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Guanine nucleotide-binding protein 1 is one of the key molecules contributing to cancer cell radioresistance

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Standard fractionated radiotherapy for the treatment of cancer consists of daily irradiation of 2-Gy X-rays, 5 days a week for 5–8 weeks. To understand the characteristics of radioresistant cancer cells and to develop more effective radiotherapy, we established a series of novel, clinically relevant radioresistant (CRR) cells that continue to proliferate with 2-Gy X-ray exposure every 24 h for more than 30 days *in vitro*. We studied three human and one murine cell line, and their CRR derivatives. Guanine nucleotide-binding protein 1 (*GBP1*) gene expression was higher in all CRR cells than their corresponding parental cells. *GBP1* knockdown by siRNA cancelled radioresistance of CRR cells *in vitro* and in xenotransplanted tumor tissues in nude mice. The clinical relevance of GBP1 was immunohistochemically assessed in 45 cases of head and neck cancer tissues. Patients with GBP1-positive cancer tended to show poorer response to radiotherapy. We recently reported that low dose long-term fractionated radiation concentrates cancer stem cells (CSCs). Immunofluorescence staining of GBP1 was stronger in CRR cells than in corresponding parental cells. The frequency of Oct4-positive CSCs was higher in CRR cells than in parental cells, however, was not as common as GBP1-positive cells. GBP1-positive cells were radioresistant, but radioresistant cells were not necessarily CSCs. We concluded that GBP1 overexpression is necessary for the radioresistant phenotype in CRR cells, and that targeting GBP1-positive cancer cells is a more efficient method in conquering cancer than targeting CSCs.

Radiotherapy is one of the major therapeutic modalities for eradicating malignant tumors. The existence of radioresistant cells remains one of the major obstacles in radiotherapy and chemoradiotherapy. Ordinary radiotherapy for cancers is composed of fractionated radiation (FR), with approximately 2 Gy of X-ray irradiation once a day, 5 days a week, over a 5–8-week period.(1) In order to develop more effective tumor radiotherapies, we established three human and one murine clinically relevant radioresistant (CRR) cell lines independently. These cells continue to proliferate under exposure to 2 Gy/day for more than 30 days *in vitro*.(2,3) The total dose to these CRR cells over the whole process added up to more than 1500 Gy. We carried out cDNA microarray analyses of differential gene expression in association with the CRR phenotype. We found that the Guanine nucleotide-binding protein 1 (GBP1) gene was overexpressed in all of the CRR cells examined.

GBP1 is a member of the large GTPase family.(4) The human large GTPase family consists of seven members, encoded by a gene cluster located on chromosome 1.(5) *GBP1* is one of the genes most strongly induced by interferons.(6) *GBP1* is highly expressed in endothelial cells, where it inhibits the proliferation and invasion of endothelial cells in response to γ-interferon and is activated by inflammatory cytokines *in vitro* and *in vivo*.(7–9) Downregulation of *GBP1* by siRNA resulted in higher levels of hepatitis C virus replication in a human hepatoma cell line, Huh-7.(10) In addition to the GTPase activity and its involvement in viral infections, *GBP1* overexpression also contributes to cell survival by inhibiting apoptosis in human umbilical vein endothelial cells after growth factor and serum depletion.(11)

Ovarian cancer cases with GBP1 protein overexpression are resistant to paclitaxel, leading to poor prognoses.(12) *GBP1* overexpression is directly associated with moderate levels of paclitaxel resistance in ovarian cancer cell lines.(13) Higher *GBP1* levels are associated with higher pathological stages, positive perineural invasion, and poorer prognosis of patients with oral squamous cell carcinoma.(14) In this study we found that GBP1 is necessary but not sufficient for cellular radioresistance *in vitro*. We carried out immunohistochemical studies on clinicopathological specimens from head and neck cancers (HNC) to confirm the relevance of GBP1 in cancer treatment. This study revealed that GBP1 is one of the key molecules contributing to radioresistance.

Materials and Methods

Cell culture and drugs. Human cancer cell lines SAS, HepG2, and KB and a mouse breast cancer cell line, MM102, were obtained from the Cell Resource Center for Biomedical Research (IDAC, Tohoku University, Sendai, Japan). We established CRR cell lines SAS-R1, HepG2-8960-R, and KB-R by exposing these parental cells to FR of X-rays for more than 5 years.(3) For the maintenance of the CRR phenotype, FR at 2 Gy was carried out every 24 h. All cells used in this study were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) and supplemented with 5% FBS (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere at 37°C in air with 5% CO_2. Acute exposure experiments were carried out with cells in the exponential growth phase, 24 h after the last maintenance irradiation. We introduced pIRES GBP1 expression vectors(13) into CRR cells by Lipofectamine 2000 (Invitrogen) and selected colonies resistant to 100 μg/mL G418 (Geneticin, Grand Island, NY, USA).

Irradiation. X-ray irradiation was carried out in a 150-KVp X-ray generator (MBR-1520R; Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper filter, at a dose rate of 1.0 Gy/min.

Cell survival assay after irradiation. Cell survival was determined by the modified high density survival (MHDS) assay.(15)

Microarray analysis. Genome-wide expression arrays (Illumina, San Diego, CA, USA) were used for the analysis of SAS (human WG6, version 3, 48 803 genes) and HepG2 (human WG6, version 1, 47 296 genes). Data were analyzed by TransGenic (Kumamoto, Japan). For MM102, a 3D-Gene mouse Oligo chip 24k (23 522 genes; Toray Industries, Tokyo, Japan) was used and the data were analyzed by Toray Industries using GeneSpring GX10 (Agilent Technologies, Santa Clara, CA, USA). HepG2 *versus* HepG2 cancer stem cell (CSC) analysis was carried out by MOGERA-Array self (Tohoku Chemical, Iwate, Japan).

Antibodies. The primary antibodies used were as follows: anti-β-actin (A5316; Sigma, St. Louis, MO, USA), anti-GBP1 (15303-1-AP; Proteintech Group, Chicago, IL, USA), purified anti-H2AX-phosphorylated (γH2AX) (Ser139; BioLegend, San Diego, CA, USA), anti-Oct4 antibody 7E7 (ab105931; Abcam, Cambridge, MA, USA), CD34 (ab8158, Abcam), anti-GBP2 N1C1 (GTX114426; GeneTex, Irvine, CA, USA), anti-GBP3 C-term (AP18451b; Abgent, San Diego, CA, USA), anti-GBP5 N1N3 (GTX106994; GeneTex), anti-TAP1 53H8 (GTX10356; GeneTex), and interleukin (IL)-15 (sc-1296; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were as follows: goat anti-rabbit IgG (H1202; Nichirei Bioscience, Tokyo, Japan), mouse anti-rat IgG (H1104; Nichirei Bioscience), Alexa Fluor 488 goat anti-mouse IgG (A11001; Invitrogen), and Alexa Fluor 594 goat anti-rabbit IgG (A11012, Invitrogen).

