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ORIGINAL ARTICLE

Expression and localization of the sodium/iodide symporter (NIS) in testicular cells

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Abstract Administration of radioiodine (I^{131}) is currently exploited for both diagnostic and therapeutic treatment of thyroid cancer. Few data are available on the sodium/iodide symporter (NIS) expression in human testis, a particular important prerequisite to predict radioiodine accumulation in the gonads of males with thyroid cancer exposed to such a treatment. In this study, we analyzed the expression of NIS in mouse, rat and human normal testis in different stages of development. By using a quantitative RT-PCR, Western blot and immunohistochemical analysis, NIS mRNA and protein were measured in both fetal and adult testicular tissues. NIS transcript was detected in both fetal and adult testis, although its expression levels were approximately 10-fold less than in thyroid gland. Western blot analysis and immunohistochemistry showed the presence of NIS protein in germinal and Leydig cells, but not in Sertoli cells with prevalent expression in the cytosol compartment of the cells. Our study demonstrates that NIS transcript and protein are expressed in normal testis. Further studies will demonstrate whether it may act as the transporter of radioiodine in normal testis of male patients with thyroid cancer.

Keywords Testis · NIS · Radioiodine · Leydig cells

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Introduction

Iodide concentrating ability is a property of normal thyroid tissue and, when maintained in tumor cells, allows the use of radioiodine (I^{131}) for both diagnostic and therapeutic purpose [1, 2]. In particular, radioiodine therapy has a fundamental role in the persistent disease, recurrences, and metastases treatment of differentiated thyroid carcinoma [2, 3]. Since this treatment is frequently associated to a normal life expectancy, the role of possible chronic complications due to the radioiodine therapy is preeminent.

It is well established that the radiotherapy may cause important damages to the male and female gonads [4]. However, it is still debated the entity of damages that I^{131}-based treatment causes to the male gonads. In particular, serious alterations to the germinal epithelium determining azoospermia or oligozoospermia with increase of serum FSH levels, decrease of serum inhibin B levels and infertility have been reported [5]. Damages to Leydig cells with low serum testosterone levels and high serum LH levels have been also observed [4, 6]. In patients treated with ^{131}I for differentiated thyroid carcinoma, there is evidence of a transitory alteration of testicular germinal

cells function, with increased serum FSH levels, reduced serum inhibin B levels, and decreased sperm motility [7–9]. Furthermore, it has been observed a Leydig cells dysfunction with a decreased testosterone/LH ratio [8]. A recent systematic review of the gonadal effects of therapeutic radioiodine in male thyroid cancer survivors confirmed the existence of frequent abnormalities in testicular function. However, they usually resolve within 18 months after administration of a single dose, unless a repeated or high cumulative radioiodine activity is used [10].

Iodide 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 [11]. NIS expression has been demonstrated also in various extra-thyroidal tissues [12]; among them, even normal testicular tissue resulted positive for the NIS transcript by RT-PCR analysis and weakly positive when tissue cores were examined by immunohistochemistry [13–15]. All these studies, however, did not investigate the exact localization of the NIS (cell type, cellular compartment).

Herein, we describe the expression and localization of the NIS in human normal testis. The study was performed also in rat and mouse testis in view of extending the investigation about other aspects of NIS expression in experimental models of rat and mouse testicular cell lines.

Materials and methods

Tissue samples

All human tissue samples used in the study had been collected with full patients' consent and institutional review board approval. They included specimens from 10 normal human testis (patients operated for testicular tumors), from a patient with spermatid maturation arrest and from 10 thyroid tissues (patients operated for differentiated thyroid tumors), used as controls. Immediately after removal, part of each tissue specimen was snap frozen in liquid nitrogen and stored at −80°C for RNA and protein isolation**;** the rest was fixed in Bouin's fixative overnight, for histological studies. Before use, representative sections of the frozen tissue were cut and examined histologically to confirm the diagnosis and to avoid the contamination of normal sample with nucleic acids from tumor surrounding tissues. The human normal testis tissue slides were purchased from BioCat GmbH (Heidelberg, Germany). Samples of mouse and rat testis tissues at different times of development [4, 10, and 18 (adult) days after birth for the mouse, 3, 7, and 14 (adult) days after birth for the rat] were also collected for histological studies. Testis sections were dehydrated in a graded series of ethanol and finally embedded in paraffin wax. Moreover, total RNA from 64 normal thyroid of patients succumbed to sudden death was purchased from Clontech (Mountain View, CA, USA).

