Radbound Repository

Radboud University Nijmegen

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/114097

Please be advised that this information was generated on 2017-12-06 and may be subject to change.

DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER DEVELOPMENT

Marion Bussemakers

DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER DEVELOPMENT

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Bussemakers, Maria Jacoba Gerarda

Differential gene expression in prostate cancer
development / Maria Jacoba Gerarda Bussemakers. - [S.L. :
s.n.] (Nijmegen : SSN). - Ill.
Proefschrift Nijmegen. - Met lit. opg. - Met samenvatting
in het Nederlands.
ISBN 90-9004078-1
Trefw.: prostaatkanker / differentiële genexpressie.

DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER DEVELOPMENT

een wetenschappelijke proeve op
het gebied van de natuurwetenschappen,
in het bijzonder de biochemie

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op
donderdag 25 juni 1992, des namiddags te 1.30 uur precies

door

Maria Jacoba Gerarda Bussemakers

geboren op 19 september 1964 te Helden

Druk SSN, Nijmegen

Promotores: Prof. Dr. H.P.J. Bloemers
Prof. Dr. F.M.J. Debruyne

Co-promotor: Dr. J.A. Schalken

The investigations presented in this thesis were performed in the Department of Biochemistry, University of Nijmegen and the Urological Research Laboratory of the Department of Urology, University Hospital of Nijmegen, The Netherlands.

These studies were financially supported by the Dutch Cancer Society (NUCC 90-01) and the Foundation for the Advancement of Urological Research (STIWU). This thesis was financially supported by the Dutch Cancer Society.

CONTENTS

		page
CHAPTER I	Genetic steps associated with prostate cancer development	7
CHAPTER II	Identification of high mobility group protein I(Y) as potential progression marker for prostate cancer by differential hybridization analysis	25
CHAPTER III	Increased expression of retroviral sequences in progressionally advanced rat prostatic tumors	43
CHAPTER IV	Differential expression of vimentin in rat prostatic tumors	53
CHAPTER V	Decreased expression of E-cadherin in the progression of rat prostatic cancer	61
CHAPTER VI	Decreased expression of E-cadherin in high grade prostate cancer	79
CHAPTER VII	Summary Samenvatting	91
LIST OF PUBLICATIONS		101
DANKWOORD		103
CURRICULUM VITAE		104

CHAPTER I

GENETIC STEPS ASSOCIATED WITH PROSTATE CANCER DEVELOPMENT

Marion J.G. Bussemakers, Frans M.J. Debruyne and Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands

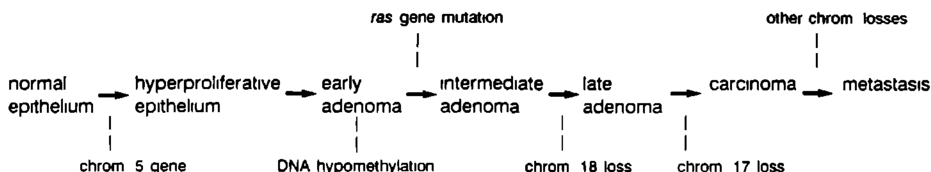
In: Oncogenes and Molecular Genetics of Urological Tumors (C.A. Olssen, Marberger, M. Eds.) SIU Monograph, 1992, Churchill Livingstone, Edinburgh, UK.
In Press

INTRODUCTION

Prostate cancer is an increasing medical problem: it is now the most commonly diagnosed cancer in the Western male population and the second leading cause of cancer deaths in man (1). Despite the increasing number of patients that manifest with clinical disease, only little is known about the mechanisms involved in the onset and progression of prostate cancer. However, it seems clear that prostate carcinogenesis is a multistep process leading first to histological prostate cancers that upon acquisition of additional malignant events develop into clinical disease (2). During the change of tumor phenotype from benign to malignant and eventually metastatic state, an accumulation of genetic changes (qualitatively as well as quantitatively) occurs (3). This often provides the target cell with the ability to circumvent controls that regulate growth thereby gaining the propensity to proliferate indefenitely. This increased proliferative capacity has two important consequences. First, there is an increased chance for the acquisition of genetic hits due to the fact that most genotoxic agents damage genes in cycling cells. Furthermore, the increased proliferative capacity is required for the outgrowth of the malignant population of cells. Genes implicated in uncontrolled proliferation are often identified as oncogenes or tumor suppressor genes. Progression to the more malignant state, i.e., acquisition of metastatic ability, requires that the cell can invade the surrounding tissue, spread through the lymphatic and/or blood circulation, extravasate and grow at secondary site, a process usually involving changes in other genes like those implicated in proteolysis, cell adhesion and cell motility.

The identification of the genetic steps associated with the onset and progression of cancer is now in the focus of molecular oncological research. The tremendous developments in molecular biology and immunology have provided a wide variety of tools to identify and study the target genes implicated in prostate carcinogenesis. Whereas a genetic cascade for the development of colon cancer development is now emerging (4), the situation for prostate cancer is still unclear. Moreover, morphological changes occuring in the etiology of prostate cancer are not unequivocally recognized, i.e., can we identify a hyperproliferative stage in the development that precedes the outgrowth into an adenocarcinoma, a carcinoma and a metastatic tumor? (Figure 1). The occurence of specific genetic changes and whether these occur early or late in this cascade is not yet known. This chapter reviews the results obtained today regarding the molecular steps associated with

prostate cancer. We divided this in the "indirect approach" in which known genes with potential relevance for cancer development are discussed, and the "direct approach" aimed at the characterization of molecular differences between aggressive- and non-aggressive cancers. Finally, we will discuss the potential of molecular biology in advanced diagnostics.



