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Sodium/iodide symporter is expressed in the majority of seminomas and embryonal testicular carcinomas

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Short title: NIS expression in testis tumours

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Abstract

Testicular cancer is the most frequent cancer in young men. The large majority of patients has a good prognosis, but in a small group of tumours the current treatments are not effective. Radioiodine is routinely used in the treatment of thyroid cancer and is currently investigated as a potential therapeutic tool even for extra-thyroid tumours able to concentrate this radioisotope. Expression of Na+/I- symporter (NIS), the glycoprotein responsible for iodide transport, has been demonstrated in normal testicular tissue. In this study, we analyzed NIS expression in a large series of testicular carcinomas. Our retrospective series included 107 patients operated for testicular tumours: 98 typical seminomas, 6 embryonal carcinomas, 1 mixed embryonal-choriocarcinoma and 2 Leydig cells tumours. Expression and regulation of *NIS* mRNA and protein levels were also investigated in human embryonal testicular carcinoma cells (NTERA) by real time RT-PCR and western blotting respectively. Immunohistochemical analysis showed presence of NIS in the large majority of seminomas (90/98) and embryonal carcinomas (5/7) of the testis, but not in Leydig cell carcinomas. Expression of NIS protein was significantly associated to the lymphovascular invasion. In NTERA cells treated with the histone deacetylase inhibitors SAHA and valproic acid, a significant increase of *NIS* mRNA (about 60 and 30 fold *vs* control, $p<0.001$ and $p<0.01$ respectively) and protein levels, resulting in enhanced ability to uptake radioiodine, was observed. Finally, NIS expression

in testicular tumours with the more aggressive behavior is of interest for the potential use of targeting NIS to deliver radioiodine in malignant cells.

Introduction

Testicular cancer represents about 1-1.5% of all human neoplasia and is the most frequent malignancy in young adult men between 15 and 40 years, representing the leading cause of cancer-related mortality and morbidity in this age group (Winter & Albers 2011). Although conventional treatments or high-dose chemotherapy are able to treat approximately 80% of these patients, it is highly desirable to identify novel effective therapeutic options provided with minimal side effects (Sonpavide *et al.* 2007; Schrader *et al.* 2009).

Radioiodine (I^{131}), used in the treatment of thyroid cancer, has recently been proposed as novel therapeutic tool even for extra-thyroid tumours, if able to concentrate this radioisotope (Riesco-Eizaguirre & Santisteban 2006; Kogai *et al.* 2006). Radioiodine concentration requires the presence and function of the Na+/I- symporter (NIS), the glycoprotein responsible for iodide transport across the basal membrane of the thyrocytes (Dohan *et al.* 2003). Thus, stimulation of NIS expression by TSH is adopted in the radioiodine-based treatment of thyroid recurrent and metastatic cancer and defects in its functional expression is a major cause of failure of such a treatment (Arturi *et al.* 2000; Schlumberger *et al.* 2007). Similarly, attempts to induce/enhance NIS expression in extra-thyroid tumour cells, to make them able to concentrate the radioisotope, may offer the opportunity of using the same therapeutic approach adopted for thyroid tumours. NIS expression has been recently demonstrated in normal testicular tissue both at transcript

and protein levels (Russo *et al.* 2011a), while only one study, analyzing a small number of samples, has been performed on neoplastic testicular tissues, showing NIS expression in 1 of 11 malignant cores examined (Wapnir *et al.* 2003).

In this study, NIS expression was investigated in 98 typical seminomas, 7 embryonal testicular carcinomas (including one mixed embryonal-choriocarcinoma) and 2 Leydig cell tumours. In addition, we attempted to stimulate *in vitro* NIS gene and protein expression and iodide uptake in testicular tumour cells. For this purpose we used an experimental model of embryonal testicular cancer, known for its high aggressiveness, testing the effects of a series of stimulators in NTERA human cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and amphotericin B were purchased from Lonza (Milan, Italy). Suberoylanilide hydroxamic acid (SAHA), Decitabine, Bortezomib and Rapamycin were obtained from Aurogene (Rome, Italy); Valproic Acid, Forskolin, 5-azacytidine, Mevinolin, Apha compound 8, Hepes, $KClO_4$, NaI and monoclonal anti β-actin antibody were from Sigma Aldrich s.r.l (Segrate, Milan, Italy). Monoclonal anti-NIS antibody MAB3562 was purchased from Prodotti Gianni (Milan, Italy), anti-human NIS monoclonal antibody N2750 was from United States Biological (Swampscott, MA), PVDF membrane and ECL plus were from VWR (Milan, Italy), Trizol was from

Invitrogen (Carlsbad, CA, USA), nuclease-free H_2O was from GIBCO (Milan, Italy) and horseradish peroxidase-conjugated anti-mouse antibody was from Transduction Laboratories (Lexington, KY, USA). The High Capacity cDNA Reverse Transcription kit, TaqMan Fast Universal PCR master mix, FAM dye-labelled probes, Assay-on-Demand Gene Expression Products and β-actin and were obtained from Applied Biosystems (Foster City, CA, USA). Hank's balanced salt solution from EuroClone (Celbio, Pero, Milan, Italy), carrier free NaI from PerkinElmer (Monza, Milan, Italy).

