Short Communication

NKX3.1 Expression Is Lost in Testicular Germ Cell Tumors

Rolf I. Skotheim,* Kemal S. Korkmaz,[†]
Tove I. Klokk,[†] Vera M. Abeler,[‡]
Ceren G. Korkmaz,[†] Jahn M. Nesland,[‡]
Sophie D. Fosså,[§] Ragnhild A. Lothe,*[†] and
Fahri Saatcioglu[†]

From the Department of Genetics, Institute for Cancer Research, and the Departments of Pathology[‡] and Medical Oncology and Radiotherapy,[§] The Norwegian Radium Hospital and University of Oslo, Oslo; and the Department of Cell and Molecular Biology,[†] Institute of Biology, University of Oslo, Oslo, Norway*

NKX3.1 **is a homeobox gene which exhibits prostate and testis specific expression. Loss of NKX3.1 expression has been implicated in prostate development and tumorigenesis, but the role of NKX3.1 in testis biology is not known. Here we show that NKX3.1 expression is dramatically down-regulated in testicular cancer of germ cell origin. Immunohistochemical analysis on a tissue microarray containing 510 testicular tissue samples indicate that NKX3.1 is expressed at high levels in normal germ cells and in carcinoma *in situ*, but is sharply decreased or absent in most seminomas and all embryonal carcinomas. However, NKX3.1 is expressed in a subset of the more differentiated nonseminomas. We provide evidence that these changes in NKX3.1 protein levels are mainly due to transcriptional effects. These results suggest that NKX3.1 is essential for normal testis function and that its loss of expression is highly associated with the invasive phenotype of testicular germ cell tumors.** *(Am J Pathol 2003, 163:2149–2154)*

Testicular germ cell tumor (TGCT) is the most common malignancy among adolescents and young adult men in Western industrialized countries, and the incidence has increased dramatically over the past fifty years.[1,2] TGCTs are subgrouped into two main histological types, seminomas and nonseminomas, both of which develop from premalignant non-invasive carcinoma *in situ* (CIS, or intratubular malignant germ cells). Seminomas resemble the CIS cells, but are not constrained within the tubules. Whereas seminomas are believed to only rarely differentiate, nonseminomas develop through an undifferentiated but pluripotent embryonal carcinoma stage, which frequently differentiates into cells and tissue types of all three primary germ layers at various stages of differentiation (somatically differentiated teratomas and extra-embryonally differentiated choriocarcinomas and yolk sac tumors). Although several chromosomal regions and a few genes are known to be frequently altered, the molecular mechanisms involved in testicular tumorigenesis remain mostly unknown.[3] One of the genes that has highly restricted expression to the testis, along with the prostate, is the homeobox gene NK3 transcription factor-related locus 1 (*NKX3.1*).[4−6] The gene product of *NKX3.1* is a positive effector of differentiation in prostate cancer,[4,6] and the gene is under both androgen and 17β-estradiol regulation.[4,5,7,8] A large proportion of human prostate carcinomas have lost the NKX3.1 protein expression, and this is most commonly seen in the advanced stages.[6] In mice, deletion of the *Nkx3.1* gene results in developmental defects in the prostate, leading to hyperplasia and dysplasia.[9−12] In testis, however, little information is currently available on the possible deregulation of *NKX3.1* expression during testicular dysgenesis or tumorigenesis. In this study, we have assessed *in situ* NKX3.1 expression using a tissue microarray representing normal testis, CIS, and invasive TGCT of all histological subgroups and clinical stages. In a small series of tumors we further evaluated whether the altered protein expression could be explained by transcriptional regulation.

Materials and Methods

Tissue Microarray

We used sections from a tissue microarray block that we previously constructed to contain 510 testicular tissue cores punched from formalin-fixed and paraffin-embedded testicular specimens from 279 individuals.[13] The 279 cases included 278 orchiectomy specimens from patients diagnosed with TGCT of adolescents and young adult males and one testicular autopsy from a patient with no known history of cancer. The patients had TGCT of various clinical stages, including 174 with localized TGCT and 104 with metastatic disease. The numbers of tissue cores acquired from the various histological subtypes were as follows: normal testis, 28; CIS, 21; seminoma, 184; embryonal carcinoma, 102; choriocarcinoma, 16; yolk sac tumor, 69; and teratoma, 90. The use of the testicular tissue samples was approved by the Regional Committee for Medical Research Ethics in Norway.

