ERCC1 and XPF expression in human testicular germ cell tumors

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Abstract. Nucleotide excision repair (NER) is one of the factors influencing the cellular sensitivity to anticancer drugs. In the present study we compared the expression of the NER proteins ERCC1 and XPF in 24 testicular germ cell tumors (TGCT) with the corresponding normal testicular tissue of the same patient. Using immunoblotting, we demonstrated in TGCT a significant increase of ERCC1 expression compared to the normal tissue. There was no significant increase in XPF expression in TCGT. Based on histological characteristics TGCT are subgrouped into seminomas and non-seminomas, the latter being clinically more aggressive. Investigating ERCC1 levels in seminomas we found a slightly increased expression compared to normal tissue, which was, however, not significant. Similarly, no significant difference was observed for XPF levels in seminomas compared to normal testis tissue. In non-seminomas, however, we found a significant increase in the expression of ERCC1 ($p<0.017$) and XPF ($p<0.03$) when compared with the corresponding normal tissue. Comparing seminomas with non-seminomas, we observed a significant increase in the expression of ERCC1 ($p<0.05$) and XPF ($p<0.007$) in the non-seminomas. Furthermore, a correlation between the expression of ERCC1 and XPF was observed. Our data demonstrate that non-seminomas are characterized by an increased expression of ERCC1 and XPF protein compared to seminomas and the normal testis tissue. The data indicate a possible up-regulation of ERCC1 and XPF during TGCT progression.

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Introduction

Testicular germ cell tumors (TGCT) are relatively rare, accounting for less than 1% of all male malignancies. However, they are the most common solid malignant tumors in men 20-40 years of age (1,2). TGCT are a heterogeneous group of neoplasms derived from the germ cell lineage (3). Histologically TGCT can be divided into seminomas and non-seminomas, and the latter is further subdivided into yolk sac carcinoma, embryonal carcinoma, teratoma and choriocarcinoma (4,5). Seminomas are composed of uniform cells, non-seminomas contain one or more histological subtypes. It is generally accepted that both types arise from a common progenitor, named carcinoma *in situ*. At the time of diagnosis about 25% of seminoma and about 60% of non-seminoma patients suffer from metastatic disease. Remarkable progress has been made in the medical treatment of advanced TGCT. Whilst seminomas can be cured by dissection and radiation therapy, non-seminomas require chemotherapy, with cisplatin being the gold standard. Using cisplatin-based combination chemotherapy even more than 80% of patients with metastatic TGCT can be cured (6,7). This is in stark contrast to the often poor therapeutic response in most other solid tumors (8). The molecular basis for the extreme sensitivity of TGCT to cisplatin-based chemotherapy remains poorly understood (9).

DNA repair is one of the factors influencing the cellular sensitivity/resistance towards cisplatin (10). Nucleotide excision repair (NER) and interstrand crosslink (ICL) repair play a central role in the repair of cisplatin-induced DNA intrastrand adducts and interstrand crosslinks (11,12). The DNA repair protein ERCC1 associates with the xeroderma pigmentosum group F (XPF) protein to form a nuclease that functions in NER and ICL repair (13,14). In cells *in vitro*, ERCC1 expression has been shown to be associated with resistance to cisplatin. This prompted investigations as to the influence of ERCC1 on resistance of tumors to cisplatin chemotherapy, and it was shown that in tumor tissues of patients with cancer of the lung, head, neck and ovary high levels of ERCC1 mRNA or protein correlate with a poor response towards cisplatin-based chemotherapy (15-20). Collectively the data suggest that the ERCC1 expression level is a useful marker of tumor cell resistance to cisplatin and its analogs.

Table I. Tumor characteristics of patients included into the present study.