Cell culture

NTERA cells, the only commercial available cell line of human embryonal testicular carcinoma, were purchased from LGC Standards (Sesto San Giovanni, Milan, Italy), cultured in DMEM containing FBS 10% (v/v), penicillin (0.1 mg/ml), streptomycin (2.5 μg/ml), amphotericin B (2.5 μg/ml) and were maintained at 37 ºC in a humidified atmosphere (5% CO_2).

Tissue samples

A retrospective series of 107 patients operated at the Policlinic of Modena for testicular tumours who underwent inguinal orchifunicolectomy was analyzed: 98 seminomatous tumours (typical seminomas) and 9 non-seminomatous tumours including 6 embryonal carcinomas, 1 mixed embryonal-choriocarcinoma and 2 Leydig cells tumors. Tumours were histologically classified according to World Health Organization criteria (Eble *et al.* 2004). The Tumour staging (TNM), that represents the validated standard tool to describe tumour extent and includes prognostic information on the probability of disease control,

was assigned using the current guidelines (Edge *et al.* 2009). Specimens' aliquots were fixed in Bouin's fixative overnight for histological studies. Review of patients' charts was carefully performed to collect the clinical features of each case, as described in Table 1.

Ethics Statement

All human tissue samples used in the study were collected with full patients' informed written consent and approval from the Policlinic of Modena ethic committee.

Immunohistochemistry

The presence of NIS in testicular tumour tissues was analyzed by immunohistochemistry as described previously (Navarra *et al.* 2010). Dewaxed 4-µm sections were first incubated with 6% H_2O_2 for 10 min at room temperature to block endogenous peroxidase activity. Then, they were immersed in a citrate buffer (pH 6) for 30 min at 98 °C and incubated at room temperature overnight with the monoclonal anti-NIS antibody N2750 diluted 1:100. The avidin-biotin complex was applied using an automatic system (Benchmark, Ventana, Tucson, AZ, USA) and staining was visualized using diamino-benzidine chromogen. The sections were lightly counterstained with Carazzi's hematoxylin and dehydrated, before being mounted and examined by two pathologists, who expressed concordant opinions for all the cases examined. A rate >10% of cells staining associated with at least moderate intensity was used to indicate positivity, 10-50% moderate, >50% high.

Analysis of mRNA levels

Levels of *NIS* mRNA were determined with real-time quantitative RT-PCR, as previously described (Sponziello *et al.* 2010). Briefly, total RNA was extracted from cells treated with various compounds at various incubation times using the Trizol method, according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed in a 20 µl reaction volume using the High Capacity cDNA Reverse Transcription kit following the instructions of the manufacturer. After 1:5 dilution, the cDNAs were amplified using an Applied Biosystems 7900HT Fast Real-Time PCR Sequence Detection System and fast quantitative PCR thermal cycler parameters. Each tube contained, in a total of 20 µl, 2 µl of cDNA, 10 µl of TaqMan Fast Universal PCR master mix, and 1.0 µl of a pre-developed primer/probe mixture for each gene to be measured. All values were normalized to β-actin as endogenous control, with similar results. The experiments were repeated at least three times. Reactions, results determination and expression and normalization were performed as previously reported (Sponziello *et al.* 2010).

Analysis of protein levels

Extraction of total proteins was performed as previously described (Celano *et al.* 2008). Briefly, fifteen µg of proteins were run on a 7.5% SDS-PAGE gel and transferred to PVDF membrane with the Mini Trans Blot system (Bio-Rad Laboratories S.r.l, Milan, Italy) (2 h at 225 mA). Membranes were blocked with TTBS/milk (TBS, 1% Tween 20 and 5% non-fat dry milk) for 1 h at room temperature and incubated overnight with the affinity-purified anti-NIS monoclonal antibody MAB3562 diluted 1:250. The membranes were washed once for 15-min and twice for 5-min in TTBS, and incubated with

160 horseradish peroxidase-conjugated anti-mouse antibody diluted 1:10000 in TTBS/milk.
161 After one 15-min and two 5-min washes in TTBS, the protein was visualized by
162 chemiluminescence using the Western blot detection system ECL Plus. Monoclonal
163 mouse β-actin antibody was used as an internal control.
164
165 **Iodide uptake**
166 Iodide uptake by NTERA cells was measured as previously described (Weiss *et al.* 1984).
167 Briefly, cells were seeded into 12-well plates and treated with SAHA 3 μM and valproic
168 acid 3 mM for 48h. Then, the culture medium was aspired and cells washed twice with 1
169 ml Hank's balanced salt solution (HBSS) supplemented with Hepes (10 mM, pH 7.3).
170 ^{125}I-uptake was initiated by adding to each well 500 μl of HBSS containing 0.1 μCi/ml
171 carrier free labeled NaI and 10 μM NaI. In half of the wells, used as control for specific
172 uptake, this buffer also contained 100 μM $KClO_4$, a NIS inhibitor. After 30-40 min at 37
173 °C in a humid atmosphere, the radioactive medium was aspirated and cells were washed
174 twice with 1 ml of ice-cold HBSS. The amount of iodide uptake was determined after
175 incubation with 1 ml of 95% ethanol to each well for 20 min and transfer into vials for
176 counting with a γ-counter. The NIS-specific radio-iodine uptake was normalized using
177 data of cell viability measured with MTT assay (data not shown). Each experiment was
178 carried out in triplicate.
179
180 **Statistical analysis**
181 The results are expressed as means ± SD, and the one-way ANOVA followed by the
182 Tukey-Kramer multiple comparisons test was adopted to determine the significance of

