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TITLE: Rit42 is an Energy-Governor Molecule Which Prevents Over-Synthesis of ATP

PRINCIPAL INVESTIGATOR: Win-Jing Young, Ph.D.
Ivan Ding, M.D.
Paul Okunieff, M.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14627

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Win-Jing Young, Ph.D.
Ivan Ding, M.D.
Paul Okunieff, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Universitv of Rochester
Rochester, New York 14627

E-Mail:
ivan@radonc.medinfo.Rochester.edu

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13. ABSTRACT *(Maximum 200 Words)*
RTP/DRG/Cap43/Rit42 gene is highly expressed in normal prostate cells, but is weakly expressed in prostate cancer cells. The inducible expression by hypoxia is low in normal prostate cells, but is significantly increased in prostate cancer cells. In this study, we report that radiation and various anti-androgens including hydroflutamide, Casodex, and cyproterone acetate greatly induce RTP/DRG/Cap43/Rit42 mRNA expression in a time- and dose-dependent manner. The effect is observed in both human breast tumor (MCF-7) and in prostate cancer cells (LNCaP), but not in cultured normal prostate epithelial cells. The in vitro growth rate of MCF-7 cells was markedly reduced after stable transfection of the Rit42 gene using a tetracycline-inducible system. A yeast two-hybrid study demonstrated that RTP/DRG/Cap43/Rit42 protein binds with cytochrome C oxidase VIb. In this work, RTP/DRG/Cap43/Rit42 gene expression was found to be induced by hypoxia, implying that its association with cytochrome C oxidases may play an important role in regulation of synthesis and consumption of ATP in tumors. Involvement of this gene in the control of cell growth and metabolism, as well as the high inducibility of the gene in tumor cells by hypoxia, radiation, or anti-androgens suggest that RTP/DRG/Cap43/Rit42 might be a novel stress response protein.

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Rit42 is an Energy Governor Molecule, which Prevents Over-synthesis of ATP

INTRODUCTION

Rit42 is expressed in normal breast tissue, but not in breast cancer (1, 2). As is generally known, ATP is the energy source for most cellular chemical reactions. We believe that Rit42 provides a switch that, when turned off, allows the cancer cells to generate ATP at excessively fast rates. Conversely, if Rit42 could be turned on in breast cancer cells, ATP would not be generated at a fast rate, and breast tumor cells would deplete their ATP stores in minutes resulting in either death, or at best, very slow growth. In addition, restoration of controls on ATP synthesis rates would allow the tumor to oxygenate to normal levels by decreasing oxygen consumption. This will greatly improve the tumor's response to radiation and many chemotherapies including the main drugs, doxorubicin, paclitaxel, and cytoxan.

PRELIMINARY RESULTS

We previously proposed that Rit42 controls the rates of synthesis and utilization of energy-rich ATP on the basis that the overexpression of Rit42 gene caused MCF-7 breast tumor cell growth inhibition in vitro (3). Therefore, we postulated that Rit42 acted like the governor on an engine during breast tumor cell growth. Loss of the governor might result in higher ATP utilization rates. In cancer cells, the expression of this molecule is suppressed, such that the energy produced can be maximized for rapid tumor cell growth. By introducing that Rit42 molecule into breast cancer cell MCF-7, we predicted Rit42 overexpressed MCF-7 cells should have: 1) a reduction of the metabolic rate, 2) decreased oxygen utilization, and 3) slower growth rates. Additionally, Rit42 would prevent hypoxia by reducing oxygen utilization, and therefore should also improve radiation response of breast cancer. However, our in vivo data did not support this hypothesis.

