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Human equilibrative nucleoside transporter 1 gene expression is associated with gemcitabine efficacy in advanced leiomyosarcoma and angiosarcoma

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Background: The expression of human equilibrative nucleoside transporter 1 (hENT1), the major gemcitabine transporter into cells, has been thoroughly investigated as a predictive marker of response to gemcitabine in pancreatic cancer and biliary tract cancers. Since gemcitabine is widely used in the treatment of leiomyosarcoma and angiosarcoma, we investigated the correlation between hENT1 expression and gemcitabine efficacy in these sarcoma subtypes.

Methods: We retrospectively identified 71 patients affected by advanced angiosarcoma (26) or leiomyosarcoma (45) treated within five Italian referral centres for sarcoma; among them, 49 patients (15 angiosarcoma, 34 leiomyosarcoma) were treated with gemcitabine. All tumour samples were analysed for hENT1 expression by real-time PCR. Median $2^{-\Delta Ct}$ value was used as the cutoff to dichotomise patients into 'high' expression and 'low' expression groups. Kaplan–Meier analysis was performed to estimate progression-free survival (PFS) and overall survival (OS).

Results: We found a significant association between high hENT1 expression levels and favourable outcome in terms of PFS and OS compared to cases with low hENT1 expression in leiomyosarcoma treated with gemcitabine (PFS: 6.8 *vs* 3.2 months, $P=0.004$; OS: 14.9 *vs* 8.5 months, $P=0.007$). In addition, hENT1 overexpression correlated with a significant improvement in PFS (9.3 *vs* 4.5 months; $P=0.02$) and OS (20.6 *vs* 10.8 months; $P=0.001$) in angiosarcoma patients treated with gemcitabine.

Conclusions: Our study suggests that higher hENT1 expression are associated to gemcitabine efficacy both in patients with advanced leiomyosarcoma and angiosarcoma.

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The human equilibrative nucleoside transporter 1 (hENT1) is a transmembrane protein that is thought to be responsible for the intracellular uptake of the prodrug gemcitabine into tumour cells (Ueno *et al*, 2007). Other identified transporters belong to the human concentrative nucleoside transporter family (hCNTs), especially hCNT1 (Bhutia *et al*, 2011) but hENT1 is generally considered to be predominantly involved in gemcitabine transport. The first study exploring the relationship between hENT1 and gemcitabine efficacy was published in 2004 (Spratlin *et al*, 2004) and, during the following decade, several pre-clinical studies and clinical investigations confirmed the hypothesis that hENT1 overexpression might serve as a predictive biomarker for the efficacy of gemcitabine (Michalski *et al*, 2008; Pérez-Torras *et al*, 2008; Farrell *et al*, 2009; Maréchal *et al*, 2009, 2012; Morinaga *et al*, 2012; Nakagawa *et al*, 2013). In particular in a series of 105 patients with pancreatic adenocarcinoma treated with gemcitabine, the high tumour expression of hENT1 was associated with a significant improvement in overall survival (OS) (25.7 *vs* 8.4 months), progression-free survival (PFS) (12.6 *vs* 5.8 months) and Disease-Free Survival (20.4 *vs* 9.2 months; Giovannetti *et al*, 2006). Similar results have been obtained for biliary tract cancers in a retrospective analysis of 44 patients with locally advanced or metastatic cholangiocarcinoma undergoing treatment with gemcitabine, where higher hENT1 positivity was correlated with a better PFS (6.0 *vs* 2.0 months) and OS (11.0 *vs* 5.0 months; Borbath *et al*, 2012).

Our group has previously demonstrated that patients affected by locally advanced biliary tract cancers, who were hENT1 positive, showed significant benefits in terms of PFS (6.33 *vs* 2.83 months) (Santini *et al*, 2011). Moreover, we identified hENT1 as a negative prognostic marker in patients with ampullary and gastric cancer post radical resection and as a potential predictive factor to select patients who could benefit from chemotherapy postoperatively (Santini *et al*, 2008, 2010; Perrone *et al*, 2010.

