold in tumor compared with
normal tissue (Fig. 3). This semi-quantitative method shows that mRNA levels are a
statistically significant surrogate for Nox1 protein expression (p = 0.0095, Mann-Whitney
test).

**Correlation of Nox1 expression and oncogenic mutations in K-Ras**

The protooncogene K-Ras is mutated in codons 12, 13 or 61 in about half of colon
cancers, and mutations first appear at the adenoma stage, at a time similar to increases in
Nox1 expression. These mutations inhibit the GTPase activity of the Ras protein, rendering
it constitutively in the active, GTP-bound form. The sequence of genomic K-Ras DNA was
obtained from the 23 tumors shown in Table I. Mutations in codons 12, 13 or 61 were seen
in 52% of patient samples (Table II). A strong correlation was seen between high level Nox1
expression and mutations in codons 12 and 13. Eighty percent of tumors with these
mutations (8/10) showed a 2-fold or greater elevation in Nox1 mRNA when compared with
the normal tissue. Seventy percent (7/10) had 5-fold or greater levels. Conversely, 88% (7/8)
of the tumors with greater than a 5-fold increase in Nox1 mRNA compared with their paired
control also contained an oncogenic mutation in K-Ras. Only 2 tumors showed mutations at
codon 61 (and 1 of these showed an additional mutation), confirming another report that
very few K-Ras mutations in colon occur in codon 61. Mutations at this position did not
show elevated Nox1, but the small sample size makes this finding inconclusive. Thus, K-
Ras mutations in codons 12 and 13 correlate strongly with elevated Nox1 expression in
colon cancer.

**Comparison of Nox1 expression in large and small intestine of wild type and K-RasG12V
transgenic mice**

To establish experimentally the link between activating mutations in K-Ras and Nox1
expression, we quantified Nox1 mRNA in transgenic mice expressing K-RasG12V in the
intestinal epithelium. As in human, Nox1 expression in normal mice generally increased at more distal locations in the intestinal track. Nox1 mRNA was elevated in K-Ras^G12V^ mice compared with wild-type mice (Fig. 4): Nox1 mRNA was increased 8-fold compared with wild-type in the small intestine, and 2-fold in large intestine. The smaller increase in Nox1 in the large intestine of the transgenic mice was due to the relatively higher level of Nox1 in wild-type large intestine tissue compared with the very low levels normally seen in wild-type small intestine. Nox1 protein expression paralleled the Nox1 mRNA levels as judged by immunofluorescence, using a Nox1 polyclonal antibody raised in rabbit (data not shown).

**Correlation of Nox1 expression and p53 mutation**

To determine whether there was a correlation between Nox1 levels and another mutation commonly seen in colon cancer, we analyzed the human normal/tumor paired samples for the presence of loss-of-function mutations in the p53 gene. Mutation of p53 was assessed by subcloning the tumor p53 gene into a yeast expression vector and evaluating its function in yeast using an assay, in which active p53 produces a white colony whereas functionally defective p53 produces a red colony. As shown in Table II, p53 was functionally normal in adenomas, but was defective in 67% (12/18) of carcinomas. The presence of mutant p53 did not correlate with Nox1 expression (Table II).

**Discussion**

The studies presented here demonstrate elevation of Nox1 mRNA and protein in colon neoplasia in humans when compared with matched normal adjacent colon tissue from the same patient. Nox1 mRNA is overexpressed by 2-fold or greater in more than half of colon neoplasias. Increased Nox1 mRNA was detected in all stages of colon cancer examined, including adenoma, confirming an earlier report. We did not see any meaningful trend towards increased expression with increasing tumor stage, consistent with other reports showing a loss of Nox1 expression in more de-differentiated tumors. This indicates that overexpression occurs early in neoplastic progression, including the precancerous adenoma stage, and is not associated with transition from adenoma to carcinoma or progression to more advanced stages of carcinoma. However, overexpression of Nox1 may play an important role in colon cancer from the earliest stage of adenoma through advanced disease, possibly by a variety of mechanisms. Contrary to our findings and those of Fukuyama, a recent report found little difference in Nox1 mRNA expression in normal versus neoplastic colon. However, visual inspection of the data from Figure 1 of this study reveals a trend to be present (higher Nox1 in tumors versus normals is noted in ~50% of dot blots, see their Fig. 1a). Nevertheless, although a trend was also present in their averaged data (see their Fig. 2c) statistical significance was not seen, possibly due to the use of densitometry to measure differences in hybridization blots in this experiment. An additional experiment quantifying mRNA by RT-PCR was also performed and also failed to demonstrate statistical significance. The reason for this discrepancy is not clear, but it should be noted that mRNA levels from left or right colon were reported as pooled values rather than individual patient tumor:normal ratios, and this might have caused outlier values (e.g., such as the one very high value seen in our Table I) to obscure real differences. From our data we conclude that when Nox1 levels are compared between tumor and normal tissues from the same patient and limited to the same region of the colon, a clear increase in Nox1 mRNA expression is seen in early stage neoplasias including adenomas, as well as in some more advanced adenocarcinomas. Our mRNA expression data were confirmed by immunofluorescence and immunohistochemical evaluation of a subset of the same tumor:normal pairs used for mRNA evaluation. Significantly more Nox1 protein expression was detected in colon cancer tissue when compared with normal adjacent tissue samples. The Nox1 protein distribution in the colon cancer samples was not uniform, showing a more concentrated expression in...
apical-like structures. The significance of this Nox1 protein expression pattern in tumor cells is not clear.