1. <u>Rit42 transfectants did not alter tumorigenicity of parental MCF-7 breast tumor cells</u>

$10X10^6$ Rit42 transfected MCF-7 clone (SN22) and its vector control were grown in the nude mice. The tumor formation rate and tumor sizes were measured and recorded at 20 days after inoculation of tumor cells. As shown in Table 1, no significant difference in tumor formating frequency among parental, vector and Rit42 positive clone SN22, regardless doxycyclin treatment. Tumor size ranged from 100-250 mm^3, no significant difference was observed among each tumor cell line tested. Although SN22 grew slower than vector control in vitro in our previous study, the Rit42 mediated breast tumor growth inhibition was not observed in our animal study. In order to confirm this observation, we plan to further investigate the role of Rit42 in tumor development and progression in other tumor models. Future experiments will be:

a. Transfection of Rit42 gene into more malignant breast tumor cell lines, such as, MDA-MB231 or MDA-MB435 to confirm the ability of Rit42 tumor suppression. Because MCF-7 parental cells did not form aggressive tumor in nude mice, more malignant tumors are need using as parental cell.

b. Transfection of Rit42 gene into other histological types of malignant tumor cells, such as, colon and esophageal adenocarcinomas, to determine the antitumoral ability of Rit42.

Table 1: Tumor formation in the nude mice after inoculation of Rit42 transfectants

	Dox (-)	Dox (+)
MCF-7	75%	80%
Vector	80%	83%
SN22	60%	66%

N = 4-8 mice, 5 mg Tetracyclin was given intragastrically foı 3 days

2. <u>Radiation induced Rit42 mRNA expression in breast tumor cell lines</u>

During our investigation, one of our collaborators, Dr Salnikow (NYU, New York) reported that several stress-related agents induce Rit42, particularly by hypoxia (4, 5). He also found that the induction by hypoxia of the HIF-1 dependent genes Rit42 was the highest in the most aggressive prostate cancer cells compared to less malignant cancer cells. Because these advanced prostate cancer cell lines have lost p53 function, they suggested that this shifts a balance from p53 to HIF-1 transcriptional regulation, and a high ratio of HIF-1-dependent:p53-dependent transcription was a marker of the advanced malignant phenotype. Since we did not observed that Rit42 had direct antitumoral effects in our Rit42-transfected MCF-7 model, we planed to examine if Rit42 can play a role as a hypoxia inducible protein in breast cancer shown by Dr Salnikow. We found that not only hypoxia induced Rit42 mRNA expression, radiation also caused elevation of Rit42 mRNA in these breast tumor cells. As shown in Figure 1, using a series of breast tumor cell lines with different malignant phenotypes (MCF-7 as a parental; ML-20 is β-glacosidase gene transfected MCF-7 cell; FGF1 and FGF4 transfected MCF-7 clones, and FGF1 transfected MCF-7 clone metastasized to lung), we found that more malignant tumor lines (FGF1 transfected MCF-7 primary tumor and FGF1/MCF-7 lung metastatic cells) had higher levels of Rit42 mRNA after radiation compared to the parental and ML-20 cells. This indicates that Rit42 is very sensitive to hypoxia and radiation. Although the expression of the hypoxia-responsive Rit42 under normoxic conditions did not differ dramatically in all of the tumor cell lines, the inducible levels correlated with tumor cell malignant potential.

3. *Rit42 transfected MCF-7 tumor cells had altered sensitivity to Taxanes and radiation, and increased therapeutic resistance did not correlate with G2/M phase*

Since radiation induced Rit42 gene expression in more malignant cancer lines it suggests that rit42 could offer tumor cells a protective effects to the harmful microenvironment (4, 6). We then determined the effects of Rit42 overexpression on tumor cell responses to chemoagents and radiation, using Rit42 transfected MCF-7 and vector control in clonogenic assay. Rit42 positive clone (SN22) had decreased Taxanes response after doxycyclin addition (Fig 2a and 2b). Similarly, SN22 clone had slightly increased radiation resistance compared to vector control (Fig 2c). The Rit42 mediated elevation of drug and/or radiation resistance was particularly obvious when 150nM Taxol was combined with radiation (Fig 2d). It indicates that Rit42 overexpressing cells had tendency to obtain ability of drug and radiation resistance. This phenotype may render tumor cells had better survival ability in a harmful microenvironment.

In general, alteration of G2/M phase in tumor cells plays an important role to determine drug or radiation sensitivity. However, we did not observe significant change for G2/M fraction in Rit42 transfectants compared with vector control after Taxol (Fig 3a) and Taxotere (Fig 3b) treatment. Taxanes can significantly induced G2/M arrest and increase radiation sensitivity. It suggested that Rit42-mediated drug and/or radiation resistance was not associated with the alteration of cell cycle.