During the last few years, some investigators reported that hENT1 might potentially act as a prognostic rather than as a predictive marker. Indeed, a significant association between hENT1 expression and clinical outcome was demonstrated in patients with resected pancreatic cancer (Kim *et al*, 2011) and in chemotherapy-naive gallbladder adenocarcinoma patients (Espinoza *et al*, 2016); on the contrary no evidence for hENT1 as a prognostic biomarker was found in pancreatic cancer patients treated with gemcitabine-based neoadjuvant chemoradiotherapy (Kawada *et al*, 2012).

Up to now, translational hENT1 data have been reported from three controlled clinical trials in pancreatic cancer. Two retrospective studies conducted in the adjuvant setting, demonstrated a significant correlation between hENT1 and benefit from gemcitabine treatment after surgical resection (Farrell *et al*, 2009; Greenhalf *et al*, 2014), whereas the third study did not confirm its predictive value in a prospective series of metastatic patients treated with gemcitabine (Poplin *et al*, 2013).

hENT1 as prognostic/predictive biomarker has been also investigated in patients affected by non-small cell lung cancer and metastatic bladder cancer and its expression was significantly associated with gemcitabine treatment efficacy (Oguri *et al*, 2007; Matsumura *et al*, 2011).

No data concerning hENT1 expression in soft tissue sarcomas (STSs) are available, although gemcitabine is an active drug in different sarcoma subtypes, including leiomyosarcoma (Pautier *et al*, 2012) and angiosarcoma (Hensley *et al*, 2002, 2008; Stacchiotti *et al*, 2012).

The aim of this study is to retrospectively determine the gene expression levels of hENT1 in samples derived from advanced leiomyosarcoma and angiosarcoma patients and, for those treated with gemcitabine, to define the correlation between the expression of hENT1 and gemcitabine efficacy in terms of PFS and OS.

MATERIALS AND METHODS

Study population. We retrospectively identified a series of 71 metastatic or locally advanced consecutive patients affected by angiosarcoma (26 cases) and leiomyosarcoma (45 cases) treated at the Istituto Nazionale Tumori of Milan, Second University of Naples, University of Palermo, Treviso General Hospital and Campus Bio-Medico University of Rome from January 2010 to December 2014. We included in the analysis only leiomyosarcoma and angiosarcoma patients with an updated follow-up and in which there was adequate naive tumour tissue to evaluate hENT1 expression.

Among the 71 patients identified, 49 patients (15 angiosarcoma and 34 leiomyosarcoma) received gemcitabine (1000 mg/m^2 once weekly for 3 consecutive weeks out of every 4 weeks). Diagnosis was reviewed and confirmed by expert sarcoma pathologists (APDT, PC). The study was approved by the Biomedical Ethics Committees of the centres providing tumour tissue and an informed consent was signed by recruited patients.

A description of clinicopathologic characteristics of the entire patient cohort are summarised in Supplementary Table 1.

For all 71 patients, formalin-fixed paraffin-embedded (FFPE) surgical specimens, obtained from naive tumour at the time of diagnosis were collected. hENT1 expression levels were analysed in leiomyosarcoma and angiosarcoma patients both treated (49 patients) and not treated with gemcitabine (11 leiomyosarcoma and 11 angiosarcoma patients). All patients not treated with gemcitabine received anthracycline based regimens.

Quantitative real-time PCR. FFPE sections were treated with xylene to remove paraffin; the tissue was subsequently incubated overnight at 56 °C with Proteinase K (Qiagen, UK) to allow sample lysis. Total RNA was extracted using the Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA was treated with DNAse (DNAse Turbo, Applied Biosystems, Foster City, CA, USA) to avoid genomic DNA contamination. The concentration and purity of the isolated RNA (A260/A280 ratio between 1.8 and 2.0 were accepted) were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA). cDNA was synthetised using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's recommendations. mRNA levels were measured by qRT-PCR performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). In all samples, hENT1 (Hs01085704_g1) expression levels were normalised to the endogenous housekeeping gene GUSb (Hs99999908_m1) using the ΔCT calculation. Three technical replicates of all samples and ddH_2O, as non-template control, were performed and analysed for every reaction mix. Moreover, sample triplicates with Ct values over 35 with a ΔCt standard deviation over 0.25 were excluded to remove poor quality samples from analysis. PCR cycling included the following steps: 1 cycle at 95 °C for 10 min, 45 times at 95 °C for 15 s and 60 °C for 1 min.

