The loss of NKX3.1 expression in testicular – and prostate – cancers is not caused by promoter hypermethylation

Guro E Lind1, Rolf I Skotheim1, Mario F Fraga2, Vera M Abeler3, Rui Henrique4, Fahri Saatcioglu5, Manel Esteller2, Manuel R Teixeira6 and Ragnhild A Lothe*1,5

Address: 1Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway, 2Cancer Epigenetics Laboratory, the Spanish National Cancer Centre (CNIO), Madrid, Spain, 3Department of Pathology, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway, 4Department of Pathology, Portuguese Oncology Institute, Porto, Portugal, 5Department of Molecular Biosciences, Institute of Biology, University of Oslo, Oslo, Norway and 6Department of Genetics, Portuguese Oncology Institute, Porto, Portugal

Email: Guro E Lind - guroli@ulrik.uio.no; Rolf I Skotheim - r.i.skotheim@labmed.uio.no; Mario F Fraga - mfraga@cnio.es; Vera M Abeler - v.m.abeler@labmed.uio.no; Rui Henrique - rmfhenrique@hotmail.com; Fahri Saatcioglu - fahris@bio.uio.no; Manel Esteller - mesteller@cnio.es; Manuel R Teixeira - mteixeir@ipoporto.min-saude.pt; Ragnhild A Lothe* - rlothe@radium.uio.no

* Corresponding author

Published: 03 February 2005
Molecular Cancer 2005, 4:8 doi:10.1186/1476-4598-4-8
This article is available from: http://www.molecular-cancer.com/content/4/1/8

© 2005 Lind et al; licensee BioMed Central Ltd.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Recent studies have demonstrated that the NKX3.1 protein is commonly down-regulated in testicular germ cell tumors (TGCTs) and prostate carcinomas. The homeobox gene NKX3.1 maps to chromosome band 8p21, which is a region frequently lost in prostate cancer, but not in TGCT. Mutations have not been reported in the NKX3.1 sequence, and the gene is hypothesized to be epigenetically inactivated. In the present study we examined the methylation status of the NKX3.1 promoter in relevant primary tumors and cell lines: primary TGCTs (n = 55), intratubular germ cell neoplasias (n = 7), germ cell tumor cell lines (n = 3), primary prostate adenocarcinomas (n = 20), and prostate cancer cell lines (n = 3) by methylation-specific PCR and bisulphite sequencing.

Results and Conclusions: Down-regulation of NKX3.1 expression was generally not caused by promoter hypermethylation, which was only found in one TGCT. However, other epigenetic mechanisms, such as modulation of chromatin structure or modifications of histones, may explain the lack of NKX3.1 expression, which is seen in most TGCTs and prostate cancer specimens.

Background
The protein expression of the homeobox gene NK3 transcription factor related locus 1 (NKX3.1) is highly specific for the prostate and the testis [1-3], and is frequently lost in cancers of these two tissue types [1,4,5]. NKX3.1 is located in chromosome band 8p21 [2,6,7], a region that undergoes frequent allelic imbalance in prostatic intraepithelial neoplasia (PIN) and prostate carcinomas [8,9]. In mice, targeted disruption of Nkx3.1 leads to prostatic epithelial hyperplasia and dysplasia [10,11], and over-expression of exogenous NKX3.1 suppresses growth and tumorigenicity in human prostate carcinoma cell lines [12]. However, the expression levels and possible role for NKX3.1 during prostate cancer progression in humans is still being debated [13-15]. No gene mutations of NKX3.1 have been found [6], and NKX3.1 is therefore believed to be
epigenetically inactivated in the cases with loss of protein expression [1,5,16]. Only one study has reported NKX3.1 protein expression in testicular germ cell tumors (TGCTs), however the series analyzed was large, including a total of more than 500 samples, and NKX3.1 was found absent in all embryonal carcinomas and present in only 15–20% of the seminomas as well as among the differentiated histological subtypes of germ cell tumors [5].

