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Expression of Histone Deacetylases 1, 2 and 3 in histological subtypes of testicular germ cell tumours

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Summary. In this study we aimed to evaluate the protein expression of class I histone deacetylases (HDAC) in testicular germ cell tumours (GCT) and to analyse differences between the histological subtypes of testicular GCT. 325 testicular GCT were included in a tissue microarray with each histological subtype of the tumour being separately represented on this array. Expression of class I HDAC isoforms 1, 2 and 3 was assessed by immunohistochemistry.

While HDAC2 and 3 were highly expressed in all histological subtypes of GCT, HDAC1 was almost consistently expressed at lower levels. We observed significant differences in the expression of the respective HDACs between seminoma and non-seminoma GCT tissue components. Interestingly, choriocarcinomas showed generally high expression values for all three class I HDAC isoforms. Relevant correlations with clinicopathological parameters could not be demonstrated.

Contrasting published findings on other tumour entities, no immediate practical diagnostic or prognostic value for HDAC1-3 in GCT could be inferred. However, the high expression levels might still be indicative for a treatment response to HDAC inhibitors which ought to be evaluated in further studies.

Key words: HDAC, Immunohistochemistry, Germ cell tumour, Seminoma, Embryonal carcinoma

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Introduction

Testicular germ cell tumours (GCT) are among the most common malignant tumours in young to middle-aged men. In contrast to current trends in several other malignancies, the incidence of testicular germ cell tumours is rising, especially in highly developed countries (Jemal et al., 2009). Fortunately, the prognosis of these tumours is generally considered excellent, with the most common histological subtype, the classical seminoma, being sensitive to therapeutic intervention even at late stages. However, many GCT still cause metastatic disease, which is less easy to treat and associated with a poorer outcome. In this situation novel therapeutic options are highly warranted.

Histone deacetylases (HDAC), comprising four classes with eighteen isoforms, modulate the deacetylation of nuclear core proteins, including histones, and lead to a tighter wrapping of the DNA around the histone core. This deacetylation of histone proteins results in reduced gene transcription (Minucci and Pelicci, 2006). Class I HDACs have been reported to be included in four distinct multiprotein complexes (Hayakawa and Nakayama, 2011). These complexes bind to the chromatin and take part in the regulation of cell proliferation and cell differentiation (Brehm et al., 1998; Meraner et al., 2008; Montgomery et al., 2009).

Differential expression of class I HDAC isoforms has been described in several malignant tumour types. In prostate cancer and in renal cell carcinomas class I HDAC isoforms 1-3 (HDAC1-3) have been shown to be highly expressed and to be associated with unfavourable tumour characteristics (Fritzsche et al., 2008; Weichert et al., 2008c). The findings in prostate cancer were further validated by cell line experiments (Wang et al., 2009).

HDAC1 has been associated with tumour cell proliferation and HDAC3 has been demonstrated to influence the migratory potential of tumour cells via repression of E-cadherin in ovarian carcinomas (Hayashi et al., 2010). Similar results have been reported for HDAC2 in endometrial carcinomas, where it was associated with tumour proliferation and with a higher tumour grade (Fakhry et al., 2010). Recently, Adams et al. showed a high expression of the three class I HDACs in Hodgkin and Reed-Sternberg cells in classical Hodgkin's lymphoma (Adams et al., 2010). In contrast to most other studies a lower expression of HDAC1 was prognostically unfavourable in this cohort. Weichert et al. concluded, supported by various studies from his and other groups, that class I HDAC expression was typically high in locally advanced, dedifferentiated, strongly proliferating tumours and in as much associated with adverse prognosis (Weichert et al., 2008a-d; Weichert, 2009).

In this study, the protein expression pattern of class I HDAC isoforms 1, 2 and 3 was carefully analyzed in a well-characterized cohort comprising more than 300 primary testicular germ cell tumours via immunohistochemistry on tissue microarrays (TMA).