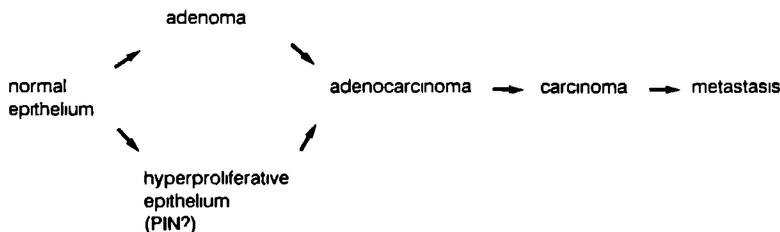


Figure 1: *Upper*: schematic presentation of the various stages as occuring during the progression of colorectal cancers and the molecular steps associated with this progression (4); *Lower*: schematic presentation of the various stages as they may occur in prostate cancer. The genetic events associated with the progression of prostate cancer remain to be established. (PIN = prostatic intraepithelial neoplasia).

THE "INDIRECT APPROACH" : STUDY KNOWN GENES WITH POTENTIAL RELEVANCE FOR CANCER DEVELOPMENT

Genes implicated in cell growth

The most striking advantage a cancer cell has over a non-cancer cell is its unlimited growth potential which is presumably associated with a corrupted cell cycle control. Basic studies on the mechanisms of growth control might give us clues which genes are implicated in the onset and progression of cancer. There are several groups of genes that are involved in growth control. To gain insight which of these genes are implicated in carcinogenesis, one has to consider several hundreds of genes like (proto)-oncogenes, tumor suppressor genes, genes encoding growth factors, growth factor receptors and transcription factors.

Oncogenes and tumor suppressor genes

For prostate cancer several proto-oncogenes have been studied at different levels: proto-oncogenes can be activated to oncogenes by mutations (qualitative changes) but also by changes in mRNA or protein expression levels (quantitative changes) (3). The possible involvement of the *ras*-family of oncogenes and proteins were extensively studied for prostate cancer. Peehl and associates (5) reported on the presence of an activated Ki-*ras* oncogene in a primary prostate cancer. Recently, however, it was shown that the frequency of *ras*-mutations in prostate cancer is rather low (6, 7). Studies at the *ras* mRNA levels in the Dunning R-3327 rat prostate adenocarcinoma model system showed no clear correlation of over-expression of the *ras*-oncogenes with tumor progression (8, 9) nor did studies in human prostatic carcinoma cell lines (10). Viola *et al.* (11) studied the *ras* gene product, p21, and showed an inverse relation between $p21^{ras}$ expression level and histological tumor grade. Furthermore, $p21^{ras}$ levels correlated with the frequency of nodal metastases. However, the monoclonal antibody used in these experiments was shown to be nonspecific in subsequent studies (12). More recently, Sumiya *et al.* (13) found a higher expression of $p21^{ras}$ in high grade and high stage tumors although in high stage tumors $p21^{ras}$ expression did not correlate with survival. Several other oncogenes were studied at the mRNA level in the Dunning model system (9, 14) or in human prostatic carcinoma cell lines (10) but no clear correlation of expression of any of the oncogenes studied with tumor progression was shown. Interestingly, Fleming *et al.* (15) and Buttyan *et al.* (16) found increased levels of *myc* expression

in high grade prostate cancers. The implications of these findings are still unclear.

Until now, no consistent genetic changes affecting (proto-)oncogenes are evident for prostate cancer. Similarly no consistent genetic changes are found in tumor suppressor genes: point mutations in p53, a tumor suppressor gene shown to be involved in several human cancers, were shown to be rare in human prostate cancers although in several prostate cell lines mutations in p53 were found (17). Loss of retinoblastoma (Rb) gene expression, presumably associated with a promoter deletion, was found in only one out of seven human prostate cancers and in one out of three cell lines, suggesting that Rb mutations are not common in prostate cancer. However, reintroduction of a normal Rb gene into DU145 cells (human prostate cancer cell line with a mutated Rb gene) resulted in the reversion of the tumorigenic phenotype of the cells (18).

In conclusion, *ras* and p53 mutations are by far not as frequently found in prostate cancer as in other solid tumors. However, future studies have to reveal whether prostate tumors containing *ras* and/or p53 mutations represent more aggressive subpopulations.

Growth factors and growth factor receptors.

The prostate is known to contain large amounts of growth factors like basic fibroblast growth factor (bFGF), transforming growth factor-ß (TGF-ß), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF). Also the receptors for these growth factors are present in the prostate gland (review (19)). Studies by Mori and associates (20) showed that bFGF is expressed at elevated levels in benign prostate hyperplasia (BPH) compared to normal prostatic tissue, suggesting that this growth factor might be involved in benign growth disorders of the human prostate. The fact that bFGF has been shown to be mitogenic to cultured prostate epithelial cells (21) provides additional evidence for a possible role of bFGF in abnormal growth of the prostate. TGF-ß2 is also expressed at elevated levels in BPH compared to normal prostate (20). TGF-ß1 is highly expressed in poorly-differentiated rat prostate tumors when compared to well-differentiated tumors (22). Considering its ability to induce angiogenesis (23) cell motility (24) and deposition of the extracellular matrix (25), TGF-ß1 might contribute to a more malignant phenotype (see next paragraph). EGF is expressed in both normal and malignant prostate tissue but especially the elevated levels of EGF-receptor and mRNA (26) in human prostate cancers when compared to normal prostate, might indicate a role for

EGF in the complex process of prostate carcinogenesis.

