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# Association of Nox1 and Vinculin With Colon Cancer Progression

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**Nox1 mRNA, protein, and activities were compared in the paired primary and metastatic colon adenocarcinoma cell lines SW480 and SW620, and in normal colon tissues and colon cancer tissues. Our results demonstrated that Nox1 levels were higher in the primary SW480 cells than that in metastatic SW620 cells and were not associated with colon cancer progression. We further discovered that vinculin protein level in SW620 was much higher than that in SW480 cells, whereas E-cadherin was lower. We conclude that vinculin and E-cadherin, but not Nox1, may serve as biomarkers for colon cancer progression.**

**Keywords:** Nox1, Vinculin, Adhesion, Colon cancer progression

## INTRODUCTION

NADPH oxidase enzyme family contains seven members and generates reactive oxygen species (ROS) (1). Recent studies have shown that Nox1 plays crucial roles in cancer development including transformation and invasion, (2, 3) hence Nox1 was proposed to be a therapeutic target (4, 5). A number of Nox1 inhibitors have been developed for various diseases (6–8), among which diphenyliodonium is an irreversible nonspecific inhibitor (9), VAS2870 is a Nox1 inhibitor identified via high-throughput screening (10), and apocynin is a natural compound that was widely used to inhibit Nox family activities (11, 12).

Nox1 is expressed in various cell types and tissues including colon epithelial cells, vascular smooth muscle cells, endothelium, uterus, placenta, prostate, osteoclasts, and retinal pericytes (13–17), and it is overexpressed in a number of cancer cells including colon cancer, gastric cancer, and prostate cancer (2, 18). Expression of Nox1 seems to be relatively high in colon epithelial cells than that in other normal cell types (19). In colon cancer cells, the known function of Nox1 is conflicting: one study suggested that Nox1 was downstream of 12-HETE and was responsible for cell proliferation but not for cell spreading (20); another study suggested that Nox1 was responsible for cell invasion (6). In this study, we investigated whether Nox1 was associated with colon cancer progression using cell lines, as well as normal and cancerous human colon tissues. Our data suggested that Nox1 was not a suitable biomarker; instead, vinculin and E-cadherin maybe associated with colon cancer progression.

## MATERIALS AND METHODS

### Normal colon and tumor tissues and cell culture

The tissue array was obtained from Biomax.com (Cat. # CO1501), which followed our approved exempted IRB protocol. SW480 and SW620 were kind gifts from Dr. Vi Chiu (Department of Medicine, University of California Irvine, Irvine, CA, USA). Both cells were maintained in DMEM media containing 1% penicillin/streptomycin, 5% fetal bovine serum, and 5% newborn bovine serum at 37°C incubator with 5% $CO_2$.

### Immunohistochemistry and immunofluorescence

The tissue slides were baked and rehydrated by immersing for 5 min sequentially in the following solutions: ClearRite3 (three times), 100% ethanol (two times), 95% ethanol (two times); 70% ethanol (two times), 50% ethanol, and 1× PBS. Tissues were blocked by 10% goat serum (in 1× PBS) at room temperature for 15 min. Nox1 antibody (10 mg/mL purified IgG) was diluted 1:20 in 10% goat serum and incubated with tissues at 4°C overnight. The tissue slide was washed five times in 1× PBS containing 0.1% Tween 20 and then incubated with anti-rabbit polymer HRP antibody (DAKO, K4011) at room temperature for 30 min. After five times washes, DAB chromogen was added and incubated for approximately 1 min. Tissue was washed and counterstained with Harris modified hematoxylin solution for 10 min. Tissue staining was viewed and photographed under a Nikon microscope.

For immunofluorescence the procedure is similar. After incubation with the primary antibody, the cells on the coverslips were incubated with FITC-labeled anti-rabbit secondary antibody (room temperature for 45 min) and then DAPI