Materials and methods

Patients

A total of 325 patients diagnosed with testicular germ cell tumours were retrieved from files of the Institute of Surgical Pathology of the University Hospital Zurich, from 1990-2003. Tumours were classified according to the 2004 WHO Classification. The series included a total of 94 mixed GCTs (49 with and 45 without a seminoma component), 4 spermatocytic seminomas, 207 classic seminomas, 19 pure embryonal carcinomas and 1 pure mature teratoma. The 94 cases of mixed GCT included the following components: seminoma, embryonal carcinoma, yolk sac tumour, choriocarcinoma and teratoma. The number of all histological components included in this study are shown in Table 1. Follow-up data was available for 195 patients. Four patients died from GCT and 22 had disease progression. Serum levels of lactate dehydrogenase (LDH), human chorionic gonadotropin (HCG) and alpha fetoprotein (AFP) were known for 113 to 139 patients respectively. Eighteen samples of non-neoplastic testicular tissue and sixteen samples of intratubular germ cell neoplasia unclassified (IGCNU) were derived from tumour-adjacent tissue. The project has been approved by the local ethics committee (ref. number StV 25-2008).

Tissue microarray (TMA)

Formalin-fixed paraffin-embedded tissue from the tumours was used according to tissue availability. Following expert review (AB), suitable areas for tissue retrieval were identified and marked on haematoxylin and eosin sections, subsequently punched out of the donor paraffin block using a tissue arrayer and finally inserted into a recipient block. The punch diameter for each core was 0.6 mm. All histological tumour types were represented by two tissue cores. If more than one histological tumour subtype was present in a patient (mixed GCT), each histological subtype was separately punched out (two cores) and represented on the TMA. The final TMA consisted of two paraffin blocks. Four cases were lost during processing. The evaluation of the immunostaining included 436 different histological tumour components.

Histology and immunohistochemistry

Three-micron thick sections of the TMA blocks were mounted on glass slides (SuperFrost Plus; Menzel, Braunschweig, Germany), deparaffinized, rehydrated and stained with haematoxylin and eosin using standard histological techniques. HDAC1 (Abcam, Cambridge, UK, prediluted polyclonal rabbit IgG antibody (ab15316) directed against a c-terminal HDAC1 specific peptide), HDAC2 (Abcam, monoclonal mouse IgG antibody (ab51832), directed against a synthetic peptide corresponding to amino acids 447-462 of human HDAC2) and HDAC3 (Becton Dickinson, Franklin Lakes, USA, monoclonal mouse IgG antibody, clone 40, directed against a synthetic peptide corresponding to amino acids 309-425 of human HDAC3 (c-terminus)). Antibodies were used at dilutions of 1:11, 1:5000 and 1:500 respectively. All antibodies used have been validated for specificity by western blotting and specific siRNA knockdown previously (Weichert et al., 2008a-d).

Ventana Benchmark autostainers (Ventana Medical Systems, Tucson, AZ, USA) were used with Ultraview detection kit and HRP-DAB serving as chromogen.

As positive controls we used prostate cancer cases with known HDAC positivity, as well as endothelial cells as positive internal controls. For negative controls a slide without primary antibody was used

Semiquantitative Evaluation of Immunoreactivity

All immunostainings were uniformly evaluated by two pathologists and a medical student (AH, GK, FRF).

Table 1. Median immunoreactive scores (IRS) of HDAC 1-3 in the different histological compartments of testicular germ cell tumours.

Tissue type (n)	HDAC1	HDAC2	HDAC3
Non-malignant testicular tissue (18)	4	8	8
IGCNU (16)	0	12	12
Seminoma (249)	2	9	8
Embryonal carcinoma (89)	6	8	12
Yolk sac tumour (47)	6	8	12
Teratoma (42)	6	8	12
Choriocarcinoma (9)	9	8	12

IGCNU: intratubular germ cell neoplasia unclassified.