The Instrumedics (Instrumedics, Hackensack, NJ) tape-transfer method was used to transfer 4-μm sections of the tissue microarray block to glass slides. Hematoxylin and eosin-stained tissue microarray sections were evaluated to check the consistency with the originally assigned histology. Histological classification was performed according to the recommendations of the World Health Organization.[14] Distinction of CIS from normal tissue was assisted by immunohistochemical staining of a parallel section using anti-PLAP antibodies, targeting germ cell and placental alkaline phosphatases, extensively present in CIS but not in normal germ cells.[15]

Rabbit Polyclonal Antiserum

NKX3.1 antiserum was raised in rabbits using a recombinant NKX3.1 protein spanning the whole coding region produced in *Escherichia coli* (Korkmaz CG, Korkmaz KS, Onody T, Seller WR, Loda M, Saacioglu F, submitted manuscript). The antiserum was purified on IgG affinity columns before use.

Immunohistochemistry on the Tissue Microarray

Tissue microarray sections were stained with the biotin-streptavidin-peroxidase method (Supersensitive Immunodetection System, LP000-UL, BioGenex, San Ramon, CA). Each section was deparaffinized and rehydrated, and high temperature antigen retrieval was achieved by microwaving twice for five minutes at 900W in 10 mmol/L citrate buffer (pH 6.0). The slides were then incubated with 1% hydrogen peroxide (H_2O_2) for 10 minutes to block the endogenous peroxidase activity before incubation with the polyclonal NKX3.1 antibody (affinity-purified rabbit antiserum against NKX3.1, 1:150) or the monoclonal PLAP antibody (clone 8A9, 1:20; IgG1k, Novocastra Laboratories Ltd., Newcastle, UK) for 30 minutes at room temperature. Afterward, the sections were incubated for 20 minutes with multilink biotinylated anti-immunoglobulins (1:30; BioGenex) and 20 minutes with streptavidin peroxidase (1:30; BioGenex). Finally, the sections were stained for 5 minutes with 0.05% of the peroxidase substrate 3k,3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in 0.05 mol/L Tris-HCl buffer (pH 7.6) containing 0.01% H_2O_2, before being counterstained with hematoxylin, dehydrated, and mounted. Negative controls consisted of replacement of the primary polyclonal NKX3.1 antibody with the IgG fraction of a non-immunized rabbit and replacement of the PLAP antibody with mouse myeloma protein of the same subclass and concentration. These controls were negative as expected.

The NKX3.1 immunostaining was nuclear, though weak cytoplasmic staining was also seen in some of the testicular tissues. Tissue cores with staining of more than 5% of the relevant nuclei were considered positive. A tumor was considered positive when one or more of the tissue cores from that specific tumor were positive. Equally, when more than one tissue core of a specific histological component of a tumor was present on the array, that specific component was considered positive if at least one of the samples was scored positive.

Quantitative RT-PCR

For real-time reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA was isolated by use of the Trizol reagent (Invitrogen, Carlsbad, CA) from 12 testicular tissue samples (four of which are present on the tissue microarray), including two from morphologically normal testicular parenchyma adjacent to TGCT, one CIS, seven seminomas, and two embryonal carcinomas. From each RNA sample, first-strand cDNA was synthesized in an oligo(dT)-primed polymerization with SuperScript II reverse transcriptase (Invitrogen). The cDNA was then used as template in quantitative PCR, using LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland), to evaluate the mRNA expression levels of *NKX3.1* (primers: 5′-GGCCTGG-GAGTCTCTTGACTCCACTAC-3′ and 5′-ATGTGGAGC-CCAAACCACAGAAAATG-3′) and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control transcript (primers: 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′). All samples were analyzed simultaneously, and the experiment was repeated twice with consistent results. For each of the amplifications, standard curves were constructed for both *NKX3.1* and *GAPDH* using plasmid templates containing the respective fragments. A calibrated expression value for *NKX3.1* was then calculated by dividing its expression value by that of *GAPDH*. For each sample, the average of the three runs was divided by the average expression from the normal samples to get changes in expression relative to the normal tissues.