differences using the GrafPAD Software for Science (San Diego, CA, USA). Patients were all uniformly followed-up at our Institution. The association between protein NIS expression and clinico-pathological parameters was calculated using contingency table methods and tested for significance using the Pearson's chi-square test. A probability (p) value <0.05 was considered statistically significant.

Results

Clinical and pathological features

A total of 107 testicular tumour tissues were evaluated: histological types included 98 seminomatous tumours (90 fixed in formalin specimens and 8 fresh/not fixed in formalin tissue), 7 embryonal carcinomas (all fresh/not fixed in formalin tissue) and 2 sex cord/gonadal stromal tumours (Leydig cell tumours, both fresh/not fixed in formalin tissue). Eighty-eight tumours (82.3%) are classified as Stage I, 10 (9.3%) as Stage II and 9 (8.4%) as Stage III. There was a complete accordance of the two pathologist in attributing the grading of each sample. In 18 cases (17%) we detected lymphovascular invasion. The clinical and pathological findings of the patients are listed in Table 1.

Expression of NIS in human testicular cancer tissues

Expression of *NIS* mRNA was evaluated in the available samples of fresh frozen testicular tumours. We observed detectable levels of *NIS* mRNA in 5 of 8 seminomas, in 5 of 7 embryonal carcinomas while in Leydig cell tumours *NIS* resulted absent (Fig.1).

All tumours were analyzed by immunohistochemistry to evaluate the expression of NIS protein. NIS protein staining was detected in the cell plasma membrane in the majority of the cases with intense staining (Fig.2). As shown in fig.2, in 64 seminomas and 5 embryonal carcinomas we observed more than 50% of cell stained. Twenty-six seminomas presented moderate to weak staining, while 8 seminomas, 2 embryonal carcinomas and both Leydig cell tumours were negative (Fig.3). Interestingly, NIS protein expression was significantly associated to the lymphovascular invasion ($p<0.005$) but not with the other clinical and pathological parameters, as reported in Table 2. In the samples in which both RNA levels and tissue slice could be examined, concordance in the positivity of NIS mRNA and protein was observed, except for one seminoma positive for NIS mRNA expression and only weak staining of the protein.

Stimulation of NIS expression in testicular embryonal carcinoma cells

Expression of *NIS* mRNA was then evaluated in the embryonal human testicular carcinoma cells NTERA. A series of molecules, including Suberoylanilide hydroxamic acid (SAHA), Decitabine, Bortezomib, Rapamycin, Valproic Acid, Forskolin, 5-azacytidine, Mevinolin and Apha compound 8, known to stimulate NIS expression in thyroid cells (Frölich *et al.* 2008), were tested at various doses and incubation times (Table 3). The strongest stimulating effect was observed with the histone deacetylase inhibitors (HDACi) SAHA and valproic acid. Subsequently, we conducted dose-response and time-course analysis of selected dosages of SAHA and valproic acid: the greatest increment of the levels of *NIS* mRNA was observed after 24 h treatment with SAHA 3 μM and valproic acid 3 mM, about 60 fold and 30 fold over control, respectively (Fig.4).

We next examined the expression of NIS protein in NTERA cells exposed to the same HDAC inhibitors. As shown in fig.5, a specific band of approximately 90 kDa, corresponding to human NIS protein was detected in the total protein extracts of NTERA cells in basal condition and after treatment with SAHA 3 μM or valproic acid 3 mM, with the strongest effect observed in NTERA after 48 h of incubation (Fig.5).

Radioiodine uptake in NTERA cells

In order to test whether stimulation of NIS protein by HDACi determined an increase of its function, radioiodide uptake experiments were performed in the cells treated with SAHA and valproic acid (3μM and 3 mM, respectively). After 48 h of treatment, we observed a significant increase of the uptake with both compounds (Fig.6).

Discussion

Testicular cancer, the most common malignancy occurring in young males, is a highly curable tumour even in patients with metastatic disease. Indeed, seminomas, the most frequent histotype, have a high radiosensitivity, so that combination of orchiectomy and adjuvant radiotherapy on the para-aortic and ipsilateral iliac lymph nodes, the standard therapy adopted in the last 60 years, has reduced the risk of relapse to 1-3%, resulting in a global survival rate close to 100% (Warde *et al.* 2002). In the less radiosensitive nonseminomatous tumours, including embryonal cell carcinomas, yolk sac tumor, choriocarcinoma and teratoma, the chemotherapy, mainly based on 3 to 4 cycles of PEB (cisplatin, etoposide, bleomycin), is the alternative choice. However, resistance to such a

treatment often arises (Krege *et al.* 2001; Castillo-Avila *et al.* 2009). Recently, it was reported the description of some molecular mechanisms potentially involved in the pharmacological resistance and developed by the more aggressive tumours of the testis (Looijenga *et al.* 2011). Novel therapeutic strategies are therefore urgently required for those tumours resistant to the current treatment.