A unique feature of the prostate gland is its dependence on androgens. Furthermore, growth and differentiation of benign and malignant prostatic epithelial cells are regulated by androgens. This suggests an important role for androgens and the androgen receptor in prostate carcinogenesis. Experiments have shown the androgen-regulated DNA synthesis in the ventral prostate (27) and some genes encoding growth factors and some non-growth factor proto-oncogenes are under androgenic control: androgen ablation (by castration) leading to an active process of cell death, is associated with increased levels of c-*myc*, c-*fos* and TGFß1 mRNA (28, 29) and also an increased number of receptor binding sites for TGFß1 and EGF (30, 31). Upon castration and readministration of androgens, transient increases in steady-state levels of several genes like c-Ha-*ras*, c-Ki-*ras*, c-*myc*, c-*fos* and bFGF have been observed (32). These results suggest a complex regulation of gene expression by androgens and indicate that inappropriate androgenic regulation of proto-oncogenes and growth factors may contribute to the progression of prostate cancer. A role for the androgen receptor in prostate cancer has not been established: using monoclonal antibodies it was shown that loss of androgen receptor expression is not associated with the progression of prostate cancer. No correlation with tumor grade or stage was evident (33).

The importance of a diffusable factor or factors synthesized in response to androgens by prostate stromal cells was already reported by Cunha *et al.* (34). Tissue recombinations studies indicated that these factors induce the proliferation and/or differentation of prostate epithelial cells. Using this technique, Chung and associates (35) showed that fibroblasts can play a role in the development of prostate cancer. They also showed that the expression of six extracellular matrix genes was decreased upon transformation (36), suggesting not only the involvement of paracrine growth factors but also of the extracellular matrix (see also next paragraph) in prostate carcinogenesis. Additional evidence for the presence of diffusible factors was reported by Djakiew and colleagues (37), who showed the stimulation of prostate epithelial cell growth by factors secreted by prostate stromal cells. Furthermore, paracrine growth stimulation is not only implicated in local growth of the prostate but also in the formation of metastasis: PC3 cells were shown to secrete a factor that stimulates the growth of bone cells, indicating that this factor might play a role in osteoblastic metastases as seen in prostatic cancer (38).

Genes implicated in invasion and metastasis

During the progression of a tumor to a metastatic state, cancer cells have to acquire the ability to invade locally into small blood vessels and/or lymphatic systems; if the cells survive the host defence/surveillance mechanisms, they have to extravasate and move into surrounding organ tissue and finally proliferate to give rise to clinical apparent metastases (39). The acquisition of metastatic properties is probably due to changes in the expression of genes involved in cell attachment and cell motility. One might expect increasing levels of degradative enzymes (like proteases) or decreasing levels of their inhibitors (like TIMP = tissue inhibitors of metalloproteinases). The expression levels of cell adhesion molecules may change and also the expression of components that aid the cells in escaping host defence mechanisms might be important. For prostate cancer, the genes involved in the acquisition of metastatic abililty are largely unknown. Of the genes that might be important in cell attachment and cell motility, only few were studied: increased levels of plasminogen activors were shown in human prostatic cancers (40) and in rat model systems (41); secretion of collagenase by a rat prostatic epidermoid carcinoma in culture has been reported (42) and also elevated activities of elastase and a chymotrypsin-like protease were found in metastatic Dunning tumors (43). Recently, it was shown that E-cadherin, a calcium-dependent cell adhesion molecule is down regulated in invasive rat prostate cancers (44). The association of changes in the expression of different components of the extracellular matrix with a transformed phenotype of rat prostate fibroblasts (36) was already mentioned above. To be complete on our knowledge on invasion or metastasis related genes, it should be mentioned that fibronectin was found to be down modulated in metastatic prostate cancer cells (45) although these observations were obtained by a direct approach (differential hybridization analysis).

THE "DIRECT APPROACH" : IDENTIFY MOLECULAR CHARACTERISTICS OF PROGRESSIONALLY ADVANCED PROSTATE CANCER CELLS

Loss of heterozygosity

The low mitotic index of prostate tumors and the difficulty to grow prostate cancers in culture, made studies on chromosomal changes associated with the onset and progression of prostate cancer rather difficult. Nonetheless, the involvement of

some specific chromosomal changes have thus been identified. As summarized by Brothman and associates (46), loss of chromosomes 1, 2, 5, 11 and Y, trisomy of chromosomes 7, 14, 20 and 22, and structural changes involving chromosomal segments 2p, 7q and 10q are the most common changes reported. Of these, deletions of 7q and 10q were found in late stage cancers (47, 48). These findings, however, are all based on small numbers of patients. A less complicated technique that overcomes the problems associated with the low mitotic index in prostate cancer, is allelotyping: using DNA probes that recognize Restriction Fragment Length Polymorphisms (RFLPs), one can identify deletions of (parts of) chromosomes. Using this technique, the frequent deletion of the long arm of chromosome 17 in colon cancer was revealed, leading to the identification of p53 as a potential tumor suppressor gene (49). Also a candidate tumor suppressor gene on chromosome 18 was identified after an initial lead obtained from RFLP analysis (50). RFLP analysis now provides a technique to test the observations on chromosome deletion in prostate cancer as mentioned above in a large group of patients and also to include all known chromosomal loci that are thus far reported to contain potential tumor suppressor genes. Carter *et al.* (51) showed that whereas the loss of chromosome 10 indeed frequently occured (30 % of cases studied) an as yet unreported loss of chromosome 16 was observed even more frequently. These results suggest that chromosome 10q and 16q should be studied in more detail to identify a relation between allelic loss and progression of the tumor and to identify candidate tumor suppressor genes located on these chromosomes.

