Research Paper

Heterogeneity of chondrosarcomas response to irradiations with X-rays and carbon ions: A comparative study on five cell lines

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ABSTRACT

Objectives: Chondrosarcomas are malignant bone tumors considered as resistant to radiotherapy. To unravel mechanisms of resistance, we compared biological responses of several chondrosarcomas to X-ray irradiations in normoxia and hypoxia. Since hadrontherapy with Carbon-ions gave interesting clinical outcomes, we also investigated this treatment *in vitro*.

Methods: Five human chondrosarcoma cell lines were used and cultured in normoxia or hypoxia. Their sensitivities to irradiations were determined by carrying out survival curves. DNA damage was monitored by γH2AX expression. Apoptosis was assessed by cell cycle analysis and Apo2.7 expression, and by evaluating PARP cleavage. Senescence was evaluated using SA β-galactosidase assay. Necrosis, and autophagy, were evaluated by RIP1 and beclin-1 expression, respectively. Mutations in relevant biological pathways were screened by whole-exome sequencing.

Results: X-ray radiations induced death in some chondrosarcomas by both apoptosis and senescence (CH2879), or by either of them (SW1353 and JJ012), whereas no death was observed in other cell lines (FS090 and 105KC). Molecularly, p21 was overexpressed when senescence was elicited. Genetic analysis allowed to identify putative genes (such as TBX3, CDK2A, HMGA2) permitting to predict cell response to irradiations. Unexpectedly, chronic hypoxia did not favor radioresistance in chondrosarcomas, and even increased the radiosensitivity of JJ012 line. Finally, we show that carbon ions triggered more DNA damages and death than X-rays.

Conclusions: Chondrosarcomas have different response to irradiation, possibly due to their strong genetic heterogeneity. p21 expression is suggested as predictive of X-ray-induced senescence. Surprisingly, hypoxia does not increase the radioresistance of chondrosarcomas, but as expected Carbon ion beams are more effective that X-rays in normoxia, whereas their efficiency was also variable depending on cell lines.

1. Introduction

Chondrosarcoma (CHS) family represents a heterogeneous group of malignant bone mesenchymal tumors characterized by the production of a chondroid matrix. While considered as rare cancers, they account for about 25% of bone tumors and represent the second most common malignant primary bone sarcoma after osteosarcoma. They typically affect adults, with a peak of incidence age around 40 to 60 years. The main prognostic factor of conventional CHS is the histological grade [1]. Grade 1 chondrosarcomas are characterized by a very low metastatic potential, and a re-classification of these types as "atypical cartilaginous tumors" has been recently suggested [2]. At contrary, high grade chondrosarcomas are marked by an increased metastatic

potential and a bad prognostic. The 10-year survival is of 64% and 29% for grade 2 and 3, respectively [3]

CHS are among the most difficult bone tumors to treat. Surgical resection is currently the only primary treatment modality, due to their resistance to conventional chemotherapy and radiotherapy [4]. While curettage is acceptable for grade 1 chondrosarcomas, wide excision is usually required for higher grade chondrosarcomas. Adjuvant radiotherapy may be proposed to patients presenting an inoperable tumor, or when its excision could not be complete. However, the benefit of radiotherapy to treat CHS remains unclear. Indeed, conventional radiotherapy using X-rays is considered as poorly effective to treat these tumors despite the weak number of studies done in CHS [5]. By comparison with other kind of tumors, several hypothesis have been

proposed to explain the radioresistance of chondrosarcomas. For instance, the slow growth and the low percentage of dividing cells may favor DNA damage repair, and consequently reduce death induced by radiations. Also, the poor vascularity of tumors maintains a hypoxic microenvironment that could reduce the DNA damages induced in cells by radiations [6–9]. The absence of bystander response could also contribute to the radioresistance of chondrosarcomas [10].

In contrast, hadrontherapy by carbon ions (C-ions) gave good outcomes in the management of chondrosarcomas, notably when they were inoperable due to their location in the skull base [11–13]. Hadrontherapy consists to use accelerated charged particle beams (such as protons or carbon ions). Charged particles demonstrated an increase in energy deposition with penetration depth up to a sharp maximum at the end of their range, known as the Bragg peak beam. This feature makes heavy ion beams possess an excellent dose distribution, allowing precise localization of a sufficient dose in the target lesion, while minimizing the damage to the surrounding normal tissues [14]. In addition to this ballistic advantage, charged particles have higher linear energy transfer (LET). Compared with sparsely ionizing radiation such as X-rays, high-LET particle radiation has a higher relative biological effectiveness (RBE) [15,16]. Therefore, hadrontherapy with carbon ions is considered as more effective than conventional radiotherapy for inducing DNA damages, cell cycle arrest and cell death of tumor cells [15,17,18].

In this *in vitro* study, we genetically characterized five commonly used CHS cell lines by whole exome-sequencing, and compared their sensitivities to radiations with X-rays and carbon ions.

2. Material and methods

2.1. Cell culture

Grade 2 central chondrosarcoma-derived cell lines (SW1353, JJ012, FS090, 105KC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), or kindly provided by Dr. J. A. Block (Rush University medical center, Chicago, USA) [19,20]. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) (DMEM and FBS from Fisher Bioblock scientific, Illkirch, France) and antibiotics. Grade 3 central chondrosarcoma cells (CH2879) were gifted by Dr A Llombart-Bosh [21], and cultured in Roswell Park Memorial Institute 1640′s medium (RPMI, Lonza AG, Verviers, Belgium) supplemented with 10% FBS and antibiotics. Cells were incubated at 37 ˚C in a humidified atmosphere containing 5% CO_2 in normoxia (21 % O_2) or hypoxia (1% O_2). The identity of cell lines was confirmed using STR profiling with the GenePrint 10 System (Promega). Cell cultures were regularly tested for mycoplasma contamination by PCR.

2.2. Irradiations

For X-ray radiations, media were replaced prior to irradiations, and cells were horizontally irradiated using Cegelec Cx 225 machine (25 keV, 13 mA, 1.32 Gy/min) at RecHadron facility (Caen, France).