Differences in the evaluation were discussed at a multiheaded microscope until consensus was reached. Nuclear staining of HDAC isoforms was scored by applying a semiquantitative immunoreactivity scoring (IRS) system that incorporates the percentual area and the intensity of immunoreactivity results in a score ranging from 0 to 12. The intensity was graded as absent (0), weakly positive (1), moderately positive (2) or strongly positive (3). The percentage of positive cells was scored as no cells (0), less then 10% of cells (1), 10-50% of cells (2), 51-80% of cells (3) or more than 80% of cells being stained respectively. For disease-free survival analyses of the 106 pure seminomas with follow-up data, cases exhibiting an IRS from 0-6 were lumped in a HDAC low group whereas cases with a higher IRS (7-12) were designated HDAC high group. This cut-off for the dichotomisation (lumping in two groups) was chosen to allow for a better comparability with previous works.

Statistical analyses were performed using SPSS 18. We applied frequency analyses for the primary assessment of data. Bivariate correlations according to Spearman were used to analyse correlations of IRS-scores with each other and with clinicopathological parameters. A Kaplan Meier curve with log rank test was used for the survival analyses of HDACs in pure seminomas. Mann-Whitney-U-tests were applied to analyse for differences in the HDAC expression between seminomatous and non-seminomatous tumours. The level of significance was set at 5%.

Results

All three HDACs displayed a typical distinct nuclear staining pattern. Median IRS-scores for the different histological tumour components are depicted in Table 1. Median IRS values of HDAC2 and 3 were higher than