Monoclonal antibodies

The development of the hybridoma technology (52) offered the ability to produce antibodies detecting specific antigens. Using tumor cell extracts it is possible to raise antibodies against tumor specific antigens. Although the use of monoclonal antibodies as progression markers has an established use in many disciplines, for prostate cancer research it is still an underexplored approach. Whereas the isolation of several prostate cancer monoclonal antibodies was reported (53, 54, 55), none of them was shown to be useful as progression markers. Although TURP-27 (55) has only limited value as progression marker, it was interesting to learn that the antigens recognized by this antibody are related to those recognized by HNK-1 (56). HNK-1 recognizes antigens related to the neural cell adhesion molecule (N-CAM). Recently, a new monoclonal antibody specific to prostate cancer, PD41 was described (57). Its

value for diagnosis and the nature of the antigen remain to be established.

Differential hybridization analysis

Another direct approach to identify molecular differences associated with prostate tumor development is based on the comparison of steady-state mRNA populations, such as differential or subtraction hybridization analysis. These techniques enable the identification of genes expressed at different levels but do not allow the identification of genes aberrantly expressed. Furthermore, differential or subtraction hybridization analysis has several advantages: one can screen for genes which are upregulated as well as down regulated; the reagents which become available, i.e., cDNA clones, are easy to characterize by DNA sequence analysis and computer-assisted database comparison; finally, usefulness of the reagent in diagnosis can be evaluated immediately by RNA *in situ* hybridization. The techniques of differential and subtraction hybridization have been succesfully used in identifying genes induced by growth factors (58, 59, 60) or genes differentially expressed when comparing normal *versus* malignant tissue (61, 62). For metastasis, few reports are available, most of them reporting on down regulation of gene expression during progression. In melanoma cells with low metastatic potential, NM23 is down regulated (63); in metastatic mammary adenocarcinoma WDNM1 and -2 are down regulated (64, 65); and for rat metastasizing prostate cancer cells a down modulation of fibronectin was shown (45). These results indicate that as in tumorigenesis, suppressor genes might also be involved in metastasis (metastasis suppressor genes). For prostate cancer, we recently identified two cDNA clones that are overexpressed in metastatic rat prostate cancers (66). One of those was found to be rather specific for metastasizing tumors and upon DNA sequence analysis was shown to be identical or related to High Mobility Group protein I (Y). HMG-I(Y) is a small, non-histon, nuclear protein implicated in transcription and replication processes. Its overexpression in dedifferentiated, fast-proliferating cells was reported earlier (67). The value of HMG-I(Y) as progression marker needs further investigation. The second cDNA clone contained rat-specific LTR-like sequences and is not likely to be useful for human diagnosis. Interestingly, Liu & Abraham (68), studying differential gene expression in human prostatic cancer cell lines, identified a cDNA containing human endogenous retroviral sequences spliced to human calbindin. The possible role of retroviral sequences in prostate cancer, however, remains unclear.

DISCUSSION AND PERSPECTIVES

The major questions in prostate cancer, i.e., what is the cellular origin of cancer and which molecular steps are implicated in its development, are essentially still open. There are some indications of the importance of as yet unidentified tumor suppressor genes and several progression markers are under investigation. However, the question remains whether these markers can be used in a routine setting or if they will require more advanced technology.

Interphase cytogenetics

The frequent loss of chromosomes 10 and 16 might prove to be useful progression markers in prostate cancer diagnosis. Since RFLP analysis is a technique with little feasibility for a pathological setting, interphase cytogenetics might be a better approach (69). This technique involves the *in situ* hybridization of interphase nuclei using chromosome specific probes and enables the study of numerical chromosomal aberrations. Thus a study on a large group of patients with superficial bladder cancer showed the frequent loss of chromosome 9 and the frequent gain of chromosome 1 (70). Until now, the chromosome specific probes used, often recognize the centromeric region of the chromosome, thereby not allowing the detection of arm deletions. The use of cosmid clones might enable the analysis of more specific regions for over/under representation of the genome in cancer cells. Of further importance for interphase cytogenetics is the definition of the area of interest, especially considering the tumor cell heterogeneity in prostate cancer. Since it is not clear yet whether reliable *in situ* interphase cytogenetics (i.e., on frozen- and paraffin-embedded sections) will be possible, the nuclei preparations need to be made from pathologically defined sections. However, the technique is potentially powerful to study gross genetic aberrations associated with prostate tumor progression.

RNA in situ hybridization

Progression markers identified by differential/subtraction hybridization analysis, are isolated as cDNA clones. If they represent known genes and if antibodies against these genes are available, immunohistochemical studies can be performed - on fresh and/or archival material - to establish the importance of these progression markers. If, however, an unknown gene is identified or if an antibody is not available, one

might consider RNA *in situ* hybridization to study primary prostate samples. Since RNA molecules are extremely sensitive to degradation, the use of RNA *in situ* hybridization requires careful tissue handling. This aspect of the technique may impair its use in a routine setting.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) technique has many applications and many publications have already shown its potential (overview: 71). The technique is based on the exponential amplification of small amounts of target sequences and one needs only little material in contrast to most standard protocols. For a diagnostic approach one might even consider the use of tissue-sections (72). PCR is mainly a simple procedure; only the adequate choice of the primers is of great importance and since the technique is very sensitive, one must realize that minor contaminations might already interfere with the results of the experiment. Also the quantification of the amplification is still cumbersome.

