Induced resistance to ifosfamide in osteosarcoma cells suggests a more aggressive tumor profile

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A B S T R A C T

Aims: Osteosarcoma (OS) is the most common primary malignant bone sarcoma among children and adolescents. Treatment is based on neo-adjuvant and adjuvant chemotherapy, using the standard drugs cisplatin, methotrexate, doxorubicin, and ifosfamide (IFO). Due to the high capacity of tumor resistance, the current work aimed to analyze genes related to cycle control and cell differentiation in OS cells sensitive to and with induced resistance to IFO. This was to assess whether the differentiated expression of these genes may affect resistance to the drug IFO used in OS treatment, and thus establish possible biomarkers of disease progression.

Materials and methods: In this work, the treatment-sensitive OS U2OS lineage was used, and the same lineage was submitted to the process of induction of IFO resistance. These cells were evaluated by MTT, migration and proliferation assays and submitted to gene expression analysis.

Key findings: The results demonstrate that after induction of resistance to IFO, resistant U2OS cells show a more aggressive tumor behavior, with greater capacity for cell migration, proliferation, and invasion compared to sensitive cells. Gene analysis indicates that resistance-induced cells have differentiated expression of the genes EPB41L3, GADD45A, IBD3, OXCT1, UBE2L6, UBE2A ALPL, and EFNB2. Our results suggest new perspectives on possible resistance biomarkers, especially the genes EFNB2 and EPB41L3, given that these genes have rarely been studied their expression linked to osteosarcoma. They show how the resistance induction model can be useful for studies on tumor cell behavior.

1. Introduction

Osteosarcoma (OS) is one of the most common types of primary bone tumors and most often affects the epiphysis region of long bones [1]. Approximately 20–25% of patients present metastases at diagnosis, a factor that implies worse survival of these patients, among whom only 1 in 4 patients has a 5-year disease-free survival. Overall, about 30% of metastatic tumors do not respond to chemotherapy [2].

The most commonly used anticancer agents are doxorubicin (DOX), ifosfamide (IFO), cisplatin (CIS), and high doses of methotrexate (MTX) [3,4]. The drug IFO is an alkylating agent that, due to its phosphoric mustard metabolites, intercalates in the double strand of DNA, generating cross-links in the N-7 region of the guanine base, thus leading to cellular apoptosis [5,6].

Drug resistance can be defined as intrinsic resistance, when tumor cells already have mutations in genes responsible for anti-apoptotic activities, drug efflux, cell migration, and other activities [7], or acquired resistance that occurs due to the selective process generated by
drug treatment. These two forms of resistance can occur in OS [8].

In this context, the current study aimed to induce resistance to IFO in the U2OS lineage of human osteosarcoma to assess how these cells behave toward the drug IFO and to analyze the gene expression of EFNBR, EPB41L3, GADD45A, ALPL, OXCT1, IER3, UBE2A, and UBE2L6 involved in tumor resistance, in both sensitive and resistant cells. EPB41L3 is a tumor suppressor that inhibits cell proliferation and promotes apoptosis, in addition to encoding a protein that assists in the organization of the actin cytoskeleton; GADD45A is responsible for cell cycle control and stimulates DNA repair by excision of bases; IER3 influences the activation of the ERK pathway, which is responsible for cell proliferation, survival, and differentiation; OXCT1 plays a role in the metabolism of ketone bodies and is used by tumor cells as a source of ATP; the genes UBE2L6 and UBE2A are part of the family of enzymes that assist in the ubiquitination process, and they are responsible for protein degradation mediated by proteasomes and which is highly important in tumorigenesis; in this case the E2 group of enzymes are related to tumor progression involving mechanisms of DNA repair in tumor cells, apoptosis, and signaling of oncogenic pathways, among others; ALPL is a gene that plays a fundamental role in the formation of osteocytes and mineralization of the bone matrix; and, finally, EFNBR regulates axon orientation, angiogenesis, and epithelial cell migration.

In this context, it is of great importance to understand how dysregulation in the expression of these genes may be related to the degree of tumor aggressiveness and the process of resistance to the drug IFO.

In this work, we demonstrated the process of inducing resistance to IFO and the expression of genes that are differentially expressed in the absence and presence of the drug, suggesting that IFO leads to a process of selective pressure on resistant cells; when it is removed from the culture, the cells return to being sensitive to the drug. Furthermore, the genes involved in the process of cell cycle regulation and differentiation present a difference between cells that have been induced to be resistant to IFO and cells that are sensitive.