Expression of the NIS, the protein which actively transport the iodide into the thyrocytes, in extra-thyroidal tumour tissues has been exploited for its potential use to target radioiodine in malignant cells for diagnosis and/or treatment of the disease (Riesco-Eizaguirre & Santisteban 2006; Kogai *et al.* 2006). Therefore, induction of NIS expression in cancer cells to deliver radioiodine is currently being explored for many types of extra-thyroid neoplasia (Hingorani *et al.* 2010). While encouraging results have been obtained in some preclinical models, unresolved issues are still present about the feasibility of a gene therapy-based approach on humans (Haberkorn *et al.* 2003). Equally promising are the attempts to stimulate endogenous NIS expression in those tumour cells, from thyroid and non-thyroid cancers, with detectable levels of *NIS* mRNA (Kogai *et al.* 2006). The feasibility of such an approach has been addressed in various tumours, including prostate cancer. In a previous report, expression of the NIS has been detected in the more aggressive forms of prostate tumours, suggesting a potential use as target for a therapy with radioiodine as well as biomarker for identifying individuals with biologically active disease (Navarra *et al.* 2010). Interestingly, even in breast cancer NIS expression was detected in the more aggressive 'triple-negative' samples (Renier *et al.* 2009), at variance with thyroid cancer, in which lymph node metastatic tissues have usually reduced or lost NIS expression (Arturi *et al.* 2000). In the only other study

addressing this issue in testicular tumours, no information was provided according to the histotype, the clinical characteristics of the patient(s) and the localization of the NIS in the specimen examined (Wapnir *et al.* 2003).

In this study we demonstrate that NIS is expressed in the plasma membrane of the large majority of seminomas and embryonal carcinomas of human testis, while is absent in 2 Leydig cell cancer. Our data also demonstrate a significant association of the expression of NIS protein with the lymphovascular invasion, a well-known marker of aggressiveness. We believe that the association between NIS expression in the tumour cells and the lymphovascular invasion may reflect the different biological aggressiveness of testis tumours suggesting the presence of the NIS as an unfavorable prognostic factor. Thus, the majority of the aggressive seminomas and embryonal carcinomas express the NIS protein so that may be considered, in case of refractoriness to the standard treatment, potential candidate to an alternative radioiodine-based therapeutic strategy. Since the embryonal carcinomas, for their refractoriness to the current treatment, represent potential candidates for such a novel therapeutic approach, we chose the NTERA cells, the only available commercial human embryonal carcinoma cell line, to attempt to stimulate NIS expression in tumour cells. Our present findings reveal that NIS expression may be enhanced in vitro by HDAC inhibitors. Histone acetylation is a known epigenetic mechanism of regulation of gene expression and its alteration has been reported in many human cancers (Chi *et al.* 2010). In many cell lines of thyroid and non-thyroid cancer, HDAC inhibitors have been successfully tested to induce radioiodine uptake due to increased NIS expression (Puppin *et al.* 2005; Russo *et al.* 2011b; Liu & Xing 2012). The same result was obtained in the NTERA cells in the present study, showing that at least *in*

vitro embryonal testicular tumour cell susceptibility to radioiodine administration may occur and suggesting the possibility to use the radioiodine after pharmacological induction of NIS expression even in this rare tumour histotype. It is noteworthy that these drugs are being tested in clinical trials at doses compatible with those effective *in vitro* (www.clinicaltrials.gov).

In conclusion the present data demonstrate that NIS is expressed in the large majority of seminomas and embryonal carcinomas of human testis, including those with a more aggressive phenotype (i.e. with lymphovascular invasion). Its presence in the plasmamembrane compartment of the tumour cells suggests that it may serve as potential carrier of radioiodine for an ablative treatment of cancer tissue.

Declaration of interest, Funding and Acknowledgements

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. This work was supported by grants from the Italian Ministry of Instruction, University (PRIN COFIN 2008) (to D.R.).

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www.clinicaltrials.gov

Figure legends

Fig. 1 Expression of NIS mRNA in testicular tumours

NIS mRNA levels assayed by RT-PCR in seminomas, embryonal testicular carcinomas and in Leydig cell fresh frozen tumours.

Fig. 2 Expression of NIS in testicular carcinoma tissues

Immunohistochemistry of NIS in testicular tumors. In seminomas and embryonal carcinomas NIS staining is detected in both cytosol and plasma membrane of cancer cells. Thyroid hyperfunctioning adenoma is used as positive control; one Leydigioma, one embryonal carcinoma and one seminoma negative for NIS mRNA expression are shown as negative controls. Experiments were performed using a primary monoclonal anti-human NIS antibody diluted 1:100 as described in Methods.