RNA extraction, cDNA synthesis, and real time PCR

Total RNA was extracted from the mouse and human testis tissues, as described previously [16]. RNA extracted from human fetal testis tissue slides were purchased from BioCat GmbH. Briefly, the samples were treated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two microgram of total RNA were reverse transcribed in a 20 µl reaction volume and quantitative PCR (Q-PCR) analysis of NIS mRNA expression was performed on each cDNA samples in an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers and probes were purchased from Applied Biosystems as Assays-on-Demand Gene Expression Products Quantification of mRNA was expressed, in arbitrary units, as the ratio of the sample quantity to the quantity of the calibrator. All values were normalized to three endogenous controls, *GAPDH*, *β-actin* and $\beta_2 M$, with similar results.

Proteins preparation and western blot

Total proteins were extracted from thyroid tissues as described previously [17]. Briefly, tissues and confluent cells were collected, homogenized, and centrifuged at 14,000×*g* for 15 min. The proteins of human adult normal testis were purchased from the BioCat GmbH. All proteins (30 µg/lane) were run on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham S.r.l., Milan, Italy). Membranes were blocked in TBST 1×/milk (TBS, 1% Tween 20 and 5% non fat dry milk) and incubated overnight with primary anti-NIS polyclonal antibody [18] in TBST 1× (1:1,000 dilution). After three 5-min washes in TBST 1×, the membrane was incubated with peroxidase-conjugated anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark). After three 5-min washes in TBST 1×, the protein was visualized with an enhanced chemiluminescence western blot detection system (ECL plus, Amersham Pharmacia biotech.). Monoclonal mouse β-actin antibody (Sigma Chemical Corp.) was used as an internal control.

Immunohistochemistry

For light microscopy immunohistochemistry, 3 µm-thick paraffin sections from human adult normal testis, and from rat and mouse testis at different times of development were studied by immunoperoxidase staining. For each sample one section was stained with Hematoxylin/Eosin (H/E). For immunolocalization of NIS, the sections were dewaxed,