2. Materials and methods

2.1. Cell culture and MTT assay

Human osteosarcoma U2OS lineage cells (BCRJ code:0304/ATCC: HTB-96) were cultured with DMEM high glucose medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS). The cells were maintained at 37°C and 5% CO₂ and cell culture was performed in the absence of antibiotics. The alkylating agent IFO >98% (Sigma-Aldrich®) C₇H₆N₂O₄P, with a molecular weight of 261.09 g/mol, was solubilized with DMSO and kept in stock solution of 1 mM divided into aliquots of approximately 10 ml, protected from light at a temperature of –20°C.

Before starting the IFO resistance induction protocol, an initial IC₅₀ was performed to determine the percentage of cellular metabolic activity in an IFO concentration gradient (5.0 μM; 10 μM; 15 μM; 20 μM; 30 μM and 40 μM); this assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the mitochondrial activity of the cells [9].

The initial IC₅₀ value was obtained from a nonlinear regression calculation.

For the MTT assay, a total of 1 × 10⁴ IFO-treated cells were used per well in 96 plates, with a final volume at 150 μl (cells with DMEM medium and 10% FBS). Positive control consisted of cells and DMEM medium with 10% FBS, without treatment, and negative control consisted of only cells with medium and dimethyl sulfoxide (DMSO). After adding the IFO concentrations, the plate was kept in the chamber under the same culture conditions for 24 h, and then all the medium was removed and 10 μl of MTT (4 mg/mL) were added. The plate was incubated again for another 4 h with the addition of 60 μl of DMSO for 30 min and then read on a microplate spectrophotometer (BioTek Eon Microplate Spectrophotometer S N 265483) at an absorbance of 570 nm.

2.2. Induced resistance

The induction resistance protocol was performed [10]. When cells reached confluence between 70% and 80%, they received 20% of the concentration of 25 μM IFO and were incubated for 24 h under the same culture conditions. They were then washed with phosphate-buffered saline 1X (PBS), and when they reached the same confluence as the previous step, they received 20% of the initial IC₅₀ value and the whole process was repeated three times. Cells were treated with 20%, 40%, 60%, 80%, and 100% of the 25 μM value. After four months of resistance induction, the U2OS R+ lineage was obtained.

A new MTT assay, as mentioned above, was performed to verify the sensitivity of the U2OS lineage after resistance induction (R+) compared to the sensitive U2OS lineage (S). In this new MTT assay, the IFO concentrations used were 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, and 60 μM. The U2OS R+ line was maintained in culture with 35 μM of the drug, and some of the cells that went through the resistance induction protocol began to be cultivated in the absence of the drug (U2OS R-).

2.3. Migration assay

For the migration test, a total of 2.5 × 10⁵ cells per well were used. After reaching 100% confluence, three parallel lines were made with the aid of a 200 μl tip; then the culture medium was removed and washed once with PBS [11]. Culture medium was added to each well without the addition of FBS, with the exception of the positive control, which had the addition of 2% FBS.

The cells were incubated at 37°C, and the measurements were photo-documented at previously established time intervals, starting from the moment after the incision of the lines (time 0 h), followed by intervals of 24, 48, and 72 h after the incision, always at the same previously marked points and corresponding to the location with the absence of cells at time 0 h. The number of cells was counted using the ImageJ® software, where the cell migration capacity was measured in relation to the groups of sensitive cells (S) and resistant cells (U2OS R+).

2.4. Survival test without membership

Cells were seeded in a quantity of 2.5 × 10⁵ cells per well containing LB Agar covering the entire surface of a 6-well plate [12]. The cells were maintained in culture for 6 days in a humidified chamber with an atmosphere of 5% CO₂ at 37°C, with changes of DMEM medium supplemented with 10% fetal bovine serum every 48 h. At the end of the culture time, the cells were subjected to an MTT test to assess cell viability.

2.5. Proliferation assay

A total of 2.5 × 10⁵ cells per well were seeded in 6-well plates for recounting. The values obtained at 0 h and 48 h were analyzed. The cells were trypsinized, washed with PBS, counted, and PDT was calculated following the formula PDT=(T₂−T₁) × log₁₀ 10 2 ÷ log₁₀ N₂/N₁, where T corresponds to the assay time and N the number of cells counted [13].