Western blot analysis. Western blot of whole cell lysates was carried out as previously described.(16)

Reverse transcription–PCR. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized by RT using SuperScriptIII Reverse Transcriptase (Invitrogen). Reverse transcription–PCR of *GBP1* was carried out using the primer pair 5′-CTGCACAGGCTTCAGCAAAA-3′ and 5′-AAGGCTCTGGTCTTTAGCTT-3′.(13) Reverse transcription–PCR of *ISG20* was carried out using the primer set 5′-ATCTCTGAGGGTCCCCAAG-3′ and 5′-TTCAGTCTG ACACAGCCAGG-3′.(17) The RT-PCR was carried out using TB SYBR gPCR Mix (Toyobo, Osaka, Japan). The PCR conditions were: 95°C for 1 min, followed by 60 cycles of 95°C for 15 s, and 60°C for 30 s using the Thermal Cycler Dice Real Time System (Takara, Shiga, Japan).

RNA interference. Lipofectamine 2000 was used for transfection. GBP1 siRNA (Hs_GBP1_8 and Hs_GBP1_9) and All-Stars Negative Control siRNA were purchased from Qiagen.

Apoptosis assay. Apoptotic cells were quantified using an annexin V–FITC apoptosis detection kit (BioVision, Mountain View, CA, USA). Cells (5×10^5) were collected 48 h after irradiation and were analyzed by a FACScan (Cytomics FC500; Becton Dickinson, Mountain View, CA, USA).

Immunofluorescence staining of culture cells. Immunofluorescence staining was carried out as previously described.(18) Images were randomly captured in a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan). We scored γH2AX foci and Oct4-positive cells by counting 50 cells in total.

Animal experiments. This study was approved by Regulations for Animal Experiments and Related Activities, Tohoku University, and carried out as described previously.(16) Atelo Gene (Koken, Tokyo, Japan) was used to deliver siRNA into animal tissues according to the manufacturer's protocol.

Immunohistochemistry. Tumor tissues were fixed in 10% formalin and immunohistochemical staining was carried out as described previously.(19)

***In situ* hybridization.** GBP1 (NM_002053) gene expression in tumor tissues was visualized using RNAscope and the HybEZ system according to the manufacturer's protocol (Advanced Cell Diagnostics, Hayward, CA, USA).

Demographic analysis. This study was approved by the committees of medical ethics, Shinshu University School of Medicine (No.354; Matsumoto, Japan) and Tohoku University, Graduate School of Medicine (No. 2011-42, 2013-1-1). We carried out immunohistochemical studies on biopsy specimens from HNC before treatment at Shinshu University Hospital and Tohoku University Hospital. The correlation between relative protein expression and clinicopathological data was analyzed using the Behrens–Fisher test.

Statistical analysis. Experiments *in vitro* were carried out in triplicate and were statistically analyzed using Student's *t*-test.

Results

Identification of *GBP1* overexpression by cDNA array analysis. We carried out a cDNA array analysis to identify genes whose expression was changed 12 h after exposure to 2-Gy X-rays (2-Gy-12 h). We compared the expression profile of CRR cells and their corresponding parental cells. In order to avoid the influence of the maintenance irradiation other than radioresistance, we also analyzed SAS-R1-NOIR cells, which were cultured CRR cells without maintenance exposure. We confirmed that NOIR cells were also radioresistant, as determined by the MHDS assay (Fig. S1a). We first compared SAS, SAS 2-Gy-12 h, SAS-R1 2-Gy-12 h, and SAS-R1 NOIR (Fig. 1a). We found 1636 genes whose expression in CRR cells was more than twice that in corresponding parental cells, regardless of irradiation. We also carried out the same analysis among the MM102 series and found 206 genes that were overexpressed in CRR cells compared with parental cells, regard-