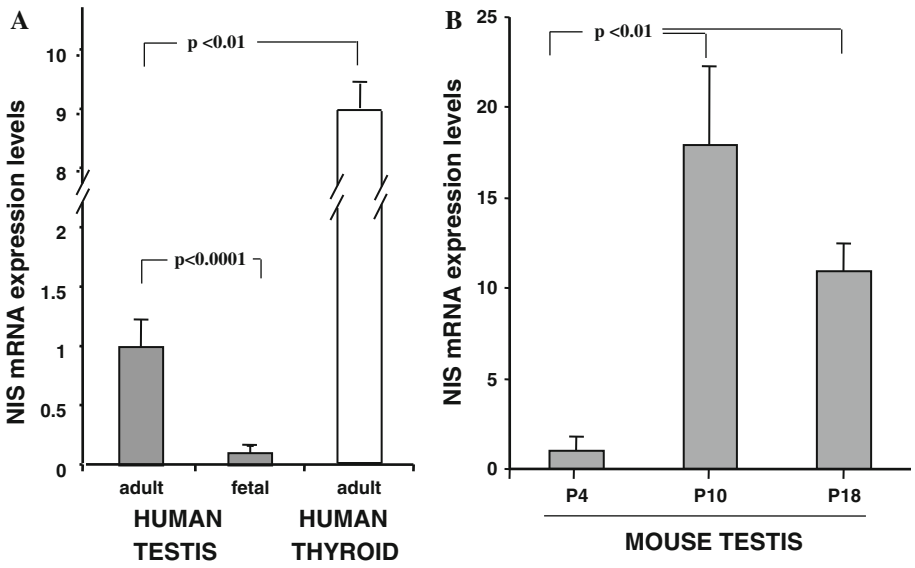

Fig. 1 Expression levels of *NIS* mRNA in normal testis and thyroid tissue. **a** *NIS* mRNA levels in human fetal and adult testis (pool of ten normal tissues) and in human thyroid (pool of normal tissues). **b** Levels of NIS *mRNA* in mice testis, four (P4), ten (P10) and eighteen (P18)-adult) days after birth. Gene expression levels were determined by RT-Q-PCR assay and normalized to 3 house keeping genes expression (mean arbitrary units ± SD), as described in "Materials and methods". Statistic analysis was performed using the one-factor ANOVA, followed by *t*-test. $P < 0.05$ is considered statistically significant

treated in 0.1 mol/l Tris–HCl buffer pH 7.6 and 0.03% H_2O_2 for 10 min at RT to block endogenous peroxidase activity and, subsequently, processed with microwave oven in 1 mmol/l ethylenediaminetetraacetic acid buffer (EDTA pH 8) (three cycles of 5 min each) followed by cooling at RT. The sections were then incubated for 30 min at RT in blocking solution (3% BSA + 0.05% Triton X-100 diluted in TRIS 1×). After removing the excess of the blocking solution, the sections were incubated for 60 min at RT with the primary polyclonal antibody anti-NIS [18] diluted 1:1,000 in CheMate™ antibody diluent (DakoCytomation, Glostrup, Denmark), washed three times in Tris–HCl 1× buffer for 5 min each time and incubated with a peroxidase-conjugated secondary antibody (En Vision System, DakoCytomation) for 30 min. Subsequently, the sections were washed two times in Tris–HCL 1× buffer for 5 min each time and one time with Tris–HCl buffer 1×, pH 7.6. Activity was visualized using diamino-benzidine chromogen (DAB, DakoCytomation). The sections were lightly counterstained with Mayer's hematoxylin, dehydrated, mounted, and examined under microscope. Negative control samples were prepared with the primary antibody omitted.

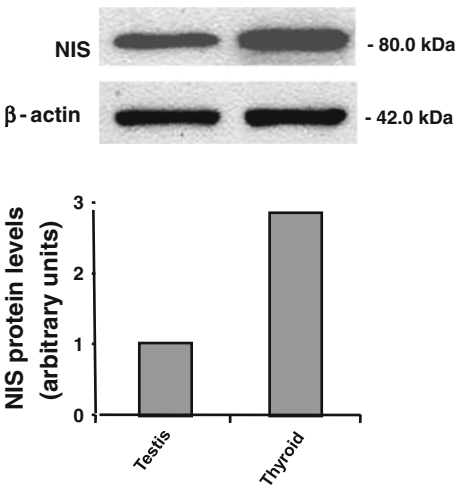

Fig. 2 Expression levels of NIS protein in normal testis and thyroid tissue**.** Western blot analysis of total protein extracts from human normal adult testis and neoplastic thyroid tissues was performed under reducing conditions. A representative of three separated experiments is shown using a polyclonal anti-NIS antibody and a monoclonal anti-human beta-actin antibody (see "Materials and methods"). In the *lower panel*, NIS protein levels measured by densitometric scanning

Results

NIS mRNA expression in testis tissues

By using a quantitative RT-PCR analysis, the expression of *NIS* mRNA was detected in both fetal and adult human normal testis tissues, with transcript levels significantly higher in normal adult testis when compared with fetal (16 weeks) testis ($P < 0.0001$). However, compared to adult thyroid tissues, the *NIS* mRNA levels in adult testis were approximately 9-fold lower ($P < 0.01$) (Fig. 1 panel A). *NIS* mRNA expression was also detected in mouse normal testis tissue at different age of development. Again, higher levels of *NIS* gene were present in adult than young mouse ($P < 0.01$) (Fig. 1 panel B).