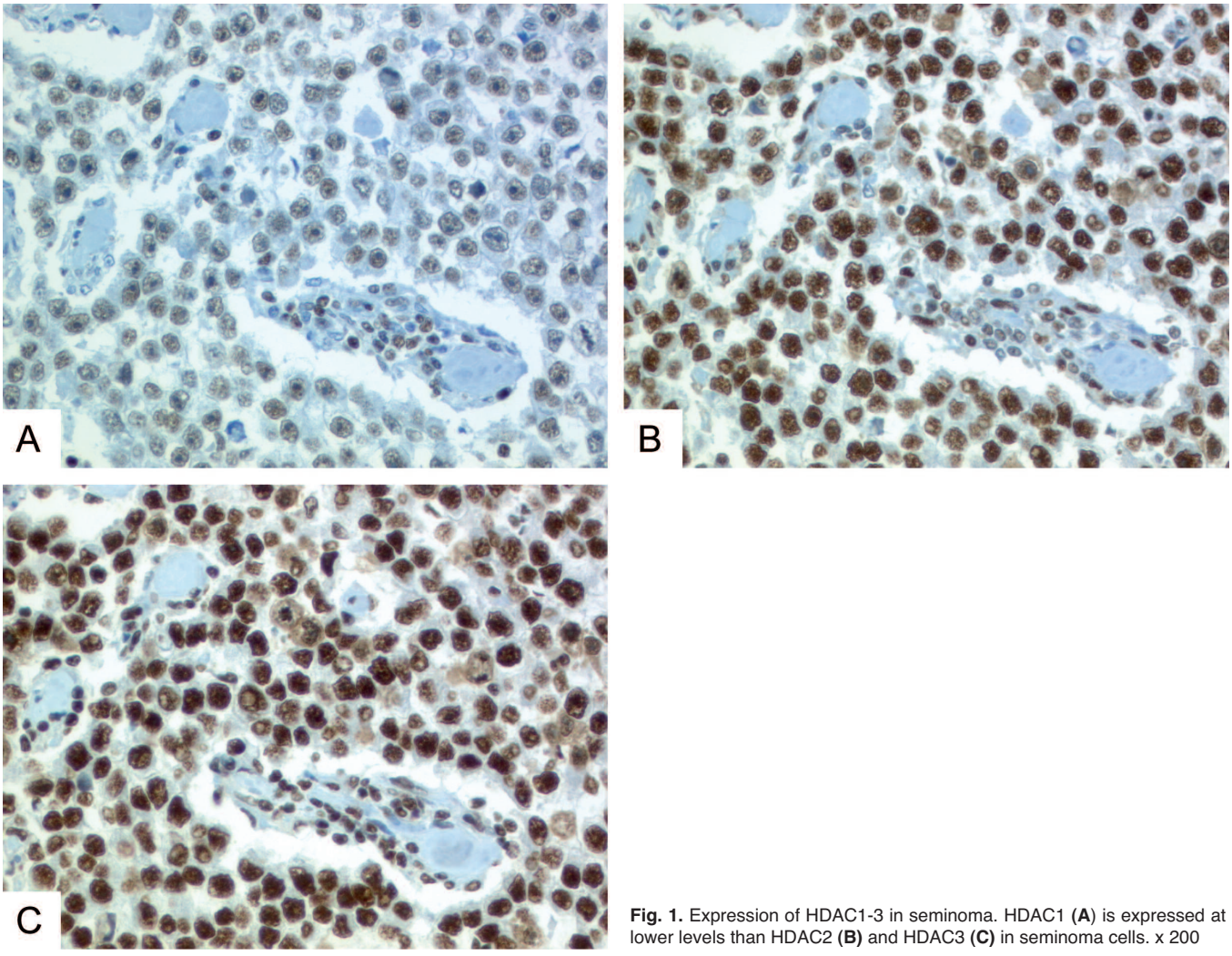

Fig. 1. Expression of HDAC1-3 in seminoma. HDAC1 (**A**) is expressed at lower levels than HDAC2 **(B)** and HDAC3 **(C)** in seminoma cells. x 200

those of HDAC1 in all histological components with the exception of choriocarcinomas. Meanwhile IRS values of HDAC3 were mainly higher or equal to those of HDAC2. Examples of staining paters of the three HDAC isoforms in components of seminoma, embryonal carcinoma and choriocarcinoma are shown in Figures 1-3. Less than 1% of seminoma components and none of the other histological tumour components were considered negative for HDAC2 and 3. Negativity rates of HDAC1 were 56% for IGCNU and 29% for the seminoma component and ranged between 5% and 6% for non-malignant tissue, embryonal carcinoma and yolk sac tumour.

Expression of HDAC2 and 3 were significantly correlated in all histological tumour components and in IGCNU. The only two significant correlations with clinicopathological parameters (tumour size, serum levels of LDH, AFP or HCG) were inverse correlations of HDAC1 in the embryonal carcinoma component with tumour size ($p=0.013$, correlation coefficient -0.282) and of HDAC1 in seminoma with HCG levels ($p=0.047$, correlation coefficient -0.192).

The disease-free survival analysis of the three HDACs in 106 pure seminomas did not reveal any prognostic value (all $p>0.4$, curves not shown).

For each HDAC isoform the differences in expression between seminomatous and non-seminomatous components were evaluated. While HDAC1 and 3 were expressed at significantly higher levels in the non-seminomatous components (each $p<0.001$), HDAC2 was expressed at significantly higher levels in the seminomatous component ($p<0.001$).

Discussion

In recent years several studies on HDACs in various malignancies have been published. This is certainly attributable to the potential therapeutic importance of