2.6. RNA extraction, cDNA synthesis, and real-time qPCR

To evaluate the gene expression profile, the qPCR of the groups U2OS R-, U2OS R+, and U2OS S was performed. Total RNA from U2OS S, U2OS R+ and U2OS R - cells was extracted using the TRizol method (Invitrogen, Osaka, Japan). Integrity and quantification were determined using qubit (Invitrogen) and nanodrop (Thermo Scientific Nanodrop 2000) and the ratios 260/280 and 260/230 in the range of approximately 2.0 were considered.
To remove contaminating genomic DNA, the preparation (10–100 μg) was subsequently incubated with 10 units of RNase-free DNASE I (Promega, M6101). RNA at a concentration of 1 μg/μL was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher USA), according to the manufacturer’s manual. Therefore, a sensitive and reproducible quantitative PCR based on SYBR green using the StepOnePlus Real time PCR System@ (Applied Biosystems) was developed. The analysis was performed using the 2^(-△△Ct) method [14]. All samples were normalized using endogenous control for GAPDH. Table 1 shows the sequence of resistance genes used. All reactions were performed in biological and experimental triplicate.

2.7. Data analysis

Statistical analyses were performed using the GraphPad Prism 8.0.1 program. The IC_{50} value was obtained by means of a non-linear regression, and the differences between the groups of cells were compared using two-way ANOVA and the Tukey post-hoc test. Gene expression was determined, as shown in Fig. 1 A. The resistance induction protocol was determined, as shown in Fig. 1 A. The IFO (Promega, M6101). RNA at a concentration of 1 μM was obtained by unpaired t-test.

The statistical difference in the gene expression of S cells in relation to R+ and R-cells was represented by p < 0.0001 (**). The significance value was determined from p < 0.05.

3. Results

3.1. Initial IC_{50} and induced resistance

To obtain the U2OS lineage that was resistant to IFO, an initial MTT assay was performed, where the concentration of IFO used in the resistance induction protocol was determined, as shown in Fig. 1A. The IC_{50} from the U2OS lineage before induction was 26.77 μM. Subsequently, a new MTT was performed in cells that were induced to resistance to IFO (U2OS R+) and also in sensitive cells (U2OS S).

The IC_{50} of the U2OS R+ group was 37.13 μM, while in the S group the IC_{50} was 33.12 μM, as shown in Fig. 1 B. After confirming the resistance in U2OS R+ cells, these cells were cultivated in the absence of the drug (U2OS R-) to verify that, even without IFO stimulation, these cells remained resistant. In a new MTT, the U2OS R-group presented an IC_{50} of 20.89 μM; the U2OS R+ group had an IC_{50} of 38.97 μM, and the U2OS S group an IC_{50} of 32.24 μM (Fig. 1 C and 1D). These results indicate that although the process of inducing resistance occurred as expected, this resistance only persists when cells are maintained in culture in the presence of the IFO drug, while when resistance is induced and subsequently cultured without the drug, these cells again become sensitive to IFO treatment.

3.2. Migration assay

After 24 h, there was a statistical difference between U2OS R+ and U2OS S cells (+), and the same pattern of statistical difference occurred at 48 h and at 72 h. The U2OS R+ group continued to show a greater migration capacity p < 0.0001 (**), showing that the U2OS R+ cell group had a higher migration capacity compared to the U2OS S cell groups (Fig. 2). In Fig. 3, it is possible to observe the cell migration in vitro at the initial time (0 h), 24 h, 48 h, and 72 h respectively.

3.3. Survival test without adherence

After six days of cultivation, counting performed using the Neubauer chamber and the MTT assay demonstrated that the number of cells increased compared to the beginning, the mean U2OS R+ cells to 3.36 x 10^7 and the U2OS S cells to 3.86 x 10^7 cells at the end of the experiment. This suggests that the IFO-sensitive and resistant osteosarcoma U2OS lineages have the ability to survive without plaque adherence and remain capable of cell proliferation. Fig. 4 shows the adherence of U2OS S cells and U2OS R+ cells, revealing their degradation potential.

3.4. Proliferation assay

The group of U2OS R+ cells showed a shorter proliferation time compared to U2OS S cells, with P value < 0.0001 (**), as indicated in Fig. 5. The proliferation capacity of the U2OS R+ group was on average 21 h, while the proliferation of U2OS S occurred in an average time of 30 h.