NIS protein expression in testis tissues

We then analyzed the expression of NIS protein in both human and mouse testis tissue, using thyroid tissue proteins

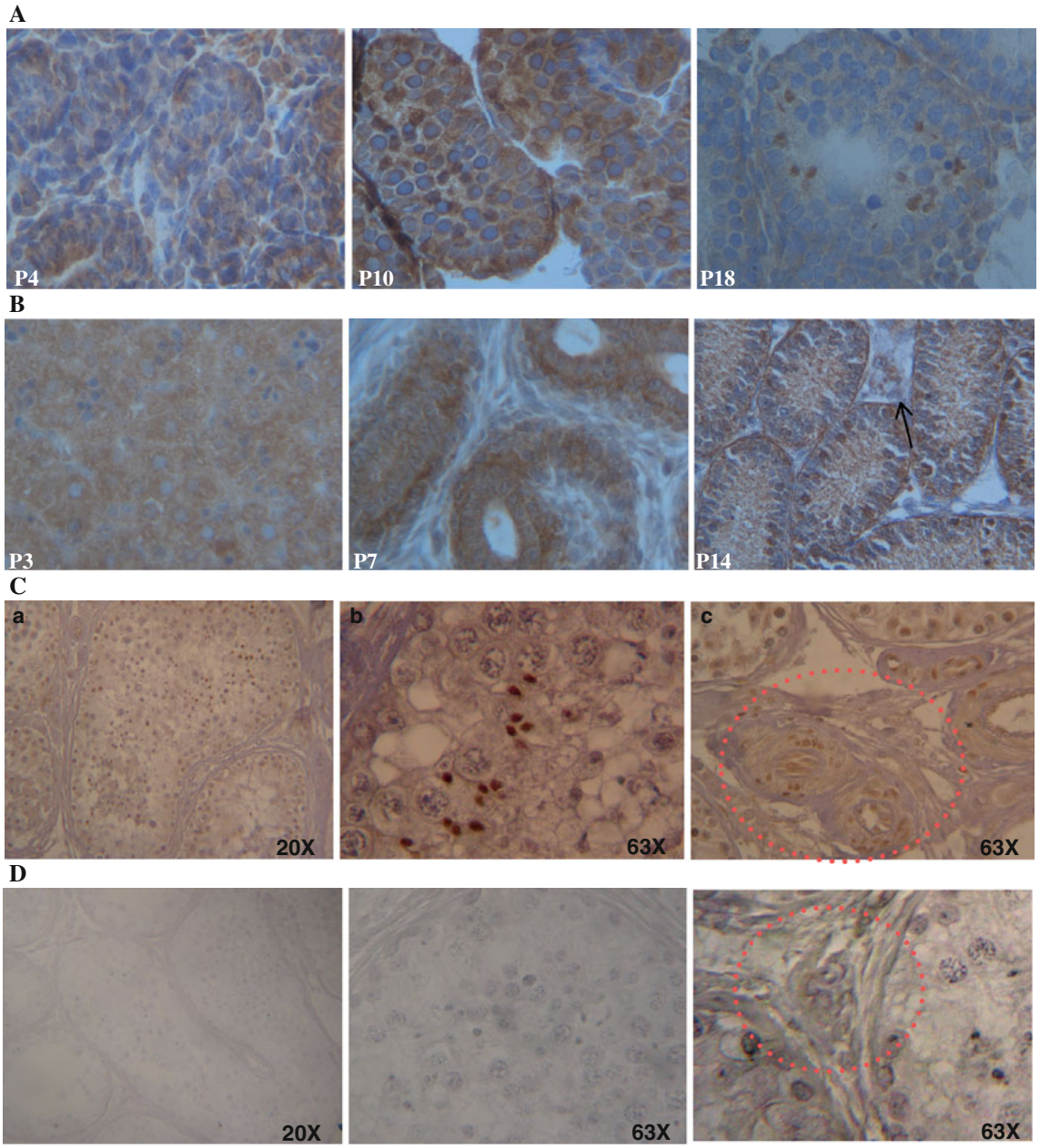

Fig. 3 Immunohistochemical staining of NIS protein in mouse (rat) and human testis tissue. **a** NIS protein in mouse testis at four (P4), ten (P10) and eighteen (P18) days after birth; NIS immunoreactivity is visible in the spermatids-rich zone of the testis and in Leydig cells. At P18, NIS positive cells are more intense than at P4 and P10; (magnification 63×). **b** NIS protein in rat testis at three (P3), seven (P7), and fourteen (P14) days after birth; spermatids and Leydig cells (*arrow*) show positive staining (magnification 63×). **c** NIS protein in human adult testis. NIS is located in seminiferous tubules in normal testis and particularly in mature spermatic cells (**a** and **b**) and in Leydig cells (**c**) (magnification 20× and 63×, as indicated). **d** In a patient with spermatid maturation arrest NIS staining is absent in spermatic cells, while Leydig cells are positively stained (magnification 20× and 63×, as indicated)

