Growth arrest-specific 5 lncRNA as a valuable biomarker of chemoresistance in osteosarcoma

Simone Polvani[a], Filippo Martignano[b], Guido Scoccianti[c], Adriano Pasqui[d], Anna Rita Palomba[e], Silvo Conticello[b], Andrea Galli[a], Ilaria Palchetti[f], Chiara Caporalini[g], Lorenzo Antonuzzo[d,h], Domenico Andrea Campanacci [c,i] and Serena Pillozzi[d]

Osteosarcoma is the most common primary malignant bone tumour in children and teenagers, and it is characterised by drug resistance and high metastatic potential. Increasing studies have highlighted the critical roles of long noncoding RNAs (lncRNAs) as oncogenes or tumour suppressors as well as new biomarkers and therapeutic targets in osteosarcoma. The growth arrest-specific 5 (GAS5) lncRNA can function as a tumour suppressor in several cancers. The present study aimed to validate GAS5 and other chemoresistance-associated lncRNAs as biomarkers in a cohort of primary osteosarcoma samples, to obtain predictive information on resistance or sensitivity to treatment. The GAS5 and a panel of lncRNAs related to chemoresistance [SNGH1, FOXD2-AS1, deleted in lymphocytic leukemia (DLEU2) and LINC00963] were evaluated in a cohort of osteosarcoma patients enrolled at the Careggi University Hospital. Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections and the expression levels of the lncRNAs were quantified by qPCR. A bioinformatic analysis on deposited RNA-seq data was performed to validate the qPCR results. Clustering analysis shows that GAS5 could be linked to the expression of isoforms 02 and 04 of the lncRNA DLEU2, whereas the DLEU2 isoform 08 is linked to the lncRNA LINC00963. We found that GAS5 is significantly increased in patients with a good prognosis and is expressed differently between chemosensitive and chemoresistant osteosarcoma patients. However, the results obtained are not concordant with the in-silico analysis performed on the TARGET osteosarcoma dataset. In the future, we would enlarge the case series, including different disease settings. *Anti-Cancer Drugs* **33: 278–285**

Anti-Cancer Drugs 2022, **33**:278–285

Keywords: biomarkers, chemoresistance, growth arrest-specific 5, long non coding RNA, osteosarcoma

[a]Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Florence, [b]Core Research Laboratory, ISPRO, Firenze, [c]Orthopaedic Oncology Unit, [d]Medical Oncology Unit, Florence, [e]Histopathology and Molecular Diagnostic Unit, Careggi University Hospital, [f]Department of Chemistry Ugo Schiff, Univerisity of Florence, Sesto Fiorentino, [g]Pathology Unit, Anna Meyer Children's University Hospital, [h]Department of Experimental and Clinical Medicine, University of Florence and [i]Department of Health Sciences, Orthopaedic Oncology Unit, Careggi University Hospital, University of Florence, Florence Italy

Correspondence to Serena Pillozzi, PhD, Medical Oncology, Careggi University Hospital, largo Brambilla 3, 50134, Florence, Italy
Tel: +39 05549648; e-mail: serena.pillozzi@unifi.it

Received 12 July 2021 **Revised form accepted** 27 September 2021

Introduction

Osteosarcoma is the most common bone tumour and it is more frequent in children and young adults affecting mostly the metaphysis of the long bones [1]. Its incidence rate is approximately one to three cases per million each year [2] and more than 20% of patients with osteosarcoma develop metastases, frequently to the lung [3]. For patients with nonmetastatic osteosarcoma, the survival rate has improved, reaching 60% at the fifth year after the introduction of neoadjuvant chemotherapy and the combination of surgery and chemotherapy (CT); unfortunately, overall survival (OS) is only 20% when metastases occur [4]. Doxorubicin, cisplatin and methotrexate are commonly used CT drugs in osteosarcoma treatment with mixed results; indeed, patients treated with these drugs often undergo local recurrence and metastatic dissemination caused by the onset of drug resistant-tumour cells. Consequently, the development of novel therapies is one of the main goals of osteosarcoma oncology research; this aim could be achieved with a better understanding of the molecular basis of osteosarcoma development and acquisition of chemoresistance, that unfortunately are still largely unknown [5]. Long noncoding RNAs (lncRNAs) are defined as nonprotein-coding RNAs longer than 200 nucleotides [6]; they can regulate gene expression through diverse mechanisms, including epigenetic silencing, mRNA splicing, lncRNA-mRNA, lncRNA-miRNA and lncRNA-protein interaction [7]. Emerging evidence shows that lncRNAs may play complex and extensive roles in tumour development acting either as oncogenes or tumour suppressors [1]. Similar to what occurs in many other tumours, lncRNAs might be implicated in osteosarcoma chemoresistance mechanisms [8–10].

The well-characterised tumour suppressor growth arrest-specific 5 (GAS5) has been documented in a wide

0959-4973 DOI: 10.1097/CAD.0000000000001263

variety of human malignancies and loss of GAS5 expression is linked to tumorigenesis and disease progression, as well as to patients' prognosis. The general consensus is that low GAS5 expression may predict poor survival and therapy resistance in many types of cancer [11–16]. The potential role of GAS5 in osteosarcoma is largely unknown. Recently is emerging a role of GAS5 as a competing endogenous RNA, sponging several mi-RNA such as (1) miR-23a-3p, to promote phosphatase and tensin homolog expression and suppressing cell growth and invasion by regulating the phosphoinositide 3-kinase (PI3K)/AKT pathway [17], (2) miR-203a, sequestering away from tissue inhibitor of metalloproteinase 2 [18], (3) miR-663a that promotes osteosarcoma development through targeting MYL9, ras homolog family member B axis [19,20] or ZBTB7A under endoplasmic reticulum stress [21] and (4) miR-221 suppressing cell growth and epithelial-mesenchymal transition (EMT) [22].