The experiments with carbon ion radiations have been performed using ion beams at the Grand Accélérateur National d'Ions Lourds (GANIL, Caen, France) in the D1 experimental area managed by interdisciplinary research CIMAP-CIRIL platform. The culture flasks were completely filled with media to allow irradiation in a vertical position. The cells were irradiated with a ^{13}C beam with an initial energy of 75 MeV/u (LET = 33.7 keV/μm; 2 Gy/min). All irradiations were carried out at room temperature. The control groups were sham-irradiated.

2.3. Survival assay, and calculation of D_{10} and BRE

For clonogenic survival assays, cells were irradiated at 80% of

confluency, trypsinized and plated in culture dishes at 750 cells/cm^2 in triplicates. After 10–14 days, cells were stained with 0.1% crystal violet solution containing 1% ethanol. Only colonies containing more than 50 cells were counted. At least two parallel samples were scored in three to five repetitions performed for each type of irradiation. Survival fractions were determined from the following formula: number of colonies in irradiated groups / number of colonies in control groups. Since FS090 cell line did not form colony, adherent cells were also counted, and survival fraction calculated as the ratio of number of adherent cells in irradiated plates on number of cells in control plates. D_{10} (dose required to reduce the surviving fraction to 10%) was estimated based on survival curves, and relative biological effectiveness (RBE) was calculated by dividing the D_{10} obtained with X-rays to D_{10} obtained with C ions radiation.

2.4. Protein extraction and western-blot

Total proteins were extracted using RIPA buffer and Western-blot performed as previously described [22]. The following primary antibodies directed against p21 (sc-397), β-actin (sc-47,778), γH2AX (sc-101,696), RIP1 (sc-7881) and HIF-2 (sc28706) from Santa Cruz Biotechnology and that against PARP (#5246) and beclin-1 (#3495) from Cell signaling. Antibody against HIF-1 (#610,959) was purchased from BD Sciences. Beta Actin (sc-47,778, Santa Cruz) was used to verify that similar protein amounts were loaded in all lanes.

2.5. Flow cytometry

Flow cytometry experiments were done on Gallios flow cytometer (Beckman Coulter, Villepinte, France) at FACS facility (SFR 146, Caen, France). A minimum of 10,000 cells were analyzed in each sample, and at least three independent experiments were done. Data analysis was performed using Kaluza software.

For cell cycle analysis, cells were harvested by trypsinization and fixed with 70% ethanol. Thereafter, they were incubated in phosphate buffer saline (PBS) containing 20 μg/mL RNase (Invitrogen, Cergy-Pontoise, France) and 50 μg/mL propidium iodide (PI) (Sigma Aldrich, St Quentin Fallavier, France). DNA content was evaluated by measurement of fluorescence by flow cytometry.

For apoptosis evaluation, harvested cells were stained with phycoerythrin (PE)-conjugated antibody directed against Apo2.7 (clone 2.7 7A6) according to the manufacturer's condition (Beckman Coulter, Villepinte, France). This antibody reacts with a 38-kDa mitochondrial membrane protein (7A6 antigen), which is detectable in non-permeabilized cells in the early apoptotic state [23]. Apo 2.7 expression was evaluated by measurement of PE fluorescence.

2.6. Senescence-associated β-galactosidase (SA-β-Gal) assay

Senescence was assessed using senescence SA-β-Gal kit according to the manufacturer's instructions (Merck Millipore, Molsheim, France). Briefly, cells were fixed with 1 mL of fixative solution for 15 min at room temperature and incubated overnight with SA-β-Gal staining solution at 37 ˚C without CO_2. The SA-β-Gal positive cells were detected under microscope (magnification X100) and considered as positive when blue staining was evident in cytoplasm.

2.7. Whole-exome sequencing

Genomic DNA was isolated using NucleoSpin Tissue (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions and quantified on a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) at Proteogen facility (SFR ICORE 146 – Caen - France). DNA integrity was checked on 1.2% agarose gel.

Whole-exome sequencing was performed by Integragen Genomics (Evry, France) on HiSeq 2000 (Illumina) after exome capture using

SureSelect Human All Exon (Agilent technologies). Sequencing reads alignment to the human reference genome hg19/ GRCh37 and variant calling were processed using the CASAVA v1.8 pipeline (Illumina).

2.8. Variants analysis

Variants were annotated using Annovar software [24]. Filtering was performed following strict criteria and consisted of removing any low confidence variants (QPhred < 30 and read depth < 10) and excluding variants with a minor allele frequency (MAF) > 0.001 reported in 1000 Genomes Project, in NHLBI GO Exome Sequencing Project (ESP) or in Exome Aggregation Consortium (ExAC) datasets. Variants present in the Integragen Reference Database with a MAF > 0.01 were excluded as they must correspond to false positives related to the technology.

Human genes annotated in four relevant biological processes were retrieved from AmiGO 2 tool [25]. Seven genes belong to cellular response to X-ray (GO:0071481), 305 to DNA repair (GO:0006281), 614 to apoptotic process (GO:0006915) and 24 to cellular senescence (GO:0090398). Only missense, nonsense, stop loss, indels and essential splice acceptor and donor site variants in these genes were considered. Pathogenicity was predicted using SIFT [26], MutationTaster [27] and PolyPhen-2 [28] tools.

2.9. Statistics

Survival analyses are expressed as mean ± SEM of 3 independent experiments. Statistical significances were determined with Student's *t*-test. *: *P*-values ≤ 0.05; **: *P*-Value ≤ 0.1. Western blots and cell pictures show representatives from at least 3 independent experiments.

3. Results

3.1. Chondrosarcoma cell lines had different sensitivities to X-rays

First, we compared the sensitivity to X-ray radiations of five human cell lines derived from grade 2 and 3 chondrosarcomas. Survival curves were obtained by clonogenic assay (Fig. 1A) and cell counting (Fig. 1B).

As expected, survival fractions after irradiation with X-ray decreased exponentially with increasing doses. However, CHS lines responded with various sensitivities, as confirmed by the estimation of the dose required to reduce the cell survival fraction to 10%, called D_{10} (Table 1). CH2879 were the most sensitive cells, with a D_{10} inferior to 5 Gy. JJ012 and SW1353 cells presented an intermediate response, with a D_{10} comprise between 5 and 7.5 Gy. Finally, FS090 and 105KC cells were the most resistant with a D_{10} superior to 7.5 Gy.