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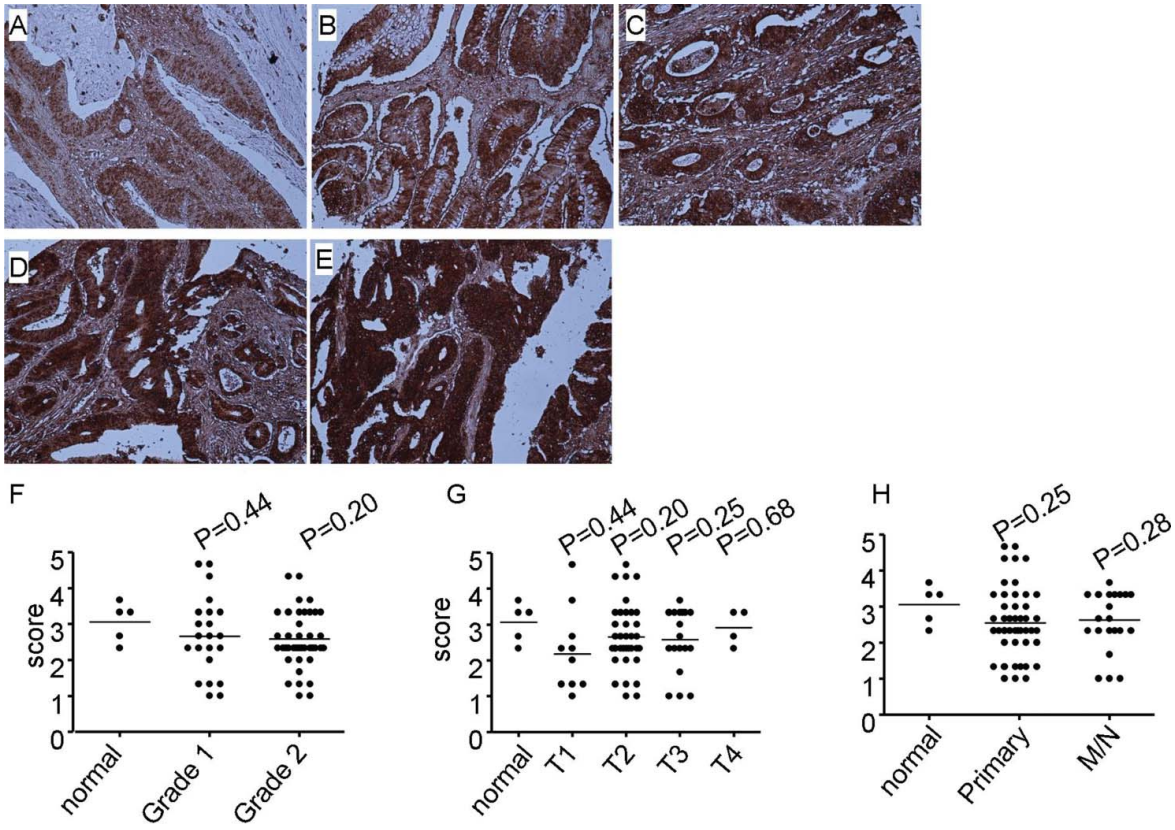


Figure 1. Immunohistochemistry detection of Nox1 in colon cancer cells and tissues. A–E, Representative IHC slides showing score of 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E). F–H, Graphics of the IHC scores received for normal tissue and various colon cancer tissues at various grades (F), stages (G), and status of metastasis (H).



(10 $\mu$g/mL) for 10 min. Cells were washed 5 times in 1× PBS containing 0.1% Tween 20 and then water, and then mounted to a slide using fluorescence compatible mounting solution. Photos were taken by a Nikon microscope.

### Western blot, qRT-PCR, and Nox1 activity measurement

$\alpha$-Tubulin antibody was purchased from Sigma (Saint Louis, MO); Nox1 antibody was generated using a C-terminal synthesized peptide (sequence NIVGHAALNFDKATDIV) as antigen to immunize rabbits. The antibody was purified using protein A/G agarose beads. Validation of the antibody was performed using *E. coli* expressed Nox1 protein (data not shown). E-cadherin and vinculin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers for Nox1 and actin are the same as previously described (22); primers for vinculin are vin1: 5′-GCAGAT CCAAAT GGTGGA CCG-3′ and vin2: 5′GTGCTT GCT-CAA TCTTGC CC-3′. Primers for E-cadherin followed a previous study (28).

### Cell adhesion assay

SW480 and SW620 cells were seeded in 96-well plates at about 7,500 cells per well on Day 1. On Day 3, cells were trypsinized using different concentration of diluted trypsin (in 1× PBS) for 20 min. The floating cells at the end of the 20-min incubation were collected and defined as "detached cells," the rest of cells that were still attached to the plate wells at the end of the 20-min incubation were defined as "attached cells." The attached cells were completely trypsinized and counted. For time course analysis, trypsin was diluted 1:4 in 1× PBS, and detached cells were collected at 3, 6, 9, 12, 15, 18, and 21 min of incubation; cell number was counted. The attached cells were also counted after complete trypsinization.