	Seminoma (n=16)	Non-seminoma (n=8)	All (n=24)
Median age (years)	34 (25-47)	34 (26-44)	34 (25-47)
Tumor size (cm)	4.7 (2.0-7.5)	3.0 (1.6-5.5)	4.5 (1.6-7.5)
pT1	9	2	11
pT2	7	6	13
Median follow-up time (months)	105 (46-124)	71 (36-106)	103 (36-124)

Using *in vitro* models, it has been shown that testis tumor cell lines are characterized by reduced levels of ERCC1 and XPF compared to cell lines derived from other types of cancer (21). However, to date no data are available on the expression of ERCC1 and XPF in testicular tumors, although as mentioned above for non-seminomas cisplatin belongs to first-line therapy. Therefore, we aimed at investigating the expression level of ERCC1 and XPF in testicular germ cell tumors (TGCT) and compared it with the corresponding normal testicular tissue of the same patient. We also compared the expression of ERCC1 and XPF in seminomas versus non-seminomas and investigated whether the expression levels of ERCC1 and XPF proteins are correlated. Finally we asked whether there is a correlation between ERCC1 and XPF and clinical parameters such as tumor size and TNM stage. Our data show that ERCC1 and XPF are up-regulated in non-seminoma TGCT.

Materials and methods

Patients and specimens. Tissue samples were obtained under sterile conditions from 24 patients with primary testis tumors who underwent orchiectomies at our Department between 1998 and 2002. Samples from testis and tumor tissue (~5x5x5 mm) were shock-frozen in liquid nitrogen and stored at -80˚C. The diagnosis of seminoma or non-seminoma was based on hematoxylin and eosin sections. Patient specific data are listed in Table I.

Cell lines. Origins of the 833K human TGCT cell line and MGH-U1 human bladder cancer cell line were described previously (22,23). The cell lines were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (PAA) and 5% antibiotics (penicillin/ streptavidin). Cells were cultivated at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Preparation of tissue extracts. Deeply frozen testis tissue was homogenized in buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM EDTA, 1 mM ß mercaptoethanol, 5% glycerol and protease inhibitors (10 μg/ml aptotinin, 10 mM bestatin, 10 mM leupeptin, 1 mM pepstatin and 0.1 mM PMSF) using an Ultra Turrax homogenizer. After homogenization the lysate was centrifuged to remove debris. The supernatant was snap-frozen in liquid nitrogen and stored at -80˚C. The protein concentration of the supernatant was determined by the Bradford method using RotiQuant reagent (Roth).

Immunoblotting for ERCC1 and XPF proteins. For immuno-blotting, 50 μg extract protein were separated by electro-phoresis on SDS 10% polyacrylamide gels. Protein (50 μg) of MGH-U1 bladder cancer cells and 833K testis tumor cell were run on each blot to allow for comparison between independent blots. Proteins were transferred to Whatman nitrocellulose membrane (Roth) overnight in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) in a Bio-Rad Mini-Protean 3 Electrophoresis Cell at a constant voltage of 30 V. Primary antibodies used were as follows: XPF 1/5000 dilution of polyclonal antibody RA1 raised against residues 571-905 of human XPF protein (24); ERCC1 1/1000 dilution of monoclonal antibody 3H11 (Neomarkers); RPA 1/5000 dilution of monoclonal antibody 9H8 (Neo-markers). Membranes were incubated with the primary antibody overnight at 4˚C, followed by incubation for 1 h with either 1/2000 peroxidase-labeled anti-rabbit IgG or 1/5000 dilution of peroxidase-labeled anti-mouse IgG (Dako). Proteins were visualized by chemiluminescence using a solution consisting of 0.1 M Tris HCl pH 8.6, 0.25 mg/ml luminol, 0.1 mg/ml hydrocoumarine acid, 0.01% H_2O_2. For quantification of the protein signals SynGene software was used. The expression levels of ERCC1 and XPF were normalized to RPA protein expression. RPA was chosen because it is a nuclear protein involved in several housekeeping aspects of DNA metabolism, and there was relatively little variation in RPA for cell lines from different tumor types (21).

