BRIEF COMMUNICATION

Relationship Between p53 Mutations and Inducible Nitric Oxide Synthase Expression in Human Colorectal Cancer

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Inducible Ca^{2+}-independent nitric oxide synthase (NOS), also referred to as NOS2, which is expressed in a variety of human cancers *(1–4)*, can generate mutagenic concentrations of nitric oxide (NO) in mice *(5)*. NOS2 is the most active isoform among the three known nitric oxide synthases *(6)*, which also include the neuronal (NOS1) and endothelial (NOS3) isoforms. Only NOS2 is capable of producing sustained NO concentrations in the micromolar range *(7)*.

We investigated the hypothesis that NO generated by NOS2 is capable of inducing mutations in the p53 (also known as TP53) gene and contributes to human colon carcinogenesis. We analyzed 118 sporadic colon tumors for NOS2 expression and p53 gene mutations. Colon tumors and surrounding normal tissues were collected from the Cooperative Human Tissue Network and the Department of Pathology, University of Baltimore, with the approval of local boards governing research on human subjects, as described previously *(3)*. The expression of NOS2 was increased in various tumors (Fig. 1, A and B) throughout the right, left, and sigmoid colon. Adenomas showed the highest average NOS2 activity. The NOS2 activity declined with advancing tumor stage and was seen at the lowest level in metastatic tumors (Fig. 1, A). NOS2 activity correlated with NOS2 protein expression. Immunohistochemical analysis localized NOS2 protein mainly in tumor-infiltrating mononuclear cells and less frequently in en-