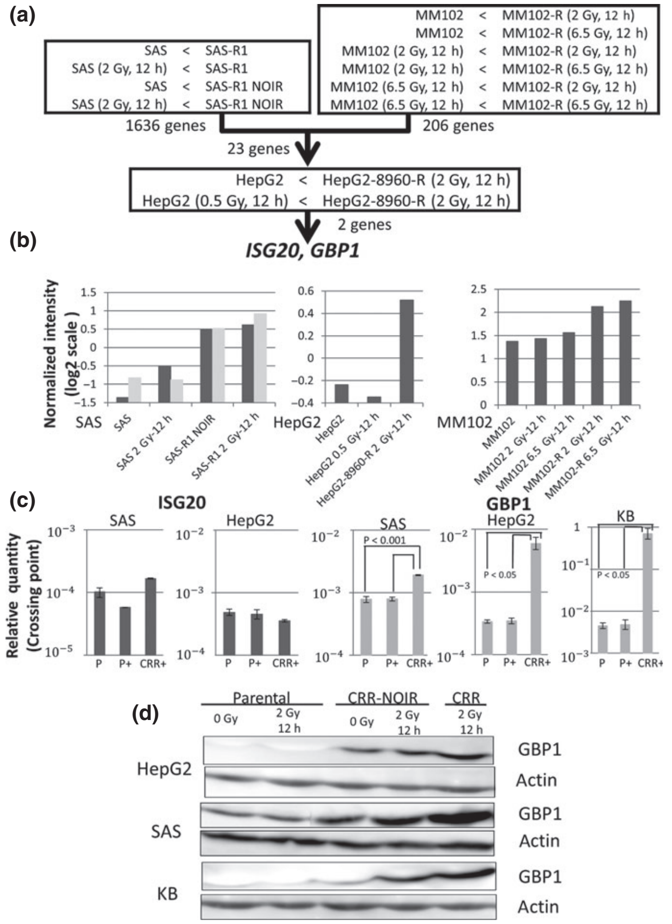

Fig. 1. Overexpression of guanine nucleotide-binding protein 1 (*GBP1*) in clinically relevant radioresistant (CRR) SAS, HepG2, KB, and MM102 cells. (a,b) Compared to corresponding parental cells, overexpression of *GBP1* and *ISG20* was found in all CRR cells. SAS cells had two different *GBP1* target probes. (c) *GBP1* but not *ISG20* overexpression was reconfirmed in CRR cells by RT-PCR. In all the combinations of cell lines, CRR cells 12 h after 2-Gy irradiation (CRR+) showed higher *GBP1* gene expression than parental cells without irradiation (P) and parental cells 12 h after 2-Gy irradiation (P+). (d) GBP1 protein expression in CRR cells and CRR-NOIR cells 12 h after 2-Gy irradiation or without irradiation were higher than their parental cells. NOIR cells were without irradiation for over 30 days, in order to avoid the influence of maintenance radiation. RNA from three independent exposure treatments was combined for the cDNA microarray and RT-PCR analysis. cDNA microarray analysis of the SAS group was carried out in duplicate.

less of irradiation. Compared with the corresponding parental cells, 23 genes were commonly overexpressed in SAS-R1 and MM102-R cells. We finally confirmed that two genes, *GBP1* and *ISG20*, were commonly overexpressed in CRR cells of the HepG2 series (Fig. 1a,b).

Verification of *GBP1* overexpression by RT-PCR. Compared with their corresponding parental cells, *GBP1* expression was commonly higher (Fig. 1c) in HepG2-8960-R as well as SAS-R1 cells, whereas *ISG20* expression was not.

Verification of GBP1 protein overexpression by Western blotting. All of the CRR cells examined showed higher GBP1 expression than their corresponding parental cells and NOIR cells (Fig. 1d). HepG2 and KB showed only a trace amount of the GBP1 protein, irrespective of irradiation. GBP1 protein expression was higher in NOIR cells than in parental cells. GBP1 expression in SAS-R1 NOIR and KB NOIR tended to be higher after 2-Gy irradiation than without irradiation. SAS, the most radioresistant line, showed the highest expression of GBP1 protein among the parental cells. These results confirmed that GBP1 protein expression was correlated to radioresistance. We picked up proteins directly connected with GBP1 on the network such as GBP2, GBP3, GBP5, TAP1 and IL-15 in reference to COXPRESdb (http://coxpresdb.jp/). No associations were observed among their protein levels (Fig. S2d).

Decreased radioresistance by *GBP1* gene knockdown. In order to determine whether *GBP1* was involved in radioresistance or not, the MHDS assay was carried out after cells were transfected with two types of GBP1 siRNA, GBP1_8 and GBP1_9 (Fig. S2a). During this period of experiments, *GBP1* gene expression was suppressed (Fig. S2b). In all of the CRR cells examined, *GBP1* knockdown cancelled radioresistance to the levels of their parental cells (Figs 2 and S1b). Furthermore, *GBP1* knockdown increased radiosensitivity in parental cells. These indicate that GBP1 is one of the key molecules commonly involved in coping with radiation exposure, both in CRR and parental cells. Because suppression with GBP1_8

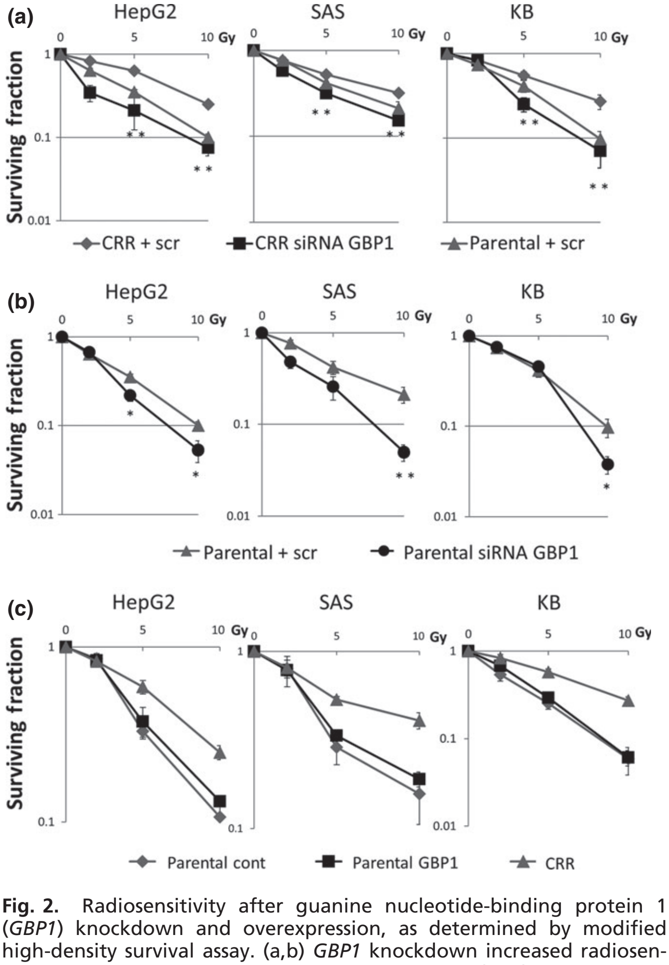

Fig. 2. Radiosensitivity after guanine nucleotide-binding protein 1 (*GBP1*) knockdown and overexpression, as determined by modified high-density survival assay. (a,b) *GBP1* knockdown increased radiosensitivity both in clinically relevant radioresistant (CRR) and parental cells. *GBP1* knockdown was carried out with Hs_GBP1_8. (c) Parental cells transfected with *GBP1* (Parental GBP1) did not show significant changes in radioresistance compared to parental cells with an empty vector (Parental cont). Exponentially growing cells were seeded in 25-cm^2 flasks and incubated for 24 h; cells were then irradiated and incubated for another 72 h. Subsequently, 10% of the cells in each flask were seeded into another flask and incubated for a further 72 h and live cells were counted. scr, scramble siRNA.

was stronger than with GBP1_9, further experiments were carried out using GBP1_8.

Association of *GBP1* overexpression and radioresistance. To determine whether GBP1 directly participates in the establishment of the radioresistant phenotype, we transfected parental cells with a GBP1 expression vector. We confirmed GBP1 protein overexpression in all transfected parental cells (Fig. S2c). The expression level was even higher in transfected cells of SAS and KB compared to their CRR cells. In all the parental cells, *GBP1* transfection did not show significant change in radioresistance (Fig. 2c).