Fig.3 Immunohistochemical results of NIS intensity in seminoma and embryonal testicular carcinomas

Bars represent the percentage of total seminomas or embryonal carcinomas with absent, moderate or high intensity staining, evaluated in immunohistochemical experiments as indicated in Methods.

Fig.4 Expression of NIS mRNA in NTERA cells

NIS mRNA levels assayed by RT-PCR in NTERA cells exposed for 4-8-24 h to SAHA 0.3 and 3 μM (***$p<0.001$ *vs* control), valproic acid (VPA) 0.3 and 3 mM (**$p<0.01$ *vs*

control). Data are means ± SD of 3 experiments using ANOVA followed by the Tukey-Kramer multiple comparisons test.

Fig.5 Expression of NIS protein in NTERA cells

Western blot analysis was performed under reducing conditions using a monoclonal anti-NIS antibody and a monoclonal anti-human β-actin antibody. A representative of three separated experiments is shown. A specific band of approximately 90 kDa, corresponding to human NIS protein, was detected in the total protein extracts of carcinoma testicular cells and increased after treatment with HDAC inhibitors.

Fig.6 SAHA and valproic acid increase radio-iodine uptake in NTERA cells.

NTERA cells were treated with SAHA 3 μM and valproic acid 3 mM for 48 h. Radio-iodine uptake was evaluated as described in Materials and Methods section. Each bar represents the mean value (±SD) of three different experiments. ***, statistical significance compared to untreated cells ($p < 0.001$).

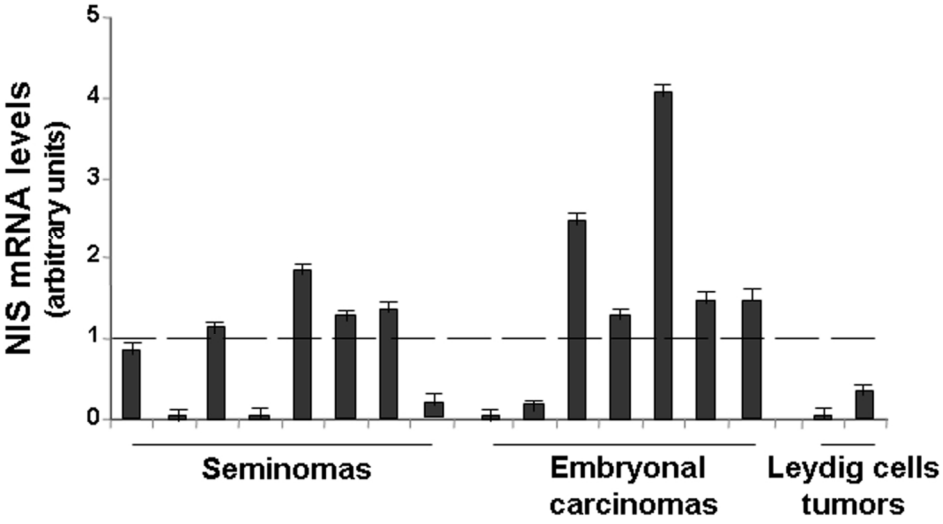

Figure 1

Fig. 1 Expression of NIS mRNA in testicular tumours
NIS mRNA levels assayed by RT-PCR in seminomas, embryonal testicular carcinomas and in Leydig cell fresh frozen tumours.

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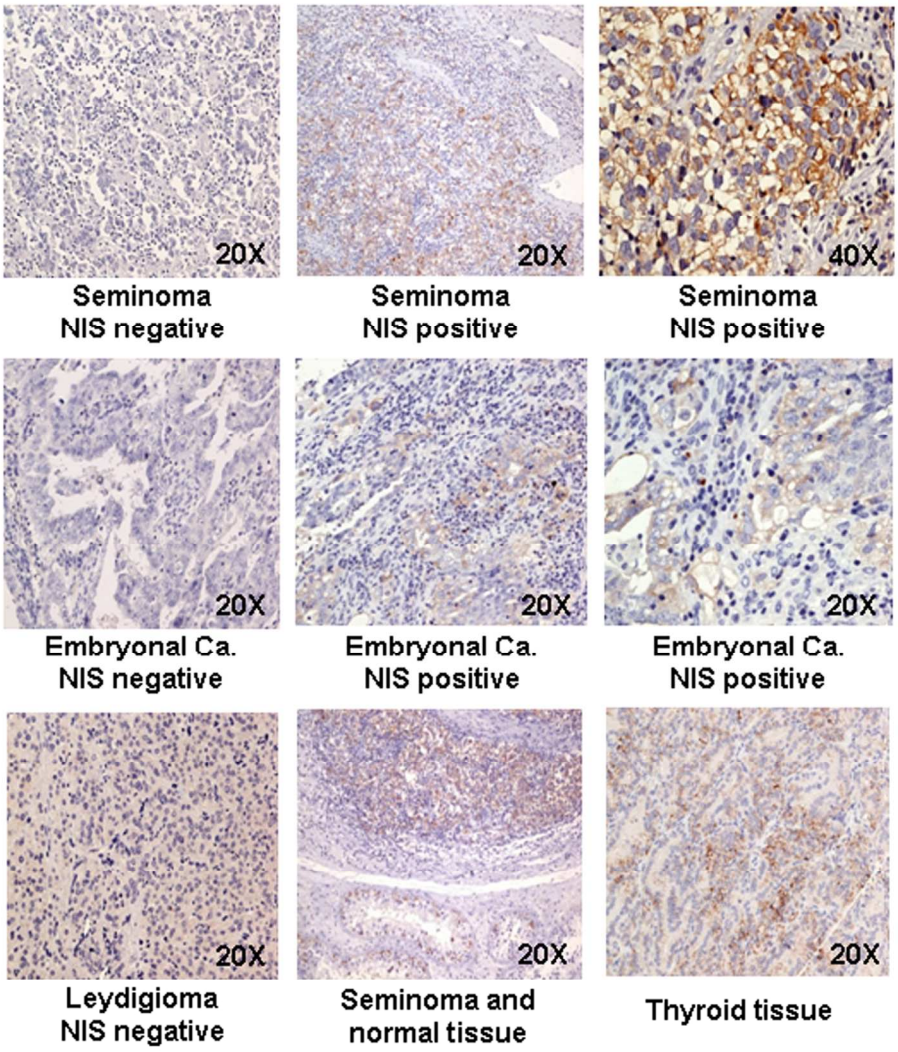

