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Inhibition of the mammalian target of rapamycin sensitizes U87 xenografts to fractionated radiation

J.N. Sarkaria, B.L. Carlson, A.C. Mladek, J.S. Eshleman. *Mayo Clinic, Department of Oncology, Rochester, USA*

Rapamycin is a highly selective inhibitor of mTOR signaling pathways, and rapamycin analogs are currently being evaluated in Phase I and II clinical trials as single agent therapy for recurrent GBM. The mammalian target of rapamycin (mTOR) modulates key signal transduction pathways in malignant gliomas that promote cell survival, tumor proliferation and angiogenesis, and these cellular processes may contribute to the clinical radioresistance of glioblastomas. Therefore, in anticipation of possibly using rapamycin in primary GBM patients, the combination of rapamycin and radiation was evaluated in U87 malignant glioma xenografts in a regrowth delay assay. Compared to sham irradiation, treatment of U87 flank xenografts with 4 Gy $\times$ 4 fractions delivered over 18 days was ineffective in these radioresistant tumors (regrowth delay of -0.2 $\pm$ 3 d, mean $\pm$ 95% CI). In contrast, radiation combined with rapamycin was significantly more effective than rapamycin alone (regrowth delay 19.1 $\pm$ 6.3 d). Rapamycin also sensitized U87 xenografts to a single 11 Gy dose of radiation, although the degree of sensitization was less profound than with fractionated radiation. This rapamycin-mediated sensitization could result from disruption from either tumor- or host-dependent processes. To distinguish between these possibilities, the combination of rapamycin and radiation was evaluated *in vitro* in U87 spheroids and monolayer culture. Similar to the xenograft studies, fractionated radiation alone had minimal effect on spheroid regrowth compared to control treatment (regrowth delay of 5.0 $\pm$ 3.8 d), while radiation combined with rapamycin was significantly more effective than rapamycin alone (regrowth delay of 10.2 $\pm$ 3.2 d). Interestingly, rapamycin had no effect on clonogenic survival following a single dose of radiation in U87 cells growing in monolayer culture.

Taken together, these data suggest that rapamycin disrupts an mTOR-dependent signaling pathway important for recovery following ionizing radiation specifically in cells growing in the context of a solid tumor mass either *in vivo* or *in vitro*. More importantly, these results provide a strong rationale for the clinical evaluation of the combination of rapamycin and radiation in patients with glioblastoma multiforme.

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Transcriptional response to ionizing radiation (IR) in normal and tumor cells

N.N. Khodarev[1], J.O. Park[1], T. Darga[1], E. Nodzenski[1], B. Roizman[2], R.R. Wechselbaum[1]. *[1]University of Chicago, Radiation and Cellular Oncology, Chicago, USA; [2]University of Chicago, The Marjorie B. Kovler Viral Oncology Laboratories, Chicago, USA*

The objective of radiation therapy is to deliver a lethal dose to cancer cells but attenuate the toxic effects of IR on adjacent normal tissue. Undesirable sequels of radiotherapy are the development of tumor resistance and normal tissue damage. Profiling of RNA on DNA arrays provide a method to study the response of thousands of genes to ionizing radiation. We used this approach to profile the transcriptional response to various doses of irradiation in primary human embryonic lung (HEL) fibroblasts and the U87 human malignant glioblastoma cell line and in U87 tumor xenograft implanted in mice. Doses ranged from 1 to 3 Gy, which are commonly employed in radiotherapy to 10 Gy which is frequently employed to study biochemical responses of mammalian cells to IR and in radiosurgery of some brain tumors. Data acquisition and filtration were optimized with receiver operator characteristic (ROC) approach for control and reduction of false positive signals. Data confirmation was based on semi-quantitative conventional RT-PCR and real-time PCR using SYBR Green. Data showed relatively low complexity of transcriptional response to ionizing radiation in the terms of temporal patterns of genes, induced by different doses. More then 90% of all responding genes were separated in 4 major expressional clusters. Functional classification showed non-random distribution of different functional groups in different expressional clusters. This analysis may give temporal specificity to intervention strategies by identifying genes or clusters with specific temporal patterns that may be selected as molecular targets for radiotherapy modification. An example of such a gene is Epidermal Growth Factor Receptor (EGFR). We observed a 4-5 fold induction of the EGFR gene following exposure to 1 or 3 Gy, at the 1 or 5 hour time points, and 6-7 fold induction at all doses at 24 hours. Anti-EGFR agents such as tyrosine kinase inhibitors currently employed in the clinical setting, can be combined with a defined ionizing radiation regimen to enhance its effect. Data suggest that modification of radiotherapy regimens to account for both radiation-dose and timing specificity of cellular responses may significantly enhance clinical outcome of IR treatment. Data also provide valuable information for the selection of possible targets for optimized temporally -modulated combined radiotherapy.