### Statistics analysis

Statistic analysis was performed using Prism 4 software. For comparing the significance of the difference between normal and various stages of tumors, two-tailed unpaired *t* test method was used. The same method was also used to compare the difference in mRNA levels of Nox1, E-cadherin, and vinculin.

Table 1. Distribution of Tumor Stages for Tissue Array

| Grade | 1 | 2 | 3 | Ukn* | Subtotal | Total |
|---|---|---|---|---|---|---|
| # | 22 | 42 | 1 | 4 | 69 | |
| Stage | 1 | 2 | 3 | 4 | | |
| # | 11 | 35 | 19 | 4 | 69 | |
| T-stage | 1 | 2 | 3 | 4 | | |
| # | 0 | 14 | 35 | 20 | 69 | |
| N/M | 0 | 1,2 | | | | |
| # | 46 | 23 | | | 69 | |
| Normal tissue | | | | | 5 | 74 |

*Ukn: unknown.

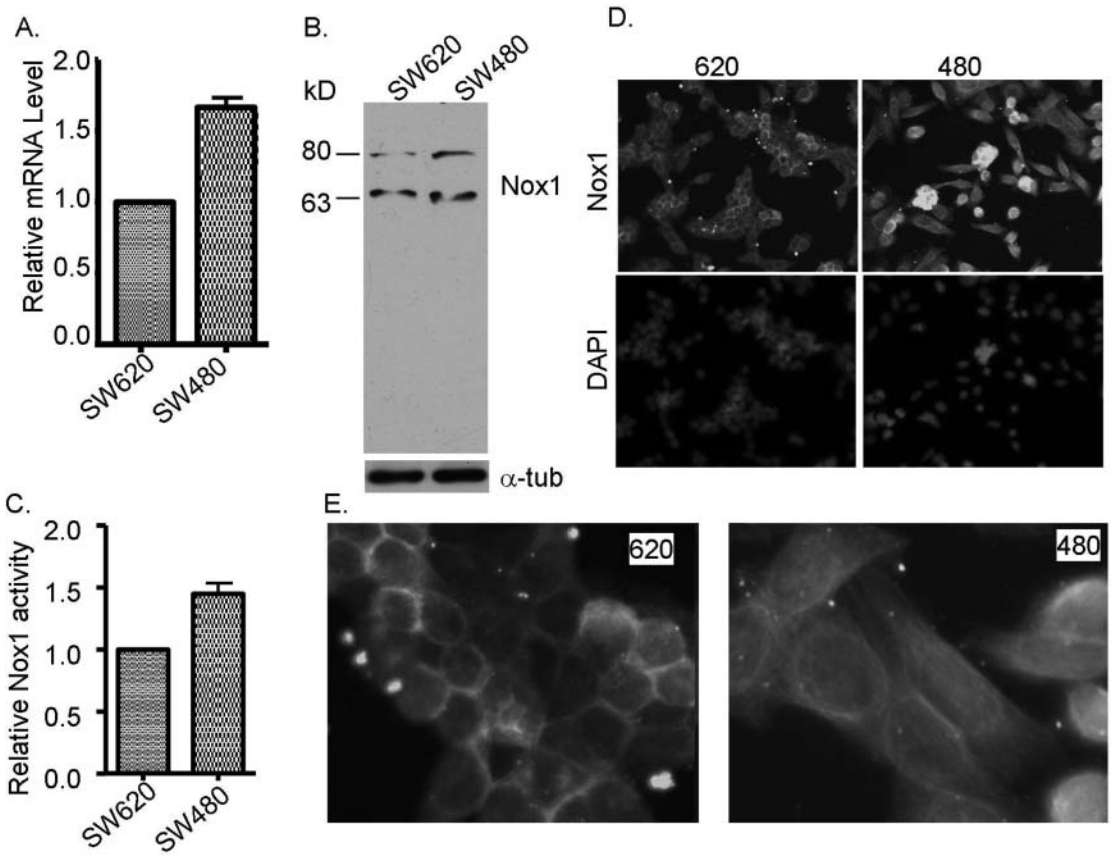


Figure 2. Expression of Nox1 in SW620 and SW480 cells. A, Relative mRNA levels measure by qRT-PCR. B, Western blot showing two bands for Nox1 using our custom-made antibody; $\alpha$-tubulin was used as a loading control. C, Relative Nox1 activity in SW480 and SW620 cells. D, Immunofluorescence of Nox1 in SW480 and SW620 cells (original 20×). E, Enlarged areas from D to show subcellular localization of Nox1 in SW480 and SW620 cells.