In the present study, for the first time, we have evaluated the clinical value of GAS5 tumour-suppressor lncRNA in improving patients' prognosis and prediction of response to chemotherapy in a pilot cohort of primary osteosarcoma.

Materials and methods

Primary osteosarcoma

A total of 10 osteosarcomas formalin-fixed paraffin-embedded (FFPE) samples were obtained from patients enrolled at the Orthopaedic Oncology Unit of Careggi University Hospital and enrolled in this study after informed written consent. The main clinical and pathologic characteristics of the patients are summarised in Table 1. FFPE sections (10–20 µm thickness) of the tumours have been set up by the Histopathology and Molecular Diagnostic Unit, Careggi University Hospital, and were used for total RNA extraction.

RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was extracted from FFPE sections using the Tri Reagent (Millipore, Milan, Italy) solution. The quantity and the quality of RNA were evaluated using a Nanodrop spectrophotometer. Whenever possible, 500 ng of RNA from each sample was retro-transcribed with the 'High Capacity cDNA Reverse Transcription Kit' (Thermofisher, Milan, Italy); the resulting cDNA was then used for quantitative real-time reverse transcription PCR (qPCR) analysis with 'GoTaq Sybr' (Promega, Milan, Italy) on a Rotor-Gene Q (Qiagen). The relative quantification was performed using LinRegPCR software and the data were normalised to glyceraldehyde-3-phosphate dehydrogenase. The primers used are listed in Table 2.

Statistical analysis

Statistical analysis was performed using Jamovi and R on $\log_2$ transformed data. The hierarchical clustering heatmaps were created with the pheatmap library [23] using the Euclidean distance. *t*-Test was used to evaluate statistical differences of qPCR data.

In silico gene expression analysis

Open access RNA-seq raw-counts data and survival data from 92 OS patients have been downloaded from TARGET (https://ocg.cancer.gov/programs/target/data-matrix). Raw counts (produced with Kallisto [24]) have been analyzed via Deseq2 [25] and normalised using the 'vst' algorithm from the Deseq2 R package.

Vst normalised values have been used for survival analysis performed via coxph R package with default settings.

Results

Clinicopathologic features of osteosarcoma patients

A total of 10 osteosarcoma FFPE samples were obtained from patients enrolled at the Orthopaedic Oncology Unit of Careggi University Hospital and used in this study after informed written consent. The clinicopathologic features of the 10 enrolled patients (five male and five female) are reported in Table 1. The mean age of the patients is 12.3 years (range 7–15), two patients had tumour localised in the proximal tibia, two in the proximal humerus and six in the distal femur; eight patients had osteoblastic osteosarcoma whereas two were telangiectatic osteosarcoma; the mean value of follow-up was 99.5 months (range 7–166).

Expression analysis of growth arrest-specific 5 and other long noncoding RNAs in osteosarcoma patients by quantitative real-time reverse transcription PCR

The tumour suppressor lncRNA GAS5 is linked to the chemoresistance of several cancers [9] whereas data on osteosarcoma are limited (e.g. its association to the resistance to therapy is to date unreported) so we evaluated its expression in our cohort of osteosarcoma patients. As reported in Table 3 and summarised in Fig. 1 the lncRNA GAS5 is expressed in all osteosarcoma samples analysed with a mean expression value of –3.62 (range –5.52; –1.90).

We next evaluated the expression of a panel of lncRNAs [SNGH1, FOXD2-AS1, deleted in lymphocytic leukemia (DLEU2) and LINC00963] that have been reported in the literature to be involved in chemoresistance mechanisms (Fig. 2a and Table 3). For the DLEU2 gene, we evaluated the expression of two isoforms (DLEU2 02-04 and DLEU2 08). We have decided to study two isoforms to this because it was not possible to design a primer suitable for the DLEU2 isoforms that emerged from bioinformatics analysis and because DLUE2 has at least seven linear transcript variants, generated by alternative splicing [26], and their roles might be cancer specific.

Whereas GAS5 is detectable in all amplifiable samples, the other lncRNAs are often poorly expressed in the cohort.

Table 1 Clinicopathologic features of the patients enrolled with overall survival

Patient ID	Sex	Age (years)	Histology	Location	Follow-up (months)	Response
1	Female	15	T	PH	112	DOD
3	Female	9	T	DF	19	DOD
4	Male	15	O	DF	7	DOD
5	Male	13	O	PT	64	DOD
6	Female	13	O	PH	99	CDF
7	Female	7	O	DF	166	CDF
8	Male	12	O	DF	135	CDF
9	Female	12	O	DF	135	CDF
10	Male	14	O	DF	143	CDF
11	Male	13	O	PT	115	CDF

CDF, continuously disease free; DF, distal femur; DOD, died of disease; O, osteoblastic; PH, proximal humerus; PT, proximal tibia; T, telangiectatic.

Table 2 Sequences of qPCR primers

Gene name	Primer forward	Primer reverse	Lenght (bp)
FOXD2-AS1	AAGCGATCAGCTCCCTTAGC	CAGACGCGTGGTGGTTATCT	184
SNHG1	TTGCTGCCTTTCTTACATGATCC	AGACACGAAGTGGAGTTATGGGAA	132
GAS5	CTTGCCTGGACCAGCTTAAT	CAAGCCGACTCTCCATACCT	122
LINC00963	GGTAAATCGAGGCCCAGAGAT	ACGTGGATGACAGCGTGTGA	100
DLEU2 02-04	GTCCGAGAGTATAGCGCCAC	ATTAAACCGACTGCGCCAGC	120
DLEU2 08	AAAGATGGTCCCTGTCAGCA	TCCTTCAAGCTTCTACCACAC	130
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	66

DLEU, deleted in lymphocytic leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SNHG, small nucleolar RNA host gene.