Statistical analysis. For statistical analyses SPSS 17.0 software was applied. The expression of ERCC1 and XPF in testis cancer tissue and the corresponding testis tissue was quantified and presented as relative units. Differences in the expression in testis tissue and tumor tissue was calculated by Wilcoxon test. Differences in the expression of seminomas versus non-seminomas or in dependence of histophathological parameters were calculated by Mann-Whitney U test. Differences were considered statistically significant at p<0.05. A correlation between the expression of ERCC1 and XPF was determined and the linear regression (R^2 linear) was calculated.
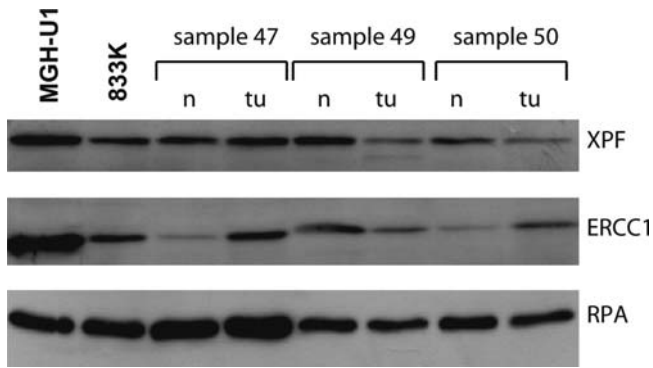
Figure 1. Immunoblot analysis of XPF and ERCC1 proteins in extracts of the bladder cancer cell line MGH-U1, the testis tumor cell line 833K and in tumor tissue (tu) and corresponding tissue (n) of three patients. RPA was used as a loading control.

Figure 3. Expression of ERCC1 (A) and XPF (B) in testis tumor tissues (seminomas, non-seminomas) and the corresponding testis tissue, as determined by immunoblot analysis. The values represent the amount of antigen determined by computer-aided quantification of the bands. Box plots show medians (central lane), 25 and 75 percentiles (lower and upper side of the box) and minimum and maximum (lower and upper bars). Extreme values have been excluded from the graph.
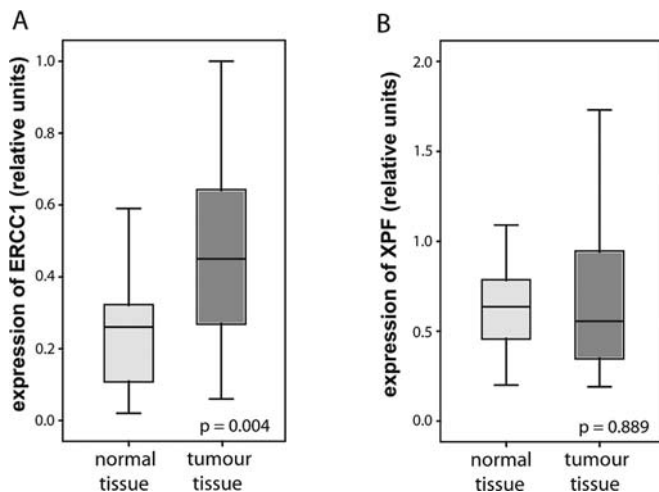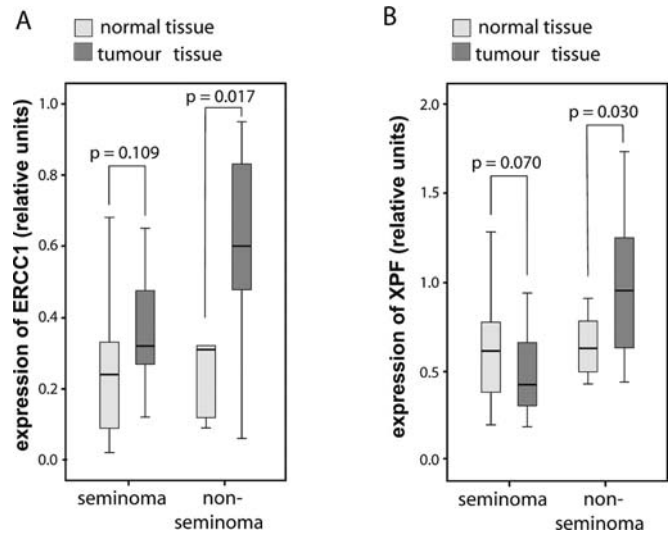