4. *Rit42 transfected breast tumor cells overexpressed angiogenic-related chemokine mRNA*

Several angiogenic-related chemokines mRNA was induced in SN22 and SN28 Rit42 positive clones (Fig 4). Elevation MCP-1, Rantes (C-C family chemokine) and IL-8 (C-X-C family chemokine) mRNA were found in both two clones even without Doxycyclin treatment. Because overexpression of angiogenic growth factors and related cytokines have been documented to have radioprotective effects for tumor and endothelial cells (7), it is likely that Rit42-mediated the induction of angiogenic–related chemokines may provide tumor cells more resistance phenotypes to radiation or chemotherapeutic agents.

5. *Rit42, as a stress-inducible protein, associated with hypoxia maker EF5 staining in breast tumor tissues*

Another interesting observation was that the association between Rit42 protein expression and hypoxia maker EF5 staining in both murine and human breast tumor tissues. Frozen murine mammary tumor tissues (MCa-4) were stained with anti-Rit42 antibody shown in orange color (Fig5a). The same tumor section was subsequently stained with anti-EF5 antibody (8) shown in red color (Fig5b). It is clearly demonstrated that Rit42 and EF5 colocalized in tumor tissues (Fig5c). More hypoxic areas in tumors had stronger staining for both markers. It indicated that it is likely Rit42 can be used as hypoxia maker to identify the tumor hypoxia. Using human primary breast tumor tissue paraffin sections, we also found that strong Rit42 immunoreactivity was localized in tumor cells compared to normal mammary glands (Fig6 a). Strong Rit42 staining was not only found in poorly

differentiated (Fig 6b) and well-differentiated breast adenocarcinoma (Fig 6c), but positive staining was also observed in lymphatic metastatic tumor cells (Fig 6d).

KEY RESEARCH ACCOMPLISHMENTS

CONCLUSIONS

Cyclic hypoxic stress, such as that known to occur in tumors, exerts an extremely powerful selection against p53-dependent cell death. Tumor cells that survive severe hypoxic stress acquire resistance to radio- and chemotherapy through the selection of p53 mutant cells. The use of Rit42 as a hypoxia marker could provide a method of selecting patients that might benefit from anti-hypoxia therapies as well as aid in the evaluation of methods for tumor hypoxia. Our exciting observation that the Rit42 gene was colocalized with a validated hypoxia marker EF5 clearly demonstrated that the possibility of application of Rit42 as an intrinsic hypoxia marker. Localized areas of tumor hypoxia detected by EF5 are assessed in frozen sections by immunohistochemical identification of sites of 2-nitroimidazole metabolism (9). A pentafluorinated derivative (EF5) of etanidazole was i.v. injected one hr prior to tumor freezing and has been shown to be well distributed through even poorly perfused regions of the tumors. Regions of high EF5 metabolism were then visualized immunohistochemically using a fluorochrome (Cy3) conjugated to the ELK-51 antibody. This antibody is extremely specific for the EF5 drug adducts that form when the drug is incorporated by hypoxic cells. However, the requirement for intravenous injection of EF5 as well as technical limitations in its quantification limit the future application of this hypoxia maker in the clinic. If Rit42 localizes in a pattern that mimics EF5, these limitations are avoided. However, several questions remain: 1) Can an endogenous hypoxia marker be used in clinical samples to determine the tumor oxygenation? 2) If so, can we use this marker as a biological marker to monitor treatment response after chemotherapy or radiotherapy? Currently we have RO1 application pending for investigating this possibility.