Table 3 Expression levels [as $\log_2$(RQ)] of lncRNAs (FOXD2-AS1, SNHG1, GAS5, LINC00963 DLEU2 02-04 and DLEU2 08) for osteosarcoma patients. Data are represented as arbitrary fluorescence units normalised to GADPH

Patient ID	FOXD2-AS1	SNHG1	GAS5	LINC00963	DLEU2 02-04	DLEU2 08
1	−7.22	−7.96	−5.52	−5.12	−5.80	−5.43
3	−2.16	−6.57	−4.43	−2.63	−4.14	−2.78
4	NA	NA	−3.92	NA	NA	−11.30
5	−8.62	−21.81	−3.59	−6.78	NA	NA
6	NA	−9.45	−3.21	NA	NA	2.15
7	−5.13	−13.93	−1.90	NA	−1.52	NA
8	−15.11	−11.51	−3.60	−2.42	−6.07	NA
10	NA	NA	−2.81	NA	−5.96	−12.61
Mean	−7.65	−11.87	−3.62	−4.24	−4.70	−6.00
Median	−7.22	−10.48	−3.60	−3.87	−5.80	−5.43
SD	4.83	5.52	1.08	2.09	1.94	6.10
Minimum	−15.11	−21.81	−5.52	−6.78	−6.07	−12.61
Maximum	−2.16	−6.57	−1.90	−2.42	−1.52	2.15

DLEU, deleted in lymphocytic leukemia; SNHG, small nucleolar RNA host gene.

Specifically, the lncRNA FOXD2-AS1 is expressed in most of the samples (six out of eight) analysed with a mean expression value of −7.65 (range −15.11;−2.16); lncRNA SNHG1 has a mean expression value of −11.87 (range −21.81;−6.57), (six out of eight samples); the lncRNA LINC00963 is detectable at meaningful levels in half of the samples (mean expression value of −4.24 with a range −6.78;−2.42). Finally, the two isoforms of lncRNA DLEU2 have a similar, although in a different scale, expression; specifically, the DLEU2 02-04 has a mean expression value of −4.70 (range −6.07;−1.52) and DLEU2 08 has a mean expression value of −6.00 (range −12.61;2.15), both were detectable in 60% of the samples.

With the R-studio software, using the Euclidean distance, we built a heatmap that clustered patients according to the gene expression values [$\log_2$(RQ)] found through the qPCR. As shown in Fig. 2b, we found a clear separation of patients according to gender and histology and a cluster association of GAS5 with the 02-04 isoforms of the lncRNA DLEU2, whose 08 isoform is instead associated to LINC00963. The results obtained showed how genes that are reported in the literature as oncosuppressors (GAS5, DLEU2 02-04) cluster together.

Association between long noncodingRNAs expression level and chemoresistance

Given the importance of chemoresistance in the progression of osteosarcoma, we evaluated the relation of these lncRNAs to the outcome in the patients. Given that all patients underwent the same therapeutic treatment, the population was divided into the two subgroups 'poor responders' and 'good responders' (Table 4). We observed a statistically significant difference only for GAS5, whose expression is increased in the 'therapy-sensitive' group ($P = 0.038$) (Fig. 3a).

Hierarchical clustering of lncRNA in 'therapy-sensitive' and 'therapy-resistant' groups shows the association of GAS5 to LINC00963 (Fig. 3b).

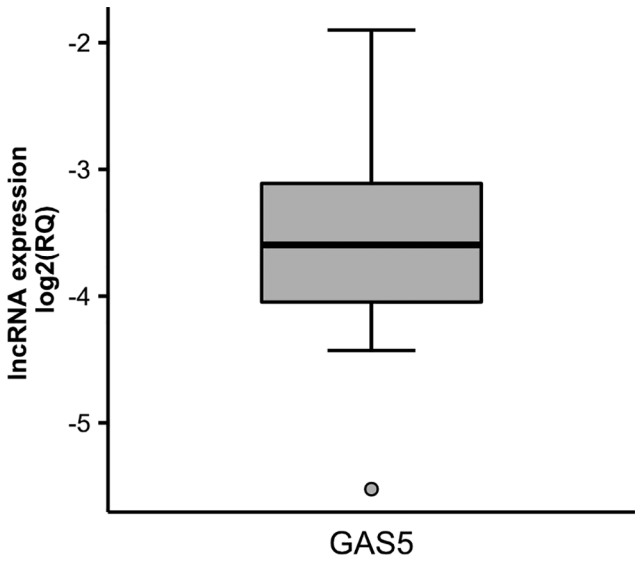
In-silico analysis

Subsequently, we retrieved RNA-seq data from 92 osteosarcoma patients from the TARGET repository, with the aim of extending our analysis to larger datasets. Unfortunately, GAS5 over-expression is weakly associated with poorer progression-free survival (PFS, hazard ratio = 1.6; $P = 0.00065$) and OS (hazard ratio = 1.57; $P = 0.00261$), in discordance with our previous observations on primary samples of our cohort (Table 5). Considering the high heterogeneity of osteosarcoma, we stratified the patients based on the recruitment protocol reported by the uploaders, with the aim of obtaining cleaner subsets of patients. We separately evaluated PFS and OS in four protocols with at least 10 patients: AOST0331, AOST06B1, IHRT and P9851. Among these, GAS5 is significantly associated with poor survival only in AOST0331. Notably, AOST0331 and AOST06B1 show very large hazard ratio values compared to IHRT and P9851 (Table 5). Such differences among protocols suggest that GAS5 might play different roles in different disease settings, which could explain the disagreement between our wet-lab results and in-silico analysis.