Recently, two methods were developed, enabling the (relative easy) detection of point mutations. The technique of Single Strand Conformation Polymorphism PCR (SSCP-PCR) is based on the fact that single stranded DNA when run under non-denaturing conditions forms a secondary conformation that will change due to (point) mutations (73). The various conformations have a different motility in a non-denaturing gel and can be discriminated accordingly. Thus, Suzuki *et al.* (74) studied a great number of mutations in the *ras*-oncogene. When studying a gene with known hot-spots for mutation, the technique can be extremely useful to analyse large numbers of patients. The second technique, Denaturing Gradient Gel Electrophoresis (DGGE), is based on the fact that due to changes in nucleotide sequence, DNA-fragments have different melting point characteristics which can be visualized when run in a denaturing gel (75). Recently, the slightly modified technique of Constant Denaturant Gel Electrophoresis (CDGE), was used to rapidly screen for p53 mutations in breast carcinomas (76). Considering the many applications and possibilities of the technique, PCR based technology is likely to become a routine instrument in molecular (uro)pathological analysis.

In conclusion, there are only few markers available yet that have potential use in the prediction of the metastatic ability of prostate cancer cells. Considering the increasing number of patients that are diagnosed with prostate cancer, the

identification of additional molecular markers for prostate cancer is of great importance. Also the development and/or improvement of discriminative screening techniques that can be used in a routine setting, should be stimulated, i.e., future studies should aim at a screening program for prostate cancer in which the prediction of the aggressiveness of the individual tumor has a crucial role.

OUTLINE OF THE THESIS

In this thesis, molecular biological approaches aiming at the identification of new molecular markers that may improve the prediction of the aggressiveness of prostatic tumors, are described. For the studies described in **Chapters II** through **V**, the Dunning R-3327 rat prostatic cancer model system was used. This model system consists of more than ten sublines, representing the various stages of prostate tumor progression and being characterized by parameters such as tumor growth rate, histology, androgen-dependency and metastatic ability. In **Chapter VI** is reported on a study using human prostate tumors to test findings obtained in the animal model system.

As a first approach to identify new molecular markers for progressively advanced prostate tumors, the technique of differential hybridization analysis was applied to compare steady-state mRNA levels of the hormone-dependent, well-differentiated, non-metastasizing Dunning R-3327-H tumor and the hormone-independent, anaplastic, metastasizing Dunning R-3327-MatLyLu tumor. The first experiment revealed three cDNA clones that were overexpressed in the progressionally advanced MatLyLu tumor (**Chapter II**). The mRNA expression patterns of two of the cDNA clones showed some similarities and further characterization of these clones revealed the relation between both cDNA clones (**Chapter III**). A second attempt to identify differentially expressed genes, revealed the overexpression of vimentin in the anaplastic, invasive tumor lines of the Dunning R-3327 model system. This overexpression of vimentin raised some questions since vimentin is usually specifically expressed in tissues of mesenchymal origin, however, the prostate is of epithelial origin and the exclusive expression of cytokeratins would be expected (**Chapter IV**).

The second approach that was used to reach our goal was an indirect one: the expression of a known gene with potential releveance for carcinogenesis was studied.

As already mentioned in the introduction, several hundreds of genes may be considered of being implicated in carcinogenesis. The studies described in **Chapter V** and **VI** deal with the expression of the Ca^{2+}-dependent cell adhesion molecule E-cadherin in the Dunning R-3327 model system and human tumors, respectively. E-cadherin is involved in cell-cell interactions and is indicated to play an important role in invasion and metastasis. The E-cadherin expression was studied at both mRNA and protein levels in order to substantiate the possible involvement of E-cadherin in the invasive and/or metastatic behaviour of prostatic tumors.

REFERENCES

1 Boring, C.C., Squires, T.S., and Tong, T. Cancer Statistics 1991. CA, 41: 19-36, 1991.

2 Carter, H.B., Piantadosi, S., and Isaacs, J.T. Clinical evidence for and implications of the multistep development of prostate cancer. J. Urol., 143: 742-746, 1990.

3 Nicolson, G.L. Quantitative variations in gene expression: possible role in cellular diversification and tumor progression. J. Cell. Biochem., 46: 277-283, 1991.

4 Fearon, E.R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, 61: 759-767, 1990.

5 Peehl, D.M., Wehner, N., and Stamey, T.A. Activated Ki-*ras* oncogene in human prostatic adenocarcinoma. Prostate, 10: 281-289, 1987.

6 Carter, B.S., Epstein, J.I., and Isaacs, W.B. *Ras* gene mutations in human prostate cancer. Cancer Res., 50: 6830-6832, 1990.

7 Gumerlock, P.H., Poonamallee, U.R., Meyers, F.J., and DeVere White, R.W. Activated *ras* alleles in human carcinoma of the prostate are rare. Cancer Res., 51: 1632-1637, 1991.

8 Cooke, D.B., Quarmby, V.E., Petrusz, P., Mickey, D.D., Der, C.J., Isaacs, J.T., and French, F.S. Expression of *ras* proto-oncogenes in the Dunning R-3327 rat prostatic adenocarcinoma system. Prostate, 13: 273-288, 1988.

9 Bussemakers, M.J.G., Isaacs, J.T., Debruyne, F.M.J., Van de Ven, W.J.M., and Schalken, J.A. Oncogene expression in prostate cancer. World J. Urol., 9: 58-63, 1991.

10 Rijnders, A.W.M., Van der Korput, J.A.G.M., Van Steenbrugge, G.J., Romijn, J.C., and Trapman, J. Expression of cellular oncogenes in human prostatic carcinoma cell lines. Biochem. Biophys. Res. Comm., 132: 548-554, 1985.

11 Viola, M.V., Fromowitz, F., Ovarez, S., Deb, S., Finkel, G., Lundy, J., Hand, P., Thor, A., and Schlom, J. Expression of *ras* oncogene p21 in prostate cancer. N. Engl. J. Med., 314: 133-137, 1986.