3.5. Gene expression profile

To assess whether the U2OS R+ group has different levels of expression of resistance genes compared to the U2OS S group, a qPCR was performed with the genes EFNB2, ALPL, TMPO, UBE2L6, UBE2A, EPB41L3, GADD45A, OXCT1, and IER3. We also evaluated whether the resistance-induced cells maintained the same gene expression profile when the IFO stimulus (U2OS R-) was removed.

The U2OS S lineage presented the genes ALPL, UBE2A, IER3 and EPB41L3 up-regulated in comparison with U2OS R+ and U2OS R-cells (p < 0.0001). In contrast, these same genes were down-regulated in the U2OS R+ and U2OS R-groups, and there was no significant difference between them, as shown in Fig. 6 A-D.

As regards gene UBE2A, there was a high expression in the U2OS S group in relation to the other groups of resistant cells (p < 0.0001). When compared to the groups of resistant cells, it was observed that the U2OS R+ group continued to show a high expression of UBE2A when compared to U2OS R+ (p < 0.0001). The same level of expression between the cell groups was repeated with OXCT1, as shown in Fig. 6 E-F.

Analysis of GADD45A showed a high expression in the U2OS S group (p < 0.0001), although cell groups U2OS R+ and U2OS R-presented a lower expression of this gene when compared to U2OS S group. U2OS R+ presented a significant difference (p = 0.0442) in relation to U2OS.

Table 1

| Ref. No. | Gene   | Sequence 5’ – 3’ Product length (bp) |
|---------|--------|--------------------------------------|
| 262967769 (fwd) | EPB41L3 | 5’- AGT GAG TTC TGC TTG GCA CCA AAG-3’ (fwd) 24 |
| 262967770 (rev) |          | 5’- AAA TGC ATC TCT GCT TCT GGC G3’ (rev)`, 24 |
| 262967775 (fwd) | GADD45A | 5’- CAT GTG CAT GGG TGT GAA CCA 3’ (fwd) 24 |
| 262967776 (rev) |          | 5’- AGT GAC ATG GAC TGT GGT CAT 3’ (rev)`, 24 |
| 260377267 (fwd) | IER3    | 5’- TCT TCT TGG TGC TCA CCA TGG -3’ (fwd) 24 |
| 262967728 (rev) |          | 5’- GCT CCG AAG TCA GAT TAA AGG GCT 3’ (rev)`, 24 |
| 262967781 (fwd) | OXCT1   | 5’- GACAGTGGATGAGCTACAGAAG 3’ (fwd) 22 |
| 262967772 (rev) |          | 5’- CACGCAAGCTGTGACAAATA 3’ (rev) 20 |
| 262967771 (fwd) | UBE2L6  | 5’- AGT ATC TGT CTA AGG CCT CCA TGA T3’ (fwd) 24 |
| 262967772 (rev) |          | 5’- GAG TCT TCC AGT TCT CAC TGA TGC 3’ (rev)`, 24 |
| 262967789 (fwd) | UBE2A   | 5’- AGAACACTGGCTGGATTG3’ (fwd) 20 |
| 262967790 (rev) |          | 5’- AGGAGTGGAGGGTACACCA 3’ (rev)`, 20 |
| 262967785 (fwd) | EFNB2   | 5’- CTTCGCGAGACGACTTGAATG 3’ (fwd) 21 |
| 262967786 (rev) |          | 5’- GCTGGTGCACCTGACTCACT 3’ (rev) 22 |
| 262967767 (fwd) | GAPDH   | 5’- CAT GTG CGT CAT GGG TGT GAA CCA 3’ (fwd) 24 |
| 262967768 (rev) |          | 5’- AGT GTG GCC ATG TGC TGT GGT CAT 3’ (rev)`, 24 |
R-, indicating that in resistance-induced cells that are later cultivated without the drug that is responsible for generating selective pressure, GADD45A starts to have its expression down-regulated (Fig. 6 G). In the same way, TMPO was up-regulated in U2OS S and, although it presented expression in the U2OS R+ and U2OS R-groups, there was not any significant difference between them (Fig. 6 H). Finally, we had a different result from the expression levels presented up until now: the expression of EFNB2 was down-regulated in U2OS S and gained an up-regulated profile in U2OS R+ and U2OS R-cells, where its expression was approximately 150 times greater in these cell groups than in U2OS, a finding that can be seen in Fig. 6 I.