Relationship between suppressing *GBP1* and cell death. As *GBP1* knockdown increased radiosensitivity, we examined the association of GBP1 expression and radiation-induced cell death by FACS. The frequency of apoptosis induced by 10-Gy X-rays was significantly higher in CRR cells with siGBP1 than with scrambled RNA in all cell lines examined (Fig. 3a). DNA double-strand breaks (DSBs) induced by 2-Gy X-rays were determined by γH2AX staining. *GBP1* knockdown increased DNA DSBs by irradiation (Figs 3b,c and S3a,b).

Relationship between suppression of GBP1 and CSCs. We isolated surviving CSCs by exposure to long-term 0.5-Gy FR for 82 days from HepG2 cells.(20) Microarray analysis showed that the mRNA level of *GBP1* was significantly higher in HepG2 CSCs than in HepG2 cells (Fig. 4a). Additionally, the protein level of GBP1 was higher in HepG2 cells after FR than in non-radiated HepG2 cells (Fig. 4b). Therefore, we examined the relationship between Oct4 expression and long-term FR. The frequency of Oct4-positive cells was higher in cells exposed to 0.5-Gy FR every 12 h for 30 days than in parental cells and was the highest in CRR cells (Fig. 4c). We examined whether Oct4-positive CSCs coincided with GBP1-positive cells by immunostaining. Even in CRRs, Oct4-positive cells

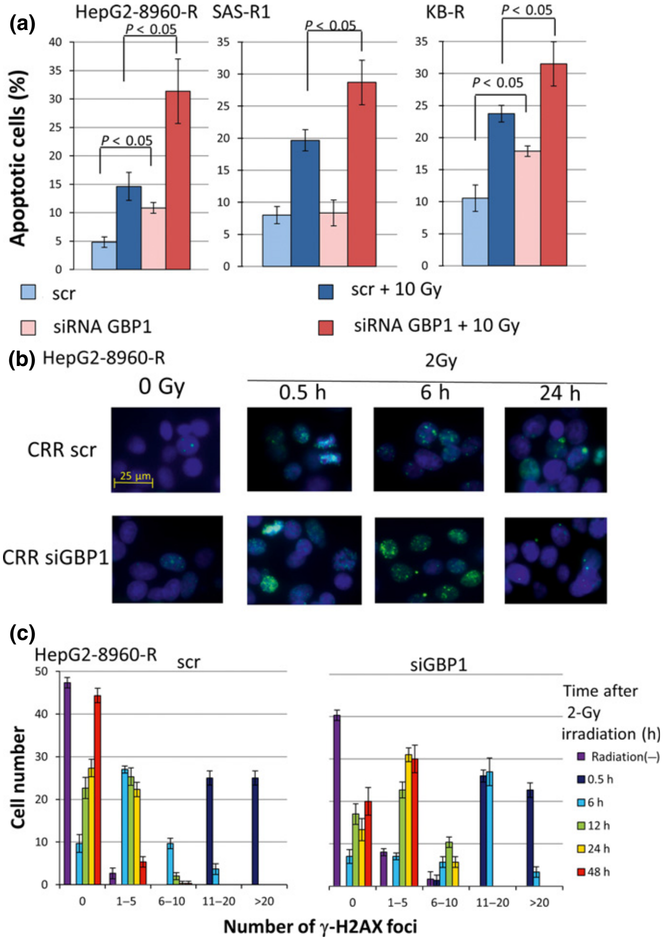

Fig. 3. Relationship between suppression of guanine nucleotide-binding protein 1 (GBP1) and cell death. (a) Apoptotic cells, as determined by annexin V staining, increased both in clinically relevant radioresistant (CRR) and parental cells after 10 Gy of irradiation. Knockdown of GBP1 increased the frequency of apoptotic cells, both in CRR and parental cells regardless of irradiation. (b,c) Knockdown of GBP1 increased the number of γH2AX foci (DNA double-strand breaks, green) induced by 2-Gy X-rays in CRR cells. In total, 50 cells were counted for the number of γH2AX foci at 0, 0.5, 6, 12, 24, and 48 h after X-ray irradiation. The nucleus was counterstained with DAPI (blue). scr, scramble siRNA.

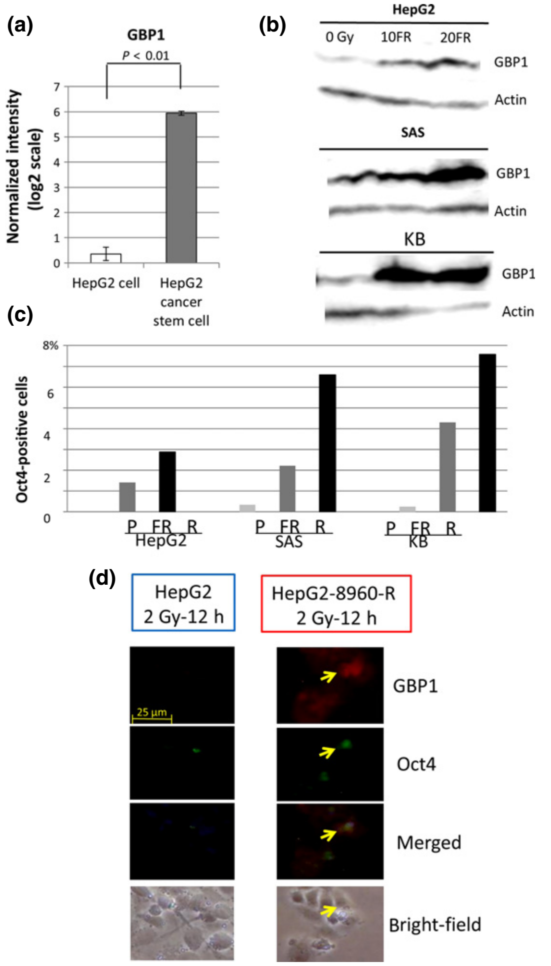

Fig. 4. Relationship between suppression of guanine nucleotide-binding protein 1 (GBP1) and cancer stem cell markers. (a) Microarray analysis revealed significantly higher *GBP1* gene expression in HepG2 cells than in HepG2 cancer stem cells (CSC). (b) GBP1 protein expression tended to correlate with the accumulation of fractionated radiation with 0.5-Gy X-rays every 12 h (FR). (c) The fraction of GBP1-positive cells was higher in cells with FR for 30 days than in Parental cells (P) and the highest in clinically relevant radioresistant (CRR) cells (R). (d) The number of Oct4 (green) and GBP1 (red) cells was higher in CRR cells than parental cells. Immunofluorescence of GBP1 staining was stronger in CRR cells than their parental cells but not all CRR cells were Oct4-positive.

constituted less than 8% of total cells (Fig. 4d). In contrast, GBP1 was positive in all CRR cells.