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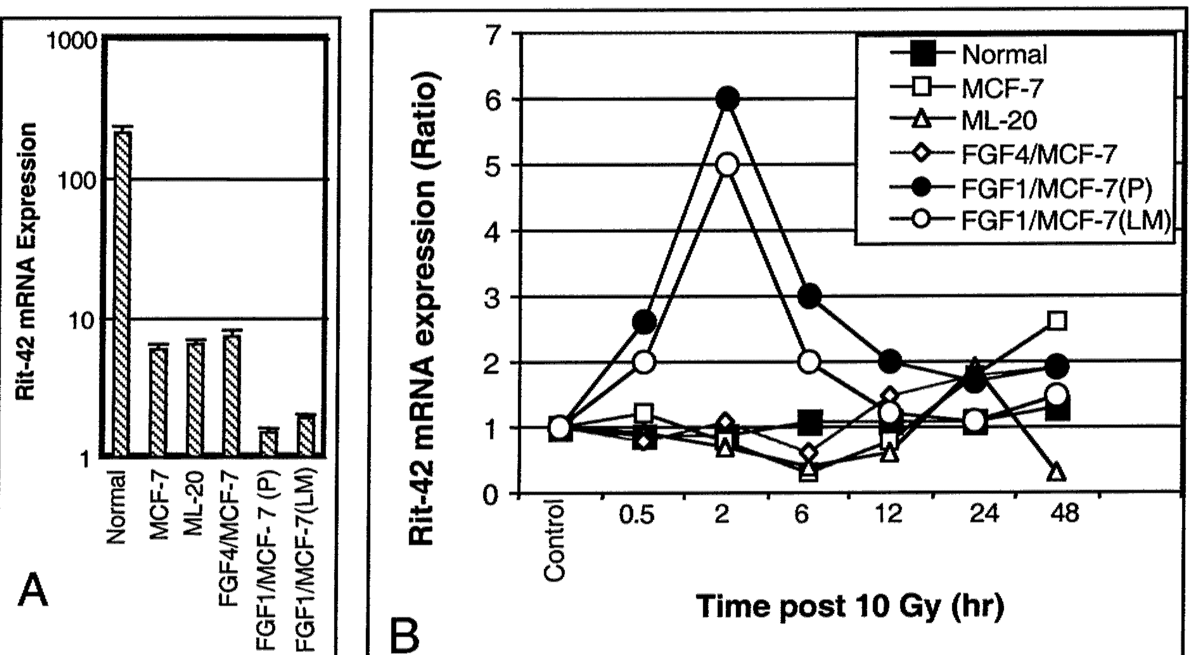