During the last decade, epigenetic changes in cancer have been frequently reported and are now recognized to be at least as common as genetic changes [17]. The best characterized epigenetic mechanism is DNA hypermethylation, in which cytosines located within selected CpG sites in the gene promoters become methylated, thereby inactivating gene expression. Several tumor suppressor genes are inactivated by such promoter hypermethylation in various cancer types [18,19]. In the present study we have performed methylation-specific PCR (MSP) and bisulphite sequencing of the \( \text{NKX3.1} \) promoter in TGCTs and prostate adenocarcinomas to examine whether this mechanism may explain the commonly observed loss of NKX3.1 protein.

**Results**

Only one out of 54 TGCTs and none of the prostate adenocarcinomas (n = 20), intratubular germ cell neoplasias (n = 7), normal testis tissues (n = 4), or the cell lines (n = 6) displayed methylation when analyzed with MSP (Figure 1a). Bisulphite genomic sequencing of the tumors and cell lines showed that all cytosines at non-CpG sites were converted to thymine (Figure 1b). Only one sample demonstrated overall methylation in the \( \text{NKX3.1} \) sequence, and this was the same sample that was positive for methylation from the MSP analysis. Interestingly, all the samples that were sequenced, including the normal blood, unmethylated cell lines, and primary tumors, displayed some extent of methylation (the majority below 25%) at the cytosine in CpG number 21 (base 1914762, +1 bp from transcription start). We detected a possible polymorphism in base 1914730 (+33 bp from transcription start and 15 bp upstream of the coding sequence). In previous sequences this site has been described as a guanine (Gene bank accession number NT_023666, and AF24770). In the cell lines, 5/6 contained adenosine in this position, but all except the germ cell tumor cell lines NCCIT and TERA2 were heterozygotes. In contrast, all 5 primary tumors sequenced were homozygous for the adenosine.

**Figure 1**

**Representative results of the methylation analyses of the \( \text{NKX3.1} \) promoter.** (A) Methylation-specific PCR. A visible PCR product in Lanes U indicates the presence of unmethylated alleles whereas a PCR product in Lanes M indicates the presence of methylated alleles. The left panel illustrates the methylation status of selected TGCTs and the testicular cancer cell lines. Note the methylation of sample # 2110. The right panel shows the unmethylated status of primary prostate cancers and prostate cancer cell lines. Abbreviations: NB, normal blood (positive control for unmethylated samples); IVD, in vitro methylated DNA (positive control for methylated samples); neg, negative control (containing water as template); U, lane for unmethylated MSP product; M, lane for methylated MSP product. (B) Bisulphite sequencing. The bisulphite sequence allows a positive display of 5-methyl cytosines in the gene promoter as unmethylated cytosines appear as thymines, while 5-methylcytosines appear as cytosines in the final sequence. The left chromatogram represents a part of the unmethylated \( \text{NKX3.1} \) promoter in the germ cell tumor cell line TERA2, including 11 CpG sites marked by underlined letters. The right chromatogram represents the unmethylated prostate cancer cell line DU145. Both sequences have been generated by reversing the respective anti-sense sequences by use of the software "Chromas".
Discussion

We have previously reported that the protein expression of NKX3.1 is virtually ubiquitously lost in TGCTs [5]. This was done using a tissue microarray containing 510 testicular tissue samples. NKX3.1 expression is known for 25 of the TGCTs now analyzed for promoter hypermethylation and 22 showed complete absence of protein. The down regulation of NKX3.1 in TGCT has also been detected at the mRNA level, both by quantitative RT-PCR [5] and from an oligonucleotide microarray study including 20 of the present TGCTs (Skøtheim et al., submitted). This simultaneous down regulation of both protein and mRNA levels of NKX3.1 is consistent with epigenetic regulation, which is further supported by the fact that mutations have not been detected in the NKX3.1 gene [6]. DNA promoter hypermethylation is the best-characterized epigenetic change in cancer, and can be associated with gene silencing. It was therefore of interest to analyze the methylation status of the NKX3.1 promoter in TGCT and prostate cancer samples. However, with the exception of a single TGCT, the NKX3.1 promoter was unmethylated in the samples analyzed. The methylated TGCT was classified as a yolk sac tumor, and has also been demonstrated to have promoter hypermethylation of several other genes that are generally unmethylated in TGCTs (Lind et al., unpublished). We therefore consider this sample not to be representative for the general TGCT epigenotype, nor for the general epigenetic profile of yolk sac tumors. Thus, we do not regard promoter hypermethylation as the general mechanism of NKX3.1 down-regulation neither in TGCT nor in prostate carcinomas.