Figure 2. Expression of ERCC1 (A) and XPF (B) in testis tumor and the corresponding normal testis tissue determined by immunoblot analysis. The values represent the amount of antigen determined by computer-aided quantification of the bands. Box plots show medians (central lane), 25 and 75 percentiles (lower and upper side of the box) and minimum and maximum (lower and upper bars). Extreme values have been excluded from the graph.
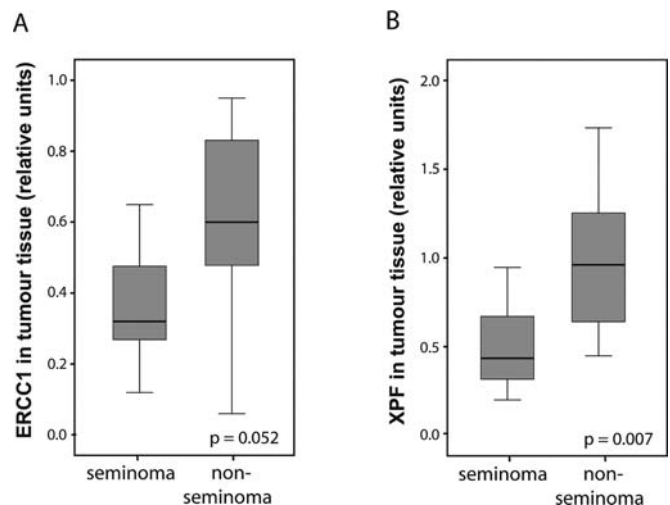

Figure 4. Expression of ERCC1 (A) and XPF (B) in seminomas compared with non-seminomas, as determined by immunoblot analysis. The values represent the amount of antigen determined by computer-aided quantification of the bands. Box plots show medians (central lane), 25 and 75 percentiles (lower and upper side of the box) and minimum and maximum (lower and upper bars). Extreme values have been excluded from the graph.

Results

Using quantitative immunoblotting, we investigated the expression of ERCC1 and XPF protein in 24 testis tumors and their corresponding normal testicular tissues. Examples of a representative immunoblot are shown in Fig. 1. Normalizing to RPA, we found a significant difference ($p<0.004$) in the expression of ERCC1 in tumor compared to the normal tissue. The relative ERCC1 expression increased from 0.26 to 0.45 (Fig. 2). For XPF protein no significant difference in the expression level in tumor versus normal tissue was detected.

Of the 24 tumors examined 16 samples were seminomas and 8 samples were non-seminomas. We therefore investigated whether there is a difference in the expression of ERCC1 and XPF with respect to testis tumor subtype. We found no significant difference in expression levels of ERCC1

in seminoma when compared to normal corresponding testis tissue (Fig. 3A). However, the ERCC1 protein expression was significantly increased (by ~2-fold) in non-seminoma compared to normal tissue ($p<0.017$). Similarly, no significant difference was found in expression levels of XPF when comparing seminoma with normal testis tissue (Fig. 3B). In non-seminoma XPF protein was significantly ameliorated in the tumor compared to normal tissue ($p<0.03$). When we
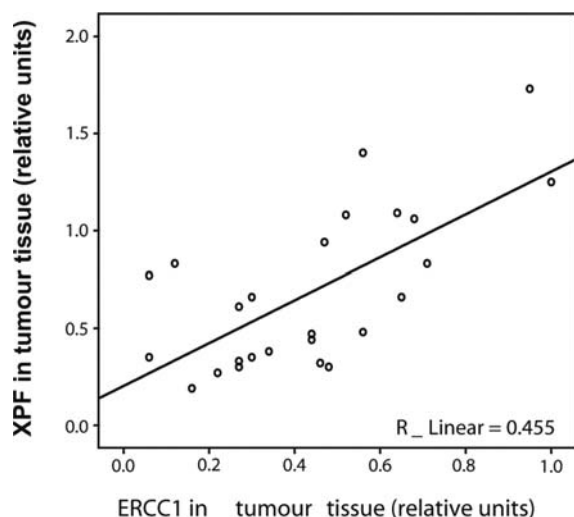
Figure 5. Correlation between the expression level of ERCC1 and XPF in testis tumor tissue. Expression values were determined by immunoblot analysis and computer-aided quantification. The expression of ERCC1 correlated with the expression of XPF (R^2 linear = 0.455).

compared ERCC1 and XPF levels in seminomas with non-seminomas we found significantly higher levels for ERCC1 ($p<0.05$) and XPF ($p<0.007$) in the non-seminoma tumor tissues (Fig. 4).