4. Discussion

In the current study, the induction of resistance in cells of the U2OS lineage using IFO was demonstrated. However, MTT assays showed that in the absence of the drug, resistant cells regained sensitivity to the treatment. These results suggest that the cultivation of resistant cells without the drug can generate a re-sensitization of the cells, classifying the previously observed resistance as temporal or reversible [15]. There are not many data in the literature to explain this change in the behavior of resistant cells in the absence and presence of the drug in osteosarcoma studies, so this work may provide new data that lead to gaining a better understanding of the complexity of tumor resistance in this type of cancer.
By evaluating the IC$_{50}$ of the U2OS lineage at the beginning of the tests and the IC$_{50}$ obtained later in the new MTT assay, it was possible to observe an increase in the dose of IFO that maintains 50% of the cells with metabolic activity. This result suggests that U2OS S cells show tumor heterogeneity and intrinsic resistance to IFO, which is probably due to the fact that groups of tumor cells are composed of multiple clones of tumor cells that compete with each other for selective advantage [16]. Drug-sensitive tumor cell populations may have a small subpopulation of treatment-resistant cells, evidencing once again the heterogeneity within a cancer cell population [15]. Another hypothesis for the increase and variation in resistance of the U2OS S lineage suggests that it would be due to the phenomenon of phenotypic plasticity, which can occur spontaneously in tumor cells [17]. According to He et al. (2011), the J82 lineage of drug-sensitive bladder cancer was able to develop a highly tumorigenic phenotype without the presence of selective pressures, that is, without drug treatment. This phenomenon is called phenotypic plasticity and the same was observed in vivo, corroborating the data described in this work.

Even with this variation in drug resistance observed in the U2OS S lineage, migration, proliferation, and survival-free assays demonstrate that the U2OS R+ lineage has a more aggressive and resistant tumor phenotype. This result was expected, since OS cells with induced resistance have a greater capacity for migration and invasion compared to parental cells [18,19]. In this study, it was observed that both sensitive and resistant groups formed cell aggregates in the adherence-free survival assay. OS cells with metastatic potential in vivo form cell aggregates.
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the group of resistant cells. Optical microscopy view. 10x magnification.

The expression profile was similar in U2OS R+ and U2OS R-cells, with no significant difference between them, but the expression levels in the U2OS S group were significantly different in relation to the other groups.

Ephrin B2 (EFNB2) is variably expressed in tumor cells, and its blockage has been seen to inhibit angiogenesis in solid tumors. Overexpression of ephrin B2 has also been correlated with a worse response to chemotherapy and radiotherapy. As it is an important mediator of the invasion, migration and angiogenesis of tumor cells, which are characteristic of metastatic cells, its use as a critical biomarker in prognosis can be observed [20].

Ephrins exert their biological functions by bonding with Eph receptors, which include the biggest family of tyrosine kinase receptors. As well as signaling via receptor, the ephrins can act independently from the Eph receptor by means of reverse signaling, when the cytoplasmic domain of ephrin is phosphorylated, resulting in the activation of cell signaling. Both reverse signaling and signaling that depends on the receptor have been implicated in the development and progression of cancer [22].

Little is known about the expression of this gene in OS. The results obtained have demonstrated a greater capacity for migration and proliferation in U2OS R+, indicating more aggressive tumor behavior. The high level of expression of the R+ and R-lineages in comparison with the S lineage corroborates the data found in the literature.

Brueheim et al. (2009) demonstrated that OS xenografts with an unsatisfactory response to IFO treatment showed a low expression of GADD45A, relating this fact to tumor resistance to IFO. These data corroborate the current study, where only sensitive cells showed positive expression of GADD45A. This gene is responsible for regulating the cell cycle and apoptotic processes in response to physiological and environmental stress, which in OS and other tumors usually show methylation in the CpG region due to epigenetic mechanisms [23,24]. Tumors that lack GADD45A expression have greater angiogenesis and cell migration activity [25].

Corroborating this information, the present study showed that U2OS R+ cells, in addition to not showing gene expression of GADD45A, also demonstrated resistance to IFO and a high capacity for cell migration and proliferation when compared to U2OS S cells. However, the U2OS S group showed upregulation of GADD45A and a less aggressive tumor profile.

Genes such as ALPL and EPB41L3 were downregulated in IFO-resistant OS xenografts [26], and this profile was also repeated in the current study, where both U2OS R+ cells showed resistance to IFO and low expression of these genes, while U2OS S cells sensitive to IFO had high levels of expression. Levels of ALPL in blood plasma are often associated with primary bone lesions and, because of this, it has been shown to be a potential biomarker for the follow-up of OS [27]. Elevated levels of ALPL were related to low disease survival [27]; however, in the current study, low ALPL expression was reported in the most sensitive lineage, while the resistant lineage showed high levels of this gene. This reinforces the idea that further studies are needed in these IFO-sensitive and resistant lineages, involving the related pathways, evaluating the genes and how they interact with each other.