To confirm hENT1 protein expression, immunohistochemistry with anti-hENT1 rabbit monoclonal antibody, (SAB5500117, Sigma) was performed.

***In silico* analyses.** To get preliminary information about hENT1 expression in leiomyosarcoma, we analysed the transcriptomic profiles of 106 patients from 'TCGA SARC' cohort from cBioportal. TCGA SARC represents the largest Sarcoma data set provided with OS information currently available even if is highly censored with a low OS event rate. All possible cutoff points have been explored using Cox proportional hazard models. In particular, survival analysis has been executed using the functions coxph and survfit from the R package survival. For each cutoff point, a Cox analysis of ENT1 expression level and the survival

variable has been executed. The resulting curve of hazard ratios (HRs) is shown in Supplementary Figure 3. The percentage significant cutoffs out of all investigated cutoffs is displayed at the top of the figure. Representative cutoff point to categorise leiomyosarcoma patients into 'low' and 'high' hENT1 expression was obtained by the use of the receiver operating characteristic (ROC) method.

ROC curves are the standard method to balance between sensitivity and specificity of a molecular test. Supplementary Figure 4 shows the ROC curve for the prediction of living\deceased patients status by ENT1 expression level. All analysis have been performed using cutoff finder (http://molpath.charite de/cutoff). No data set for cohorts of patients affected by angiosarcoma were available from cBioportal.

Statistical analyses. For all statistical analyses the program SPSS 17.0 (SPSS, Chicago) was used. Relative changes were obtained by normalising the hENT1 mRNA expression levels to the endogenous control using the $2^{-\Delta Ct}$ method (ABI software, Applied Biosystems). $2^{-\Delta Ct}$ median value was used as cutoff to discriminate 'high' and 'low' hENT1 expressers.

PFS was calculated as the period from the date of starting treatment to the first observation of disease progression or to death from any cause. The OS time was calculated as the period from the date of starting treatment until death from any cause or until the date of the last follow-up, at which point data were censored. Kaplan–Meier analysis was performed to evaluate OS and PFS in patients treated with gemcitabine monotherapy by stratifying cases according to hENT1 expression level. Patients who discontinued gemcitabine for any reason without evidence of RECIST progression were censored at the last tumour assessment. Patients alive were censored at the last contact. Differences in terms of OS and PFS were evaluated according to different clinical and pathological parameters according to log-rank test. The Cox proportional hazards model was applied to the multivariate survival analysis. Differences in terms of distribution of patients features were evaluated using fisher exact test and Mann–Whitney *U*-test when indicated. *P*-values <0.05 were considered statistically significant.

Table 1. Association between hENT1 expression and clinicopathological characteristics of leiomyosarcoma patients treated with gemcitabine

	High hENT1 (16 patients)	Low hENT1 (18 patients)	*P*-value
Gender (M/F)	9/7	11/7	0.523
Median age (range)	62 (21–81)	57 (34–71)	0.651
Median ECOG PS	0 (0–2)	0 (0–2)	0.645
Metastatic disease	11	16	0.214
Median number of metastatic sites (range)	2 (0–4)	2 (0–5)	0.310
Median number of previous CTs (range)	1 (0–4)	1 (0–4)	0.704
Uterine *vs* non-uterine	5/11	3/15	0.428

Abbreviations: CTs = chemotherapies; ECOG PS = Performance Status according to Eastern Cooperative Oncology Group; F = female; M = male.

RESULTS

Leiomyosarcoma

hENT1 expression. hENT1 was heterogeneously expressed in leiomyosarcoma FFPE samples (median $2^{-\Delta Ct}$ value 9.3 used as the cutoff to dichotomise leiomyosarcoma patients into 'high' expression and 'low' expression groups).

Table 1 summarises clinic-pathological characteristics of patients treated with gemcitabine and stratified for hENT1 expression.

Out-come analysis. Kaplan–Meier analysis in leiomyosarcoma cases treated with gemcitabine (34 patients) showed a significant correlation between hENT1 expression levels and outcome in terms of PFS (6.8 *vs* 3.2 months; $P=0.004$) and OS (14.9 *vs* 8.5 months; $P=0.007$) suggesting a better survival in patients with a higher hENT1 expression (Table 2; Figure 1). Conversely, in the small cohort of leiomyosarcoma patients not treated with gemcitabine (11 patients), we did not find any significant correlation between hENT1 expression and outcome (Supplementary Table 2; Supplementary Figure 1).