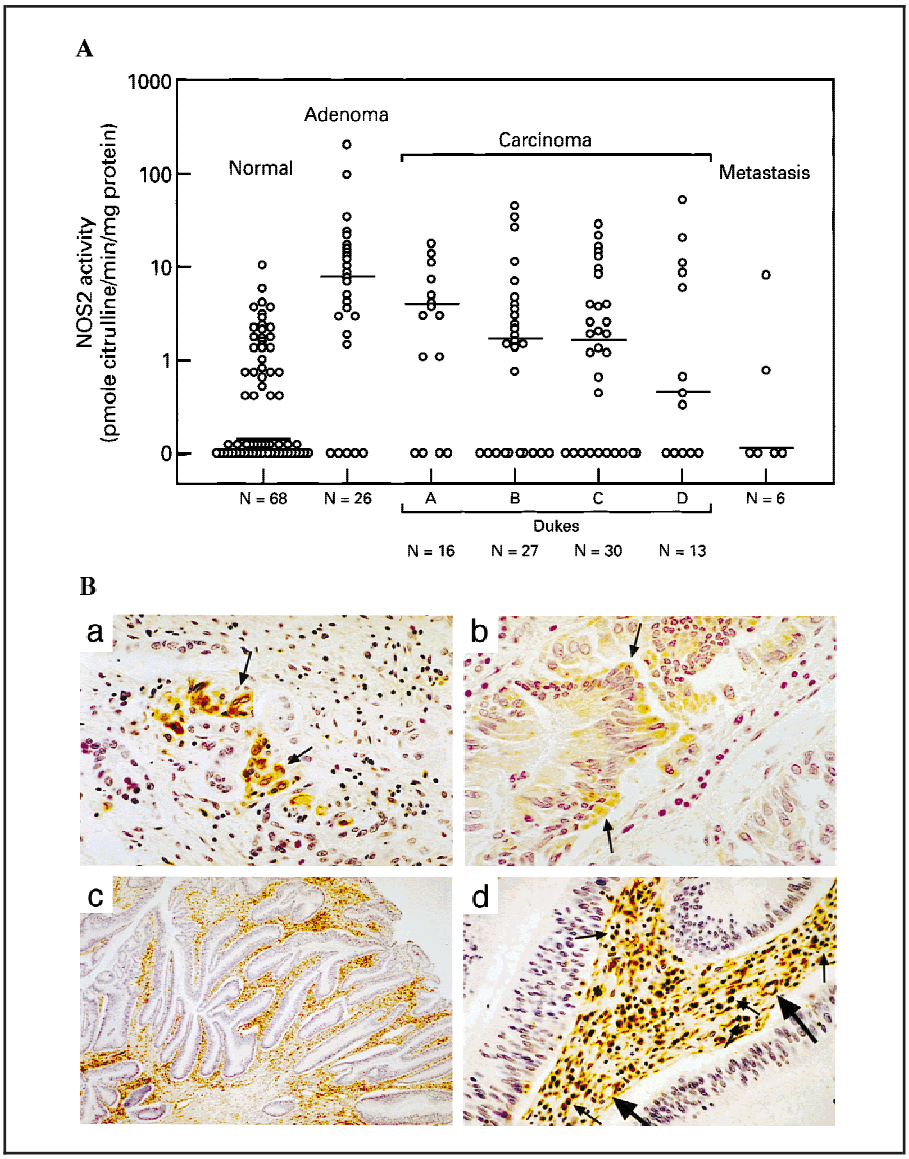

Fig. 1. Inducible Ca^{2+}-independent nitric oxide synthase (NOS2) expression in human colon tumors. **A)** Ca^{2+}-independent nitric oxide synthase (NOS) activity, characteristic of the type NOS2, is high in colon adenomas, while it is low in the surrounding normal colon tissues. The activity decreases with the progression of colorectal cancer (Dukes' stages A through D) and is lowest in colon carcinoma metastases in the liver and lung (adenomas: $P<.001$ and n = 20; carcinomas: $P = .01$ and n = 48; both versus surrounding normal colon tissues from the same patient analyzed by the Wilcoxon signed rank test for two-tailed, paired analysis). Details of the NOS2 assay, together with the NOS activities for a subset of the tissues, were reported recently *(3)*. **B)** Immunohistochemical analysis of colon tumors for NOS2 protein. In **panels a** and **b,** focal clusters of tumor cells (arrows) have cytoplasmic staining with brown chromogen indicating NOS2. In **panel c,** scanning magnification shows extensive staining of tumor interstitium. **Panel d** is a higher magnification detail of panel c that shows heavy staining of mononuclear cells (small arrows) and endothelial cells (large arrows). Hematoxylin counterstain; original magnifications: **panel a,** ×400; **panel b,** ×630; **panel c,** ×50; **panel d,** ×400. NOS2 immunohistochemistry was performed as described previously *(3)*.

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dothelial cells within the tumors and in the tumor cells (Fig. 1, B). The decline in NOS2 activity with advancing tumor stage may be attributed to the tumor cell-induced immunosuppression of NOS2 expression in tumor-infiltrating mononuclear cells of advanced tumors *(8)*.

We then determined the p53 mutation frequency and mutation type in relation to the NOS2 activity levels in colon tumors. We confined the mutational analysis to the evolutionarily conserved region in the p53 gene. This genomic region contains about 90% of the known p53 mutations and all of the mutational hotspots at CpG dinucleotide sites *(9,10)*. We found 11 mutations among 26 adenomas (mutation frequency = 34.6%, with one tumor containing three mutations) and 44 mutations among 92 carcinomas of Dukes' stages A through D (mutation frequency = 47.8%). None of the carcinomas had multiple mutations. There were 44 missense mutations, six nonsense mutations, two insertions, two deletions, and one inversion. The predominant mutation was the G:C to A:T transition at CpG dinucleotides (n = 34, mutation frequency = 61.8%), and a significant association between these transitions and increased NOS2 activity was observed when compared with tumors with other types of mutations, e.g., transversions and frameshift mutations (P = .004; Mann–Whitney U rank sum test) (*see* Fig. 2, A). Further analysis demonstrated a convincing dose–response relationship between NOS2 activity and G:C to A:T transitions at CpG dinucleotides in carcinomas (P = .003; Mantel–Haenszel test for trend) (Fig. 2, B); the rates of all other mutations varied inversely with NOS2 activity.

Most p53 transition mutations in colorectal carcinoma occur at CpG dinucleotides that contain 5-methylcytosine *(9,10)*, and our data support the hypothesis that NOS2 activity generates the high frequency of G:C to A:T mutations at 5-methylcytosine sites in the p53 gene. The formation of deaminating NO intermediates through up-regulation of NOS2 has been documented *(11)*, and exposure of *Salmonella typhimurium*, plasmid DNA, and the p53 complementary DNA to NO donors generated mostly G:C to A:T transitions *(12,13)*. In addition, the increased formation of *N*-nitrosamines in activated macrophages *(14)* and *Corynebacterium parvum*-treated rats *(15)* indicates that autoxidation of endogenously produced NO leads to electrophilic and nitrosating agents such as N_2O_3 *in vivo*.

Endogenous NO production also causes oxidative DNA damage *(16)* as a result of stoichiometric fluxes of NO and superoxide that generate peroxynitrite *(17)*. Our observation that detectable nitrotyrosine formation is restricted to only a subset of NOS2-expressing tumor-infiltrating mononuclear cells in colon tumors *(3)* suggests that the NO to peroxynitrite pathway is not a dominant pathway in adenomas. Peroxynitrite has also been shown to cause mainly G:C to T:A and G:C to C:G transversions *(18)*, which does not match the p53 mutational spectrum of colon tumors with a predominance of G:C to A:T transition mutations. However, NO quenches both superoxide and an oxidizing intermedi-