GBP1 immunohistochemistry in xenotransplanted tumor tissues. SAS-R1 tumors transplanted into nude mice are also resistant to FR.(16) We found overexpression of both the GBP1 protein and mRNA in SAS-R1 tumors and KB-R tumors, compared with their parental cell-derived tumors (Fig. 5a). In order to determine the association of GBP1 with tumor radioresistance, we suppressed *GBP1* by AteloGene-delivered siRNA into animal tissues (Fig. S4) and irradiated with FR of 2-Gy/day for 5 days (Fig. 5b). *GBP1* suppression alone did not affect the growth or microvessel density (MVD) of SAS and SAS-R1 tumors. However, SAS-R1 tumors shrank if combined with irradiation. In parental tumors, irradiation diminished the growth irrespective of *GBP1* suppression.

GBP1 immunohistochemistry of clinical samples. Immunohistochemical study of GBP1 was possible in 45 cases of HNC before treatment. Clinicopathological features of cases are shown in Table 1. We defined a case GBP1-positive if the nucleus and cytoplasm of more than 5% of tumor cells were positive for GBP1 (Fig. 5c). Only one GBP1-positive case and seven GBP1-negative cases out of a total of 45 cases were without recurrence or metastasis 5 years after chemoradiation

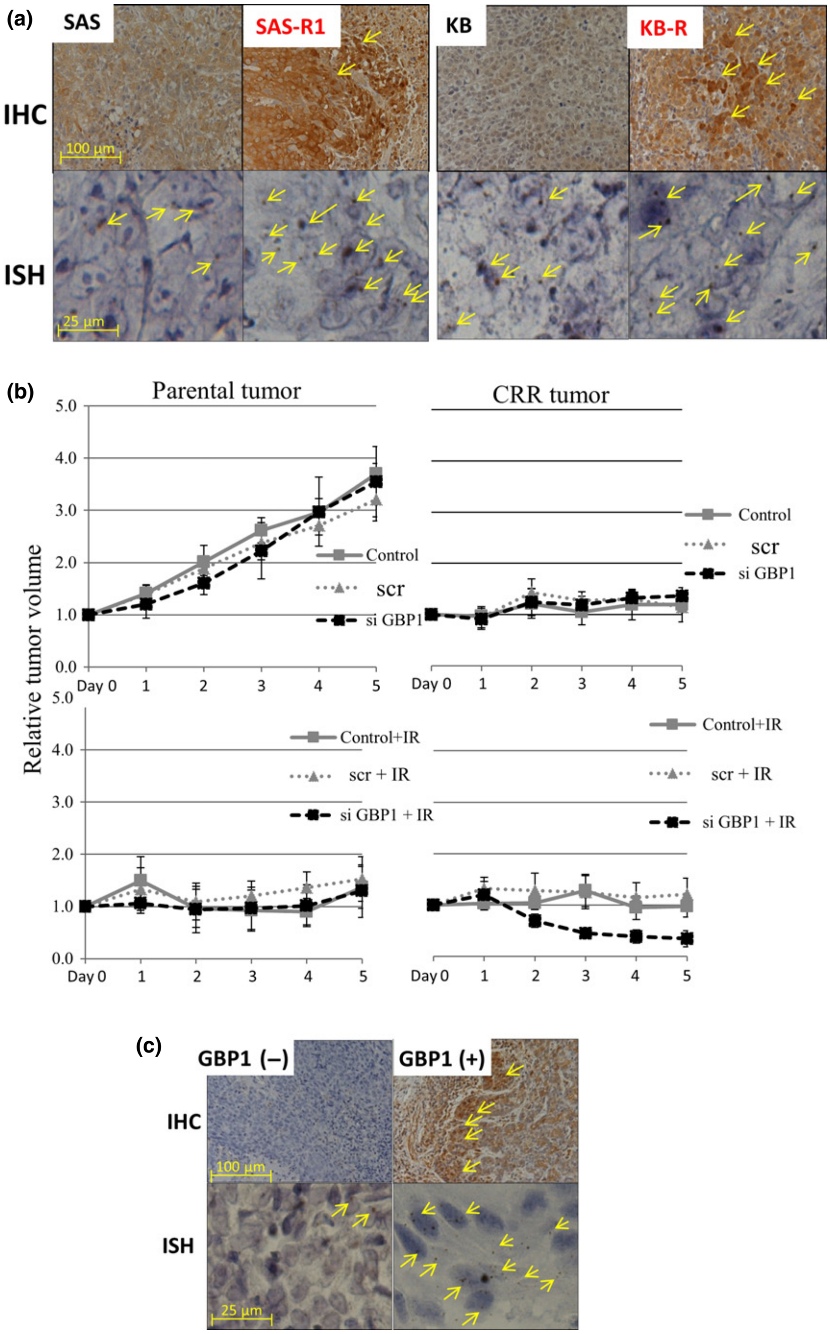

Fig. 5. Distribution of guanine nucleotide-binding protein 1 (GBP1) in tumor tissues. (a) Compared with parental tumors, overexpression of the *GBP1* gene and GBP1 protein was observed in clinically relevant radioresistant (CRR) tumor cells xenotransplanted into nude mice. (b) SAS-R1 tumors shrank due to fractionated radiation in combination with *GBP1* suppression. SAS-R1 cells (1×10^7) were injected into the right flank and SAS cells into the left flank of a mouse. (c) Representative positive staining of GBP1 in clinical cancer tissues. IHC, immunohistochemistry; IR, fractionated radiation with 2-Gy X-rays every 24h ISH, *in situ* hybridization; scr, scramble siRNA; arrows, GBP1 positive cells

Table 1. Guanine nucleotide-binding protein 1 (GBP1) immunohistochemistry and clinicopathological features of head and neck cancer