Colon cancer is associated with the accumulation of specific mutations in proto-oncogenes and tumor suppressors. For example, mutations in APC occur very early prior to adenomas and are not in themselves associated with pathological changes, whereas mutations in K-Ras are associated with the development of adenomas. Functional mutations in the tumor suppressor p53 occur late and are associated with more de-differentiated, aggressive tumors. The appearance of Nox1 overexpression as early as the adenoma stage suggested the possibility that mutational activation of K-Ras might regulate Nox1, resulting in overexpression. Herein, we demonstrate a strong correlation between Nox1 expression level and activating mutations of K-Ras. No such correlation was seen with mutations of p53, which are typically observed at later tumor stages. Indeed, 80% of tumors with K-Ras mutations in codon 12 or 13 had a 2-fold or more increase in Nox1 mRNA levels. Furthermore, most of the samples (88%) with greater than a 5-fold increase in Nox1 mRNA contained an activating mutation in K-Ras. A causal association between activating mutations in K-Ras and Nox1 expression was demonstrated by showing that elevated Nox1 mRNA was observed in the small and large intestine of transgenic mice expressing K-RasG12V in the intestinal epithelium.

The functional relationship between elevated Nox1 expression and ROS levels is not considered in the present study, because in a clinical setting, tissue collection protocols are secondary to patient welfare, and can result in variations in the handling and storage of tissue samples. Since Nox1 activity (but in our experience, not immunoreactivity) is labile and because tissues were subjected to handling and freezing, we did not attempt to quantify ROS production in patient samples. Our assumption, however, is that increased Nox1 levels will generally correlate with increased ROS. While ROS production by Nox1 is regulated not only by the expression of the catalytic subunit, but also by NOXO1 and NOXA1 expression, the rate of ROS production is also a function of the expression of these regulatory subunits. While we did not address the expression of these subunits in the present study, over-expression of Nox1 alone in mouse colon results in a marked increase in the production of ROS, measured by dihydroethidium fluorescence (McCoy, unpublished studies). This indicates that constitutive levels (at least in mouse) of regulatory subunits are not rate-limiting, and that increasing the catalytic subunit alone can result in markedly increased ROS. Thus, whether activating mutations in Ras are also increasing the levels of regulatory subunits is unknown, but increased expression of the catalytic subunit is likely to result in increased ROS.

In support of this hypothesis, activating mutations in Ras have previously been associated with increased ROS. Fibro-blasts expressing mutationaly activated Ras show increased superoxide generation and mitogenic effects are mediated by Rac1, which is efficiently activated by K-Ras. Furthermore, Nox1 was markedly overexpressed in NRK cells transformed with K-RasG12V and Nox1 overexpression in K-RasG12V cells was downstream of the ERK1,2 pathway, which is activated by Ras. In that study, stable expression of K-RasG12V not only induced cell transformation in culture, but also led to tumor formation in athymic mice. Knockdown of Nox1 in these Ras-transformed cells led to reversal of transformed appearance and protected against tumorigenesis by these cells in athymic mice. Thus, it is tempting to speculate that the tumor phenotype in human colon cancers that harbor oncogenic mutations in K-Ras may be causally related to overexpression of Nox1 and associated overproduction of ROS. As discussed in the introduction, the causal relationship between increased cell division and Nox activity appears to be complex and may depend upon the genetic background of the cell and probably other unknown factors. In addition, Nox1-derived ROS might influence the tumor phenotype not only by affecting...
mitogenic rate, but also by inhibiting apoptosis, stimulating angiogenesis and modulating extracellular matrix structure, as discussed in detail in a recent review.\textsuperscript{29} In addition, the overproduction of Nox1-derived ROS in the adenoma stage may increase the risk of colon cancer by exerting a genotoxic/mutagenic and/or proinflammatory effect. Alternatively, Nox1 overexpression could be a marker of Ras transformation that does not relate to oncogenesis. Although studies in the Ras-transformed NRK cells support Nox1 as a link between activating mutations in Ras and tumorigenesis,\textsuperscript{12} definitive experiments in animals have not yet been carried out. We are currently investigating these possibilities using Nox1 knockout mice in a mouse with a V12Ras genetic background.