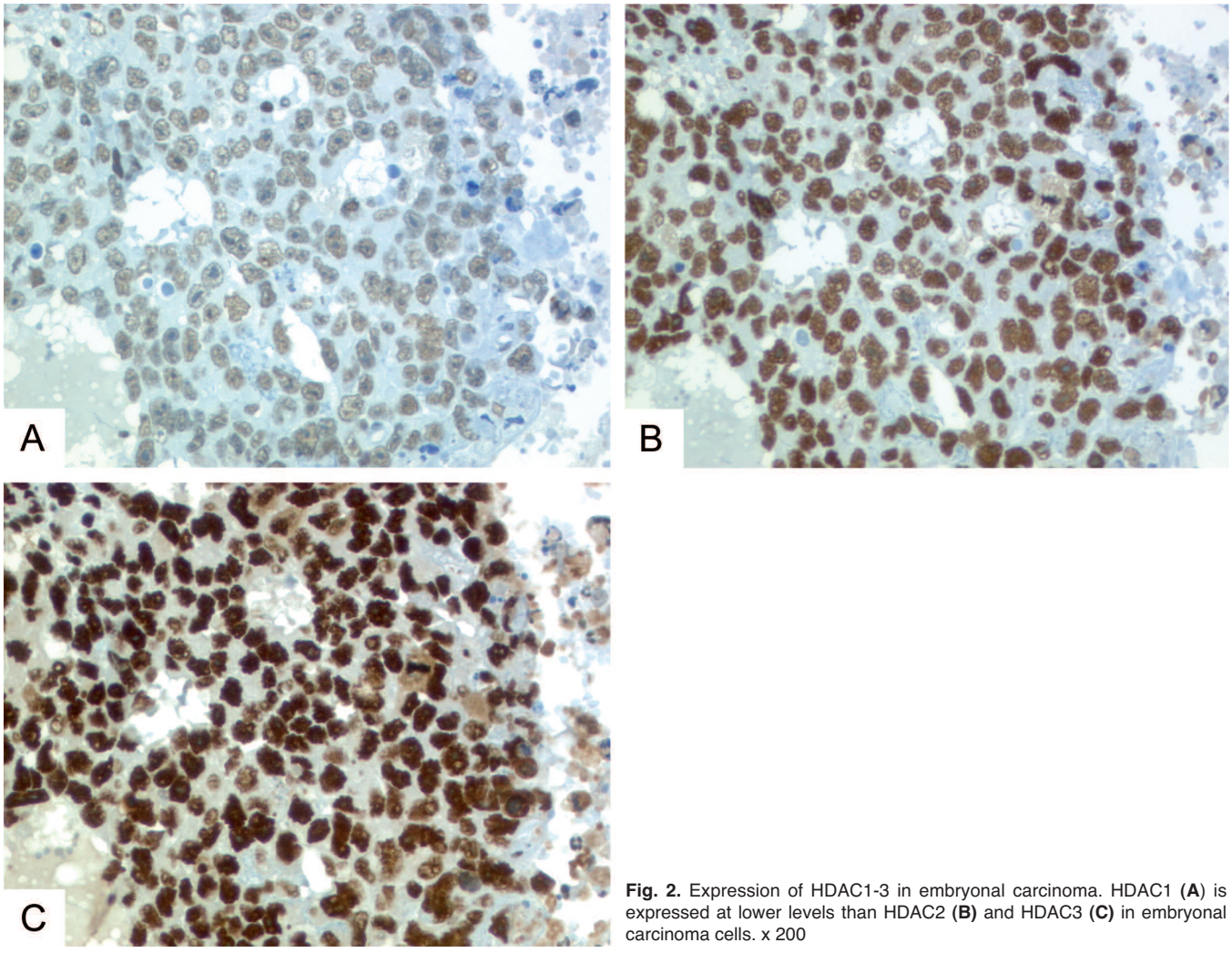

Fig. 2. Expression of HDAC1-3 in embryonal carcinoma. HDAC1 (**A**) is expressed at lower levels than HDAC2 (**B**) and HDAC3 (**C**) in embryonal carcinoma cells. x 200

histone deacetylase inhibitors (HDI). HDI constitute a relatively new group of chemotherapeutic agents, which target proteins of the HDAC family.