Figure 2

Fig. 2 Expression of NIS in testicular carcinoma tissues
Immunohistochemistry of NIS in testicular tumors. In seminomas and embryonal carcinomas NIS staining is detected in both cytosol and plasma membrane of cancer cells. Thyroid hyperfunctioning adenoma is used as positive control; one Leydigioma, one embryonal carcinoma and one seminoma negative for NIS mRNA expression are shown as negative controls. Experiments were performed using a primary monoclonal anti-human NIS antibody diluted 1:100 as described in Methods.

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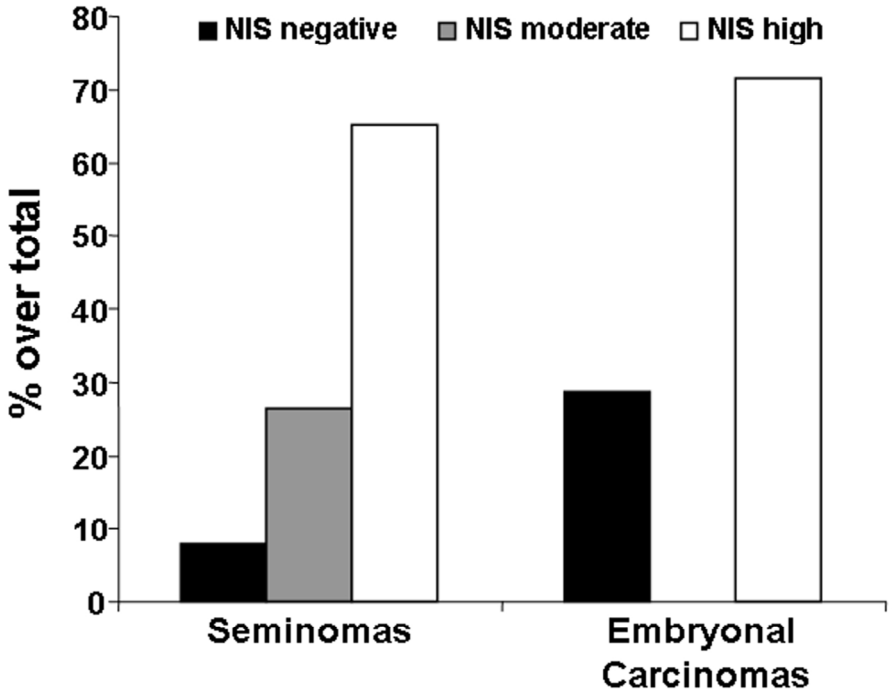

Figure 3

Fig.3 Immunohistochemical results of NIS intensity in seminoma and embryonal testicular carcinomas
Bars represent the percentage of total seminomas or embryonal carcinomas with absent, moderate or high intensity staining, evaluated in immunohistochemical experiments as indicated in Methods.

151x132mm (300 x 300 DPI)