## RESULTS

### Nox1 level is not associated with colon cancer progression

To our best knowledge, there is no published data to examine whether Nox1 can be served as a progression biomarker for colon cancer. To answer this question, we performed immunohistochemistry using Nox1-specific antibody on tissue arrays, which includes normal and cancer tissues Grades 1 to 3, Stages I –IV, and various TNM stages (Table 1), each in duplicate cores. The typical IHC results are shown in Figure 1. Each tissue received a score of 1 to 5, with 1 indicating minimum staining [Figure 1(A)] and 5 the heaviest staining [Figure 1(E)]. Each core was scored by three technicians independently and the average score was computed. There was no significant difference between normal tissue, Grade 1 and Grade 2 tumors [Figure 1(F)], with $p$ values of 0.44 (normal vs. Grade 1) and 0.20 (normal vs. Grade 2), respectively (2-tailed unpaired $t$ test). Nor was there a difference between normal and each tumor stage (Stage 1 through Stage 4) [Figure 1(G)]. In fact, there was a nonsignificant decrease in Nox1 levels in Grade 1 and Stage 1 tumors as compared with normal tissues. As shown in Figure 1(H), there was no difference in Nox1 accumulation in normal versus primary or normal versus metastatic tumors ($p$ values of 0.25 and 0.28, respectively). Here "metastatic tumors" include both lymphnode-positive tumors and distant metastatic tumors (N1, N2, and M1 tumors in TNM classification).

### Nox1 levels are higher in primary SW480 than that in metastatic SW620 cells

Because ROS plays a key role in cell motility and invasion (21), inhibition of Nox1 was previously shown to inhibit invadopodia formation in colon cancer cells (6). We next examined Nox1 expression levels in SW480 and SW620 cells. To our surprise, Nox1 mRNA level was lower (approximately 64% less, $p < .05$) in SW620 cells than that in SW480 cells, as measured by qRT-PCR [Figure 2(A)]. Nox1 protein level was also lower in SW620 cells than that in SW480 cells [Figure 2(B)]; consequently Nox1 activity was lower in SW620 cells as well [Figure 2(C)]. Note that Nox1 antibody detects two bands, one at approximately 67 kD, which is the expected Nox1 molecular weight, the other was at around 80 kD whose level was always consistent with the level of 67 kD band; hence, we speculate this 80 kD band is also Nox1 with certain posttranslational modification. Furthermore, we used immunofluorescence method to localize Nox1 protein in these cell types. As shown in Figure 2(D), again the fluorescence signal for Nox1 was stronger in SW480 cells than that in SW620 cells. In an enlarged view shown in

A.

| | SW480 | SW620 |
|---|---|---|
| VS2870 | 98.2 ± 10.9 | 25.9 ± 6.8 |
| DPI | 0.68 ± 0.17 | 0.24 ± 0.06 |

B.

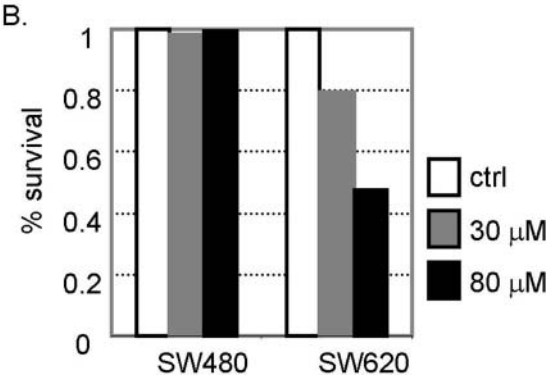


Figure 3. SW480 is more resistant to Nox1 inhibitors. A, IC50s of DPI and VAS2870 for SW480 and SW620 cell lines. B, Cell killing effect of apocynin in SW480 and SW620 lines.

Figure 2(E), Nox1 seemed to be localized at the cell–cell junction in SW620 cells while it was more diffused in SW480 cells with an enhanced perinuclear accumulation [Figure 2(E)].