Apoptosis

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Phosphorylation on Tyrosine-15 of p34Cdc2 by ErbB2 Receptor Tyrosine Kinase Inhibits p34Cdc2 Activation and Is Involved in Resistance to Taxol-Induced Apoptosis

D. Yu[1,3,4], M. Tan[1]. *[1]Univ. of Texas M.D. Anderson Cancer Center, Dept. of Surgical Oncology, Houston, USA; [2]Department of Molecular Pathology, [3]Department of Molecular and Cellular Oncology,*

Overexpression of p185ErbB2 confers resistance to taxol-induced apoptosis by inhibiting p34Cdc2 activation but the inhibitory mechanisms are not fully understood. Here we report that the inhibitory phosphorylation on tyrosine (Tyr)15 of Cdc2 (Cdc2-Tyr15-p) is elevated in ErbB2-overexpressing breast cancer cells and primary tumors independent of Wee1, Cdc25C, and p21Cip1. ErbB2 can bind to and co-localize with cyclin B-Cdc2 complexes and phosphorylate Cdc2-Tyr15. The ErbB2 kinase domain is sufficient for binding to and directly phosphorylating Cdc2 specifically on Tyr15. Increased Cdc2-Tyr15-p in ErbB2-overexpressing cells corresponds with delayed M-phase entry. An ErbB2 tyrosine kinase inhibitor and a kinase-dead ErbB2 inhibited Cdc2-Tyr15-p. ErbB2-overexpressing cells expressing the non-phosphorylatable Cdc2Y15F mutant are more sensitive to taxol-induced apoptosis than cells not expressing Cdc2Y15F. Thus, ErbB2 membrane RTK may directly phosphorylates Cdc2, which operates in the nucleus.

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Expression of nuclear BCL10 is highly correlated with the expression of nuclear NF-kB and is predictive of helicobacter pylori-dependent status in early-stage high-grade gastric malt lymphoma

S.H. Kuo[1,3], L.T. Chen[2], P.Y. Yeh[1,3], C.L. Chen[4], I.J. Su[5], J.T. Lin[6], A.L. Cheng[1,3,6]. *[1]National Taiwan University Hospital, Oncology, Taipei, Taiwan; [2]National Health Research Institutes, Division of Cancer Research, Taipei, Taiwan; [3]National Taiwan University College of Medicine, Cancer Research Center, Taipei, Taiwan; [4]China Medical College Hospital, Pathology, Taipei, Taiwan; [5]National Taiwan University Hospital, Pathology, Taipei, Taiwan; [6]National Taiwan University Hospital, Internal Medicine, Taipei, Taiwan*

Background: We have recently demonstrated that around 60% of early-stage high-grade gastric mucosa-associated lymphoid tissue (MALT) lymphomas are rendered durable tumor remission by eradication of H pylori (J Clin Oncol 2001; 19:4245 -51). However, the histologic and molecular features that help predict the H pylori-dependent state of these tumors remains elusive. The intracellular signal protein BCL10 was identified by its direct involvement in t(1;14)(p22;q32) of MALT lymphomas, and is a putative regulator of antigen-receptor-mediated NF-kB activation. Upregulation of BCL10 may trigger a constitutive NF-kB signal from the antigen receptor, and therefore may contribute to antigen-independent growth and progression of gastric MALT lymphoma.

Purpose: The present study sought to investigate the correlation between nuclear BCL10 expression and nuclear NF-kB expression, and the correlation of their expression with the tumor resistance to H pylori eradication therapy in patients with stage IE high-grade gastric MALT lymphoma.

Materials and Methods: Lymphoma biopsies of all patients, who had participated in a prospective study of H pylori-eradication for stage IE high-grade gastric MALT lymphoma, were collected. The H pylori-dependent status was verified by the results of the prospective clinical trials. There were 13 patients with H pylori-dependent, and 7 patients with H pylori-independent high-grade gastric MALT lymphoma. The expression of BCL10 and NF-kB in pre-treatment paraffin-embedded lymphoma tissues was determined by immunohistochemistry with anti- BCL10 antibody (polyclonal; 1:10; Santa Cruz Biotechnology) and anti-NF-kB RelA (p65; 1:150; Santa Cruz Biotechnology). A semi-quantitative method was used to determine the level of expression of RelA. Reactive spleen and lymph nodes tissue sections were used as the control. The co-expression of nuclear BCL10 and NF-kB activity was further analyzed by a confocal immunofluorescence microscopy.

