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Original Article

Baseline MAPK signaling activity confers intrinsic radioresistance to *KRAS*-mutant colorectal carcinoma cells by rapid upregulation of heterogeneous nuclear ribonucleoprotein K (hnRNP K)

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ABSTRACT

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is overexpressed in malignant tumors and involved in DNA damage response upon ionizing radiation (IR). Here, we investigate its role in radioresistance of colorectal carcinoma (CRC) and evaluate a pharmacological approach to enhance CRC radiosensitivity via downregulation of hnRNP K. We show that hnRNP K is overexpressed in CRC tissue specimens and upregulated in response to IR in vitro, which occurs faster in *KRAS*-mutant CRC cells. HnRNP K knockdown impairs cell survival, cell cycle progression and *KRAS*-dependent radioresistance and increases apoptosis. Using the chicken chorioallantoic membrane assay, a decrease in xenograft tumor growth and radioresistance upon hnRNP K depletion could be verified in vivo, and comparable effects were achieved by suppression of hnRNP K expression using the MEK inhibitor MEK162 (Binimetinib). In summary, *KRAS*-mutant CRC shows intrinsic radioresistance along with rapid upregulation of hnRNP K in response to IR that can effectively be targeted by MEK inhibition. Our results point towards a possible use of MAPK pathway inhibitors to decrease radioresistance of *KRAS*-mutant CRC via downregulation of hnRNP K.

Introduction

In patients with rectal adenocarcinoma, neoadjuvant radiotherapy and/or chemotherapy has been shown to significantly reduce the rate of local recurrence compared to surgery alone [1,2]. However, the tumor response to radiochemotherapy varies widely between individual patients. Efforts have therefore been made to identify biomarkers that predict responsiveness of CRC to neoadjuvant therapy, but so far none is ready for clinical practice [3,4].

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is ubiquitously expressed and involved in transcription, pre-mRNA maturation and translation processes of specific target genes in mammalian cells, including the oncogenes c-src and c-myc [5–7]. HnRNP K has the ability to re-locate from the nucleus to the cytoplasm in response to extracellular stimuli, and cytoplasmic hnRNP K levels are increased in oral squamous cell carcinoma, pancreatic ductal adenocarcinoma and melanoma, why it has been discussed as a potential prognostic biomarker [8–11]. HnRNP K regulates cell proliferation and DNA damage repair through stabilization of the spindle microtubule–kinetochore complex and as a transcriptional cofactor for p53 [12,13]. Exposure to ionizing radiation (IR) leads to rapid phosphorylation of hnRNP K by the ataxia-telangiectasia-mutated (ATM) protein kinase, preventing its MDM2-dependent ubiquitination and proteasomal degradation. Enhanced transcription of hnRNP K/p53 target genes like *CDKN1* provide the cell the required time to repair IR-induced DNA damage, and in line with that, we could show delayed repair of radiation-induced γ H2AX/53BP1-foci upon siRNA-based knockdown of hnRNP K in malignant melanoma [10,13,14].

RAS mutations are common in CRC and have pro-proliferative and survival-promoting effects [15]. Activated MAPK/ERK Kinase (MEK) downstream of RAS has been demonstrated to phosphorylate specific hnRNP K serine residues, leading to cytoplasmic

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accumulation of the protein by nucleo-cytoplasmic shuttling [16]. In our own and others' previous work, pharmacological MEK inhibition led to downregulation of hnRNP K [10,17,18].

In the present study, we assessed the role of hnRNP K in radioresistance of *KRAS*-mutant and *KRAS*-wild-type CRC. Therefore, we analyzed the expression patterns of hnRNP K in patient tumor samples and in CRC cell lines using *KRAS*mut (G12V) SW480 and *KRAS*wt Colo320 CRC cells. SiRNA-mediated knockdown as well as pharmacological inhibition of MEK signaling was used to suppress hnRNP K expression. Finally, the CAM assay was used as in vivo model to investigate possible synergistic effects of hnRNP K knockdown and therapeutic exposure to IR.

Material and methods

Ethics statement

The use of human tissue samples was approved by the ethics committee of the University of Ulm (Approval No. 162/13). CAM-experiments were carried out in compliance with European and German laws for the protection of animals used for scientific purposes.

Tissue samples and immunohistochemical (IHC) analysis

For a more detailed description of experimental procedures, please refer to the Supplementary material. We performed immunohistochemistry as previously described using monoclonal antibodies against hnRNP K, MLH-1, MSH-2, MSH-6 and PMS-2 [19]. We determined IHC scores 0–7 for hnRNP K by assessing cytoplasmic staining intensity as well as the percentage of positively stained cells as previously described [20]. Image analyses for quantification of staining intensities were performed with ImageJ software, v. 1.51 (NIH, Bethesda, USA).