### SW480 cells are more resistant to Nox1 inhibitors than SW620

Consistent with Nox1 levels in SW480 and SW620 cells, SW480 cells exhibited higher resistant to all three Nox1 inhibitors than did the SW620 cells, which was demonstrated by higher IC50s for DPI and VAS2870 for SW480 cells [Figure 3(A)]. Apocynin did not show cytotoxicity for SW480 cells up to 100 $\mu$M, but it killed about 20% and 50% of SW620 cells at 30 and 80 $\mu$M concentrations, respectively [Figure 3(B)].

### SW620 cells express higher level of vinculin and lower level of E-cadherin

To seek additional factors that may play crucial roles in colon cancer progression, we sought to compare several adhesion molecules between SW480 and SW620 cells, including integrin $\beta$1, N-cadherin, E-cadherin, and vinculin. By qRT-PCR method, we did not observe dramatic difference of N-cadherin and integrin $\beta$1 between SW480 and SW620 cells (data not shown), but we discovered that SW620 cells accumulated significant higher level of vinculin mRNA and significant lower level of E-cadherin mRNA [Figure 4(A) and (B)]. Western blot analysis confirmed that SW620 cells also accumulated about threefold higher of vinculin and about half of the E-cadherin protein as compared with SW480 cells [Figure 4(C)].

### SW620 cells are less adhesive to culture dishes than SW480 cells

We next examined the adhesive property of SW480 and SW620 cells using serial-diluted trypsin. First, trypsin was diluted into 1× PBS at 1:0 (undiluted), 1:3 and 1:5 ratio, 20 min after incubation at room temperature, the detached cells were collected and counted. The attached cells continued to be digested with undiluted trypsin for additional 30 min. Percentage of attached and detached cells was calculated and graphed in Figure 5(A). After 20 min of digestion, majority of SW480 cells (96.8%) were detached from culture dishes when treated with undiluted trypsin. About 73.6 and 23.4% of cells were still detached when digested by 1:3 and 1:5 diluted trypsin. In comparison, about 97.3, 95.5, and 92.9% of SW620 were detached from culture dishes after 20 min of digestion with 1:0, 1:3, and 1:5 diluted trypsin [Figure 5(A)]. Similarly, when cells were digested with 1: 4 diluted trypsin and counted at various time points, again more SW620 cells were detached than SW480 cells after the time course [Figure 5(B)]. For example, after 9 min of digestion, only approximately 4.1% of SW480 cells were detached; in comparison, about 80% of SW620 cells were detached at the same condition [Figure 5(B)].

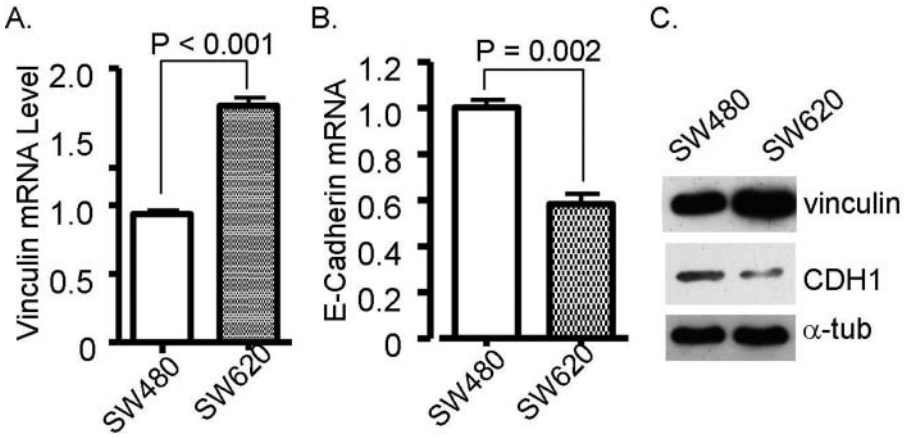


Figure 4. Expression of vinculin and E-cadherin in SW620 and SW480 cells. A, Relative vinculin mRNA determined by qRT-PCR. B, Relative E-cadherin determined by qRT-PCR. C, Western blot detection of vinculin and E-cadherin in SW480 and SW620 cells.