12 Samowitz, W.S., Paull, G., and Hamilton, S.R. Reported binding of monoclonal antibody RAP-5 to formalin-fixed tissue sections is not indicative of *ras* p21 expression. Hum. Pathol., 19: 127-132, 1988.

13 Sumiya, H., Masai, M., Akimoto, S., Yatani, R., and Shimazaki, J. Histochemical examination of expression of *ras* p21 protein and R1881-binding protein in human prostatic cancers. Eur. J. Cancer, 26: 786-789, 1990.

14 Cooke, D.B., Quarmby, V.E., Mickey, D.D., Isaacs, J.T., and French, F.S. Oncogene expression in prostate cancer: Dunning R-3327 rat dorsal prostatic adenocarcinoma system. Prostate, 13: 263-272, 1988.

15 Fleming, W.H., Hamel, A., MacDonald, R., Ramsey, E., Pettigrew, N.M., Johnston, B., Dodd, J.H., and Matusik, R.J. Expression of the c-*myc* protooncogene in human prostatic carcinoma and benign prostatic hyperplasia. Cancer Res., 46: 1535-1538, 1986.

16 Buttyan, R., Sawczuk, I.S., Benson, M.C., Siegal, J.D., and Olsson, C.A. Enhanced expression of

the c-*myc* protooncogene in high-grade human prostate cancers. Prostate, 11: 327-337, 1987.

17 Isaacs, W.B., Carter, B.S., and Ewing, C.M. Wild type p53 suppresses growth of human prostate cancer cells containing mutant p53 alleles. Cancer Res., 51: 4716-4720, 1991.

18 Bookstein, R., Shew, J.-Y., Chen, P.-L., Scully, P., and Lee, W.-H. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. Science, 247: 712-715, 1990.

19 Thompson, T.C. Growth factors and oncogenes in prostate cancer. Cancer Cells, 2: 345-354, 1990.

20 Mori, H., Maki, M., Oishi, K., Jaye, M., Igarashi, K., Yoshida, O., and Hatanaka, M. Increased expression of genes for basic fibroblast growth factor and transforming growth factor type ß-2 in human benign prostatic hyperplasia. Prostate, 16: 71-80, 1990.

21 McKeehan, W.L., Adams, P.S., and Fast, D. Different hormonal requirements for androgen-independent growth of normal and tumor epithelial cells from rat prostate. In Vitro Cell. Dev. Biol., 23: 147-152, 1987.

22 Wilding, G., Zugmeier, G., Knabbe, C., Flanders, K., and Gelmann, E. Differential effects of transforming growth factor ß on human prostate cancer cells *in vitro*. Mol. Cell. Endocrinol., 62: 79-87, 1989.

23 Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauchi, A.S. Transforming growth factor type ß: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc. Natl. Acad. Sci. USA, 83: 4167-4171, 1986.

24 Myrdal, S.E., Twardzik, D.R., and Auersperg, N. Cell-mediated co-action of transforming growth factors: incubation of type ß with normal rat kidney cells produces a soluble activity that prolongs the ruffling response to type α. J. Cell Biol., 102: 1230-1234, 1986.

25 Rizzinno, A. Transforming growth factor-ß: multiple effects on cell differentiation and extracellular matrices. Dev. Biol., 130: 411-422, 1988.

26 Morris, G.L., and Dodd, J.G. Epidermal growth factor receptor mRNA levels in human prostatic tumors and cell lines. J. Urol., 143: 1272-1274, 1990.

27 Coffey, D.S., and Williams-Ashman, H.G. Polymerization of deoxyribonucleotides in relation to androgen-induced prostate growth. Arch. Biochem. Biophys., 124: 184-198, 1968.

28 Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. Cascade induction of c-*fos*, c-*myc*, and heat-shock 70 K transcripts during regression of the rat ventral prostate. Mol. Endocrinol., 2: 650-657, 1988.

29 Kyprianou, N., and Isaacs, J.T. Expression of transforming growth factor-ß in the rat ventral prostate during castration-induced programmed cell death. Mol. Endocrinol., 3: 1515-1522, 1989.

30 Kyprianou, N., and Isaacs, J.T. Identification of a cellular receptor for transforming growth factor-ß in rat ventral prostate and its negative regulation by androgens. Endocrinology, 123: 2124-2131, 1988.

31 St.Arnaud, R., Poyet, P., Walker, P., and Labrie, F. Androgens modulate epidermal growth factor receptor levels in the rat ventral prostate. Mol. Cell. Endocrinol., 56: 21-27, 1988.

32 Katz, A.E., Benson, M.C., Wise, G.J., Olsson, C.A., Bandyk, M.G., Sawczuk, I.S., Tomashefsky, P., and Buttyan, R. Gene activity during the early phase of androgen-stimulated rat prostate regrowth. Cancer Res., 49: 5889-5894, 1989.

33 Van der Kwast, T.H., Schalken, J.A., Ruizeveld-de Winter, J.A., Van Vroonhoven, C.C.J., Mulder, E., Boersma, W., and Trapman, J. Androgen receptors in endocrine-therapy-resistant human prostate cancer. Int. J. Cancer, 48: 189-193, 1991.

34 Cunha, G.R., Chung, L.W.K., Shannon, J.M., Taguchi, O., and Fujii, H. Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions. Rec. Progr. Horm. Res., 39: 559-598, 1983.

35 Chung, L.W.K., Chang, S.-M., Bell, C., Zhau, H.E., Ro, J.Y., and Von Eschenbach, A.C. Co-inoculation of tumorigenic rat prostate mesenchymal cells with non-tumorigenic epithelial cells results in the development of carcinosarcoma in syngeneic and athymic animals. Int. J. Cancer, 43: 1179-1187, 1989.