Histone deacetylase inhibitors can alter the epigenetic configuration of tumour cells by inhibiting HDACs and other oncogenic proteins. Apart from their inhibition of HDACs, HDI are also thought to enhance the expression of Cancer/Testis antigens, which are potential targets for anti-cancer vaccinations and are also of importance in testicular tumours (Karpf, 2006). Previous studies have demonstrated that HDI's such as valproic acid (VPA) and suberoylanilide hydroxamic acid, or repression of HDAC1, can increase radiosensitivity and can lead to growth arrest, further differentiation or apoptosis of tumour cells (Chinnaiyan et al., 2005; Blaheta et al., 2005; Zhang et al., 2010a). Concordantly, experiments on prostate, endometrial and pancreatic cancer cell lines, all of them with known high expression levels of class I HDACs, are promising in terms of effective HDI induced tumour suppression (Lehmann et al., 2009; Wang et al., 2009; Fakhry et al., 2010; Schuler et al., 2010). Likewise, in cell lines from lung, colon and thyroid cancer a combination of HDI and a RAS inhibitor resulted in reduced tumour cell proliferation (Biran et al., 2011). These findings strongly suggest that tumours with HDAC over-expression might be successfully treated with HDI or via stimulation of endogenous HDAC inhibiting proteins (Riccio, 2010).

With these findings it is not surprising that HDI have entered clinical trials as therapeutic options for several malignant tumours e.g. prostate cancer and have already been approved for the treatment of some hematologic malignancies (Zhang et al., 2010; Beumer and Tawbi, 2010). The development of more specific HDI to tackle exactly the specific HDAC expressed by the tumour is now considered of major importance for more individualized cancer therapies (Noureen et al., 2010). Data on HDI in testicular tumours is very limited.