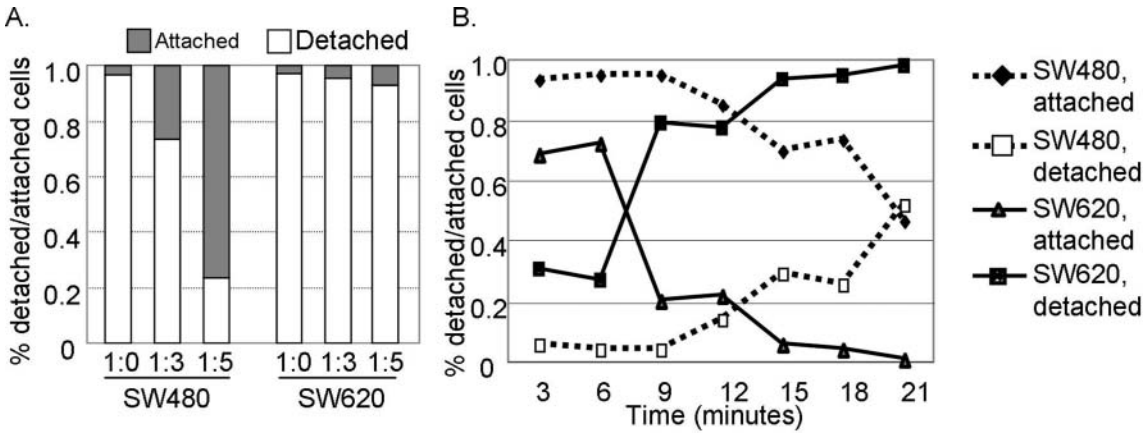


Figure 5. SW480 cells are highly adhesive to culture dishes. A, Dose-dependent trypsinization of SW480 and SW620 cells. Trypsin dilution is indicated at the bottom of the graph, the detached cells were counted 20 min after incubation, and the attached cells were counted after complete digestion with trypsin. Relative percentages of the attached/detached cells for each treatment were graphed. B, Time course of the trypsin digestion. Trypsin was diluted 1:4 in 1× PBS and cells were counted after incubation of the indicated time periods.



## DISCUSSION

Previous studies in melanoma and K-ras transformed rat kidney cells suggest that Nox1 regulates cell invasion process (22, 23). Hence, we set out to study whether Nox1 can be used as a progression marker for colon cancer by performing IHC for 74 normal and tumor tissues at various stages. Our data showed that in normal colon tissues there was relatively high accumulation of Nox1 protein, which is not significantly different to those found in colon cancer tissues in various stages. Nox1 levels were not associated with colon cancer progression. We further confirmed our results by comparing Nox1 levels in a paired primary and metastatic colon cancer cells obtained from the same patient, SW480 and SW620, which are often used as colon cancer progression cell models. Nox1 levels are actually lower in primary SW480 cells than that in metastatic SW620 cells. As a consequence, SW480 cells exhibited higher resistance to Nox1 inhibitors. These results prompted us to seek additional molecules that may be responsible for colon cancer invasion. As would be expected (24, 25), loss of E-cadherin was found in SW620 cells. More interestingly, we discovered a significant increase of vinculin accumulation in SW620 cells as compared with SW480 cells. We further characterized that SW480 cells are tightly attached to culture dishes as compared with SW620 cells. Taken together, these results suggest that Nox1, as it is normally highly expressed in normal colon tissue, may not be a good biomarker for colon cancer progression; rather, the conventional adhesive molecule E-cadherin and vinculin maybe better choices.

Vinculin is a molecule localized at cell–cell junction and plays a key role in controlling cell motility (26). vinculin exhibits very different function in 2D and 3D cell culture models: in 2D model, less vinculin renders cells to become more motile and more invasive; however, in 3D model, the outcome is opposite: more vinculin renders cells more invasive (27). Our data in this study clearly show that although Nox1 is not associated with colon cancer progression, metastatic SW620 cells accumulated about threefold of vinculin protein than did the SW480 cells, which may contribute to the invasiveness of SW620 cells. Hence, vinculin might be a biomarker for colon cancer progression. More detailed study on human colon cancer tissues will be needed to confirm this finding.

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## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Nox1 | NADPH Oxidase 1 |
| DAPI 4'-6 | Diamidino-2-phenylindole, dihydrochloride |
| DPI | Diphenyliodonium chloride |
| ROS | Reactive oxygen species |
| IHC | Immunohistochemistry |

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