Cell culture and transfection experiments

CRC cell lines SW480 and Colo320 were purchased from Leibniz-Institut DSMZ (Braunschweig, Germany) and transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Mannheim, Germany), Silencer® Select hnRNP K (sequence: 3′-AUAAUCAUAGGUUUCAUCGta; 5′-CGAUGAAACCUAUGAUUAUtt) and Silencer® Select negative control siRNA #1 (both from Life Technologies, Waltham, USA). Transfected cells were harvested after 48 h or underwent further treatment according to the experimental protocol.

Western blot analysis and subcellular fractionation

We performed immunoblotting according to standard methods. Used antibodies and dilution concentrations are listed in Supplementary Table 1. Digital image acquisition was performed using the myECL™ Imager system (Thermo Scientific, Westham, USA). Densitometriy was performed using ImageJ software, v. 1.51 (NIH, Bethesda, USA) and greyscale value ratios were calculated with non-irradiated cells from the identical experiment as a reference. Subcellular fractionation experiments were carried out using the Cell Fractionation Kit- Standard (ab109719, Abcam, Cambridge, UK).

In vitro X-ray irradiation

Exposure of cells to 240 kV X-rays was performed by using the YXLON Maxishot (Hamburg, Germany) with a 3 mm beryllium filter. To guarantee equal surrounding conditions, we stored control cells under equivalent conditions at room temperature during irradiation experiments.

Mutation analyses

Mutation analysis for *KRAS* codons 12, 13 and 61 and codon 600 of *BRAF* from FFPE material and cell lines was performed as previously described using the Pyromark Q24 pyrosequencing platform and the IVD marked therascreen® KRAS and BRAF pyro® kits (Qiagen, Hilden, Germany) [19].

Immunofluorescence (IF) microscopy

IF stainings were performed as previously described [21] using TexasRed-conjugated Phalloidin and a rabbit monoclonal antibody to hnRNP K. A Zeiss Axioimager 2i fluorescence microscope and the ISIS fluorescence imaging system (MetaSystems) were used for image acquisition. Exposure times were adjusted according to the highest staining intensity.

Flow cytometry

Flow cytometric analyses were carried out using the BD FACSCalibur system; image acquisition and data analysis were performed with BD CellQuest Pro software (from BD, Heidelberg, Germany). All experiments were performed in triplicate.

Clonogenic survival assay

CRC cells were seeded in 6-well plates and allowed to incubate for 24 h before treatment/irradiation. Colonies were fixed, stained and counted manually using a Zeiss STEMI SV8 stereomicroscope. Each experiment was performed in quadruplicate.

Chicken chorioallantoic membrane (CAM) assay

We performed the CAM tumor xenograft assay as previously described [22]. CRC cells were seeded onto the CAM and allowed to grow for 4 days. After fixation, tumors were embedded in paraffin, cut into 5 μm-slices and mounted on poly-L-lysine-coated slides. Immunohistochemical staining was applied using primary antibodies against hnRNP K (Biozol, Eching, Germany) and Ki-67 (DAKO, Hamburg, Germany). Each experiment was performed at least in quadruplicate.

Statistics

Differences between immunohistochemical expression levels, greyscale ratios from western immunoblotting and BrdU-Assays were calculated by t-test or ANOVA (Kruskal-Wallis-test) followed by Dunn's multiple comparison post-test using GraphPad Software where applicable (GraphPad, La Jolla, USA). Differences among treatment groups of colony formation assays and Annexin V/PI experiments were tested for significance with ANOVA (Kruskal-Wallis-test) followed by Dunn's multiple comparison post-test by using SigmaPlot 12.0 (Systat Software, Erkrath, Germany). Based on the linear-quadratic model of cell survival ($S = e^{-\alpha*D-\beta*D^2}$), the parameters α and β were calculated by non-linear regression analysis using SigmaPlot 12.0 (Systat Software, Erkrath, Germany). P values below 0.05 were considered as statistically significant.

Results

Strong hnRNP K expression and association with an infiltrative tumor growth pattern in CRC tissue specimens

CRC tissue specimens from 56 patients, comprising all UICC stages and histopathological differentiations, were immunostained for hnRNP K. Detailed clinic-pathological sample characteristics are summarized in Table 1. Cytoplasmic hnRNP K expression was significantly stronger in CRC tissue compared to normal mucosa (Fig. 1A I–III). No correlation was observed between UICC tumor

Table 1
Clinic-pathologic sample characteristics.

No of patients	56
No of samples	56
Age (median, range)	72 (48–96)
m/f	34/22
UICC stage	
I	17
II	15
III	17
IV	7
Localization	
Right	17
Left	28
Rectum	11
Growth pattern	
Infiltrating	20
Expanding	36
Tumor cell budding	
High-grade	16
Absent/low-grade	40
Lymphovascular invasion	
Present (L1/V1)	19
Absent (L0V0)	37
KRAS status (c. 12/13/61)	
*KRAS*mut	23
*KRAS*wt	33
BRAF status (c. 600)	
*BRAF*mut	2
*BRAF*wt	54
MSI status	
MSI	7
MSS	49
Tumor-infiltrating lymphocytes	
Present	14
Absent	42