Discussion

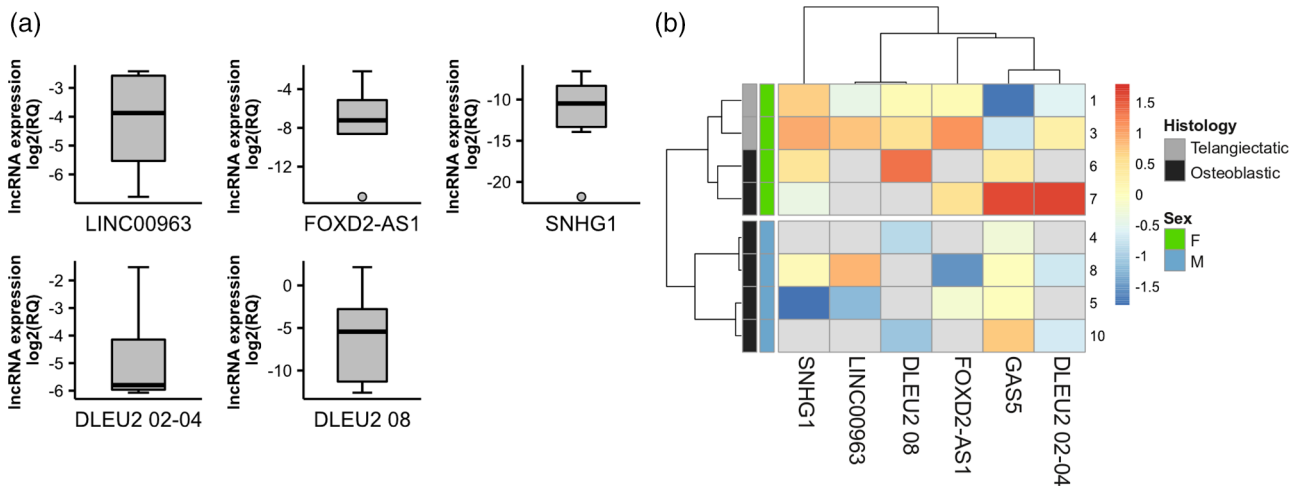
Chemoresistance is associated with poor outcomes of osteosarcoma patients and remains a major challenge in treatment. Many studies have shown the role of lncRNA in processes that confer resistance to treatment. After a literature analysis on the role of lncRNAs in cancer we identified GAS5 and other lncRNAs (SNGH1, FOXD2-AS1, DLEU2 and LINC00963) as possible lncRNAs that were associated with osteosarcoma survival and chemoresistance; hoping to find out novel biomarkers, their expression and association to clinical and pathologic data were evaluated in primary osteosarcoma. The analysis was carried out on a pilot cohort of 10 patients suffering from osteosarcoma and enrolled at Careggi University Hospital. The limited number of patients is due to the rarity of the neoplasm studied and to the need of a homogeneous cohort characterised by patients who had followed the same therapeutic schedule. Literature analysis on the role of lncRNAs in cancer identified GAS5 as a possible lncRNA associated with osteosarcoma survival. GAS5 plays the role of tumour suppressor gene in many types of cancer and is found underexpressed in breast [11], prostate [12], pancreatic [13], colorectal [14], cervical [15] and gastric cancers [16]. It plays a role as tumor suppressor gene by disadvantaging processes such as cell migration and cell proliferation and promoting cell death by apoptosis [27]. In particular, it acts on cell proliferation by raising P21 levels and lowering CDK6, Cyclin D1 and E2F1 levels [28] as well as on apoptotic processes by lowering clAP2 levels and raising P53 levels. The expression of GAS5 can be regulated by the interaction between mammalian target of rapamycin and nonsense-mediated decay [29]. In addition, in the literature there are data on the possible role of GAS5 in osteosarcoma; also in this case, it plays the role of oncosuppressor interacting with some miRNA [17–22]. In addition, recently it has been described that a downregulation of GAS5 in osteosarcoma cell lines enhances migration and invasion, along with an upregulation of EMT and increased expression of miR-21 [30]. Moreover, a genetic variant in the promoter region of GAS5 (rs145204276) is functionally associated with the susceptibility of osteosarcoma [31]. Furthermore, other lncRNAs with a possible role in cancer chemoresistance (SNGH1, FOXD2-AS1, DLEU2 and LINC00963) have been selected from the literature analysis. FOXD2-AS1 has been reported to play the role of an oncogene in many types of cancers, as it has been found to be abnormally expressed in gastric, lung, bladder, colon rectum, nasopharyngeal, oesophagus, liver, thyroid and skin cancers [32]. Its role is related to cell proliferation that promotes through its involvement in both the Wnt/β catenin and Notch signaling pathway; moreover, in bladder cancer, FOXD2-AS1 plays a role in gemcitabine chemoresistance [32]. Regarding the role of this gene in osteosarcoma, it has been reported that FOXD2-AS1, following the activation by transcription factor hypoxia-inducible factor 1-alpha, acts as an oncogene in the osteosarcoma tumorigenesis and interacts with enhancer of zeste homolog 2 to silence p21 protein [33].