Results

Various testicular tissue samples from the tissue microarray are shown in Figures 1 and 2 with representative
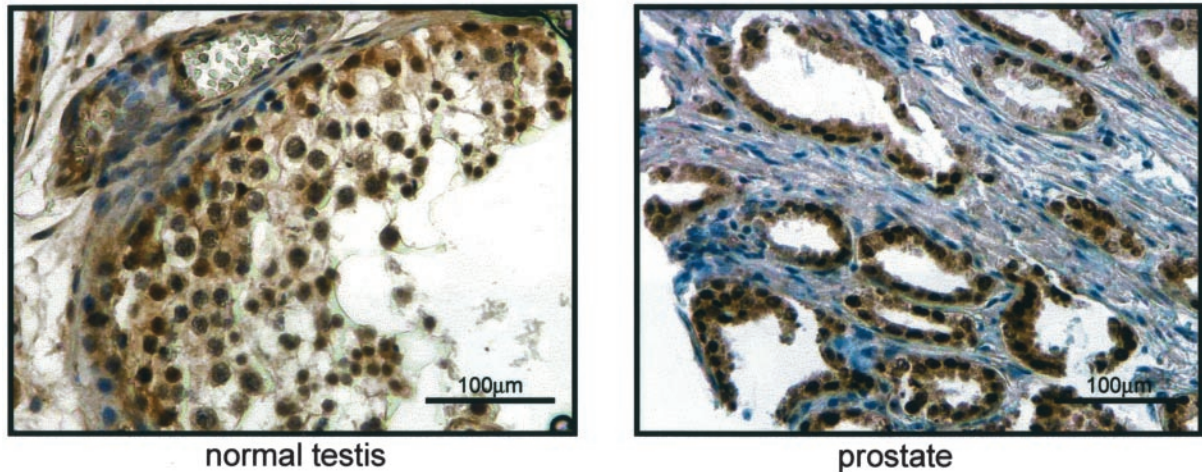
Figure 1. Normal testicular and prostate tissues are immunopositive for NKX3.1. The antibody specificity is shown demonstrating NKX3.1 positive spermatogenic germ cells within a seminiferous tubule of a non-cancerous testis and immunopositive epithelial cells in a prostatic tissue sample. The prostate tissue was included as a positive control for staining.

immunohistochemical staining of NKX3.1. Whereas all of the normal testicular tissue samples ($n = 17$) had NKX3.1 immunopositive germ cells, and all of the CIS samples ($n = 17$) were also positive, only 14% of the seminomas ($n = 150$; $P = 4 \times 10^{-13}$ [seminoma *versus* normal, two-sided Fisher's exact]) and none of the embryonal carcinomas ($n = 70$; $P = 2 \times 10^{-18}$) or the choriocarcinomas ($n = 9$; $P = 3 \times 10^{-7}$) expressed NKX3.1. Some of the differentiated nonseminomas expressed NKX3.1, including 15% of the yolk sac tumors ($n = 48$), and 20% of the teratomas ($n = 49$), which was significantly different from the complete absence of NKX3.1 in embryonal carcinomas ($P = 1 \times 10^{-4}$).

The possible correlation of NKX3.1 expression with the metastatic potential of TGCT was evaluated, and NKX3.1 expression tended to be inversely correlated with metastases in patients with seminomas. Whereas 17% of the pure seminomas without metastases at time of diagnosis ($n = 89$) expressed NKX3.1, there was no NKX3.1 expression among the pure seminomas with metastases at diagnosis ($n = 16$; $P = 0.12$). Such a link between NKX3.1 expression and metastases was not observed for the nonseminomas.

The quantitative RT-PCR analyses revealed that all analyzed tumor samples had reduced expression of *NKX3.1* mRNA compared with the normal testis tissues (Figure 3). For six of ten tumors, *NKX3.1* mRNA accumulation was less than five percentage of the average expression in the normal samples, showing that the loss of NKX3.1 in TGCT is at least in part due to loss of *NKX3.1* mRNA accumulation. Four of the tumors analyzed by RT-PCR were also present on the tissue microarray. These included two with very low expression (both <0.02 compared to normal testis, and both lacking immunoreactivity to the NKX3.1 antiserum) and two of the seminomas with intermediate expression (0.32 and 0.43, of which one was immunopositive).

Discussion

Previous reports have implicated a role for NKX3.1 in human prostate tumorigenesis, possibly as a tumor suppressor protein.[4,6,8,12,16] Although not commented on, it can be found from the data set of a recent study of multiple tumor types that the NKX3.1 protein was absent from a set of TGCTs.[17] We here demonstrate that primary TGCTs display a virtually ubiquitous loss of NKX3.1 expression. Since NKX3.1 expression is still retained in the CIS precursor lesions, this loss is highly associated with the invasive tumor phenotype. Thus, NKX3.1 expression clearly distinguishes the morphologically similar CIS and seminoma cells at the molecular level. The quantitative RT-PCR data suggest that the dramatically lower expression of NKX3.1 protein in testicular cancer compared with normal testis is mostly due to differential transcriptional regulation.