(a) Association of GBP1 expression and chemoradiotherapy resistance

		Recurrence or metastatis		Total	
		+	−		
GBP1	+	20	1	21	GBP1 positive cases were significantly more resistant to chemoradiotherapy than GBP1 negative cases (Behrens-Fisher test, P < 0.05). Relative risk, 1.345; 95% confidence limit, 1.022 −1.768.
	−	17	7	24	
Total		37	8	45	

(b) Clinicopathological features of 45 cases of head and neck cancer

Sample no.	Region	Age, years	Gender	Differentiation	TNM classification	Stage	Treatment	Total dose of irradiation (Gy)	Curative effect
GBP1(+) cases									
1	Tongue	53	Male	Well	T4N2bM0	IV	Chemoradiation→operation	40	Recurrent
2	Tongue	54	Female	Well	T2N0M0	II	Chemoradiation→operation	40	Recurrent and lymph node metastasis
3	Larynx	57	Male	Moderate	T2N0M0	II	Radiation	70	Lymph node metastasis
4	Larynx	59	Male	Moderate	T2N0M0	II	Chemoradiation	70	Recurrent
5	Buccal cavity	59	Male	Moderate	T1N2 cM0	IV	Chemoradiation→operation	70	Lymph node metastasis
6	Buccal cavity	60	Male	Well	T4N2bM1	IV	Chemoradiation→operation	40	Recurrent
7	Paranasal sinus	60	Male	Well > poor	T3N0M0	III	Chemoradiation→operation	70	Recurrent and lymph node metastasis
8	Paranasal sinus	60	Male	Moderate	T3N0M0	III	Chemoradiation→operation	50	Recurrent
9	Paranasal sinus	60	Female	Moderate	T3N0M0	III	Chemoradiation→operation	50	Recurrent
10	Paranasal sinus	61	Male	Moderate	T3N0M0	III	Chemoradiation→operation	50	Lymph node metastasis
11	Larynx	67	Male	Moderate	T2N0M0	II	Radiation	72	Recurrent
12	Paranasal sinus	69	Male	Poor	T3N0M0	III	Chemoradiation→operation	50	Pathologically no recurrent/CR over 5 years
13	Hypopharynx	69	Male	Well > moderate	T4N2 cM0	IV	Chemotherapy→operation →radiation	63	Recurrent and lung metastatis
14	Hypopharynx	71	Male	Moderate	T2N0M0	II	Chemoradiation→operation	70	Recurrent
15	Paranasal sinus	74	Male	Moderate	T4N1M0	IV	Radiation→operation	50	Recurrent
16	Paranasal sinus	75	Female	Well	T4N2bM0	IV	Chemoradiation	60	Recurrent
17	Buccal cavity	76	Male	Well	T2N0M0	II	Chemoradiation→operation	50	Lymph node and lung metastasis
18	Buccal cavity	79	Female	Well	T4N2 cM0	IV	Chemoradiation→operation	40	Recurrent and lymph node metastasis
19	Larynx	79	Male	Moderate > well	T1bN0M0	I	Radiation	66	Recurrent
20	Buccal cavity	80	Female	Well	T4N2bM0	IV	Chemoradiation→operation	60	Recurrent and lymph node metastasis
21	Larynx	81	Male	Well > moderate	T1aN0M0	I	Radiation	66	Recurrent

Table 1 (continued)

(b) Clinicopathological features of 45 cases of head and neck cancer

Sample no.	Region	Age, years	Gender	Differentiation	TNM classification	Stage	Treatment	Total dose of irradiation (Gy)	Curative effect
GBP1(−)									
22	Paranasal sinus	46	Male	Well	T3N0M0	III	Chemoradiationmoradiatin	50	Recurrent and lymph node metastasis
23	Larynx	51	Male	Well	T2N0M0	I	Radiation→operation	66	Recurrent
24	Oropharynx	54	Male	Poor	T4N2 cM0	IV	Chemoradiation→operation	77	Recurrent
25	Paranasal sinus	56	Male	Moderate > well	T3N0M0	III	Chemoradiationoradia wel	70	Recurrent
26	Paranasal sinus	57	Male	Moderate	T3N0M0	III	Chemoradiation→operation	50	Recurrent
27	Oropharynx	57	Male	Moderate	T2N3M0	IV	Chemoradiation→operation	60	Recurrent
28	Paranasal sinus	57	Male	Moderate > poor	T3N2bM0	IV	Chemoradiationmoradiatpo	50	Recurrent and lymph node metastasis
29	Paranasal sinus	60	Male	Poor	T3N0M0	III	Chemoradiation→operation	50	Pathologically no recurrent/ CR over 5 years
30	Larynx	61	Male	Moderate > well	T2N0M0	II	Chemoradiation	70	Pathologically no recurrent/ CR over 5 years
31	Rhinopharynx	61	Male	Moderate	T1N2bM0	IIB	Chemoradiation→operation	70	Pathologically no recurrent/ CR over 5 years
32	Oropharynx	66	Male	Poor	T3N3M0	IV	Chemoradiation→operation	70	Pathologically no recurrent/ CR over 5 years
33	Buccal cavity	67	Male	Well	T4N2 cM1	IV	Chemoradiation→operation	40	Recurrent and lymph node metastasis
34	Tongue	68	Male	Moderate	T1N0M0	I	Radiation	70	Pathologically no recurrent/ CR over 5 years
35	Paranasal sinus	69	Male	Moderate ~ well	T4N2bM0	IV	Chemoradiation→operation	50	Recurrent
36	Paranasal sinus	71	Male	Moderate > well	T3N0M0	III	Chemoradiation→operation	50	Pathologically no recurrent/ CR over 5 years
37	Larynx	71	Male	Well	T1N0M0	I	Radiation→operation	66	Recurrent
38	Paranasal sinus	71	Female	Well	T4N0M0	IV	Chemoradiationinustionec	50	Recurrent
39	Paranasal sinus	72	Male	Moderate	T4N0M0	IV	Chemoradiation→operation	50	Recurrent
40	Buccal cavity	74	Male	Well	T4N2bM0	IV	Chemoradiation→operation	40	Recurrent
41	Buccal cavity	75	Female	Well	T4N1M0	IV	Chemoradiation→operation	48	Recurrent
42	Paranasal sinus	76	Female	Moderate	T3N0M0	III	Chemoradiation→operation	60	Recurrent
43	Paranasal sinus	77	Male	Moderate	T4N0M0	IV	Chemoradiation→operation	50	Recurrent
44	Tongue	80	Male	Well	T2N0M0	II	Radiation	70	Recurrent
45	Larynx	82	Male	Well	T1N0M0	I	Radiation	66	Pathologically no recurrent/ CR over 5 years

CR, complete remission; GBP1, guanine nucleotide-binding protein 1.

treatment (Table 1a). GBP1-positive cases were significantly more resistant to chemoradiotherapy than GBP1-negative cases ($P < 0.05$, Behrens–Fisher test).