Fig.1. Comparison of Rit42 levels in breast tumor cell lines

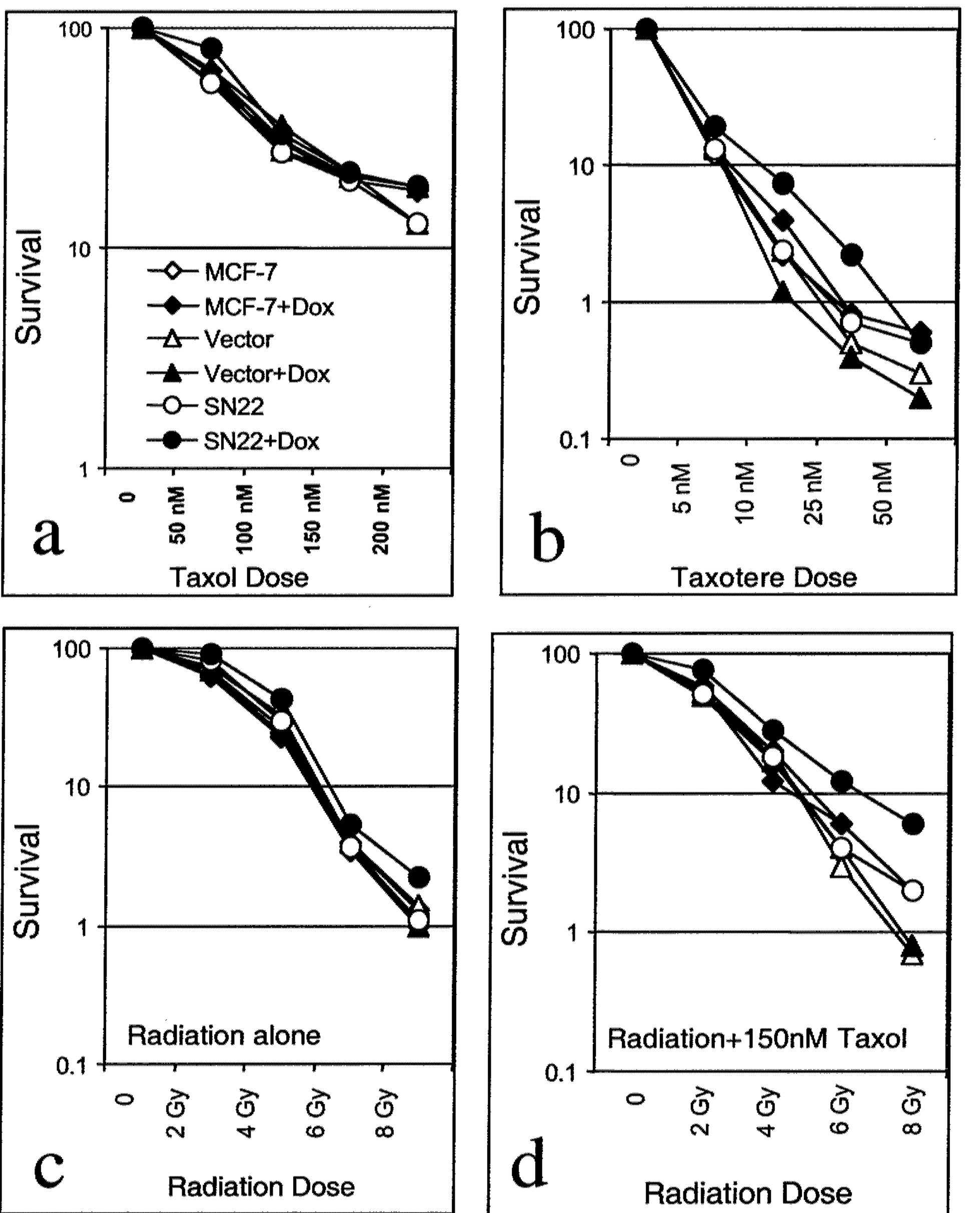


Fig 2. The effects of Rit42 overexpression on tumor cell responses to chemagents (a & b) and radiation (c & d)