Results: The aberrant nuclear BCL10 expression was detected in six (86%) of 7 H pylori-independent high-grade gastric MALT lymphomas but none in 13 H pylori-dependent cases (P<0.001). All six patients with nuclear BCL10 expression had co-expression of nuclear NF-kB, while only two of 13 patients without nuclear BCL10 expression did so (P=0.005). Interestingly, the latter two patients were found to have tumor invasion of the gastric muscularis propria. There was a significant correlation between nuclear expression of BCL10 and NF-kB activation (P<0.001). Furthermore, the nuclear co-localization of RelA and BCL10 was confirmed by confocal immunofluorescence microscopy. The frequency of nuclear translocation of RelA was also significantly higher in H pylori-independent tumors than those H pylori-dependent tumors (6 of 7=86% versus 2 of 13=15%, P=0.004).
Conclusion: The results of this study suggest that nuclear BCL10 expression is closely associated with the nuclear NF-kB expression and support the hypothesis that nuclear BCL10 may activate NF-kB. Detection of the nuclear expression of either BCL10 or NF-kB is highly useful in the prediction of H pylori-dependent state of early-stage high-grade gastric MALT lymphoma. (This work was supported by grants from NSC91-3112-B-002-009, NHRI-91A1-CANT-1 and NTUH 91-N007)

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Identification of survivin as a transcriptional target of the Wnt pathway

P. Kim[1], H. Clevers[2], D.C. Altieri[3]. [1]*Yale University School of Medicine, Boyer Center for Molecular Medicine, Department of Pathology;* [2]*University Medical Center Utrecht, Department of Immunology, and Center for Biomedical Genetics;* [3]*Yale University School of Medicine, Boyer Center for Molecular Medicine, Department of Pathology, New Haven, USA*

Survivin, a member of the inhibitor of apoptosis (IAP) family, is found in most cancers while undetectable in normal terminally-differentiated cells. Expressed in mitosis, survivin has been shown to inhibit apoptosis in cancer cells. Its expression is predictive of poor outcome in colorectal cancer, the second largest cause of cancer deaths in the United States. Most colorectal cancers have mutations in the Wnt pathway, where inactivation of the gatekeeper adenomatous polyposis coli (APC) inhibits its ability to bind and regulate beta-catenin. This allows abnormally high levels of beta-catenin to complex with T-cell factor (TCF) and transcriptionally activate target genes of the Wnt pathway. The objective of this study is to test a potential association between survivin and Wnt-dependent oncogenesis. Analysis of the survivin promoter yielded three potential TCF binding elements (TBE). Plasmids were constructed with increasing lengths of survivin promoter inserted upstream of a luciferase reporter gene. Promoter analysis resulted in 8 to 12-fold increased transcriptional activity in colorectal cancer cell line HCT-116, which has active Wnt signaling, as compared to HeLa cells, which have an inactive Wnt pathway. Co-transfecting plasmids containing TCF sites with beta-catenin cDNA in HCT-116 cells resulted in 2.5 to 4-fold increased transcriptional activity. Mutagenesis of each of the two most proximal TBE sites showed at least a 75% reduction in transcriptional activity compared to the wild-type construct. Transfection with a dominant-negative TCF-4 plasmid resulted in a 50% decrease in transcriptional activity as compared to control plasmid. Radioactive probes corresponding to each of the three TBE sites were shifted by nuclear HCT-116 extracts and were fully competed by molar excess of unlabeled probes in electrophoretic mobility shift assays. Survivin staining by immunohistochemistry is nearly absent in TCF-4 knockout embryonic mouse intestine. These data identify survivin as a direct transcriptional target of the Wnt/beta-catenin pathway. The overexpression of survivin in colorectal cancer via Wnt activation may contribute to modulation of apoptosis during linear step-wise tumorigenesis.

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Involvement of proapoptotic molecules Bax and Bak in TRAIL-induced apoptosis

R. Srivastava, K. Kandasamy. *University of Maryland, Department of Pharmaceutical Sciences, Baltimore, USA*

TRAIL/Apo-2L is a member of the tumor necrosis factor superfamily and has been demonstrated to induce apoptosis in a wide variety of cancer and transformed cells. Activation of BID, a 'BH3-domain-only' Bcl-2 family member, triggers the oligomerization of proapoptotic family members BAK or BAX, resulting in the release of cytochrome c from mitochondria to cytosol. In this study, we have shown the importance of BAX and BAK in TRAIL-induced apoptosis by studying in murine embryonic fibroblasts (MEFs) from Bax-/- Bak-/- animals. TRAIL induced cytochrome c release and apoptosis in wild type, Bid-/-, Bax-/-, or Bak-/- MEFs, but not in Bax-/- Bak-/- double knockout (DKO) MEFs. Bid, which functions upstream of cytochrome c release, was found to be cleaved in all the knock out cells except in Bid-/- MEFs. The release of cytochrome c was correlated with caspase-9 activity. TRAIL increased caspase-3 activity in all the cells except the Bax-/- Bak-/- DKO cells. TRAIL-induced mitochondrial membrane potential reduction was not observed in Bid-/-, Bax-/-, and DKO MEFs suggesting that preventing a mitochondrial membrane potential reduction alone was not sufficient to prevent TRAIL-induced apoptosis. However, microinjection of cytochrome c in DKO MEFs induced apoptosis indicating events downstream of cytochrome c are intact. Together the data suggest that activation of Bax or Bak appears to be an essential gateway to mitochondrial dysfunction required for apoptosis in response to TRAIL.