We also studied cell lines since it can be argued that presence of normal cells as well as tumor heterogeneity may mask cancer specific methylation in primary tumors. LNCaP cells have previously been demonstrated to express NKX3.1, in contrast to PC-3 and DU-145, which do not express NKX3.1 since they lack a functional androgen receptor [2]. The lack of NKX3.1 expression in PC-3 and DU-145 cells is not due to methylation. This was also the case with the germ cell tumor cell lines. From Western analysis, the cell line NCCIT had strong expression of NKX3.1 whereas both TERA1 and TERA2 had no expression (data not shown).

The polymorphism in NKX3.1 that we detected 15 bases upstream of the coding sequence has to our knowledge not been described previously. It was identified by bisulphite sequencing of the cell lines and a subgroup of primary tumors, thus caution should be taken when concluding from these results, since regular sequencing analysis is the recommended approach for describing sequence changes. As the polymorphism is located in the promoter region of NKX3.1, it has no influence on the protein structure. However, it can still have a potential role in the transcriptional regulation of NKX3.1. A polymorphism in the coding sequence is also reported for NKX3.1 [6].

All samples analyzed with bisulphite sequencing, including cell lines expressing NKX3.1, as well as non-expressing cell lines, demonstrated some degree of methylation in the cytosine in CpG number 21. As this site-specific methylation included only one CpG site, it is unlikely that it will have any regulating effect on gene expression. However, considering its intriguing location immediate upstream of the transcription start point, this possibility should not be excluded. There is also the possibility that the apparent methylation could be due to a less efficient bisulphite conversion for this site. In general, the bisulphite sequencing results showed that all cytosines at non-CpG sites were converted to thymine (Figure 1b), but sequence-specific partial resistance to this conversion may lead to methylation artifacts, but only in rare cases, as has been reported previously [20].

Conclusions

In summary, these data show that the previously reported down-regulation of NKX3.1 in TGCTs and prostate carcinomas is not caused by promoter hypermethylation. Even though the NKX3.1 promoter is unmethylated, the simultaneous down-regulation of mRNA and protein levels in TGCTs and the absence of mutations still make other epigenetic mechanisms, such as modulation of chromatin structure or modifications of histones, possible explanations for loss of NKX3.1 expression in testicular- and prostate cancers.

Materials and Methods

Primary tumors and cell lines

Included in the present study are primary TGCTs (n = 55), intratubular germ cell neoplasias (also called carcinoma in situ; n = 7), normal testis tissue (n = 4), germ cell tumor cell lines (TERA1, TERA2, and NCCIT), prostate adenocarcinomas (n = 20), and prostate cancer cell lines (LNCaP, PC-3, and DU-145). The primary TGCTs include all histological subtypes: seminomas, embryonal carcinomas, teratomas, yolk sac tumors, and one choriocarcinoma, classified according to the WHO’s recommendations [21] by a germ cell tumor reference pathologist using light microscopic examination of hematoxylin and eosin stained tissue sections. From our previous comparative genome hybridization analysis, about half of the TGCTs had a low-level copy number gain at chromosome 8, but only rarely 8p deletions [22]. Primary prostate adenocarcinomas obtained from radical prostatectomy specimens were graded according to the Gleason grading system [23] using routinely stained tissue sections. The median
Gleason score of prostate adenocarcinomas was 7 (range: 4 – 8). The prostate carcinomas were all of pTNM stage 2 and 3, and included 10 samples with 8p deletions (among other cytogenetic aberrations), 3 samples with copy number changes not involving the 8p region, and 7 samples with no copy number changes (Ribeiro et al., submitted).