36 Freeman, M.R., Song, Y., Carson, D.D., Guthrie, P.D., and Chung, L.W.K. Extracellular matrix and androgen receptor expression associated with spontaneous transformation of rat prostate fibroblasts. Cancer Res., 51: 1910-1916, 1991.

37 Djakiew, D., Tarkington, M.A., and Lynch, J.H. Paracrine stimulation of polarized secretion

from monolayers of a neoplastic prostatic epithelial cell line by prostatic stromal cell proteins. Cancer Res., 50: 1966-1974, 1990.

38 Perkel, V.S., Mohan, S., Herring, S.J., Baylink, D.J., and Linkhart, T.A. Human prostatic cancer cells, PC3, elaborate mitogenic activity which selectively stimulates human bone cells. Cancer Res., 50: 6902-6907, 1990.

39 Hart, I.R., Goode, N.T., and Wilson, R.E. Molecular aspects of the metastatic cascade. Biochim. Biophys. Acta, 989: 65-84, 1989.

40 Gaylis, F.D., Keer, H.N., Wilson, M.J., Kwaan, H.C., Sinha, A.A., and Kozlowski, J.M. Plasminogen activators in human prostate cancer cell lines and tumors: correlation with the aggressive phenotype. J. Urol., 142: 193-198, 1989.

41 Wilson, M.J., Ditmanson, J.V., Sinha, A.A., and Estensen, R.D. Plasminogen activator activities in the ventral and dorsolateral prostatic lobes of ageing Fischer 344 rats. Prostate, 16: 147-161, 1990.

42 Huang, C.-C., Wu, C.-H., and Abramson, M. Collagenase activity in cultures of rat prostate carcinoma. Biochim. Biophys. Acta, 570: 149-156, 1979.

43 Lowe, F.C., and Isaacs, J.T. Biochemical methods for predicting metastatic ability of prostatic cancer utilizing the Dunning R-3327 rat prostatic adenocarcinoma system as a model. Cancer Res., 44: 744-752, 1984

44 Bussemakers, M.J.G., Van Moorselaar, R.J.A., Giroldi, L.A., Ichikawa, T., Isaacs, J.T., Takeichi, M., Debruyne, F.M.J., and Schalken, J.A. Decreased expression of E-cadherin in rat prostate cancer cells. Cancer Res., 52: In Press, 1992.

45 Schalken, J.A., Ebeling, S.B., Isaacs, J.T., Treiger, B., Bussemakers, M.J.G., De Jong, M.E.M., and Van de Ven, W.J.M. Down modulation of fibronectin mRNA in metastasizing rat prostatic cancer cells revealed by differential hybridization analysis. Cancer Res., 48: 2042-2048, 1988.

46 Brothman, A.R., Peehl, D.M., Patel, A.M., and McNeal, J.E. Frequency and pattern of karyotypic abnormalities in human prostate cancer. Cancer Res., 50: 3795-3803, 1990.

47 Atkin, N.B., and Baker, M.C. Chromosome 10 deletion in carcinoma of the prostate. N. Engl. J. Med., 312: 315, 1985.

48 Atkin, N.B., and Baker, M.C. Chromosome study of five cancers of the prostate. Hum. Genet., 70: 359-364, 1985.

49 Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., Van Tuinen, P., Ledbetter, D.H., Barker D.F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science, 244: 217-221, 1989.

50 Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W., And Vogelstein, B. Identification of a chromosome 18q gene that is altered in colorectal cancers. Science, 247: 49-56, 1990.

51 Carter, B.S., Ewing, C.M., Ward, W.S., Treiger, B.F., Aalders, T.W., Schalken, J.A., Epstein, J.I., and Isaacs, W.B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc. Natl. Acad. Sci. USA, 87: 8751-8755, 1990.

52 Kohler, G., and Milstein, G. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 265: 493-495, 1975.

53 Webb, K.S., Paulson, D.F., Parks, S.F., Tuck, F.L., Walther, P.J., and Ware, J.L. Characterization of prostate tissue directed monoclonal antibody: alpha-Pro 13. Cancer Immunol. Immunother., 17: 7-17, 1984.

54 Lindgren, J., Pak, K.Y., Ernst, C., Rovera, G., Steplewski, Z., and Koprowski, H. Shared antigens of human prostate cancer cell lines as defined by monoclonal antibodies. Hybridoma, 4: 37-45, 1985.

55 Starling, J.J., Sieg, S.M., Beckett, M.L., Wirth, P.R., Wanab, Z., Schellhammer, P.F., Ladaga, L.E., Poleskic, S., and Wright Jr., G.L.Human prostate tissue antigens defined by murine monoclonal antibodies. Cancer Res., 46: 367-374, 1986.

56 Lipford, G.B., and Wright Jr., G.L. Comparative study of monoclonal antibodies TURP-27 and HNK-1: their relationship to neural cell adhesion molecules and prostate tumor-associated antigens. Cancer Res., 51: 2296-2301, 1991.