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Inhibition of PI3 kinase causes cell death through a PKB dependent mechanism and growth arrest through a PKB independent mechanism

A.R. Gottschalk[1,2], A. Doan[1], J.L. Nakamura[1], D.A. Haas-Kogan[1,2], D. Stokoe[2]. [1]*Radiation Oncology;* [2]*Comprehensive Cancer Center, University of California, San Francisco, USA*

PTEN is one of the most commonly inactivated genes in human cancer. The mechanism by which PTEN exerts its tumor-suppressive effects has been elucidated. PTEN regulates the activity of the PI3K pathway. PI3K transduces cellular signals by phosphorylating the 3' position of phosphoinositides thus generating the second messengers PIP2 and PIP3. PTEN antagonizes this signal by dephosphorylating the 3' position of PIP3 and PIP2. PIP3 and PIP2 activate downstream effectors of PI3K. One effector of PI3K in this pathway is PKB. PKB mediates a discrete set of responses including cell survival,proliferation, migration and angiogenesis, implicating the PI3K/PKB pathway in cancer development. LNCaP is a human prostate carcinoma cell line isolated from lymph node metastasis. LNCaP is commonly used as a model for human prostate cancer because the cells are hormone dependent and express PSA. LNCaP cells have a mutation in the PTEN gene and subsequent activation of PKB. Introduction of wt PTEN into LNCaP cells, or treatment with the PI3K inhibitor LY294002, causes inactivation of PKB, growth arrest and apoptosis. While treatment with LY294002 causes inactivation of PKB and apoptosis the exact mechanism of apoptosis has not been elucidated. To specifically test the hypothesis that inhibition of PKB is responsible for the LY294002-induced apoptosis, LNCaP cells expressing a constitutively active form of PKB were generated. PKB is activated by phosphorylation on ser-473 and thr-308. Mutating these residues to aspartic acid (S473D/T308D) mimicsphosphorylation and results in activation of PKB. PKB activity is also regulated by the PH domain. Deletion of the PH domain (∗PH) leads to increased activity. Combining the S473D/T308D mutant with the ∗PH mutant generates a constitutively active PKB (∗PH-PKB-DD). The ∗PH-PKB-DD form of PKB cannot be inhibited by LY294002. LNCaP cells expressing the ∗PH-PKB-DD were generated. Activity of the ∗PH-PKB-DD protein and the inability to be inhibited by LY294002 will be confirmed by western blot analysis. Apoptosis and growth arrest were measured in the LNCaP ∗PH-PKB-DD cells and compared with control cells expressing vector alone. Both cell lines were treated with LY294002 and proliferation and cell death were measured. Expressing constitutively active PKB in LNCaP cells abrogated apoptosis induced by LY294002 but had no effect on the G1 arrest. These data identify PKB as a potential critical target for cancer therapy.

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Claudin-1 induced apoptosis in breast tumor spheroids

R. Macek[1], T. Hoevel[2], K. Swisshelm[3], M. Kubbies[4]. [1]*Roche Diagnostics GmbH, Pharmaceutical Research, Penzberg, Germany;* [2]*Roche Diagnostics GmbH, Pharmaceutical Research, Penzberg, Germany;* [3]*Univ. Washington, Department of Pathology, Seattle;* [4]*Roche Diagnostics GmbH, Pharmaceutical Research, Penzberg, Germany*

Members of the claudin family together with occludin are the major constituents of the tight junction complex (TJ). The human homologue of the murine CLDN1, previously called SEMP1, was identified by molecular genetic analysis, and the CLDN1 mRNA was found to be downregulated or completely lost in human breast cancer cells *in vitro*. In this study we investigated the role of CLDN1 in CLDN1-transduced MDA-MB 361 breast tumor cells in adherent 2D and suspension 3D spheroid cell cultures. Retroviral-induced CLDN1 re-expression in breast cancer cells results in plasma membrane homing of the protein and reconstitution of paracellular flux inhibition, which is not dependent upon presence of occludin protein. There was