3.2. X-rays induced similar DNA damages in chondrosarcoma cell lines

We first hypothesized that the variation of sensitivities to X-rays observed between the different chondrosarcoma cell lines could be explained by a variability in the number of DNA double strand breaks

(DSB) induced by irradiations. Because γH2AX foci correspond to DSBs [29–31], we evaluated radiation-induced γH2AX level, by Western-blot (Fig. 2). We observed no correlation between γH2AX staining and sensibility to irradiations. More precisely, X-ray radiations increased γH2AX with similar kinetics in CH2879, SW1353 and 105KC, which present yet different sensibilities to X-rays, suggesting that the mechanism explaining the variability of CHS sensibilities to X-rays implies a process downstream to DNA damages.

3.3. X-rays induced apoptosis in CH2879 and JJ012 chondrosarcomas

Since variations of chondrosarcoma sensitivities to radiations could result from alterations of death pathways, we first examined apoptosis induction.

In CH2879 and JJ012 cells, X-ray radiations increased cell percentage in sub-G1 phase, PARP cleavage and Apo2.7 expression, proving that apoptosis was induced in both CHS cell lines (Fig. 3). In contrast, in SW1353, FS090 and 105KC, only a barely increase of PARP cleavage or Apo2.7 expression was observed, suggesting that X-rays weakly induce apoptosis in these cell lines.

3.4. X-rays induced senescence in CH2879 and SW1353 chondrosarcomas

Furthermore, we evaluated whether X-rays could induce senescence in chondrosarcomas. Observation of irradiated cells showed some morphologic modifications. In particular, we found that CH2879 and SW1353 cell lines became more enlarged and flattened after radiations (Fig. 4A). These morphologic changes were corroborated by the increase of the side scatter (cell size) and forward scatter (cell granularity) observed by flow cytometry (data not shown), suggesting that X-rays induced senescence in CH2879 and SW1353 cell lines. To validate this hypothesis, we performed SA-β-Gal staining, and observed blue cells only for irradiated CH2879 and SW1353 lines, confirming that X-ray induced senescence only in these both cell lines (Fig. 4B). Furthermore, since p21 is required for cellular senescence, we evaluated its protein level (Fig. 4C and D). Interestingly, basal level of p21 was high in both cell lines where X-rays induced senescence (CH2879 and SW1353), and its level is increased by irradiations in these two cell lines, but not in the others.

3.5. X-rays induced neither necrosis nor autophagy in chondrosarcomas

We also investigated necrosis and autophagy. We evaluated necrosis by monitoring the induction of RIP1 (receptor-interacting protein 1, also known as RIPK1) [32], and autophagy by beclin-1 expression [33,34]. No increase of RIP1 nor beclin-1 expression was observed, suggesting that neither necrosis nor autophagy was induced in irradiated chondrosarcomas (Fig. 5). At contrary, RIP1 and beclin-1 expressions were rather reduced in CH2879 cells. That was consistent with the induction of apoptosis in this cell line [35].
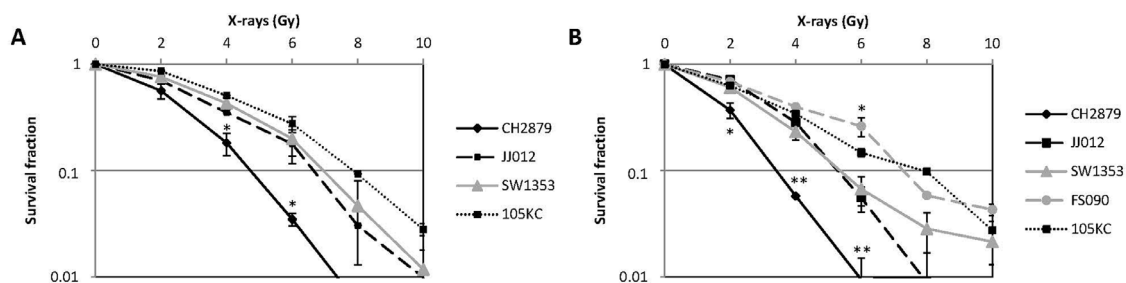

Fig. 1. Chondrosarcomas had different sensitivity to X-rays.
Chondrosarcoma cells were irradiated with increasing doses of X-rays (0 to 10 Gy). Survival fractions were determined by clonogenicity assay (A) and by adherent cells counting (B). The results of three independent experiments are shown. Data are expressed as means ± SEM.

Table 1
Efficiency of X-ray and Carbon Ion radiations in chondrosarcoma lines. Cells were irradiated with increasing doses of X-rays or carbon ions, and survival was estimated by cell counting or clonogenic assay. Data are expressed as means ± SEM of three independent experiments.

	D_{10} for X-rays (Gy)		D_{10} for C ions (Gy)		RBE	
	Cell counting	Clonogenic assay	Cell counting	Clonogenic assay	Cell counting	Clonogenic assay
CH2879	3.4 ± 0.1	4.6 ± 0.3	1.3 ± 0.2	1.6 ± 0.1	2.62	2.88
JJ012	5.4 ± 0.1	7 ± 0.8	1.5 ± 0.1	2.2 ± 0.2	3.60	3.18
SW1353	5.4 ± 0.4	7.2 ± 0.7	2.7 ± 0.1	3.1 ± 0.1	2	2.32
FS090	8 ± 0.9	ND	3 ± 0.2	ND	2.67	ND
105KC	7.9 ± 0.1	8.3 ± 0.4	2.1 ± 0.1	3 ± 0.1	3.8	2.8

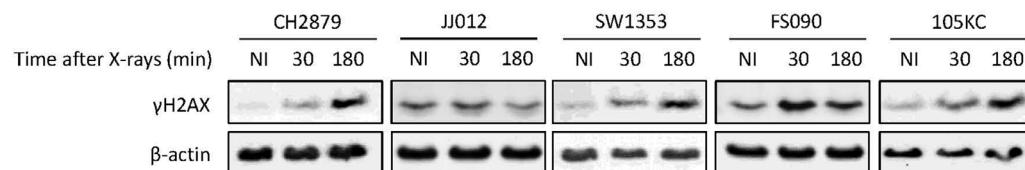ND = Not Determined (no colony formation).