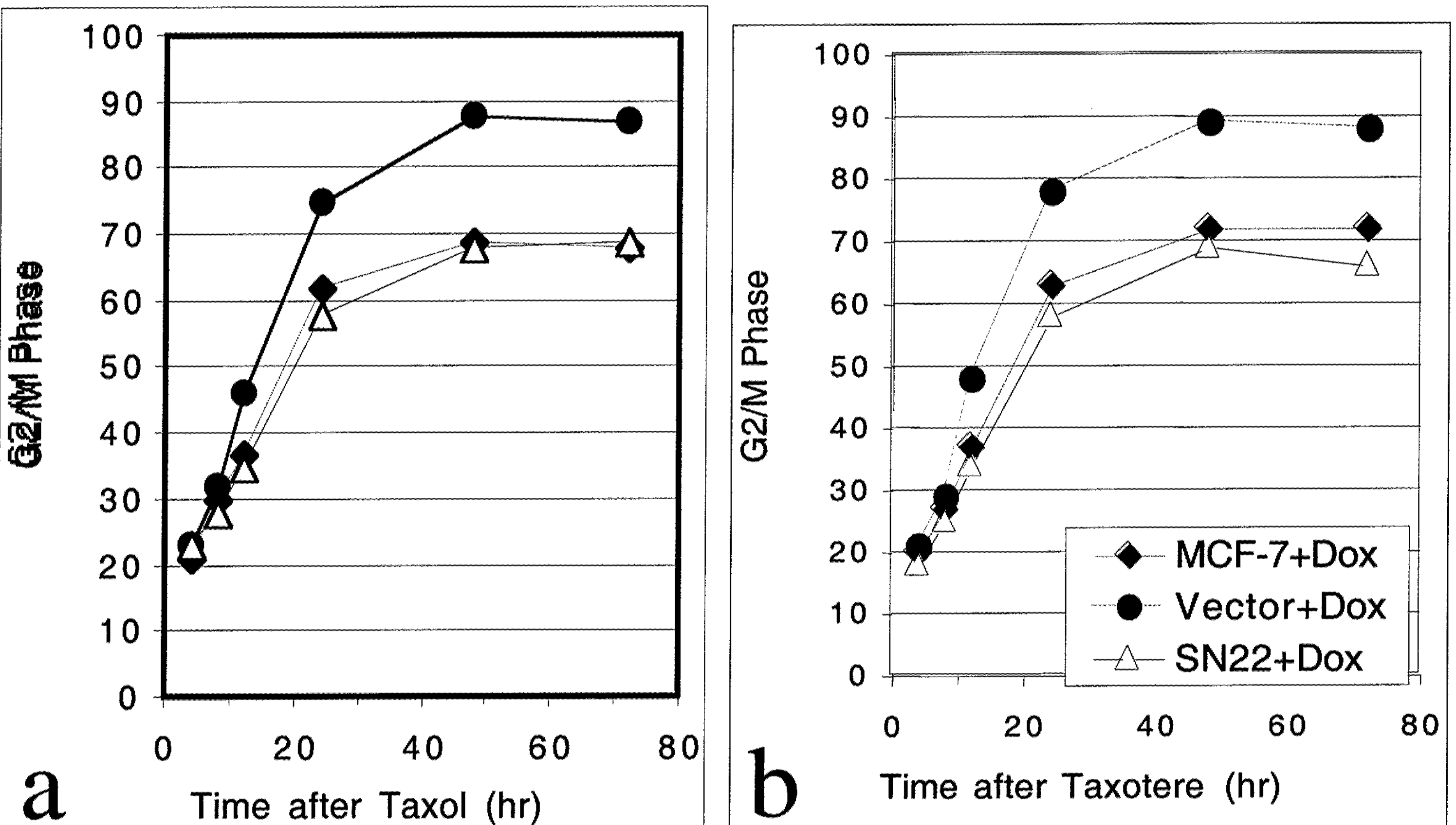


Fig 3. Alteration of G2/M phase in Rit42 transfected tumor cells after Taxol (a) and Taxotere (b) treatment