Discussion

Radiotherapy with FR is one of the major modalities for cancer treatment. Although tumors receive a large total dose, they sometimes recur and demonstrate radioresistance. In this study, we looked for genes whose overexpression was responsible for the radioresistant phenotype of CRR cells. In microarray analyses, we found two such candidate genes in common with human and murine CRR cells. We finally picked up only one gene, *GBP1*, whose overexpression in CRR cells was confirmed by RT-PCR. The translational level of GBP1 was closely correlated to the transcriptional step (Fig. 1d). The expression levels of GBP1 protein were not different between parental cells with and without 2-Gy irradiation. All the CRR and NOIR cells examined showed GBP1 overexpression, compared with their corresponding parental cells. This indicates that GBP1 protein overexpression is associated with radioresistance and is mainly regulated at the transcriptional step.

Knockdown of GBP1 cancelled radioresistance to the level of parental cells in all the CRR cells including HepG2 and KB with naturally low GBP1 expression (Figs 2a,b and S1b,c). This radiosensitization by *GBP1* gene knockdown was also observed in transplanted SAS-R1 tumors (Fig. 5b). However, parental cells with *GBP1* transfection did not demonstrate significant changes of radioresistance even in SAS and KB cells with much more GBP1 protein than their corresponding CRR cells (Figs 2c and S2c). These results indicate that GBP1 is essential to cope with radiation, but is not enough to shift the cell into CRR. Only *GBP1* knockdown in CRR cells did not enhance apoptosis but induced apoptosis through increased radiation-induced DNA DSBs in an additive manner (Figs 3 and S3a,b). Sublethal damage that is substantiated by FR is suggested to be restored by homologous recombination repair (HRR) of DSBs.(21) Therefore, prolongation of γH2AX foci in CRR cells by *GBP1* knockdown is suggested to represent the deficiency in HRR of radiation-induced DSBs.(22) A member of GTPases, RECQ5/QE DNA helicase is activated by GTP binding.(23) These suggest that GBP1 is involved in radioresistance through HRR of DSBs. However, the failure of enhancing radioresistance by *GBP1* overexpression in parental cells indicated that HRR requires multiple molecules other than GBP1.

Overexpression of *GBP1* activates class III β-tubulin and ultimately leads to paclitaxel resistance in ovarian cancer cells.(12,13) Nevertheless, GBP1 protein expression was not significantly changed by treatment with cisplatin or 5-fluorouracil (Fig. S5). Extrapolating from these findings, GBP1 plays a role in radioresistance but not in chemoresistance.

The *GBP1* gene is located at tight junctions of intestinal epithelial cells of inflammatory bowel disease and its knockdown enhances apoptosis.(24) We previously reported that inhibition of the AKT/cyclin D1 pathway suppresses acquired radioresistance through increased apoptosis. We need to study the relationship between GBP1 and the AKT/cyclin D1 pathway.(25) In this study, *GBP1* knockdown enhanced radiation-induced early apoptosis in CRR cells through the delay of DSB repair. We also reported that hyperinduction of autophagy is involved in radiation-induced cell death later than 5 days after radiation.(3) Combined induction of apoptosis and autophagy hyperinduction would be effective to conquer CRR cancers.

Recently, we reported that exposure to FR with 0.5-Gy X-rays every 12 h for 82 days enriched CSCs, owing to their radioresistance.(20) Microarray analysis showed that *GBP1* gene expression increased along with the accumulation of total dose (Fig. 4b). Immunofluorescence of GBP1 staining was stronger in all CRR cells than their corresponding parental cells but not all CRR cells were Oct4-positive (Fig. 4c). These results indicate that radiotherapy, in combination with targeting GBP1-positive cancer cells, is more efficient in impeding cancer than targeting CSCs.

The growth rate of transplanted murine mammary tumors transfected with *GBP1* is slower accompanied with lower MVD compared with those without transfection.(26) Overexpression of GBP1 in human endothelial cells decreases apoptosis but inhibits cell proliferation.(11) Patients with colorectal cancer with GBP1-positive stromal cells showed better prognosis than those with GBP1-negative cases.(27) In this study, CRR tumors revealed higher MVD compared to their parental cell tumors, suggesting that GBP1 of cancer cells is not involved in tumor vasculature. It is interesting to study the expression and role of GBP1 in normal cells, especially in endothelial cells after FR.

Reports of the association of GBP1 with chemotherapeutic taxanes are conflicting; GBP1 is a marker of paclitaxel-resistant cancer cells(12) and a marker of docetaxel-sensitive cancer cells.(28) In this study, GBP1-positive HNC was clinically resistant to radiotherapy but was not associated with resistance to cisplatin and 5-fluorouracil. These results suggest that GBP1 plays a role in radioresistance, but not in chemoresistance, and may be a useful marker for screening of specifically radioresistant tumors before treatment.

The precise mechanism of GTP hydrolyzing activity of GBP1 and its role in signal transduction remains unknown.(29,30) Our study is the first to reveal another aspect of GBP1, namely radioresistance. Our results suggest that the suppression of GBP1 transcription may overcome tumor radioresistance and that GBP1 could be used for screening radioresistant malignant tumors.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Tannock I. *The Basic Science of Oncology*, 4th edn. New York, NY: McGraw-Hill, Medical Publications, 2005.

2 Kuwahara Y, Li L, Baba T *et al.* Clinically relevant radioresistant cells efficiently repair DNA double-strand breaks induced by X-rays. *Cancer Sci* 2009; **100**: 747–52.

3 Shimura T, Kakuda S, Ochiai Y *et al.* Acquired radioresistance of human tumor cells by DNA-PK/AKT/GSK3beta-mediated cyclin D1 overexpression. *Oncogene* 2010; **29**: 4826–37.

4 Naschberger E, Bauer M, Sturzl M. Human guanylate binding protein-1 (hGBP-1) characterizes and establishes a non-angiogenic endothelial cell activation phenotype in inflammatory diseases. *Adv Enzyme Regul* 2005; **45**: 215–27.