Moreover, the correlation between hENT1 mRNA level and protein expression was confirmed by IHC analyses. Two representative IHC images show hENT1 protein expression in two tumour samples that had high and low hENT1 mRNA levels (Figure 2).

Finally, a representative example of a hENT1 overexpressing leiomyosarcoma patient that shows major radiological response to gemcitabine treatment is depicted in Figure 3.

Angiosarcoma

hENT1 expression. hENT1 expression was less variable in angiosarcoma samples compared to leiomyosarcoma (median $2^{-\Delta Ct}$ value 1.25).

Table 3 reports clinicopathological characteristics of gemcitabine treated patients grouped for 'high' and 'low' hENT1 expression.

Out-come analysis. Patients that exhibited high hENT1 expression showed a longer PFS (9.3 *vs* 4.5 months; $P=0.02$) and a significant improvement in OS (20.6 *vs* 10.8 months; $P=0.001$) compared to those characterised by a low hENT1 expression (Table 2; Figure 4). As was the case for leiomyosarcoma, no significant association was found between hENT1 expression and PFS or OS in patients not treated with gemcitabine (Supplementary Table 2; Supplementary Figure 2).

A representative image of cutaneous localisation of hENT1 overexpressing angiosarcoma originating from the maxillary sinus before and after gemcitabine treatment is shown in Figure 5.

Table 2. Association between hENT1 expression and clinical outcome in leiomyosarcoma and angiosarcoma patients treated with gemcitabine

	Leiomyosarcoma high hENT1 (16 patients)	Leiomyosarcoma low hENT1 (18 patients)	*P*-value	Angiosarcoma high hENT1 (8 patients)	Angiosarcoma low hENT1 (7 patients)	*P*-value
PFS	6.8 months (95% CI 5.0–8.6)	3.2 months (95% CI 2.6–3.8)	0.004	9.3 months (95%, CI 5.9–12.9)	4.5 months (95% CI 3.6–5.4)	0.02
OS	14,9 months (95% CI 11.4–18.4)	8,5 months (95% CI 7.1–9.9)	0.007	20.6 months (95% CI 15.4–25.7)	10.8 months (95% CI 9.3–12.3)	0.001

Abbreviations: OS = overall survival; PFS = progression-free survival.

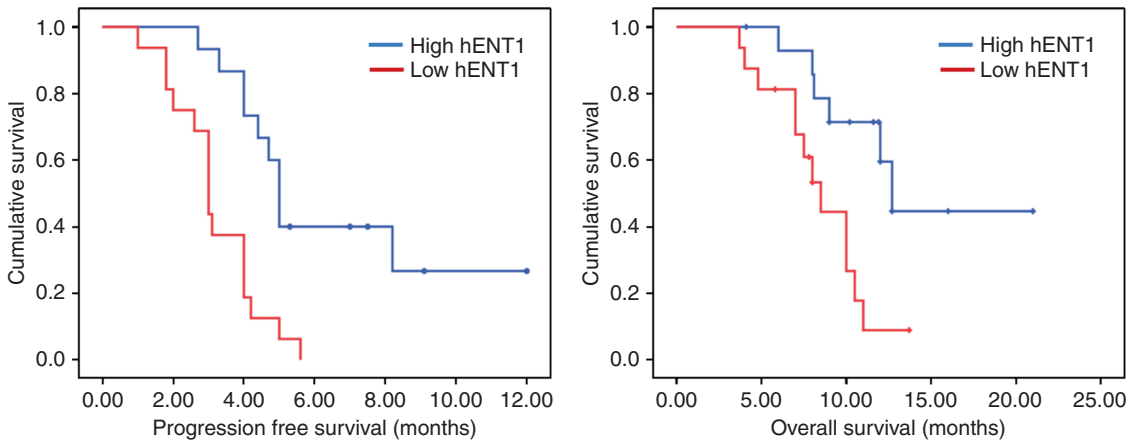

Figure 1. Kaplan–Meier curves of PFS and OS show that high hENT1 expression levels correlate with longer PFS ($P = 0.004$) and OS ($P = 0.007$) in leiomyosarcoma patients treated with gemcitabine.