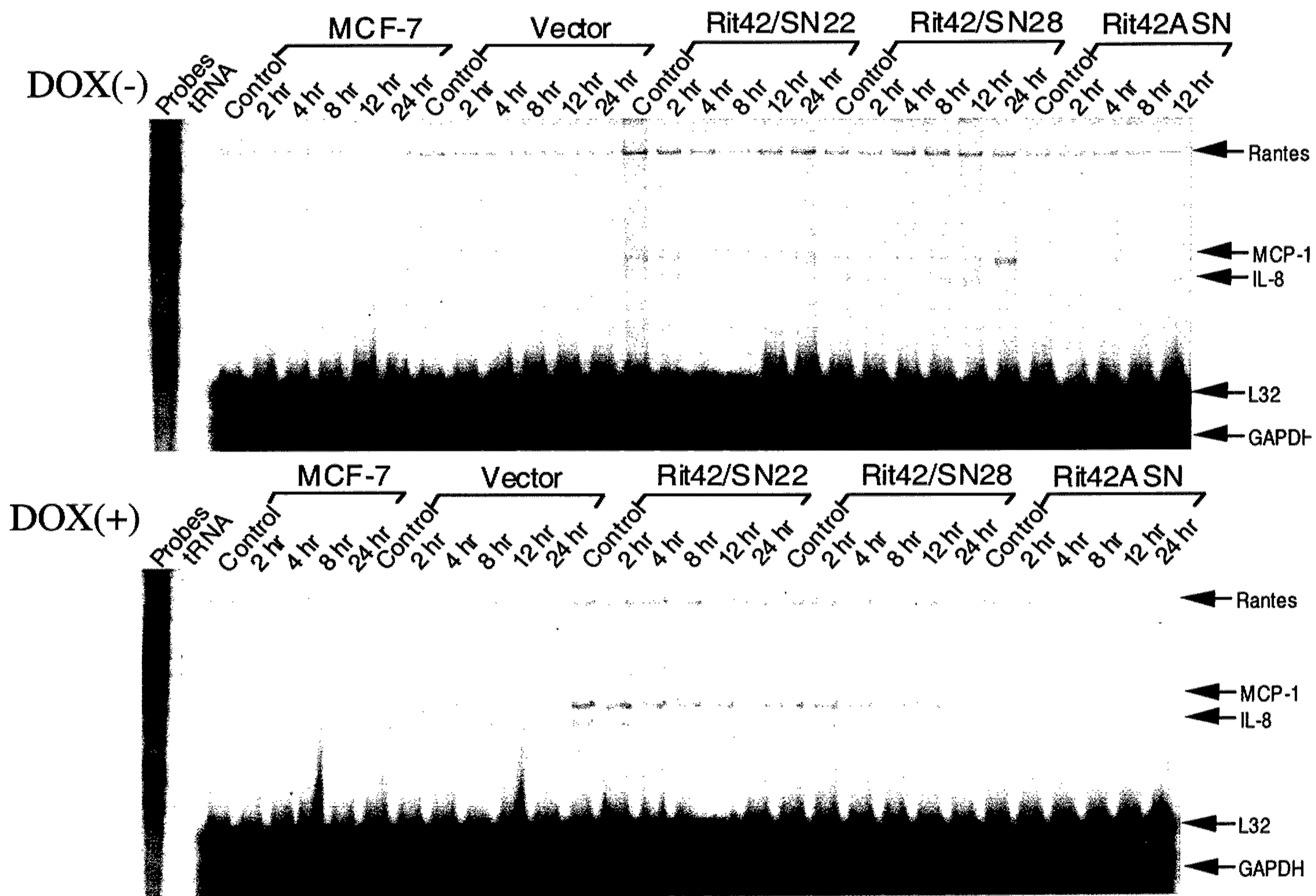
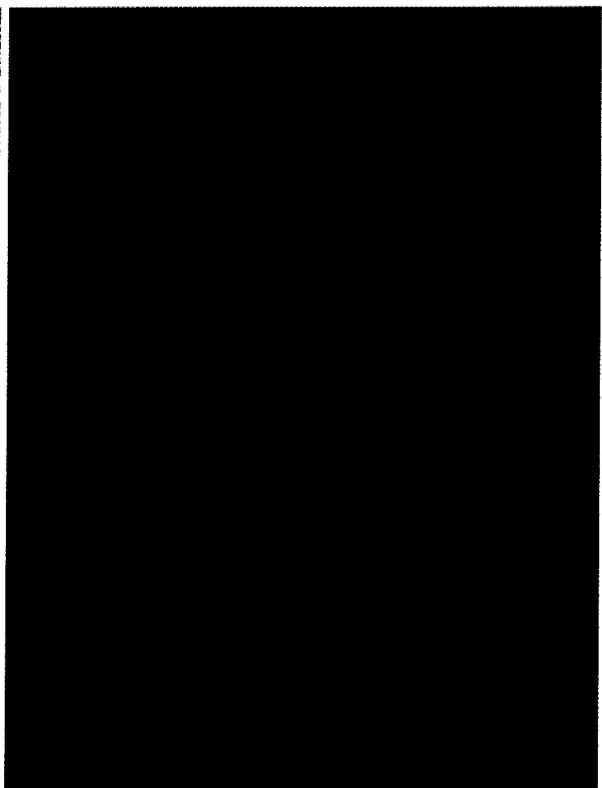


Fig 4. Induction of angiogenic-related chemokines mRNA in SN22 and SN28 Rit42 positive clones



NDRG1 EF5/Cy3 Co-localization

Fig 5. MCa-4 mammary tumor tissues were stained with anti-Rit42 antibody shown in orange color (a). The same tumor section was subsequently stained with anti-EF5 antibody shown in red color (b). It is clearly demonstrated that Rit42 and EF5 co-localized in tumor tissues (c).