5 Olszewski MA, Gray J, Vestal DJ. In silico genomic analysis of the human and murine guanylate-binding protein (GBP) gene clusters. *J Interferon Cytokine Res* 2006; **26**: 328–52.

6 Cheng YS, Colonno RJ, Yin FH. Interferon induction of fibroblast proteins with guanylate binding activity. *J Biol Chem* 1983; **258**: 7746–50.

7 Guenzi E, Topolt K, Cornali E *et al.* The helical domain of GBP-1 mediates the inhibition of endothelial cell proliferation by inflammatory cytokines. *EMBO J* 2001; **20**: 5568–77.

8 Guenzi E, Topolt K, Lubeseder-Martellato C *et al.* The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. *EMBO J* 2003; **22**: 3772–82.

9 Lubeseder-Martellato C, Guenzi E, Jorg A *et al.* Guanylate-binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. *Am J Pathol* 2002; **161**: 1749–59.

10 Itsui Y, Sakamoto N, Kurosaki M *et al.* Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. *J Viral Hepat* 2006; **13**: 690–700.

11 Pammer J, Reinisch C, Birner P, Pogoda K, Sturzl M, Tschachler E. Interferon-alpha prevents apoptosis of endothelial cells after short-term exposure but induces replicative senescence after continuous stimulation. *Lab Invest* 2006; **86**: 997–1007.

12 De Donato M, Mariani M, Petrella L *et al.* Class III beta-tubulin and the cytoskeletal gateway for drug resistance in ovarian cancer. *J Cell Physiol* 2012; **227**: 1034–41.

13 Duan Z, Foster R, Brakora KA, Yusuf RZ, Seiden MV. GBP1 overexpression is associated with a paclitaxel resistance phenotype. *Cancer Chemother Pharmacol* 2006; **57**: 25–33.

14 Yu CJ, Chang KP, Chang YJ *et al.* Identification of guanylate-binding protein 1 as a potential oral cancer marker involved in cell invasion using omics-based analysis. *J Proteome Res* 2011; **10**: 3778–88.

15 Kuwahara Y, Mori M, Oikawa T *et al.* The modified high-density survival assay is the useful tool to predict the effectiveness of fractionated radiation exposure. *J Radiat Res* 2010; **51**: 297–302.

16 Kuwahara Y, Oikawa T, Ochiai Y *et al.* Enhancement of autophagy is a potential modality for tumors refractory to radiotherapy. *Cell Death Dis* 2011; **2**: e177.

17 Hao Y, Yang D. Cloning, eukaryotic expression of human ISG20 and preliminary study on the effect of its anti-HBV. *J Huazhong Univ Sci Technolog Med Sci* 2008; **28**: 11–3.

18 Suzuki M, Suzuki K, Kodama S, Watanabe M. Phosphorylated histone H2AX foci persist on rejoined mitotic chromosomes in normal human diploid cells exposed to ionizing radiation. *Radiat Res* 2006; **165**: 269–76.

19 Toda Y, Kono K, Abiru H *et al.* Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. *Pathol Int* 1999; **49**: 479–83.

20 Shimura T, Noma N, Oikawa T *et al.* Activation of the AKT/cyclin D1/Cdk4 survival signaling pathway in radioresistant cancer stem cells. *Oncogenesis* 2012; **1**: e12.

21 Utsumi H, Elkind MM. Requirement for repair of DNA double-strand breaks by homologous recombination in split-dose recovery. *Radiat Res* 2001; **155**: 680–6.

22 Shibata A, Moiani D, Arvai AS *et al.* DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. *Mol Cell* 2014; **53**: 7–18.

23 Kawasaki K, Maruyama S, Nakayama M, Matsumoto K, Shibata T. Drosophila melanogaster RECQ5/QE DNA helicase: stimulation by GTP binding. *Nucleic Acids Res* 2002; **30**: 3682–91.

24 Schnoor M, Betanzos A, Weber DA, Parkos CA. Guanylate-binding protein-1 is expressed at tight junctions of intestinal epithelial cells in response to interferon-gamma and regulates barrier function through effects on apoptosis. *Mucosal Immunol* 2009; **2**: 33–42.

25 Shimura T, Kakuda S, Ochiai Y, Kuwahara Y, Takai Y, Fukumoto M. Targeting the AKT/GSK3beta/cyclin D1/Cdk4 survival signaling pathway for eradication of tumor radioresistance acquired by fractionated radiotherapy. *Int J Radiat Oncol Biol Phys* 2011; **80**: 540–8.

26 Lipnik K, Naschberger E, Gonin-Laurent N *et al.* Interferon gamma-induced human guanylate binding protein 1 inhibits mammary tumor growth in mice. *Mol Med* 2010; **16**: 177–87.

27 Naschberger E, Croner RS, Merkel S *et al.* Angiostatic immune reaction in colorectal carcinoma: impact on survival and perspectives for antiangiogenic therapy. *Int J Cancer* 2008; **123**: 2120–9.

28 Desarnaud F, Geck P, Parkin C, Carpinito G, Makarovskiy AN. Gene expression profiling of the androgen independent prostate cancer cells demonstrates complex mechanisms mediating resistance to docetaxel. *Cancer Biol Ther* 2011; **11**: 204–12.

29 Schwemmle M, Staeheli P. The interferon-induced 67-kDa guanylate-binding protein (hGBP1) is a GTPase that converts GTP to GMP. *J Biol Chem* 1994; **269**: 11299–305.

30 Praefcke GJ, Geyer M, Schwemmle M. Robert Kalbitzer H, Herrmann C. Nucleotide-binding characteristics of human guanylate-binding protein 1 (hGBP1) and identification of the third GTP-binding motif. *J Mol Biol* 1999; **292**: 321–32.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Radioresistance of clinically relevant radioresistant (CRR)-NOIR cells, and the effect of *GBP1* knockdown.

Fig. S2. Western blot analyses of guanine nucleotide-binding protein 1 (GBP1) and GBP1-associated proteins

Fig. S3. DNA double-strand breaks after *GBP1* knockdown in combination with irradiation.

Fig. S4. Association of guanine nucleotide-binding protein 1 (GBP1) with tumor radioresistance in SAS and SAS-R1.

Fig. S5. Guanine nucleotide-binding protein 1 (GBP1) protein expression after treatment with cisplatin or 5-fluorouracil.