We also attempted to correlate the expression levels of ERCC1 and XPF in testis tissues with clinical parameters, including tumor size and TNM stage. We found no correlation between tumor size and ERCC1 and XPF expression (data not shown). The TNM stage was available for all 24 samples analyzed and included 11 T1 and 13 T2. Data analysis revealed no correlation between tumor stage and expression level of ERCC1 and XPF (data not shown). Finally, we analyzed whether the expression of ERCC1 correlates with the expression of XPF in the tumor tissues. We found a clear correlation between the expression levels of ERCC1 and XPF in the tumor samples (Fig. 5).

Discussion

In this study we found a significant difference in the levels of ERCC1 and XPF proteins in non-seminoma versus seminoma testis tumor tissue. ERCC1 associates with XPF to form a structure-specific endonuclease that operates in NER. The ERCC1-XPF complex executes the incision in the DNA strand 5' of the DNA damage through an intrinsic endonuclease activity of XPF (13). Mammalian cells with inactivating mutations in either the ERCC1 or XPF gene manifest impaired NER and consequently increased sensitivity towards DNA damaging drugs such as cisplatin (25). On the other hand, there is increasing evidence that increased NER repair activity in tumor cells is linked with therapeutic resistance against cisplatin chemotherapy. Several investigations have examined the expression of NER mRNA and proteins in human cancer tissues. Attention has notably been payed to ERCC1, the first human DNA repair gene cloned. In gastric carcinoma,

elevated ERCC1 mRNA levels were suggested to be associated with cisplatin resistance (19). In tissue samples from patients with colorectal cancer, ovarian cancer or non-small cell lung cancer (NSCLC) low expression of ERCC1 mRNA was found to be associated with a good response to chemotherapy (17,18,20). Equally a low level of ERCC1 protein in lung cancer tissue was associated with longer patient survival after cisplatin-based chemotherapy (15). Therefore, it was proposed that the level of ERCC1 protein might serve as a powerful predictor of response to cisplatin-based chemotherapy and clinical outcome.

Although cisplatin is the gold standard in the therapy of TGCT, scarce information is available regarding the expression of DNA repair proteins in testis tumor tissue. In one single study the level of XPA protein was investigated in a series of testicular seminomas and spermatocytic seminomas. No XPA protein could be detected in the seminoma tissues while all spermatocytic seminomas were positive for the protein suggesting that spermatocytic seminomas originate from a later stage of germ cell development than seminomas (26). For ERCC1 and XPF we show here that testis tumor tissue expresses a higher level of ERCC1 and XPF compared to the corresponding normal tissue of the same patient. This abundance is characteristic for non-seminomas as seminomas display no significant difference in ERCC1 and XPF level compared with normal testis. TGCT originate from germ cells and have a common progenitor lesion, the carcinoma *in situ*, in which a series of cytogenetic and molecular alterations lead to the transformation into seminoma (5). Seminoma may subsequently develop into non-seminoma, which are more aggressively growing and have a high tendency for metastasis (27). Our data indicate that the progression of seminoma to non-seminoma may go along with increased expression of ERCC1-XPF. Of note, we could not find a significant correlation between the expression of ERCC1 and XPF and clinical parameters such as TNM stage of non-seminomas. Although the mechanisms of up-regulation is unknown, the data suggest that ERCC1 and XPF is co-regulated in the tumor tissue presumably by stabilization due to protein-protein interaction (28,29). It is noteworthy that another repair protein, Fen1, was found to be up-regulated in TGCT (30), whereas the O^6-methylguanine-DNA methyltransferase (MGMT) repairing O^6-alkylguanine adducts was found to be down-regulated (31). Therefore, the data are not in line with the supposition of a general up-regulation of DNA repair genes in TGCT. Since ERCC1-XPF is involved in the repair of cisplatin-induced DNA adducts one may anticipate that non-seminomas are resistant to cisplatin. This, however, is not the case in view of the good curability of these cancers. Therefore, it is possible that other tumors that do not respond to cisplatin-based therapy display even higher levels of ERCC1-XPF. Alternatively, one may suppose that TGCT execute very effectively the cisplatin-triggered cell death program. Work is in progress to prove these lines of arguments.

Acknowledgements

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