Fig. 2. X-rays induced DNA damages in chondrosarcomas.
Chondrosarcoma cells were irradiated with X-rays (6 Gy). Proteins were extracted before irradiation (NI) and 30 min or 3 h after X-rays. γH2AX was analyzed by Western blot. β-actin was used to compare protein loading.
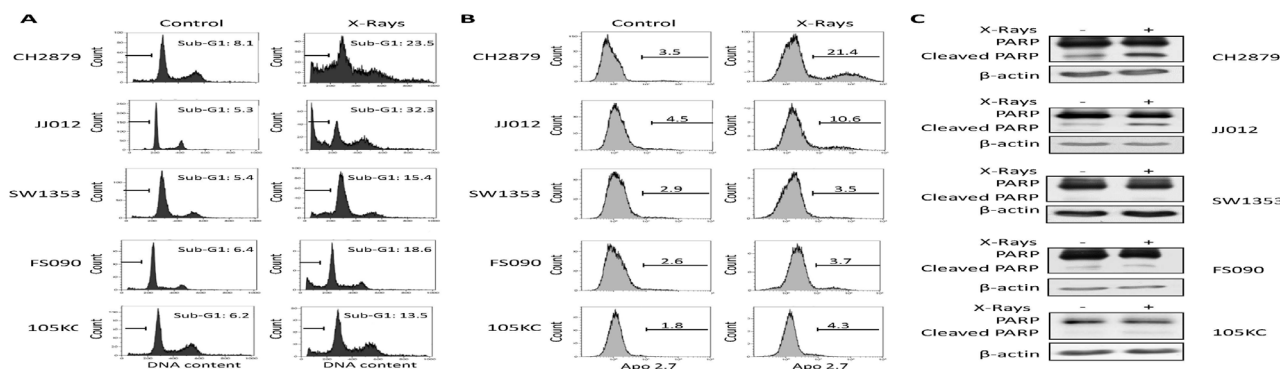

Fig. 3. X-rays differentially induced apoptosis in chondrosarcomas.
Chondrosarcoma cells were irradiated with X-rays (6 Gy). After 5 days of culture, they were fixed and DNA content was determined by flow cytometry (A). Apo 2.7 expression and PARP protein cleavage were analyzed by flow cytometry (B) or Western blot respectively (C).

3.6. The slow growth rate of CHS is not sufficient to explain their radioresistance

Since chondrosarcoma radioresistance may be explained by a low cell proliferation rate, we determined the doubling time for each cell line (Table 2). JJ012 (with a doubling time of 25 h) and 105KC (with of doubling time of 56 h) were the most and the less proliferative cells, respectively. The other cell lines (SW1353, CH2879 and FS090) had a doubling time around 40 h.

Even if 105KC cells had the slower growth rate, what may explain its weaker sensitivity to X-rays, we cannot draw any correlation between the sensitivity to X-rays and the doubling time of other cell lines.

3.7. Chondrosarcoma mutations in biologically relevant pathways

We further explore the molecular basis of the differential responses to X-rays observed in the five chondrosarcoma cell lines by analyzing their genetic profiles. 925 distinct genes involved in four relevant biological processes (cellular response to X-ray, DNA repair, apoptotic process and cellular senescence) were screened. Rare mutations were identified in 21 genes involved in DNA repair, ranging from 1 to 8 mutations per cell line (Table 3). Thirty-six mutations were identified in 33 distinct genes involved in apoptotic process, ranging from 3 to 11 mutations per cell line (Table 4). Interestingly, rare mutations in 2 genes involved in the cellular senescence were identified in both cell lines in which senescence was induced in response to X-ray irradiations,

a missense mutation in *TBX3* and a mutation that affects a splice site in *CDKN2A*, in CH2879 and SW1353 cell lines, respectively (Table 5). In addition, among genes involved in the cellular response to X-ray, one rare missense mutation was identified in *HMGA2* in CH2879, the most sensitive cell line (Table 6).

3.8. Hypoxia do not induce resistance to X-rays in chondrosarcomas

Next, we investigated the effect of hypoxia on response of chondrosarcoma to X-rays. Indeed, hypoxia is often considered as the main environmental condition responsive of the radioresistance of chondrosarcomas. Tumoral cells were incubated in hypoxia (1% O_2) for 7 days before being irradiated with X-rays and maintained under hypoxia for five supplemental days (Fig. 6). Survival were assayed. No significant differences were observed for three cell lines (SW1353, CH2879 or FS090), whereas hypoxia increased the sensibility of JJ012 to X-rays (6 Gy). Then, we investigated apoptosis by Apo2.7 in JJ012 and SW1353. Unexpectedly, hypoxia increased the induced-apoptosis in JJ012. In SW1353, no apoptosis was detected. In addition, X-rays induced senescence in SW1353, in the same extend in normoxia and hypoxia conditions, whereas no senescence was observed in JJ012.