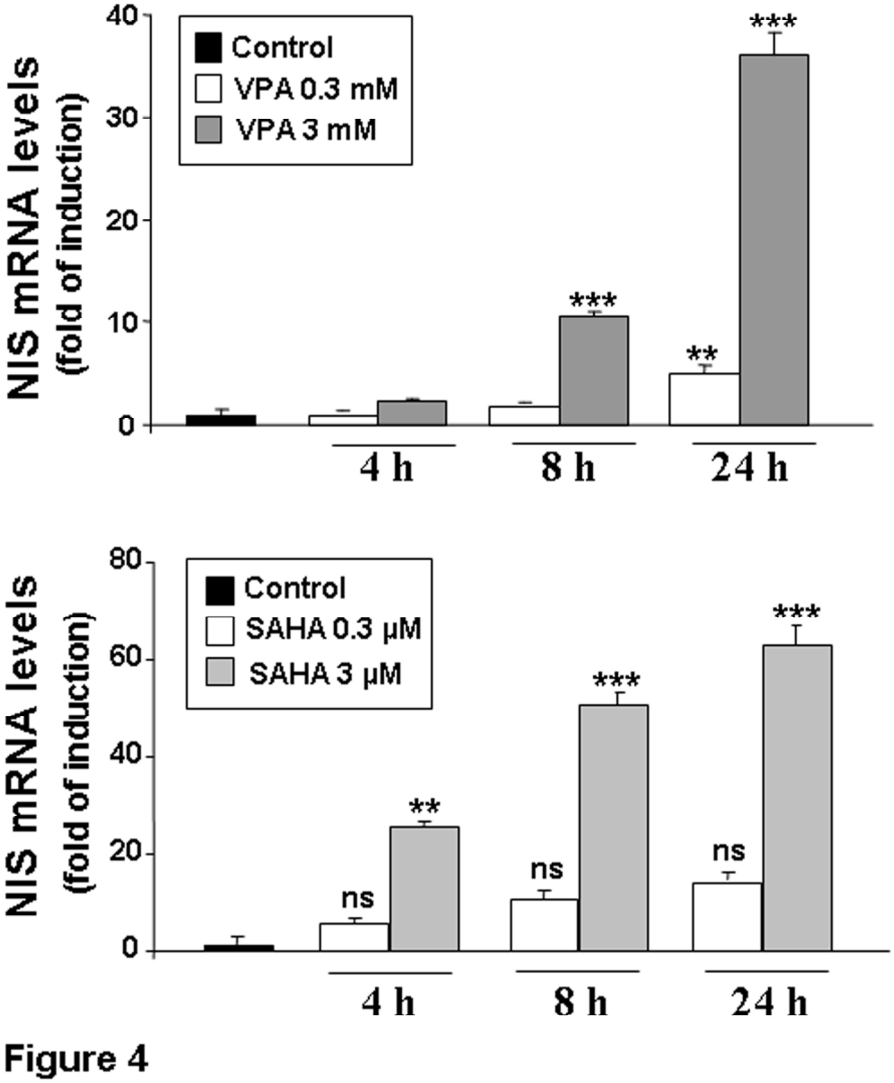

Figure 4

Fig.4 Expression of NIS mRNA in NTERA cells
NIS mRNA levels assayed by RT-PCR in NTERA cells exposed for 4-8-24 h to SAHA 0.3 and 3 µM (***p<0.001 vs control), valproic acid (VPA) 0.3 and 3 mM (**p<0.01 vs control). Data are means ± SD of 3 experiments using ANOVA followed by the Tukey-Kramer multiple comparisons test.

175x208mm (300 x 300 DPI)

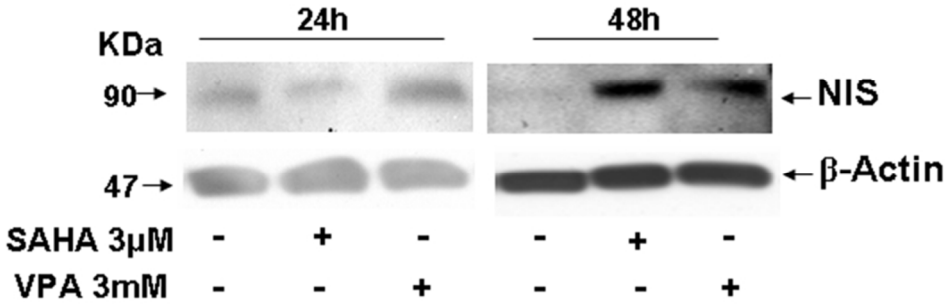

Figure 5

Fig.5 Expression of NIS protein in NTERA cells
Western blot analysis was performed under reducing conditions using a monoclonal anti-NIS antibody and a monoclonal anti-human β-actin antibody. A representative of three separated experiments is shown. A specific band of approximately 90 kDa, corresponding to human NIS protein, was detected in the total protein extracts of carcinoma testicular cells and increased after treatment with HDAC inhibitors.

78x36mm (300 x 300 DPI)