Fig. 1



Expression boxplot showing trends of long noncoding RNA (lncRNA) GAS5 levels of expression in osteosarcoma (OST) samples; the expression values are reported as $\log_2$(RQ); gray dots represent outlier values.

Fig. 2



Graphical representation of expression values of a panel of selected lncRNA. (a) Expression boxplots showing trends of individual long non-coding RNAs (lncRNAs) in osteosarcoma (OST) samples; the expression values are reported as $\log_2$(RQ); gray dots represent the outliers. (b) Expression heatmap of lncRNA among the patients, this heatmap was built using the Pheatmap function in R-studio software; the clustering between genes and between patients was created using the Euclidean distance; the color scale represents the expression values [$\log_2$(RQ)], normalised in the columns.

Table 4 **Descriptive statistic of lncRNA expression in the study population grouped according to response to therapy**

	Response	FOXD2-AS1	SNHG1	GAS5	LINC00963	DLEU2 02-04	DLEU2 08
N	Resistant	4	4	4	4	4	4
	Sensitive	4	4	4	4	4	4
Mean	Resistant	−6.00	−12.1	−4.37	−4.84	−4.97	−6.51
	Sensitive	−1·.1	−11.6	−2.88	−2.42	−4.52	−5.23
Median	Resistant	−7.22	−7.96	−4.17	−5.12	−4.97	−5.43
	Sensitive	−10.1	−11.5	−3.01	−2.42	−5.96	−5.23
SD	Resistant	3.40	8.43	0.845	2.09	1.17	4.36
	Sensitive	7.06	2.24	0.729	NaN	2.60	10.4
Range	Resistant	6.46	15.2	1.93	4.15	1.65	8.52
	Sensitive	9.98	4.48	1.70	0.00	4.55	14.8

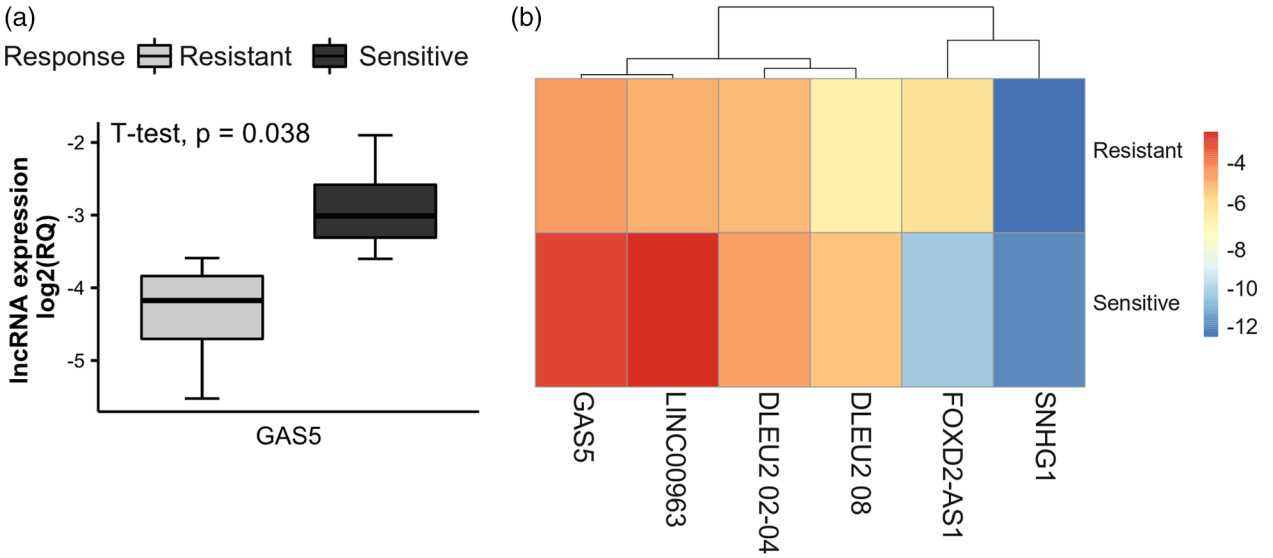
DLEU, deleted in lymphocytic leukemia; GAS5, growth arrest-specific 5; SNHG, small nucleolar RNA host gene.

Small nucleolar RNA host gene (SNHG1) is overexpressed in oesophageal squamous cells [34], lung squamous cells [35], hepatocellular carcinomas [36], colorectal [37], gastric [38] and liver cancers where it acts as a tumour promoting factor. In the liver, it promotes the development of tumour mass by inhibiting the expression of P53 [39] and the resistance to sorafenib and doxorubicin [40]. Regarding osteosarcoma, it promotes cell proliferation, metastases and invasiveness while its silencing promotes cell death by apoptosis and the G0/G1 cell cycle arrest. In osteosarcoma, it plays its role of oncogene by inhibiting the expression of miRNA-101-3p and this favours the expression of rho associated coiled-coil containing protein kinase 1 [41].

LINC00963 has been reported as an oncogene in different types of cancer such as prostate cancer and hepatocarcinoma. In prostate cancer, it has been shown that its overexpression is linked to the malignant development by acting on EGFR [42]. In hepatocarcinoma, this lncRNA acts on the PI3K/AKT signalling pathway, favouring the tumour progression [43]. There is little data in the literature on the role of LINC00963 in osteosarcoma but the available data suggest that its overexpression is linked to an unfavourable prognosis and that it facilitates proliferation and invasiveness by suppressing the miR-204-3P/FN1 axis [44].