These results being unexpected, we checked that the chondrosarcomas were able to respond to hypoxia. So, we investigated the expression of Hypoxia-Inducible Factors (HIF), which are transcription factors that respond to a decrease of available oxygen in the cellular environment (Fig. 6D). As expected, hypoxia increased HIF-1 and HIF-2
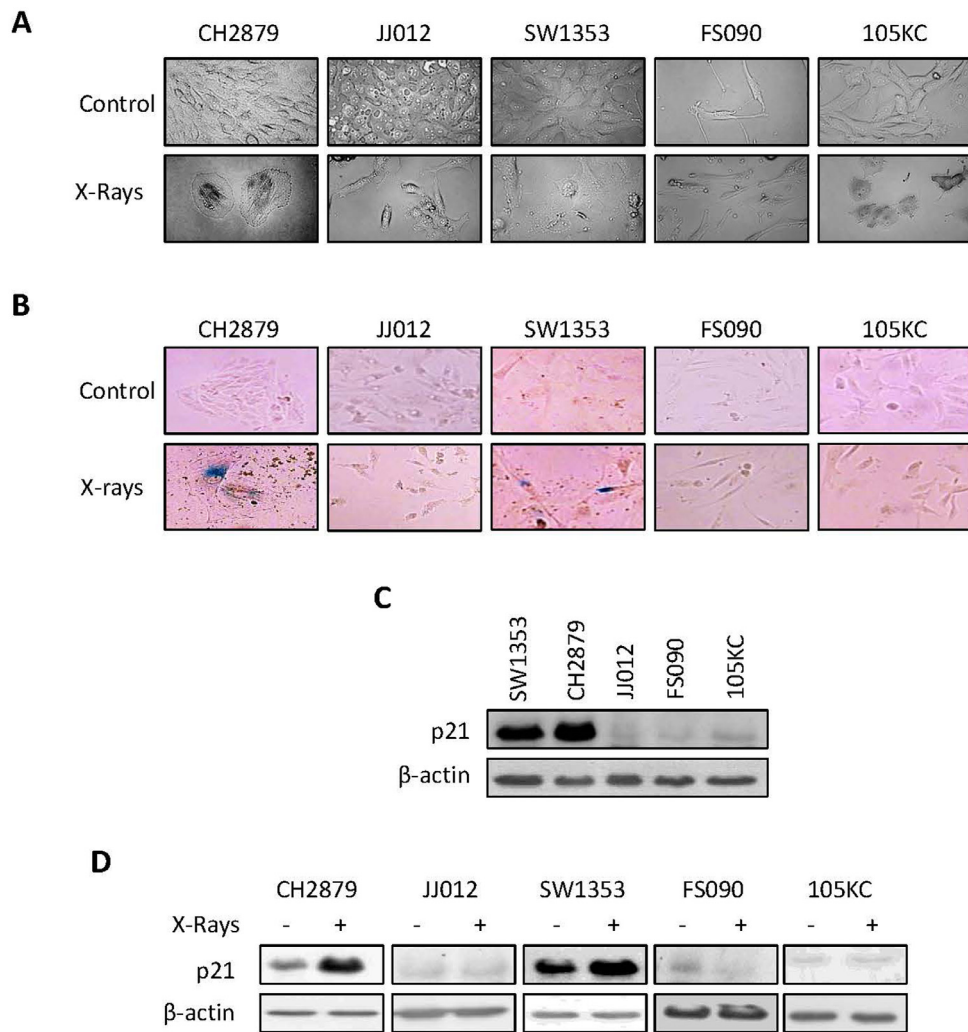
Fig. 4. X-rays induced senescence in CH2879 and SW1353.
Chondrosarcoma cells were irradiated with X-rays (6 Gy). After 5 days of culture, cells were observed by microscopy (A), or stained with senescence-associated β-galactosidase (B). Representative images are shown (magnification X100). p21 expression was evaluated by Western blot (C and D).
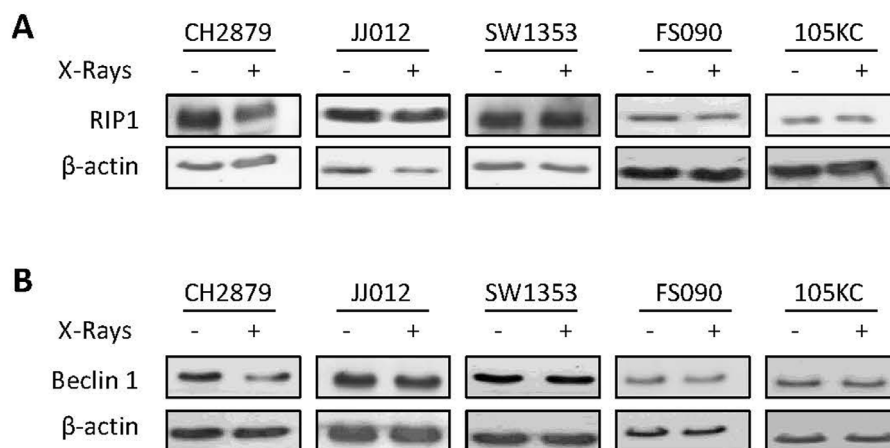

Fig. 5. Necrosis and autophagy were not induced in chondrosarcomas by X-rays.
Chondrosarcoma cells were irradiated as previously, and RIP1 (A) and beclin-1 (B) expressions were analyzed by Western blot. β-actin was used to compare protein loading.

Table 2
Doubling time of chondrosarcoma cell lines.

Chondrosarcoma cell lines	Doubling time
SW1353	36.9 h
CH2879	42.3 h
JJ012	25.9 h
FS090	40.8 h
105KC	56.1 h

expression in the five chondrosarcoma cell lines, proving that cells were able to sense the low O_2 tension.

3.9. Chondrosarcomas are more sensitive to Carbon ions than X-rays

Finally, we investigated the response of chondrosarcoma lines to Carbon ion radiations (Fig. 7). The survival fractions for all CHS cells irradiated with C-ion beam decreased exponentially with increasing doses. In addition, survival curves clearly show that C-ions radiations are more effective than X-rays. Indeed, the D_{10} was comprise, depending on the cell lines, between 3.4 and 8.5 Gy for X-ray, whereas it was estimated between 1.3 and 3.1 Gy for carbon ion beam (Table 1). Therefore, the relative biological effectiveness (RBE) values for carbon ion beams relative to X-rays at D_{10} level are comprised between 2 and 3.8 (Table 1). As observed for X-rays, the sensitivity of chondrosarcomas to carbon ion beam depends on cell line.

In addition, we analyzed DNA damage and showed that C ion beam led to more γH2AX induction in comparison to X-rays (Fig. 6C), indicating that at the same dose, either carbon ion beams induced more DSB than X-rays, or that these DSB cannot be repaired. This could explain the superior efficiency of Carbon ions to X-rays radiations.