NKX3.1 is located to chromosome band 8p21 that is frequently lost in prostate cancers.[18] Loss of NKX3.1 protein expression has been observed in up to 40% of prostate tumors with correlation to advanced tumor stages.[6] We observed a similar trend toward loss of expression of NKX3.1 in the overall sample set of TGCT. Interestingly, for the seminomas, the NKX3.1 protein was not detected in any of the tissue cores from patients with metastases at time of diagnosis. This suggests that expression of NKX3.1 may be involved in suppressing metastases arising from seminomas.

The loss of NKX3.1 expression in prostate cancer is believed to be due to epigenetic inactivation,[11] a view which is strengthened by the absence of mutations within the exons of *NKX3.1*.[19,20] In the case of TGCT, we speculate that the loss of NKX3.1 expression is due to epigenetic mechanisms based on the following arguments. Firstly, the chromosome band 8p21 has not been noted as a region with common loss of heterozygosity in this tumor type.[3] Secondly, we found immunopositivity for
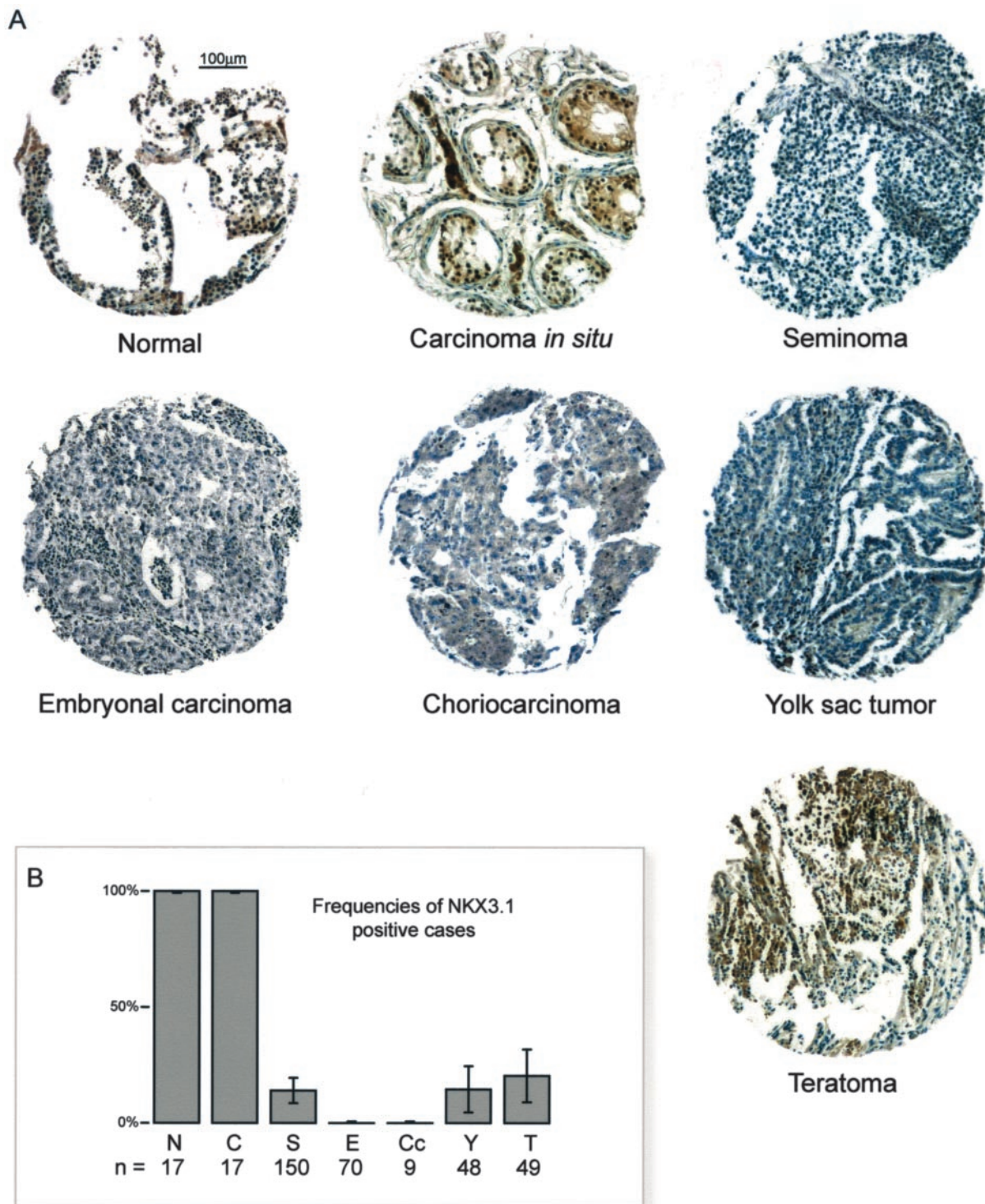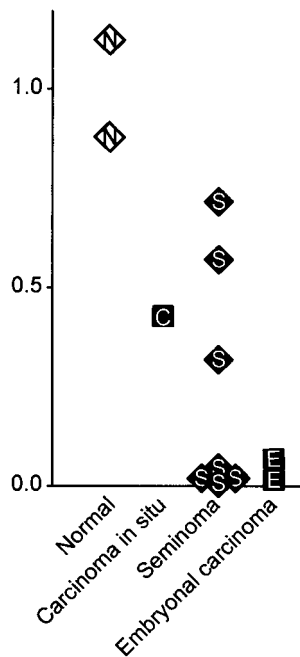
Figure 2. NKX3.1 expression analysis on a testicular germ cell tumor tissue microarray. **A:** One tissue core with representative staining is shown for each of the histological subtypes. Positive cases include the normal germ cells, carcinoma *in situ*, and the teratoma. The remaining cases were scored negative. **B:** The histogram bars represent the frequencies of NKX3.1 immunopositive cases for the subgroups of normal germ cells (N), carcinoma *in situ* (C), and the various histological subtypes of testicular germ cell tumor (S, seminoma; E, embryonal carcinoma; Cc, choriocarcinoma; Y, yolk sac tumor; T, teratoma). Error bars define 95% confidence intervals.