The gene EPB41L3 may have a dual role in OS, because despite being found in high levels in OS cells, as represented in the U2OS S group, when this gene is downregulated it leads to the process of mesenchymal epithelium, generating metastasis and a more aggressive tumor [28], corroborating the tests performed on U2OS R+ cells.

In cases of esophageal squamous cell carcinoma (ESCC), EPB41L3 undergoes the methylation process and its expression is reduced, and thus the progression of the cell cycle occurs, demonstrating that this gene plays an important role in tumor suppression [29].

In the U2OS S and U2OS R+ lineages, EPB41L3 can be evaluated later as a target for the follow-up of the disease and for characterization of a more aggressive and drug-resistant tumor, since this gene showed
differentiated expression between the sensitive and resistant lineages of OS.

Classes of genes responsible for ubiquitination, such as \( UBE2L6 \) and \( UBE2A \) [30], in addition to the \( IER3 \) gene, which is regulated in response to cellular stress and its influence on tumor progression [31,32], and the \( OXCT1 \) gene that participates in the metabolic process [33], are not well described in OS cells. All these genes were down-regulated in U2OS R+ and U2OS R-cells and up-regulated in U2OS S, but it was reported that the \( UBE2A \) gene is up-regulated in OS lineages and, when silenced, reduces the process of cell migration, invasion, and proliferation [34]. In the same way, the \( IER3 \) gene is usually up-regulated in more aggressive cancers, such as in some lineages of lung cancer [32], and in pancreatic cancer [31].

In OS xenografts, \( UBE2A \) and \( IER3 \) were up-regulated in IFO-resistant samples, which could explain why the U2OS S group has intrinsic resistance to IFO, as indicated in the metabolic activity tests, while the U2OS R+ group, despite having the \( UBE2A \) gene down-regulated, showed resistance to IFO, as was also observed for \( UBE2L6 \) and \( IER3 \). In the case of cervical cancer, the low expression of \( IER3 \) is related to the development of the tumor [35], but there are still not many data about this gene in OS. Based on its expression profile in different tumors, this gene becomes a strong candidate for further studies to better elucidate the role of \( IER3 \) in induced resistance to IFO in OS.

Finally, when analyzing the \( OXCT1 \) gene responsible for the synthesis and degradation of ketone bodies, participating in tumor progression and intercellular signaling [33], the U2OS S lineage showed high expression of this gene, while after resistance induction this gene was suppressed. Deletions in this gene that generate changes in the
5. Conclusion

When we induced resistance to IFO in the U2OS lineage, the cells presented more aggressive tumor behavior, in addition to deregulation in the expression levels of genes associated with resistance.

Among the results reported here, the behavior of resistant cells in the absence of the drug drew attention, because when subjected to the IFO sensitivity assay, these cells showed a significant reduction in resistance. However, the expression levels of U2OS R- remained similar to those of U2OS R+ cells. This result brings new data about the expression of the genes EPB41L3, IER3, OXCT1, and EFNB2 in the U2OS lineage, showing how they may play an important role in the development of resistance. Not many studies have evaluated these genes with this specific lineage. Therefore, to better evaluate how they affect the resistance and aggressiveness of the U2OS lineage, functional studies are needed with these genes as targets.

Author contribution to study

Maria Tereza de Oliveira Rodrigues,: Investigation, Writing - Original Draft, formal analysis, Visualization, Conceptualization and Methodology. Lucas Pereira da Silva: Investigation, Formal analysis, Writing - Original Draft, Visualization, Conceptualization and Methodology. Robert Edward Pogue: Conceptualization, Methodology. Juliana Lott de Carvalho: Conceptualization, Methodology. Andrea Barreto Motoyama: Conceptualization, Methodology, Resources - provided the lineage used. Thuaney de Alencar e Silva: Formal analysis, Methodology. Hilana dos Santos Sena Brunel: Resources, support in cell culture. Maria Fatima Grossi de Sá: Resources, Writing - Review & Editing. Rosângela Vieira de Andrade: Project administration, Supervision, Resources, Methodology, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the link to my data at the attach file.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101357.

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