4. Discussion

In clinical routine, chemotherapy and radiotherapy are not considered as efficient for the treatment of chondrosarcoma, and surgery still prevails as the primary treatment modality of this tumor [4]. However and surprisingly, the effective radioresistance of chondrosarcomas is not clearly described and the mechanisms responsible for this resistance are not yet well known. In the present study, using five different cell lines derived from human chondrosarcomas, we

demonstrate that these tumors form a very heterogeneous family with different sensitivity to radiations, indicating the necessity to work with several cell lines. We also show that chronic hypoxia does not favor radioresistance of chondrosarcomas, and even may increase the efficiency of X-rays for some of them. We also show that carbon ion radiations are more efficient than X-rays for each cell line, confirming the interest of this innovative therapeutic approach for chondrosarcomas.

Under normal circumstances, cells are programmed to sense DNA damage and to initiate subsequent repair. Following radiation exposure, if repair cannot be achieved, tumor suppressors trigger cell cycle arrest, preventing the damage from passing on to daughter cells. Cells arrest their growth and undergo apoptosis, senescence, or necrosis. In this study, we found that some chondrosarcoma lines undergo apoptosis (JJ012) or senescence (SW1353), or both of them after irradiations (CH2879), whereas no death was detected for other CHS lines (FS090 and 105KC). This heterogeneity of responses to radiations could be explained by genetic alterations. Rare potentially deleterious mutations in *TBX3* and *CDKN2A*, two genes involved in cellular senescence, were only identified in CH2879 and SW1353, both cell lines in which senescence was induced in response to X-ray irradiations. *TBX3* gene is overexpressed in numerous cancers. In chondrosarcoma, TBX3 is known to promote tumorigenesis [36]. TBX3 contributes to several aspects of the oncogenic process, including senescence bypass, in part through its ability to repress p14/ARF, p21, PTEN and E-cadherin [37,38]. Herein, we found that CH2879 cell line carries a p.A591V missense mutation in *TBX3* that is located in the transcriptional repression domain. Therefore, this mutation could lead to a loss of negative regulation of target genes including p21, resulting in senescence bypass. In accordance to this hypothesis, we observed higher basal and radio-induced p21 protein expression in CH2879 cell line than in those in which no senescence was induced after irradiations. This higher p21 expression is also observed in SW1353 cell line, suggesting that p21 overexpression is specific to cell lines dying by senescence after irradiations, and could be a good biomarker of senescence induced by X-ray in chondrosarcomas. However, no mutation that could explain p21 overexpression was identified in SW1353, and the functional consequence of the splice mutation in *CDKN2A* in this cell line need to be further analyzed. In addition, a p.105 L missense mutation in *HMGA2* was identified in CH2879 cell line. This oncogene encodes a chromatin-binding protein that is overexpressed in many cancers and correlates with a better response of radiotherapy in colorectal cancer [39]. This

Table 3
Rare potentially deleterious variants identified in chondrosarcoma cell lines in 305 genes implicated in DNA repair.

CHS line	Gene symbol	cDNA change	Protein change	Mutation type
CH2879	*ATR*	NM_001184:c.3194G > T (hom)	p.G1065V	missense
	CEP164	NM_014956:c.337delA (hom)[a]	p.K113fs	frameshift deletion
	CHAF1B	NM_005441:c.254_255del (het)	p.G85fs	frameshift deletion
	MLH1	NM_000249:c.545 + 2T > C (hom)	p.?	splicing
	POLD1	NM_002691:c.2954–1G > - (het)[a]	p.?	splicing
	USP28	NM_020886:c.3136C > T (hom)[a]	p.P1046S	missense
	USP43	NM_153,210:c.2390T > C (hom)	p.M797T	missense
	XAB2	NM_020196:c.1108C > T (het)	p.R370C	missense
JJ012	*ERCC1*	NM_001983:c.595G > A (het)	p.A199T	missense
	KIN	NM_012311:c.140C > T (het)	p.S47F	missense
	SLX4	NM_032444:c.1034C > A (het)	p.T345N	missense
SW1353	*ACTL6A*	NM_004301:c.524C > T (het)	p.T175I	missense
	INO80C	NM_194,281:c.409A > C (het)	p.K137Q	missense
	MUM1	NM_032853:c.1718A > G (het)	p.N573S	missense
	POLR2F	NM_021974:c.188C > T (het)	p.A63V	missense
	TOPBP1	NM_007027:c.1804G > C (het)	p.E602Q	missense
FS090	*IGHMBP2*	NM_002180:c.1478C > T (het)	p.T493I	missense
	MUS81	NM_025128:c.112C > G (het)	p.R38G	missense
	RECQL4	NM_004260:c.2653C > T (het)	p.L885F	missense
	REV3L	NM_002912:c.37A > G (het)	p.M13V	missense
105KC	*TREX1*	NM_016381:c.1072A > C (hom)	p.T358P	missense

[a] Mutations reported in COSMIC database.

hom, homozygous state ; het, heterozygous state.

Table 4

Rare potentially deleterious variants identified in chondrosarcoma cell lines in 614 genes implicated in apoptotic process.