NKX3.1 in TGCT to be correlated with positive staining of the previously analyzed DNA repair enzyme O^6-methyl-guanine-DNA methyltransferase (MGMT; data not shown).[13] This was evident both across the whole sample series and within specific histological subtypes. Because *MGMT* was recently reported to be frequently hyper-methylated in promoter sequences in TGCT,[21] this correlation suggests an epigenetic silencing mechanism

most human TGCTs. This adds to the molecular understanding of the disease, and raises the possibility that NKX3.1 expression could be used as a marker for invasive testicular tumorigenesis.

Figure 3. Testicular germ cell tumors have reduced or lost *NKX3.1* mRNA expression. Total RNA was isolated from normal testis or testicular germ cell tumors and subjected to quantitative RT-PCR analysis. The mRNA levels are shown relative to the average of the two normal testicular tissues. The experiments were run in triplicate, and the average values are shown.

also for *NKX3.1*. In fact, DNA methylation seems to be a major mechanism for regulating gene expression in TGCT.[22–24] Finally, the expression of NKX3.1 seen in a subset of the most differentiated nonseminomas indicates that the loss of its expression in TGCT is reversible and thus likely caused by epigenetic silencing. The fact that none of the 70 embryonal carcinomas expressed NKX3.1 and the general view that the embryonal carcinomas are precursors for the differentiated nonseminomas support this assumption of reversibility.

The latter observation is consistent with the function of NKX3.1 as a differentiation-specific homeobox protein. However, NKX3.1 may not only reflect the differentiation status of the tissue, but may also be an effector of differentiation itself, as suggested for the prostate gland.[17,25] In light of this, and the fact that *Nkx3.1* knockout mice are prone to prostate cancer,[9–11] the loss of NKX3.1 expression can either be a cause or a consequence of the testicular cancer phenotype. More work is needed to differentiate between these two possibilities.

A recent study of mutant mice that are deficient in both *Nkx3.1* and *Pten* demonstrated that loss of function of these two genes cooperate in prostate cancer development and progression.[11] Mice with defects in the *Pten* gene spontaneously develop germ cell tumors,[26,27] and interestingly, a human *PTEN* germ line mutation has been described in an adolescent man with synchronous testicular and extragonadal germ cell tumor.[28] We therefore speculate that there may be a link between *NKX3.1* and *PTEN* disruption also in testicular tumorigenesis.

In conclusion, we have provided evidence that the expression of the *NKX3.1* homeobox gene is lost from

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