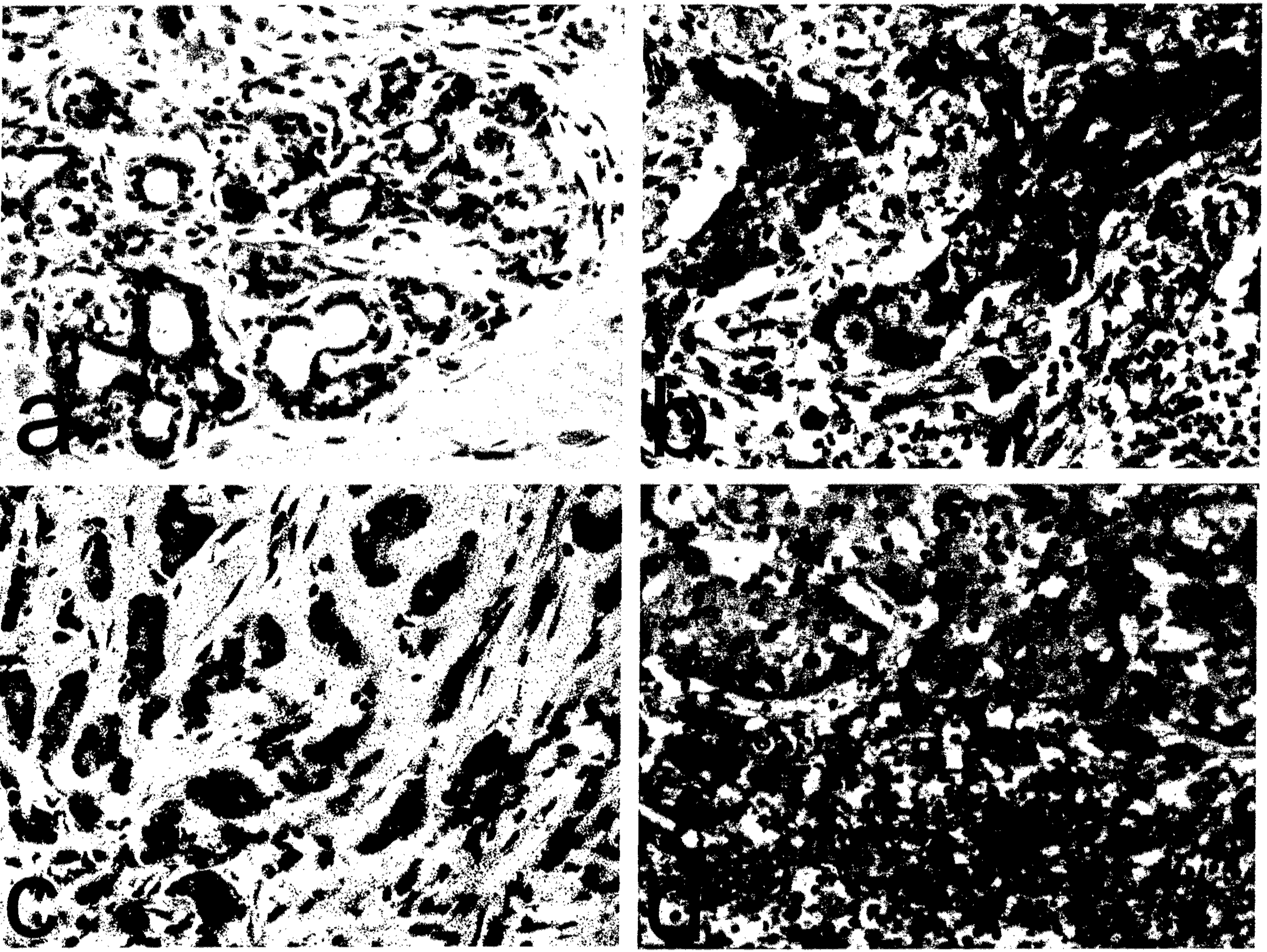


Fig 6. Rit42 immunoreactivity in paraffin sections of human breast tumor tissue is localized in tumor cells compared to normal mammary glands (a). Strong Rit42 staining was not only found in poorly differentiated (b) and well-differentiated breast adenocarcinoma (c), but positive staining was also observed in lymphatic metastatic tumor cells (d).

List of Personnel receiving pay from this research effort

No one was paid salary from this grant