CHS line	Gene symbol	cDNA change	Protein change	Mutation type
CH2879	*CTNNBL1*	NM_030877:c.166delA (het)[a]	p.K56fs	frameshift deletion
	IFNG	NM_000619:c.115–2A > - (het)	p.?	splicing
	MAP2K4	NM_003010:c.461G > A (hom)	p.R154Q	missense
	MAPK3	NM_002746:c.187G > A (hom)	p.V63M	missense
	NFKB1	NM_003998:c.949C > T (hom)	p.P317S	missense
	NLRP1	NM_014922:c.2009C > T (hom)	p.T670I	missense
	NLRP1	NM_014922:c.2056A > G (hom)	p.M686V	missense
	NME3	NM_002513:c.371A > T (het)	p.D124V	missense
	PTEN	NM_000314:c.697C > T (het)[a]	p.R233X	nonsense
	SHB	NM_003028:c.863G > A (hom)	p.R288Q	missense
	TAOK2	NM_004783:c.1900C > T (het)	p.R634W	missense
JJ012	*BCL7C*	NM_004765:c.52A > G (het)	p.I18V	missense
	MX1	NM_002462:c.1541A > G (het)	p.E514G	missense
	TP53	NM_000546:c.596G > T (hom)[a]	p.G199V	missense
SW1353	*ACIN1*	NM_014977:c.1007G > C (het)	p.R336T	missense
	BNIPL	NM_138,278:c.952C > G (het)	p.R318G	missense
	CDKN2A	NM_000077:c.151–2A > C (hom)[a]	p.?	splicing
	CHI3L1	NM_001276:c.383G > A (het)[a]	p.R128H	missense
	CIDEC	NM_022094:c.619T > C (het)	p.C207R	missense
	MAP3K10	NM_002446:c.1364G > A (het)	p.R455H	missense
	PSMF1	NM_006814:c.569C > T (het)	p.P190L	missense
	RASSF6	NM_177,532:c.937A > G (het)	p.K313E	missense
	TP53	NM_000546:c.607G > T (het)[a]	p.V203L	missense
FS090	*BRMS1*	NM_015399:c.484G > A (het)	p.E162K	missense
	GJA1	NM_000165:c.706G > A (het)	p.V236I	missense
	ITGB2	NM_000211:c.573C > A (het)	p.C191X	nonsense
	MX1	NM_002462:c.223G > A (het)	p.V75I	missense
	PSMA3	NM_002788:c.599T > C (het)	p.I200T	missense
105KC	*BRAT1*	NM_152,743:c.1564G > A (het)	p.E522K	missense
	DLC1	NM_006094:c.902C > T (het)	p.T301M	missense
	PAK2	NM_002577:c.862A > G (het)	p.K288E	missense
	PEG3	NM_006210:c.4760C > G (het)	p.T1587S	missense
	PPP2R2B	NM_181,674:c.1261A > T (het)	p.T421S	missense
	RTN4	NM_020532:c.907T > C (het)	p.S303P	Missense
	SEMA6A	NM_020796:c.2882C > A (het)	p.P961H	missense
	TNS4	NM_032865:c.683C > A (het)	p.S228Y	missense

[a] Mutations reported in COSMIC database.
hom, homozygous state ; het, heterozygous state.

Table 5

Rare potentially deleterious variants identified in chondrosarcoma cell lines in 24 genes implicated in cellular senescence.

CHS Cell line	Gene symbol	cDNA change	Protein change	Mutation type
CH2879	*TBX3*	NM_005996:c.1772C > T (hom)	p.A591V	missense
SW1353	*CDKN2A*	NM_000077:c.151–2A > C (hom)[a]	p.?	splicing

[a] Mutations reported in COSMIC database.
hom, homozygous state ; het, heterozygous state.

Table 6

Rare potentially deleterious variants identified in chondrosarcoma cell lines in 7 genes implicated in cellular response to X-ray.

CHS Cell line	Gene symbol	cDNA change	Protein change	Mutation type
CH2879	*HMGA2*	NM_001300918:c.314C > T (hom)	p.P105L	missense

hom, homozygous state ; het, heterozygous state.

suggests that the better sensitivity to X-ray irradiations of CH2879 cell line could be explain by overexpression of HMGA2 as a consequence of the missense mutation identified in this gene. Here again, further functional analyses are required to confirm the role of these mutations in radiosensitivity and senescence induced by X-rays.

Hypoxic microenvironment is thought to contribute to tumor radioresistance *in vivo* [40–42]. Low oxygen content inhibits the formation and propagation of radiation-induced reactive oxygen species (ROS) and consequently reduced DNA damages. However, our study demonstrate that unexpectedly, chronic hypoxia has no effect on the sensitivity of chondrosarcomas (JJ012, SW1353 and FS090) to X-rays, and could even increase their radiosensitivity (JJ012). An hypoxia-induced radiosensitivity was already described in the literature, and was explained by an decreased homologous recombination capacity [43]. In addition, this environmental parameter (oxygen content) cannot explain the variation of sensitivity to X-ray radiations observed in our experiments between the different cell lines, since their response was compared each time in the same condition (either at 21% O2 or at 1% O2), and since a similar level of DNA damages was observed in several chondrosarcoma lines which presented yet different sensibility to X-ray radiations, suggesting that variations of the response to X-rays between cell lines were due to intrinsic factors. Furthermore, resistance to treatments of chondrosarcomas may be also explained by slow growth rate and low percentage of dividing cells [9,8,7,6]. However, our experiments suggest that heterogeneity of chondrosarcomas to X-rays cannot be explained by a low proliferation, except, perhaps for the 105KC line.