Methylation-specific PCR

The DNA samples were initially bisulphite modified [24,25], which converts unmethylated but not methylated cytosines to uracil. All samples were subsequently submitted to MSP analysis [26] using PCR primers specific to methylated and unmethylated sequences: NKKX3.1 unmethylated sequence, sense: 5’GCAAAGTGAAGTGGTGCGTT3’, antisense: 5’CTACACACATCCACAAATCT3’, methylated sequence, sense: 5’AAAGTGAACGGTGCGCTC3’, antisense: 5’ACGCAGCTGCCCCAAAAT3’ (MedProbe AS, Oslo, Norway). The two fragments were amplified by the Fast Star DNA polymerase (Roche Ltd, Basel, Switzerland) in a reaction containing 1.5 mM Mg2+. We used a 58°C annealing temperature for both primer sets. Bisulphite treated DNA from normal blood (NB) and Ss1 methyltransferase (New England Biolabs Inc., Beverly, MA, USA) in vitro treated placenta DNA (IVD; Sigma Chemical Co., St. Louis, MO, USA) represented the unmethylated positive control and the methylated positive control, respectively. Water, replacing bisulphite treated template, was the negative control in both reactions.

Bisulphite sequencing

Bisulphite sequencing allows a positive display of 5-methyl cytosines in the gene promoter after bisulphite modification as unmethylated cytosines appear as thymines, while 5-methylcytosines appear as cytosines in the final sequence [27]. A subset of the samples (n = 11) were bisulphite sequenced, including all 6 cell lines, 3 TGCTs, and 2 prostate adenocarcinomas. Additionally, NB and IVD were bisulphite sequenced as positive controls for unmethylated and methylated sequences, respectively. The NKKX3.1 bisulphite sequence fragment (Gene bank accession number NT_023666 (minus strand), bases 1914526 to 1914961) was 436 bp long and covered 52 CpG sites in the promoter and first exon of the gene. We designed bisulphite sequencing primers (MedProbe) with the following sequences; sense: 5’ATTGGGGAAGAGGGGAATT3’, antisense: 5’CCCTCAATCCCTAATCTCC3’. The Mg2+ content of the reaction was 1.3 mM, the enzyme used was HotStarTaq™ DNA polymerase (QIAGEN Inc., Valencia, CA, USA), and the annealing temperature 52°C. The PCR fragments were eluted from a 2% agarose gel (BioRad Laboratories Inc, CA, USA) containing ethidium bromide, by the MinElute™Gel Extraction kit (QIAGEN), and sequenced with the dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) in an ABI Prism 377 Sequencer (Applied Biosystems). The bisulphite sequencing results were scored according to Melki et al. where the amount of methylcytosine of each CpG dinucleotide is quantified by comparing the peak height of the cytosine signal with the sum of the cytosine and thymine peak height signals [28].

Authors’ contributions

GEL performed the experimental analyses and statistics, interpreted the results, and drafted the manuscript. RIS did an independent scoring of the results, and contributed to manuscript preparation. MFF designed the primers used for the MSP and bisulphite treatment and contributed to manuscript preparation. VMA and RH were reference pathologists for the testicular cancer tissues and prostate tissues, respectively. FS was responsible for the Western Blot studies of the cell lines and participated in the writing of the manuscript. Parts of this work were done in the lab of ME who also contributed with scientific discussions. MRT provided the relevant selected series of primary prostate carcinomas with known genetic profiles, and contributed to manuscript preparation. RAL conceived the study, was responsible for its design and coordination, and contributed in the evaluation of the results and in preparation of the manuscript.

Acknowledgements

The study was funded by grants from the Norwegian Cancer Society supporting GEL as a Research Fellow and RIS as a Post-Doctoral Fellow (A95068 RAL). The authors are grateful to Gunnar Brunborg at the Norwegian Institute of Public Health, Oslo, Norway for providing the normal testis DNA from organ donors.