DLEU2 is a tumour suppressor gene because its downregulation is linked to malignant processes such as angiogenesis, metastasis and invasiveness. Its role as an onco-suppressant in laryngeal, oesophageal and pancreatic carcinoma has been verified, but above all, it plays a fundamental role in many types of leukaemia [45]. One of the isoforms of DLEU2 is an indicator of poor prognosis in patients with oesophageal adenocarcinoma [26]. In the literature, data on the correlation between DLEU2 and osteosarcoma are limited and are related to the phenomenon of hypoxia. Low oxygen levels suppress the transcription of DLEU2 and miR-15a and this

Fig. 3



Graphical representation of osteosarcoma (OST) cohort division based on response to treatment. (a) Boxplots of expression levels of long non-coding RNA (lncRNA) GAS5 in the treatment-sensitive group and the resistant group. (b) Expression heatmap of lncRNA among the sensitive and resistant groups; this heatmap was built using the Pheatmap function in R-studio software, the clustering between genes was created using the Euclidean distance; the color of each cell represents the mean expression value of that gene within the group.

Table 5 Progression-free survival and overall survival results based on GAS5 expression in the TARGET dataset patient cohort

	Patients	Hazard ratio PFS	*P* value PFS	Hazard ratio survival	*P* value survival
TOT	92	1.60	0.0006	1.57	0.003
AOST0331	24	2.06	0.01	4.33	0.001
AOST06B1	15	1.85	0.24	3.96	0.07
IHRT	20	1.22	0.67	2.06	0.21
P9851	17	0.92	0.85	0.77	0.58

PFS, progression-free survival.

favours the proliferation and invasion of cancer cells [46]. Noteworthy the studies reported in the literature were carried out on cell lines while our study is the first on primary samples of osteosarcoma.

Observing the clinicopathologic features of the enrolled patients, we have decided to focus on chemoresistance. Some data on the correlation between some lncRNAs and chemoresistance in osteosarcoma are reported in the literature [8]. Recent studies demonstrated that a distinctly higher expression of lncRNA FOXC2-AS1 (FOXC2 antisense RNA 1, FOXC2-AS1) was associated with a poor prognosis for osteosarcoma patients. It was also revealed that FOXC2-AS1 may have contributed to doxorubicin resistance by increasing the expression of some classical MDR (multidrug resistance) associated genes, including ABCB1 and HIF1A [47]. Overexpression of lncRNA ODRUL (OS doxorubicin resistance-related upregulated lncRNA, ODRUL) was shown in tumour tissues of osteosarcoma patients with lung metastasis and a low chemoresponse. It was reported that ODRUL may reduce sensitivity to doxorubicin in OS cells by inducing the expression of ABCB1 [48]. LncRNA LINC00161 (long intergenic noncoding RNA 161, LINC00161) was revealed to play an essential role in cisplatin-induced apoptosis, and attenuate osteosarcoma chemoresistance by targeting the miR-645-IFIT2 (interferon-induced with tetratricopeptide repeats 2, IFIT2) signaling axis [49]. LncRNA HOTTIP participated in osteosarcoma cellular resistance to cisplatin by the activation of the Wnt/β-catenin signaling pathway, which indicates a potential therapeutic approach to targeting the Wnt/β-catenin signaling pathway to reverse the resistance [50].

We compared therapy-sensitive and therapy-resistant patients and we found that the lncRNAs FOXD2-AS1, SNHG1 and LINC00963 tend to be more expressed in the group of therapy-resistant patients than in the group of sensitive patients; the lncRNA GAS5 and the isoforms of the lncRNA DLEU2 analyzed (DLEU2 02-04 and DLEU2 08) have a tendency to be more expressed in patients sensitive to therapies. The trends we found and observed are in accordance with the oncologic roles of the studied lncRNA in the literature; genes of which

a possible role of oncogene is reported tend to be more expressed in patients with osteosarcoma resistant to therapies and genes of which a possible role of tumour suppressor gene is reported tend to be more expressed in patients with osteosarcoma sensitive to therapies. However, our data on GAS5 expression were not confirmed by bioinformatics analysis.

In conclusion, we found a panel of lncRNAs expressed in primary osteosarcoma tissues, their expression is different between chemosensitive and chemoresistant patients and the lncRNA GAS5 has a significantly different expression into the two groups. In the future we have hypothesised its use as a biomarker in osteosarcoma and evaluate the expression of GAS5 could give predictive information on resistance or sensitivity to treatment of osteosarcoma patients.

Acknowledgements

Our special memory goes to Alessio Cerasola, an unforgettable guy who fights the disease with his ever-present smile.

This research was funded by Noi per Voi Odt, Ceresola family and Regione Toscana Bando Salute 2018, (Research project CUP n. D78D20000870002), grant number D78D20000870002.

Conflicts of interest

There are no conflicts of interest.