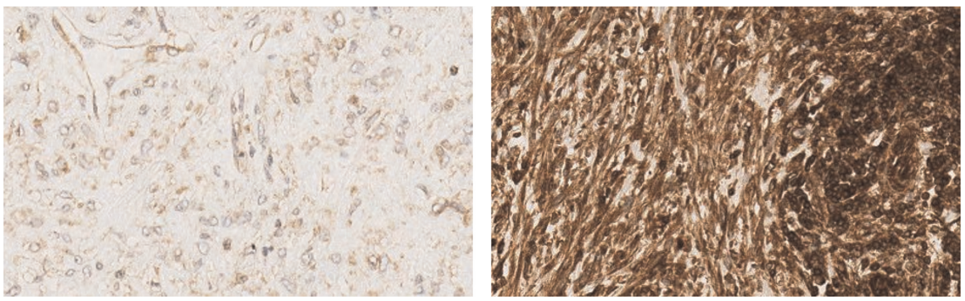

Figure 2. Representative IHC images of hENT1 protein expression in two leiomyosarcoma samples with low (*left*) and high (*right*) hENT1 mRNA level.

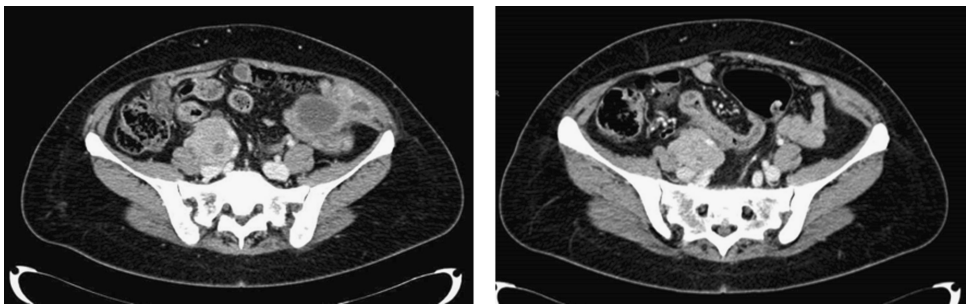

Figure 3. Baseline and 3 months CT scan in hENT1 overexpressing leiomyosarcoma patient treated with gemcitabine. This patient was diagnosed with a retroperitoneal leiomyosarcoma with multiple peritoneal nodules and lung metastasis since the first diagnosis. The patient progressed after first line anticancer therapy with adriamycin and trabectedin as second line. With gemcitabine monotherapy the patient achieved a RECIST partial response.

***In silico* analysis.** *In silico* analysis showed no significant correlation between hENT1 expression levels and patients prognosis in terms of OS in a cohort of 106 patients affected by leiomyosarcoma (HR 1.21 (0.65–2.23), $P = 0.55$) (Figure 6). No data are available for angiosarcoma patients, so far.

DISCUSSION

In this retrospective study, we found that hENT1 tumour expression was associated with clinical outcome in leiomyosarcoma and angiosarcoma patients treated with gemcitabine. In particular, patients with high hENT1 expression levels showed a better outcome in terms of PFS and OS both in leiomyosarcoma (PFS: 6.8 *vs* 3.2 months; OS: 14.9 *vs* 8.5 months) and angiosarcoma (PFS was 9.3 *vs* 4.5 months; OS 20.6 *vs* 10.8 months) compared to those with low hENT1 levels. We included also 22 patients not treated with gemcitabine to evaluate and dichotomise hENT1 expression in a larger group of leiomyosarcoma and angiosarcoma patients. In this cohort of patients not treated with gemcitabine, we did not find any significant correlation between hENT1 expression and clinical outcome.

Moreover, we performed a wide *in silico* transcriptomic analyses in a larger cohort of leiomyosarcoma patients finding no significant association between hENT1 expression and OS. Unfortunately, no complete clinical data are available such as the number of patients who received gemcitabine.

The association between hENT1 expression and gemcitabine efficacy was demonstrated in different tumour types including biliary tract cancer (Santini *et al*, 2011), cholangiocarcinoma (Borbath *et al*, 2012), bladder cancer (Matsumura *et al*, 2011) and non-small cell lung cancer (Oguri *et al*, 2007).