as control). By western blot analysis, a specific band of approximately 80 kDa, corresponding to human NIS protein was detected in the total protein extracts (BioCat GmbH) of human testis tissue, comparable, but with lower intensity (approximately 3-fold) to that observed in neoplastic thyroid tissue extracts (Fig. 2).

To add further information about the NIS localization, immunohistochemical analysis was performed in tissue slices from mouse, rat, and human testis. The results are shown in Fig. 3. A strong specific positive NIS immunostaining was observed in the seminiferous tubules of all the testis samples examined, especially in a lot of cells near the

lumen of the tubules. In mouse and rat testis tissues, increasing expression of NIS paralleled the development, with the higher number of positive cells detected in P18 adult mouse testis (Fig. 3a). Also, the Leydig cells, in the interstitial space outside the seminiferous tubules, resulted positive for NIS in both mouse and rat samples, whereas we found no positive signal for NIS in Sertoli cells (Fig. 3a, b). The same results were observed in normal human testis (Fig. 3c). At variance, in testis tissue from a patient with spermatid maturation arrest, a condition characterized by incomplete development of spermatogenesis, NIS staining was absent in germ cells, but still detectable in Leydig cells (Fig. 3d). When present, NIS staining was mainly visible in the cytoplasm and only barely in the cell plasma membranes.

Discussion

Radioiodine is selectively used as effective adjuvant therapy and in the treatment of residual and recurrent thyroid cancer [2]. As a result, radiation absorption by the testis (both beta and gamma) occurs in male patients [9, 19, 20], with an important potential impact on gonadal function and, for patients in reproductive age, also fertility. In this regard, clinical practice guidelines for male patients with differentiated thyroid carcinoma have considered in their recommendation protocols the possible alteration of semen quality following radioiodide therapy [21, 22]. At present, however, no conclusive data are reported on gonadal clinical outcomes in men subjected to this treatment [10]. Also, the molecular mechanisms underlying testicular concentration of radioiodine have been poorly investigated.

A pivotal role of the NIS in the capacity of iodide concentration in thyroid cells is well established [1]. Among the various extrathyroid tissues in which the presence of NIS, at mRNA and/or protein level, has been described, only few studies focused on the testis and other pelvis organs of reproductive axis: very few cases have been investigated, and no study has described the cell type (Leydig, Sertoli, and germinal cells) and intracellular localization of the NIS [13–15]. In a recent study, we have found that NIS is expressed in about half of prostate adenocarcinomas, but not in non-tumor hyperplastic prostatic tissues, thus excluding the possibility of radioiodine accumulation in this site [23].

In this study, we demonstrate the presence of NIS mRNA and protein in fetal and adult tissues of human, mouse and rat testis, and its localization in germinal cells during all the stages of their maturation and in Leydig cells. As expected, both mRNA and protein levels are not comparable with those present in thyrocytes, and this may explain the presence of a stronger staining in cytoplasm than in cell plasma membrane, as reported in pathological thyroid tissues with low mRNA levels [24]. According to the present data, therefore, the expression of the NIS may represent the molecular basis for the uptake of radioiodine in testicular cells, responsible for the damages observed in the reproductive function of adult male patients undergoing this treatment for thyroid cancer recurrent disease. However, the presence of low levels of NIS expression in the plasma membranes of testicular cells, as well as the absence of an organification machinery able to avoid a rapid efflux, may determine an insufficient concentration of the radioisotope thus explaining the prevalence of only transient abnormalities [12, 25].

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Conflict of interest The authors declare that the experiments comply with the current laws of the country in which they were performed and that they have no conflict of interest.

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