References

1 Wu PF, Dai ZT, Liu WD, Zhao ZX, Kong YH. Elevated long noncoding RNA HAGLROS expression correlates with clinical progression and prognosis in osteosarcoma. *Eur Rev Med Pharmacol Sci* 2019; **23**:1428–1433.
2 Pan Y, Lu L, Chen J, Zhong Y, Dai Z. Identification of potential crucial genes and construction of microRNA-mRNA negative regulatory networks in osteosarcoma. *Hereditas* 2018; **155**:21.
3 Xu J, Li D, Cai Z, Zhang Y, Huang Y, Su B, Ma R. An integrative analysis of DNA methylation in osteosarcoma. *J Bone Oncol* 2017; **9**:34–40.
4 Shi Z, Zhou H, Pan B, Lu L, Wei Z, Shi L, *et al*. Exploring the key genes and pathways of osteosarcoma with pulmonary metastasis using a gene expression microarray. *Mol Med Rep* 2017; **16**:7423–7431.
5 Rosen G, Murphy ML, Huvos AG, Gutierrez M, Marcove RC. Chemotherapy, en bloc resection, and prosthetic bone replacement in the treatment of osteogenic sarcoma. *Cancer* 1976; **37**:1–11.
6 Wang Y, Li W, Chen X, Li Y, Wen P, Xu F. MIR210HG predicts poor prognosis and functions as an oncogenic lncRNA in hepatocellular carcinoma. *Biomed Pharmacother* 2019; **111**:1297–1301.
7 Li W, Xie P, Ruan WH. Overexpression of lncRNA UCA1 promotes osteosarcoma progression and correlates with poor prognosis. *J Bone Oncol* 2016; **5**:80–85.
8 Chen R, Wang G, Zheng Y, Hua Y, Cai Z. Long non-coding RNAs in osteosarcoma. *Oncotarget* 2017; **8**:20462–20475.
9 Lambrou GI, Hatziagapiou K, Zaravinos A. The non-coding RNA GAS5 and its role in tumor therapy-induced resistance. *Int J Mol Sci* 2020; **21**:E7633.
10 Ghafouri-Fard S, Shirvani-Farsani Z, Hussen BM, Taheri M. The critical roles of lncRNAs in the development of osteosarcoma. *Biomed Pharmacother* 2021; **135**:111217.
11 Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. *Oncogene* 2009; **28**:195–208.
12 Romanuik TL, Wang G, Morozova O, Delaney A, Marra MA, Sadar MD. LNCaP Atlas: gene expression associated with *in vivo* progression to castration-recurrent prostate cancer. *BMC Med Genomics* 2010; **3**:43.
13 Lu X, Fang Y, Wang Z, Xie J, Zhan Q, Deng X, *et al*. Downregulation of gas5 increases pancreatic cancer cell proliferation by regulating CDK6. *Cell Tissue Res* 2013; **354**:891–896.
14 Yin D, He X, Zhang E, Kong R, De W, Zhang Z. Long noncoding RNA GAS5 affects cell proliferation and predicts a poor prognosis in patients with colorectal cancer. *Med Oncol* 2014; **31**:253.
15 Cao S, Liu W, Li F, Zhao W, Qin C. Decreased expression of lncRNA GAS5 predicts a poor prognosis in cervical cancer. *Int J Clin Exp Pathol* 2014; **7**:6776–83.
16 Sun M, Jin FY, Xia R, Kong R, Li JH, Xu TP, *et al*. Decreased expression of long noncoding RNA GAS5 indicates a poor prognosis and promotes cell proliferation in gastric cancer. *BMC Cancer* 2014; **14**:319.
17 Liu J, Chen M, Ma L, Dang X, Du G. LncRNA GAS5 suppresses the proliferation and invasion of osteosarcoma cells via the miR-23a-3p/PTEN/PI3K/AKT pathway. *Cell Transplant* 2020; **29**:963689720953093.
18 Wang Y, Kong D. LncRNA GAS5 represses osteosarcoma cells growth and metastasis via sponging MiR-203a. *Cell Physiol Biochem* 2018; **45**:844–855.
19 Zhao S, Xiong W, Xu K. MiR-663a, regulated by lncRNA GAS5, contributes to osteosarcoma development through targeting MYL9. *Hum Exp Toxicol* 2020; **39**:1607–1618.
20 Yao X, Li X, Luo Y, Xu X, Liu J, Bu J. LncRNA GAS5 regulates osteosarcoma cell proliferation, migration, and invasion by regulating RHOB via sponging miR-663a. *Cancer Manag Res* 2020; **12**:8253–8261.
21 Zhang L, Wang Y, Zhang L, Xia X, Chao Y, He R, *et al*. ZBTB7A, a miR-663a target gene, protects osteosarcoma from endoplasmic reticulum stress-induced apoptosis by suppressing LncRNA GAS5 expression. *Cancer Lett* 2019; **448**:105–116.
22 Ye K, Wang S, Zhang H, Han H, Ma B, Nan W. Long noncoding RNA GAS5 suppresses cell growth and epithelial-mesenchymal transition in osteosarcoma by regulating the miR-221/ARHI pathway. *J Cell Biochem* 2017; **118**:4772–4781.
23 Raivo Kolde (2019). pheatmap: Pretty Heatmaps. R package version 1.0.12. https://CRAN.R-project.org/package=pheatmap
24 Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. *Nat Biotechnol* 2016; **34**:525–7.
25 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014; **15**:550.
26 Ma W, Zhang CQ, Dang CX, Cai HY, Li HL, Miao GY, *et al*. Upregulated long-non-coding RNA DLEU2 exon 9 expression was an independent indicator of unfavorable overall survival in patients with esophageal adenocarcinoma. *Biomed Pharmacother* 2019; **113**:108655.
27 Qiao HP, Gao WS, Huo JX, Yang ZS. Long non-coding RNA GAS5 functions as a tumor suppressor in renal cell carcinoma. *Asian Pac J Cancer Prev* 2013; **14**:1077–1082.
28 Liu Z, Wang W, Jiang J, Bao E, Xu D, Zeng Y, *et al*. Downregulation of GAS5 promotes bladder cancer cell proliferation, partly by regulating CDK6. *PLoS One* 2013; **8**:e73991.
29 Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci Signal* 2010; **3**:ra8.
30 Wang Y, Ren X, Yuan Y, Yuan BS. Downregulated lncRNA GAS5 and Upregulated miR-21 Lead to Epithelial-Mesenchymal Transition and Lung Metastasis of Osteosarcomas. *Front Cell Dev Biol* 2021; **9**:707693.
31 Xu L, Xia C, Xue B, Sheng F, Xiong J, Wang S. A promoter variant of lncRNA GAS5 is functionally associated with the development of osteosarcoma. *J Bone Oncol* 2018; **12**:23–26.
32 Hu Q, Tai S, Wang J. Oncogenicity of lncRNA FOXD2-AS1 and its molecular mechanisms in human cancers. *Pathol Res Pract* 2019; **215**:843–848.
33 Ren Z, Hu Y, Li G, Kang Y, Liu Y, Zhao H. HIF-1α induced long noncoding RNA FOXD2-AS1 promotes the osteosarcoma through repressing p21. *Biomed Pharmacother* 2019; **117**:109104.
34 Zhang Y, Jin X, Wang Z, Zhang X, Liu S, Liu G. Downregulation of SNHG1 suppresses cell proliferation and invasion by regulating Notch signaling pathway in esophageal squamous cell cancer. *Cancer Biomark* 2017; **21**:89–96.
35 Zhang HY, Yang W, Zheng FS, Wang YB, Lu JB. Long non-coding RNA SNHG1 regulates zinc finger E-box binding homeobox 1 expression by interacting with TAp63 and promotes cell metastasis and invasion in Lung squamous cell carcinoma. *Biomed Pharmacother* 2017; **90**:650–658.
36 Zhang H, Zhou D, Ying M, Chen M, Chen P, Chen Z, Zhang F. Expression of long non-coding RNA (lncRNA) small nucleolar RNA Host Gene 1 (SNHG1) exacerbates hepatocellular carcinoma through suppressing miR-195. *Med Sci Monit* 2016; **22**:4820–4829.