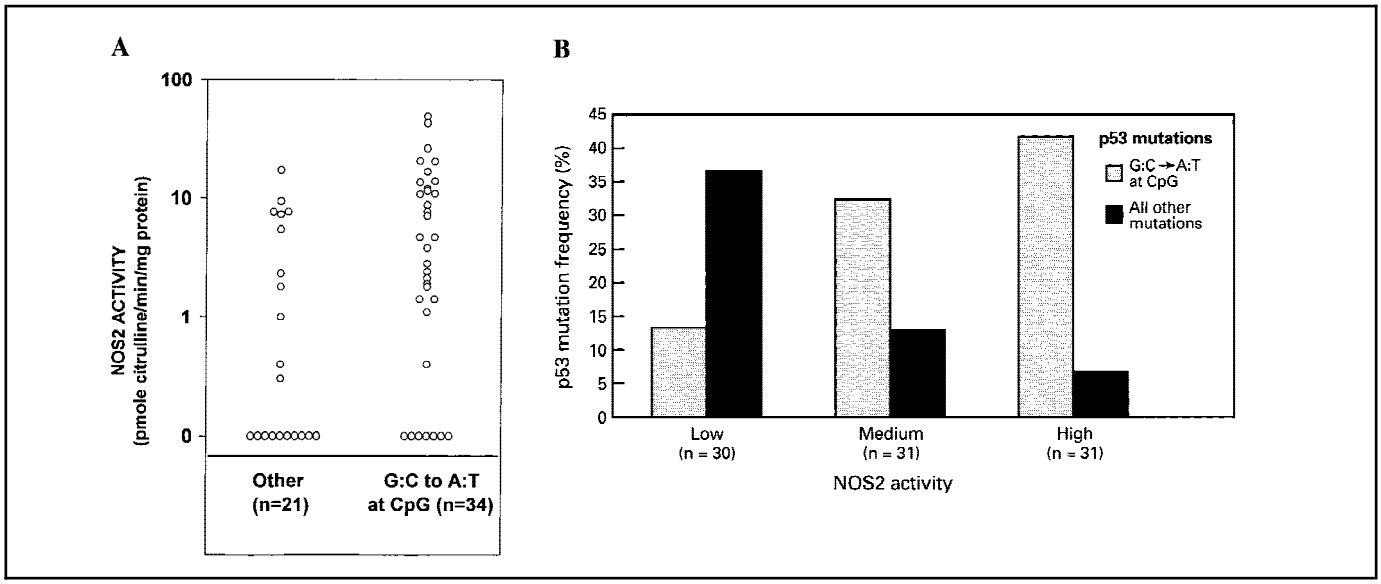

Fig. 2. Inducible Ca^{2+}-independent nitric oxide synthase (NOS2) activity and p53 mutations in human colon tumors. **A)** NOS2 activity is significantly higher in human colon tumors with G:C to A:T transitions at CpG sites in the p53 gene when compared with that in tumors with mutations at other sites in this gene (P = .004; Mann–Whitney U rank sum test). The specimens include adenomas, adenomas with carcinoma *in situ*, and carcinomas of Dukes' stages A through D. **B)** The frequency of G:C to A:T transitions at CpG dinucleotide sites is positively correlated with NOS2 activity (P = .003; Mantel–Haenszel test for trend). NOS2 activity and p53 mutation frequency and mutation type were analyzed in 92 colon carcinomas of Dukes' stages A through D containing 44 mutations. The NOS2 activity of the 92 carcinomas was divided into tertiles of the distribution, described as low (undetectable NOS2 activity), medium (range from 0.1 to 3.5 pmol citrulline/min per milligram protein as the highest NOS2 activity found in this group), and high (range from 3.7 pmol citrulline/min per milligram protein as the lowest NOS2 activity found in this group to 48.8 pmol citrulline/min per milligram protein). The frequency of both G:C to A:T mutations at CpG dinucleotides and all other mutations is shown as the percentage of tumors with a mutation for each tertile. The category ''all other mutations'' includes transversions, transitions at sites other than CpG, and frameshift mutations. The frequency of G:C to A:T mutations at CpG dinucleotide sites is highest in the group with high NOS2 activity, whereas the frequency of all other p53 gene mutations is inversely correlated with NOS2 activity. For p53 sequencing, paraffin-embedded tumor samples were dewaxed and microdissected from 50-μm sections. DNA was isolated by use of sodium dodecyl sulfate/proteinase K treatment and phenol/chloroform extraction, and the p53 coding sequence was amplified as described previously *(25)* and sequenced with the T7 sequenase kit (Amersham Life Science Inc., Arlington Heights, IL).

ate of peroxynitrite *(17)* and may, therefore, protect against superoxide toxicity. This particular NO chemistry can explain our finding that NOS2 expression is inversely correlated with the frequency of mutations other than G:C to A:T at CpG dinucleotides (Fig. 2, B).

Our investigation of primary human colon tumors establishes a strong positive relationship between the presence of NOS2 in the tumors and the frequency of G:C to A:T transitions at CpG dinucleotides. These mutations also are common in lymphoid, esophageal, head and neck, stomach, brain, and breast cancers *(9,10,19)*. Increased NOS2 expression has been demonstrated in four of these cancers *(1–4)*. Tumor-associated NO production may modify DNA directly, or it may inhibit DNA repair activities *(17)*, such as the recently described human thymine–DNA glycosylase, which has been shown to repair G:T mismatches at CpG dinucleotides *(20)*. Because NO production also induces accumulation of wild-type p53 *(21,22)*, the resulting growth inhibition can provide an additional strong selection pressure for nonfunctional, mutant p53 *(23)*. NO may, therefore, act as both an endogenous initiator and a promoter in human colon carcinogenesis. Specific inhibitors of NOS2, as demonstrated recently in an animal tumor model *(24)*, may have important chemopreventive potential in human colorectal cancer.

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NOTES

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