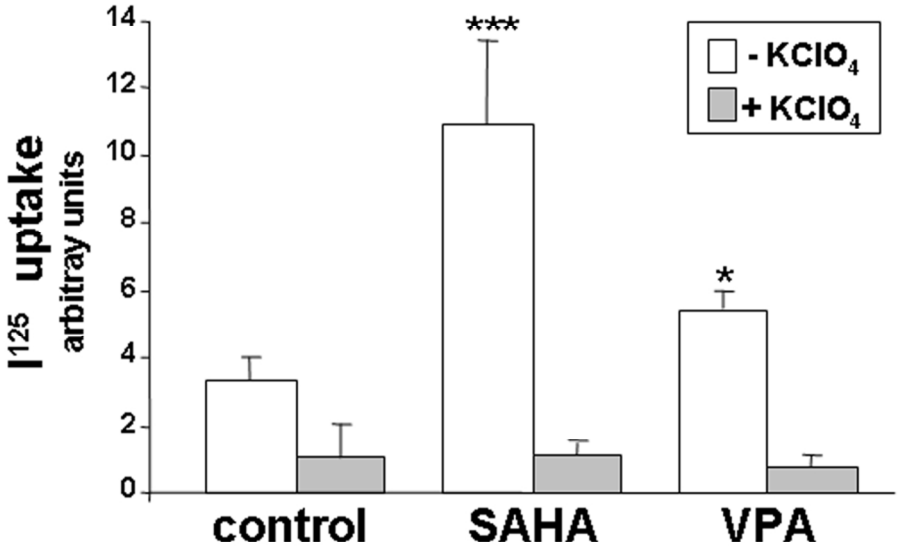

Figure 6

Fig.6 SAHA and valproic acid increase radio-iodine uptake in NTERA cells.
NTERA cells were treated with SAHA 3 μM and valproic acid 3 mM for 48 h. Radio-iodine uptake was evaluated as described in Materials and Methods section. Each bar represents the mean value (±SD) of three different experiments. ***, statistical significance compared to untreated cells ($p < 0.001$).

111x80mm (300 x 300 DPI)

Table 1. Clinical and pathological features of the 107 patients with testicular tumours analyzed

Age	**Mean±SD**	37.24±11.4	**TNM***	**T**	**T1**	82 (76.6%)
	Median	35			**T2**	20 (18.7%)
	Range	18÷73			**T3**	5 (4.7%)
Size	**Middle±SD**	4.68±2.69		**N**	**N0**	97 (90.7%)
	Median	4			**N1-N2**	10 (9.3%)
	Range	1.5÷13		**M**	**M0**	99 (92.5%)
					M+	8 (7.5%)
Histology	**Typical seminoma**	98 (91.6%)				
	Embryonal carcinoma	6 (5.5%)	**Lymphovascular invasion (+/-)**			18 (17%)/89 (83%)
	Mixed^	1 (1%)				
	Leydigioma	2 (1.9%)	**Follow up**		**Mean**	98.4 months
					Range	12÷144 months
Stage	**I**	88 (82.3%)			**Alive**	95 (89%)
	II	10 (9.3%)			**Died****	4 (4%)
	III	9 (8.4%)			**Recurrences**	8 (7%)

^Embryonal and choriocarcinoma
*TNM: Tumour Nodes Metastases
**Deceased for cause unrelated to the tumour

Table 2. Clinico-pathological characteristics of NIS+ and NIS- seminomas and embryonal testicular carcinomas

		SEMINOMAS		EMBRYONAL CARCINOMAS		LEYDIGIOMAS		TOTAL OF TUMOURS		p value*
		NIS +	NIS -	NIS +	NIS -	NIS +	NIS -	NIS +	NIS -	
TNM °	T1	74 (94.9%)	4 (5.1%)	3 (100%)	0 (0%)	0 (0%)	1 (50%)	77 (94%)	5 (6%)	
	T2-T3	16 (80%)	4 (20%)	2 (50%)	2 (50%)	0 (0%)	1 (50%)	18 (72%)	7 (28%)	n.s.
	N0	84 (92.3%)	7 (7.7%)	3 (75%)	1 (25%)	0 (0%)	2 (100%)	87 (90%)	10 (10%)	
	N1-N2	6 (85.7%)	1 (14.3%)	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	8 (80%)	2 (20%)	n.s.
	M0	86 (91.5%)	8 (8.5%)	2 (66.7%)	1 (33.3%)	0 (0%)	2 (100%)	88 (89%)	11 (11%)	
	M+	4 (100%)	0 (0%)	3 (75%)	1 (25%)	0 (0%)	0 (0%)	7 (87.5%)	1 (12.5%)	n.s.
Lymphovascular invasion	Present	10 (83.3%)	2 (16.7%)	3 (60%)	2 (40%)	0 (0%)	1 (50%)	13 (72%)	5 (28%)	
	Absent	80 (93%)	6 (7%)	2 (100%)	0 (0%)	0 (0%)	1 (50%)	82 (92%)	7 (8%)	<0.05
Recurrence of disease	Present	5 (100%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	1 (50%)	6 (75%)	2 (25%)	
	Absent	85 (91%)	8 (9%)	4 (80%)	1 (20%)	0 (0%)	1 (50%)	89 (90%)	10 (10%)	n.s.

° TNM= Tumour Node Metastases
* chi-square test
n.s. = not significative

Table 3. Compounds used to stimulate NIS expression

Compounds	Maximum concentration tested	Fold of increment of *NIS* mRNA levels
Histone deacetylase inhibitors		
SAHA	3 µM	62.8 ± 3.5
Valproic Acid	3 mM	36 ± 2.24
Apha compound 8	5 µM	5.3 ± 1.03
Proteasome inhibitor		
Bortezomib	52 nM	2.7 ± 0.35
Demethylating agent		
5-Azacytidine	5 µM	3 ± 1.1
Decitabine	5 µM	1.5 ± 0.52
Adenylate cyclase stimulator		
Forskolin	10 µM	0.8 ± 0.89
Inhibitor of hydroxymetilglutaril-coenzyme A reductase		
Mevinolin	50 µM	4.7 ± 1.7
mTOR inhibitor		
Rapamycin	20 nM	0.6 ± 0.9