Table 3. Association between hENT1 expression and clinicopathological characteristics of angiosarcoma patients treated with gemcitabine

	High hENT1 (8 patients)	Low hENT1 (7 patients)	*P*-value
Gender (M/F)	3/5	2/5	0.500
Median age (range)	59 (34–75)	64 (29–83)	0.370
Median ECOG PS	0 (0–2)	1 (0–2)	0.431
Metastatic disease	6	5	0.662
Median number of metastatic sites (range)	2 (0–4)	2 (0–5)	0.866
Median number of previous CTs (range)	1 (0–3)	1 (0–2)	0.363

Abbreviations: CTs = chemotherapies; ECOG PS = Performance Status according to Eastern Cooperative Oncology Group; F = female; M = male.

Since gemcitabine, as single agent or in combination with docetaxel, has been widely adopted also in leiomyosarcoma (Hensley *et al*, 2002, 2008; Pautier *et al*, 2012) and angiosarcoma (Stacchiotti *et al*, 2012), the identification of molecular markers such as hENT1 could help to select patients with a high likelihood of benefiting from this chemotherapy regimen.

There is no standardised protocol to define how to score hENT1 expression. Most previous studies used immunohistochemistry to evaluate hENT1 levels in tumour cells, but a clear cutoff to define hENT1 positivity is lacking as the variability in methods and antibodies leads to an absence of standardisation in the scoring systems. We have chosen the qRT-PCR to determine hENT1 expression due to its greater sensitivity and better precision.

To obtain a more homogeneous population, radiation-associated sarcomas (RAS) were excluded because this subtype differs from traditional STSs in terms of molecular biology, clinicopathological features and prognosis. Indeed, RAS are associated with higher aggressive behaviour and worse outcome compared to STSs, even if one adjusts for known prognostic factors (Gladdy *et al*, 2010). In addition, RAS differs also for treatment possibilities: repeated high-dose radiotherapy often is impossible and chemotherapy is limited by bone marrow dysfunction (Bjerkehagen *et al*, 2008).

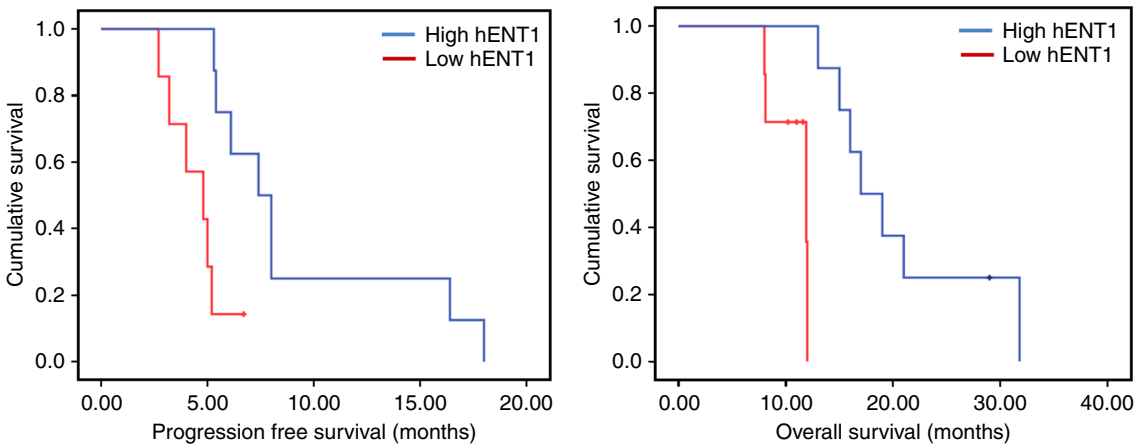

Figure 4. Kaplan–Meier curves show that high hENT1 expression levels correlate with longer PFS ($P = 0.02$) and OS ($P = 0.001$) in angiosarcoma patients treated with gemcitabine.