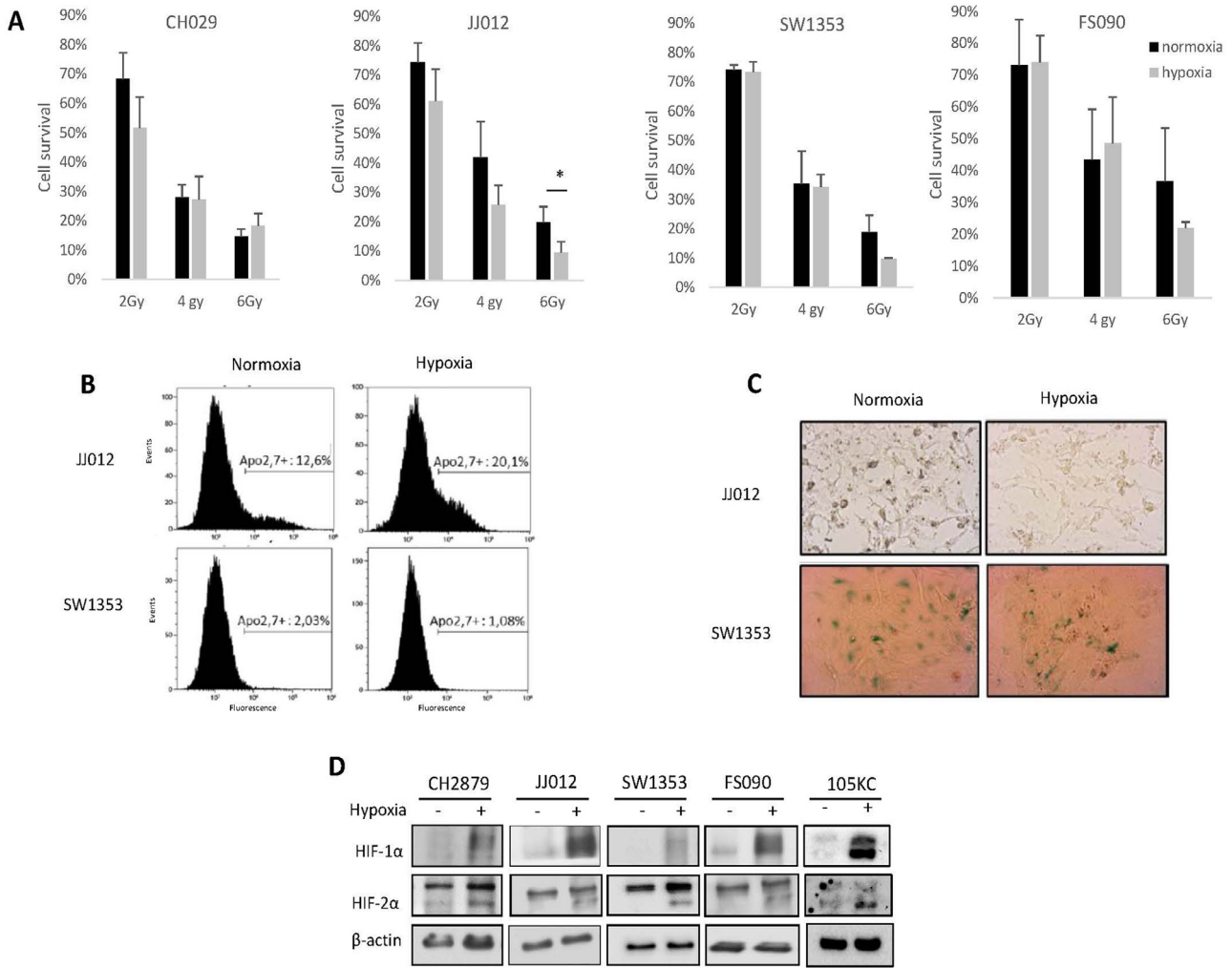Finally, we demonstrate that, as observed by radiotherapists,

Fig. 6. Chronic hypoxia does not favor radioresistance of chondrosarcomas.

Chondrosarcoma cells were maintained in normoxia (21% O2) or hypoxia (1% O2) for 7 days before being irradiated with increasing doses of X-rays. Five days later, adherent cells were counted. Values are normalized to number of non-irradiated cells for each condition. The results of three independent experiments are shown. Data are expressed as means ± SEM (A). Five days after irradiation (6 Gy), apoptosis (B) and necrosis (C) were also assayed by Apo2.7 and senescence-associated β-galactosidase staining, respectively. Expression of HIF-1 and HIF-2 were also investigated by western-blot (D).
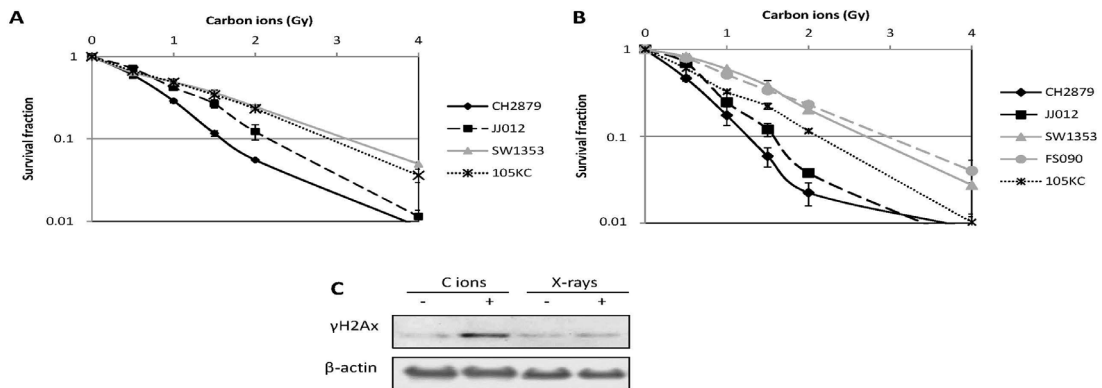
Fig. 7. Carbon ions are more effective than X-rays.

Chondrosarcoma cells were irradiated with increasing doses of Carbon ions (0 to 4 Gy). Survival fractions were determined by clonogenicity assay (A) and by adherent cell counting (B). Data are expressed as means ± SEM of three independent experiments.

SW1353 chondrosarcomas were irradiated with Carbon ions or X-rays at 4 Gy (C). Proteins were extracted 15 h after irradiation and γH2AX expression were analyzed by Western blot. β-actin was used to compare protein loading.

chondrosarcomas are more sensitive to carbon ions than to X-rays, with a RBE comprised between 2 and 4. This value is coherent with clinical data which report a RBE ranged in the same interval of doses [16,44,11]. This better efficiency of carbon ion beams compared to X-rays is probably due to the fact that carbon ions are high-LET radiations which induce mainly double strand breaks of DNA which are difficult to repair by cells [45–48]. This mechanism is likely involved also in chondrosarcomas since we observe more γH2AX after carbon ions in comparison to X-ray radiations. These *in vitro* data are consistent with clinical reports which show the therapeutic potential of carbon ions to treat some chondrosarcomas [11,12] and need to be further investigated. These *in vitro* research are indeed primordial to treat patients with doses giving the minimal side effects.

5. Conclusions

Herein, we showed that X-rays treatment elicit differential responses and death in CHS cells, confirming the importance to carry on studies using several chondrosarcoma cell lines *in vitro*. In addition, we showed that hypoxia does not increase radioresistance of chondrosarcomas. Furthermore, treatment with carbon ions gave interesting outcomes, consistent with clinical observations. We also identify p21 expression as a potential marker to predict senescence induction by X-rays radiation. However, additional investigations are required to identify markers permitting to predict the sensitivity to radiations with X-rays or Carbon ions in order to optimize chondrosarcoma treatment. Analysis of CHS mutations opens also an interesting path for elucidation of the resistance of chondrosarcoma to irradiations. Further investigations will bring more accuracy for that purpose.

Declaration of Competing Interest

Authors declare no conflct of interest regarding this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2020.100283.

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