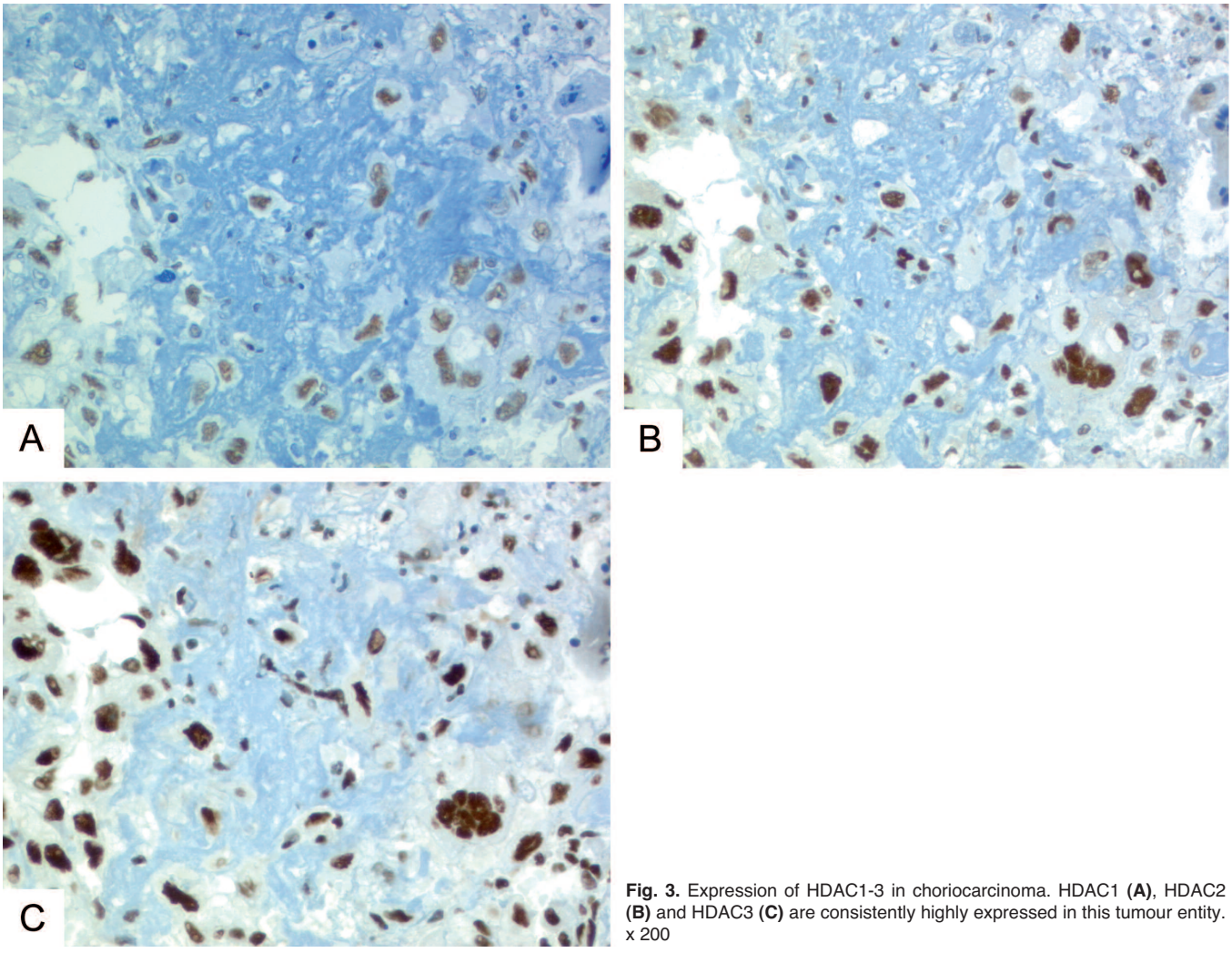

Fig. 3. Expression of HDAC1-3 in choriocarcinoma. HDAC1 **(A)**, HDAC2 **(B)** and HDAC3 **(C)** are consistently highly expressed in this tumour entity. x 200

Candelaria et al. have analyzed hydralazine in combination with the VPA in a phase II study of a small number of solid tumours, including one testicular tumour (Candelaria et al., 2007). Eighty percent of their patients, including the one with testicular cancer, showed stable disease or partial response under treatment. The importance of HDACs in proliferating testicular tissue is supported by HDI induced infertility in mice and our findings of moderate to high class I HDAC1-3 expression in normal and in neoplastic testicular tissue (Fenic et al., 2004). Taken together our results support the notion of a possible success of HDI therapies in these malignancies.

Our study is the first to assess the expression of HDAC1-3 in primary testicular GCT in a larger cohort. A previous study by Omisanjo et al. has described HDAC1 in normal testicular tissue, seminoma, teratoma and embryonal carcinoma, showing weak to moderate nuclear expression levels (Omisanjo et al., 2007). We observed higher levels of HDAC1 in non-malignant gonadal cells in comparison to seminomas, but the opposite for HDAC2. For HDAC3 there were no differences between these groups. These findings are difficult to interpret and we infer no practical value from it since the case numbers for non-malignant tissues were very low. Likewise the inverse correlations of HDAC1 with tumour size and HCG serum levels suggests a lower expression in progressing disease. This finding is interpreted as inconclusive with its low correlation coefficients, borderline significance levels and missing functional explanations.

A component-specific expression with any diagnostic usefulness could not be inferred, even with the Mann-Whitney-U-test revealing significant differences between seminomatous and non-seminomatous tumour components for the three HDACs. In fact HDAC2 and 3 were expressed at high levels in all included histological GCT components. Although HDAC1 expression possessed a higher dynamic range with high negativity rates in seminomas and IGCNU, neither a diagnostic nor a prognostic value can be proposed. Differences between histological components were not sufficiently distinct in comparison to established diagnostic markers.

Due to the mixed nature of most of the other tumours and the small number of the remaining pure non-seminomatous GCT, survival analyses for these tumours were not performed.

In conclusion, a practical or especially a diagnostic value of HDAC class one isoforms in primary testicular GCT seems very unlikely. Possibly, the high expression of these HDACs could be an indicator for a potential response to treatments with HDI. This might constitute an interesting target for further treatment-focussed studies.

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