37 Tian T, Qiu R, Qiu X. SNHG1 promotes cell proliferation by acting as a sponge of miR-145 in colorectal cancer. *Oncotarget* 2018; **9**:2128–2139.
38 Hu Y, Ma Z, He Y, Liu W, Su Y, Tang Z. LncRNA-SNHG1 contributes to gastric cancer cell proliferation by regulating DNMT1. *Biochem Biophys Res Commun* 2017; **491**:926–931.
39 Li SJ, Wang L, Sun ZX, Sun SJ, Gao J, Ma RL. LncRNA SNHG1 promotes liver cancer development through inhibiting p53 expression via binding to DNMT1. *Eur Rev Med Pharmacol Sci* 2019; **23**:2768–2776.
40 Li W, Dong X, He C, Tan G, Li Z, Zhai B, *et al*. LncRNA SNHG1 contributes to sorafenib resistance by activating the Akt pathway and is positively regulated by miR-21 in hepatocellular carcinoma cells. *J Exp Clin Cancer Res* 2019; **38**:183.
41 Deng R, Zhang J, Chen J. lncRNA SNHG1 negatively regulates miRNA-101-3p to enhance the expression of ROCK1 and promote cell proliferation, migration and invasion in osteosarcoma. *Int J Mol Med* 2019; **43**:1157–1166.
42 Wang L, Han S, Jin G, Zhou X, Li M, Ying X, *et al*. Linc00963: a novel, long non-coding RNA involved in the transition of prostate cancer from androgen-dependence to androgen-independence. *Int J Oncol* 2014; **44**:2041–2049.
43 Wu JH, Tian XY, An QM, Guan XY, Hao CY. LINC00963 promotes hepatocellular carcinoma progression by activating PI3K/AKT pathway. *Eur Rev Med Pharmacol Sci* 2018; **22**:1645–1652.
44 Zhou Y, Yin L, Li H, Liu LH, Xiao T. The LncRNA LINC00963 facilitates osteosarcoma proliferation and invasion by suppressing miR-204-3p/FN1 axis. *Cancer Biol Ther* 2019; **20**:1141–1148.
45 Wu DM, Wen X, Han XR, Wang S, Wang YJ, Shen M, *et al*. Role of circular RNA DLEU2 in human acute myeloid leukemia. *Mol Cell Biol* 2018; **38**:e00259–e00218.
46 Leng J, Song Q, Zhao Y, Wang Z. miR-15a represses cancer cell migration and invasion under conditions of hypoxia by targeting and downregulating Bcl-2 expression in human osteosarcoma cells. *Int J Oncol* 2018; **52**:1095–1104.
47 Zhu KP, Zhang CL, Shen GQ, Zhu ZS. Long noncoding RNA expression profiles of the doxorubicin-resistant human osteosarcoma cell line MG63/DXR and its parental cell line MG63 as ascertained by microarray analysis. *Int J Clin Exp Pathol* 2015; **8**:8754–8773.
48 Zhang CL, Zhu KP, Shen GQ, Zhu ZS. A long non-coding RNA contributes to doxorubicin resistance of osteosarcoma. *Tumour Biol* 2016; **37**:2737–2748.
49 Wang Y, Zhang L, Zheng X, Zhong W, Tian X, Yin B, *et al*. Long non-coding RNA LINC00161 sensitises osteosarcoma cells to cisplatin-induced apoptosis by regulating the miR-645-IFIT2 axis. *Cancer Lett* 2016; **382**:137–146.
50 Li Z, Zhao L, Wang Q. Overexpression of long non-coding RNA HOTTIP increases chemoresistance of osteosarcoma cell by activating the Wnt/β-catenin pathway. *Am J Transl Res* 2016; **8**:2385–93.