References

1. Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC, Koivisto P, Lack EE, Kononen J, Kallioniemi OP, Gellman EP. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res 2000, 60:6111-6115.
2. He WW, Sciavolino PJ, Wing J, Augustus M, Hudson P, Meissner PS, Curtis RT, Shell BK, Bostwick DG, Tindall DJ, Gellman EP, Abate-Shen C, Carter KC: A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics 1997, 43:69-77.
3. Prescott JL, Blok L, Tindall DJ: Isolation and androgen regulation of the human homeobox cDNA, NKX3.1. Prostate 1998, 35:71-80.
4. Gellman EP, Bowen C, Bubendorf L: Expression of NKX3.1 in normal and malignant tissues. Prostate 2003, 55:111-117.
5. Skotheim RI, Korkmaz KS, Klokki TI, Abeler VM, Korkmaz CG, Nesland JM, Fosså SD, Lothe RA, Saatcioglu F: NKX3.1 expression is lost in testicular germ cell tumors. Am J Pathol 2003, 163:2149-2154.
6. Voeller HJ, Augustus M, Madike V, Bova GS, Carter KC, Gellman EP: Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res 1997, 57:4455-4459.
7. Svalwell JL, Vocke CD, Yang Y, Walker JR, Grouse L, Myers SH, Gillespie JW, Bostwick DG, Duray PH, Linehan WM, Emmert-Buck MR: Determination of a minimal deletion interval on chro-
mosome band 8p21 in sporadic prostate cancer. Genes Chromosom 2002, 33:201-205.
8. Vocke CD, Pozzatti RO, Bostwick DG, Florence CD, Jennings SB, Strup SE, Duray PH, Liotta LA, Emmert-Buck MR, Linehan WM: Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21. Cancer Res 1996, 56:2411-2416.
9. Emmert-Buck MR, Vocke CD, Pozzatti RO, Duray PH, Jennings SB, Florence CD, Zhuang Z, Bostwick DG, Liotta LA, Linehan WM: Allelic loss on chromosome 8p12-21 in microdissected prostatic intraepithelial neoplasia. Cancer Res 1995, 55:2959-2962.
10. Bhatia-Gaur R, Donjacour AA, Scialvolo PJ, Kim M, Desai N, Young P, Norton CR, Gridley T, Card iff RD, Cunha GR, Abate-Shen C, Shen MM: Roles for Nkx3.1 in prostate development and cancer. Genes Dev 1999, 13:966-977.
11. Abdulkadir SA, Magee JA, Peters TJ, Kaleem Z, Naughton CK, Humphrey PA, Milbrandt J: Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol Cell Biol 2002, 22:1495-1503.
12. Kim MJ, Bhatia-Gaur R, Banach-Petrosky WA, Desai N, Wang Y, Hayward SW, Cunha GR, Cardiff RD, Shen MM, Abate-Shen C: Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res 2002, 62:2999-3004.
13. Korkmaz CG, Korkmaz KS, Manol a J, Xi Z, Risberg B, Dan ielsen H, Kung J, Sellers WR, Loda M, Sait cioglu F: Analysis of androgen regulated homeobox gene NKX3.1 during prostate carcinogenesis. J Urol 2004, 172:1134-1139.
14. Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, Carter KC, Srivastava S: Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. J Urol 2000, 163:972-979.
15. Ornstein DK, Cinquanta M, Weiler S, Duray PH, Emmert-Buck MR, Vocke CD, Linehan WM, Ferretti JA: Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. J Urol 2001, 165:1329-1334.
16. Shen MM, Abate-Shen C: Roles of the Nkx3.1 homeobox gene in prostate organogenesis and carcinogenesis. Dev Dyn 2003, 228:767-778.
17. Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002, 3:415-428.
18. Esteller M: Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 2003, 532:39-49.
19. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003, 349:2042-2054.
20. Harrison J, Storzaker C, Clark Sj: Cytosines adjacent to methylated CpG sites can be partially resistant to conversion in genomic bisulphite sequencing leading to methylation artifacts. Anal Biochem 1998, 264:129-132.
21. Mostofi FK, Sesterhenn IA: World Health Organization International Histological Classification of Tumours: Histological typing of testis tumours Berlin: Springer-Verlag; 1998.
22. Kraggerud SM, Skotheim RI, Szyman ska J, Eknæs M, Fosså SD, Stenwig AE, Petomaki P, Lothe RA: Genome Profiles of Familial/Bilateral and Sporadic Testicular Germ Cell Tumors. Genes Chromosomes Cancer 2002, 34:168-174.
23. Gleason DF, Mellinger GT: Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. J Urol 1974, 111:58-64.
24. Grunau C, Clark Sj, Rosenthal A: Bisulphite genomic sequencing: systematic investigation of critical experimental parameters. Nucleic Acids Res 2001, 29:E65.
25. Fraga MF, Esteller M: DNA methylation: a profile of methods and applications. Biotechniques 2002, 33:632-649.
26. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996, 93:9821-9826.
27. Clark Sj, Harrison, J, Paul CL, Frommer M: High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994, 22:2990-2997.
28. Melki JR, Vincent PC, Clark Sj: Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res 1999, 59:3730-3740.