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Abbreviations

- **APC**: adenomatous polyposis coli
- **CRC**: colorectal cancer
- **DAB**: 3,3′-diaminobenzidine
- **HRP**: horse radish peroxidase
- **phox**: phagocyte oxidase
- **ROS**: reactive oxygen species
- **TBS**: Tris buffered saline

References

Figure 1. Immunofluorescence detection of Nox1 protein in tumor and normal adjacent colon tissues. A representative tumor/normal pair is shown, with normal tissues on the left (a,c) and cancerous tissue on the right (b,d). Shown are histology (a,b) and Nox1 protein expression detected by immunofluorescence (c,d). Nox1 was detected using E39.1 monoclonal antibody (shown in red) and Sytox-stained nuclei are indicated in green. Magnification was 10X (a,b) or 20X (c,d). The experiment was carried out on 11 tumor/normal pairs.
Figure 2.
Immunohistochemical detection of Nox1 protein in tumor and normal adjacent colon tissues and comparison with CEA expression. A representative tumor (a–d) and normal (e–f) tissue pair is shown. Immunohistochemical staining was performed with a monoclonal antibody against Nox1 (a, c and e), an antibody against CEA (d, f) or a negative control monoclonal antibody (b). The magnification was 200× except for panel c, which was 400×.
Figure 3.
Correlation of Nox1 mRNA and protein levels. Nox1 immunofluorescence intensity (N) was measured as described in Material and methods in 11 tumor/normal pairs, and is indicated as \( N_t/N_c \) or the fluorescence ratio in tumor compared with normal tissue, shown as filled bars. mRNA levels are shown as open bars. A 2-fold or more elevation of mRNA in tumor versus normal tissue was considered as significant. Error bars represent the standard error of the mean, and the \( p \)-value comparing groups is indicated. \(*\*p = 0.0095\) (Mann-Whitney test).
Figure 4.
Nox1 expression in K-Ras$^{G12V}$ transgenic mice. Nox1 mRNA levels were measured in large or small intestinal tissue from K-Ras$^{G12V}$ mice and in control littermates by real-time PCR as described in Material and methods. Open bars show mRNA levels in wild-type animals, and filled bars show levels in transgenic mice expressing V12-Ras in the intestinal track. *p = 0.123, **p = 0.011 (student’s t-test).
**TABLE I**

Nox1 mRNA EXPRESSION IN HUMAN COLON TUMORS

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Tumor stage</th>
<th>Patient age/gender</th>
<th>Location of tissue</th>
<th>Nox1mRNA/b-Actin mRNA in Tumor</th>
<th>Nox1mRNA/b-Actin mRNA in Normal</th>
<th>Nox1 mRNA (tumor/normal)</th>
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</thead>
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<tr>
<td>449</td>
<td>IIIC</td>
<td>56/F</td>
<td>Right</td>
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<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
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</tr>
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<td>56/F</td>
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</tr>
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<td>Right</td>
<td>3.1</td>
<td>1.1</td>
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<td>Transverse</td>
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<td>0.1</td>
</tr>
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<td>Sigmoid</td>
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</table>

Quantitation of Nox1 mRNA expression is expressed as the ratio of Nox1 to β-actin, calculated prior to rounding to nearest 0.1. Hence, the ratios in the last column do not correspond exactly to the ratio of column 5 divided by column 6.
\(^1\) Indicates tissue samples used for histologic immunofluorescence analysis and reported in Figure 3.

\(^2\) Considered an outlier, and omitted in calculation of average.
### TABLE II

**CORRELATION OF Nox1 mRNA AND K-Ras AND p53 MUTATIONS IN HUMAN COLON TUMORS**

<table>
<thead>
<tr>
<th>Patient sample</th>
<th>Nox1 mRNA tumor/normal</th>
<th>K-Ras mutation</th>
<th>p53 assay (% red colonies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5.7</td>
<td>G12&lt;sup&gt;Cys&lt;/sup&gt;</td>
<td>47</td>
</tr>
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<td>70</td>
<td>1.5</td>
<td>No mutation</td>
<td>95</td>
</tr>
<tr>
<td>193</td>
<td>7.2</td>
<td>G12&lt;sup&gt;Glu&lt;/sup&gt;</td>
<td>85</td>
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<td>0.1</td>
<td>No mutation</td>
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</tr>
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<td>G13&lt;sup&gt;Glu&lt;/sup&gt;</td>
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<tr>
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<td>G12&lt;sup&gt;Val&lt;/sup&gt;/G13&lt;sup&gt;Glu&lt;/sup&gt;</td>
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</table>

Background red or white colonies (5–20%) are typically seen and may result from PCR-induced mutations or intragenic recombination.\textsuperscript{41}