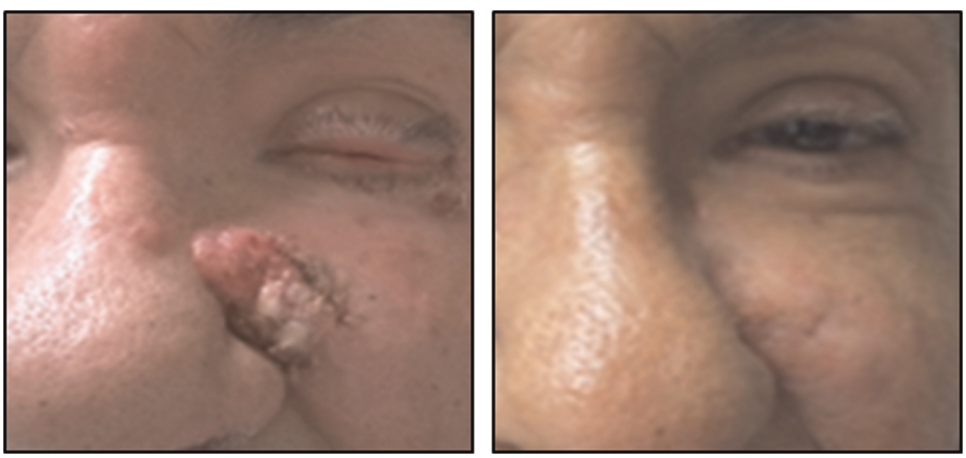

Figure 5. Tumour response in hENT1 overexpressing angiosarcoma patient treated with gemcitabine. In this patient a clinical and radiological complete response was achieved after the first three courses of gemcitabine, lasting at about 1 year. The patient is still on therapy with the same agent.

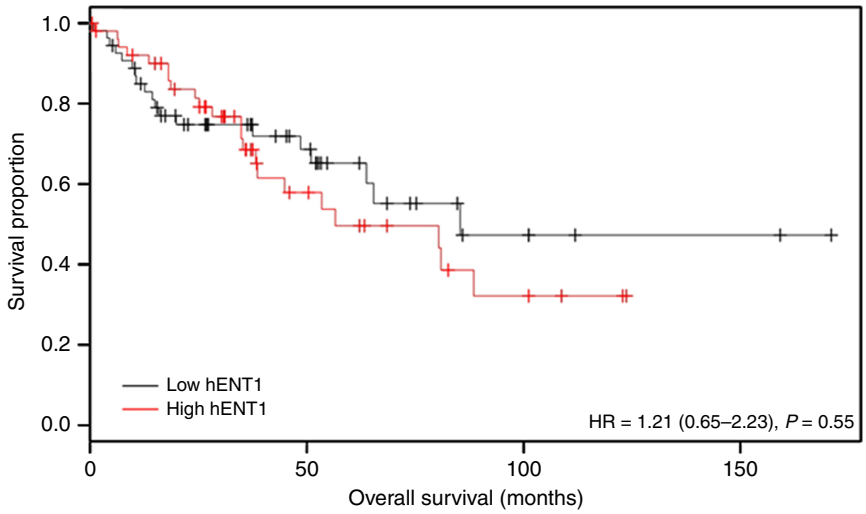

Figure 6. *In silico* analyses. Kaplan–Meier curves show that hENT1 expression levels did not correlate with OS in a cohort of 106 leiomyosarcoma patients.

The retrospective nature of the study represents a limitation as well as the low number of cases especially for angiosarcoma. Larger series, preferably within a prospective trial or randomised controlled trial are needed to confirm our results.

Moreover, hENT1 may not be the exclusive mediator for gemcitabine sensitivity. Indeed, other nucleoside transporter proteins are involved in the intracellular uptake of gemcitabine such as hENT3 and concentrative nucleoside transporters (Maréchal *et al*, 2009). Several enzymes are also implicated in the complex metabolism of gemcitabine and may contribute to the efficacy of the drug such as ribonucleotide reductase subunit 1 (RRM1, an intracellular target of gemcitabine) and deoxycytidine kinase (dCK, the enzyme that is responsible for the initial phosphorylation of gemcitabine into its active forms) (Kim *et al*, 2011). Thus, the combined expression analysis of hENT1 with these other markers could provide a greater predictive value than each factor alone.

In conclusion, we found that hENT1 expression could predict gemcitabine efficacy in leiomyosarcoma and angiosarcoma patients, and if confirmed in prospective trials, these data would allow a better patient selection and an improvement in therapeutic efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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