57 Beckett, M.L., Lipford, G.B., Haley, C.L., Schellhammer, P.F., and Wright Jr., G.L. Monoclonal antibody PD41 recognizes an antigen restricted to prostate adenocarcinomas. Cancer Res., 51:

1326-1333, 1991.
58 Lau, L.F., and Nathans, D. Identification of a set of genes expressed during G0/G1 transition of cultured mouse cells. EMBO J, 4: 3145-3151, 1985.
59 Lau, L.F., and Nathans, D. Expression of a set of growth-related immediate early genes in Balb/c 3T3 cells: coordinate regulation with *c-fos* or *c-myc*. Proc. Natl. Acad. Sci. USA, 84: 1182-1186, 1987.
60 Almendral, J.M., Sommer, D., MacDonald-Bravo, H, Burckhardt, J., Perera, J., and Bravo, R. Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol., 8 2140-2148, 1988.
61 Augentlicht, L.H., and Kobrin, D. Cloning and screening of sequences expressed in a mouse colon tumor. Cancer Res., 42. 1088-1093, 1982.
62 Matrisian, L.M., Bowden, G.T., Krieg, P., Fürstenberger, G., Briand, J.-P., Leroy, P., and Breatnach, R. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA, 83: 9413-9417, 1986.
63 Steeg, P.S., Bevilacqua, G.B., Kopper, L., Thorgeirsin, U.P., Talmadge, J.E., Liotta, L., and Sobel, M.E. Evidence for a novel gene associated with low tumor metastatic potential. J Natl. Cancer Inst., 80. 200-204, 1988.
64 Dear, T.N, Ramshaw, I.A., and Kefford, R.F. Differential expression of a novel gene, WDNM1, in nonmetastatic rat mammary adenocarcinoma cells. Cancer Res., 48: 5203-5209, 1988
65 Dear, T.N., McDonald, D.A., and Kefford, R.F. Transcriptional down-regulation of a rat gene, WDNM2, in metastatic DMBA-8 cells. Cancer Res, 49: 5323-5328, 1989.
66 Bussemakers, M.J.G., Van de Ven, W.J.M., Debruyne, F.M.J., and Schalken, J.A. Identification of high mobility group protein I (Y) as potential marker for prostate cancer by differential hybridization analysis. Cancer Res, 51: 606-611, 1991.
67 Johnson, K.R, Lehn, D.A., Elton, T.S., Barr, P.J., and Reeves, R. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG I(Y). J. Biol Chem., 263. 18338-18342, 1988.
68 Liu, A.Y., and Abraham, B.A. Subtractive cloning of a hybrid human endogenous retrovirus and calbindin gene in the prostate cell line PC3. Cancer Res., 51: 4107-4110, 1991.
69 Hopman, A.H.N., Ramaekers, F.C.S., Raap, A.K., Beck, J.L.M., Devilee. P., Van der Ploeg, M., and Vooijs, G.P. *In situ* hybridization as a tool to study numerical chromosomal aberrations in solid bladder tumors. Histochemistry, 89: 307-316, 1988.
70 Hopman, A.H.N., Moesker, O., Smeets, A.W.C.B., Pauwels, R.P.E., Vooijs, G.P., and Ramaekers, F.C.S. Numerical chromosome 1, 7, 9 and 11 aberrations in bladder cancer detected by *in situ* hybridization. Cancer Res., 51: 644-651, 1991.
71 Erhlich, H.A. (1989) PCR technology; principles and applications for DNA amplification. Stockton Press, New York
72 Coates, P.J., D'Ardenne, A.J., Khan, G., Kangro, H.O., and Slavin, G. Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J. Clin. Pathol., 44: 115-118, 1991.
73 Orita, M, Suzuki, Y., Sekiya, T., and Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics, 5: 874-879, 1989.
74 Suzuki, Y., Orita, M., Shiraishi, M., Hayashi, K., and Sekiya, T. Detection of *ras* gene mutations in human lung cancers by single-strand conformation analysis of polymerase chain reaction products. Oncogene, 5: 1037-1043, 1990.
75 Meyers, R.M., Maniatis, T., and Lerman, L.S. Detection and localization of single base changes by denaturing gradient gel electrophoresis Methods in Enzymology, 155: 501-527, 1987
76 Børresen, A.-L., Hovig, E., Smith-Sørensen, B., Malkin, D., Lystad, S., Andersen T.I., Nesland, J.M, Isselbeacher K.J., and Friend S.H. Constant denaturant gel electrophoresis as a rapid screening technique for p53 mutations. Proc. Natl. Acad. Sci. USA, 88: 8405-8409, 1991.

CHAPTER II

IDENTIFICATION OF HIGH MOBILITY GROUP PROTEIN I(Y) AS POTENTIAL PROGRESSION MARKER FOR PROSTATE CANCER BY DIFFERENTIAL HYBRIDIZATION ANALYSIS

Marion J.G. Bussemakers, Wim J.M. van de Ven (1), Frans M.J. Debruyne, and Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands, (1) Department of Biochemistry, University of Nijmegen, The Netherlands

Cancer Research 51: 606-611, 1991.

SUMMARY

One of the major problems in the diagnosis of localized prostatic tumors is to predict the aggressiveness of an individual tumor, which is presumably associated with chance to progression. In an attempt to find molecular markers that are specific for aggressive prostatic cancer cells, we compared steady-state mRNA levels of progressionally related prostatic tumors. The Dunning R-3327-H subline, a relatively benign rat prostatic tumor, was compared to the therefrom derived highly aggressive MatLyLu tumor by differential hybridization analysis. The differential screening revealed 26 complementary DNA clones that detected transcripts overexpressed in MatLyLu. Upon further screening on the entire panel of Dunning R-3327 sublines, it appeared that three clones (pBUS1, pBUS19 and pBUS30), detected transcripts specifically expressed in metastatic rat prostatic tumors. The expression pattern of pBUS19 and pBUS30 suggested a relation between these cDNAs. Nucleotide sequence analysis, however, could not yet substantiate this. Computer-assisted comparison of the DNA sequences revealed the presence of rat long terminal repeat-like repetitive elements in pBUS19. The differential expression of repetitive elements in progressionally related tumors is interesting, yet similar findings have not been reported in humans malignancies. Nucleotide sequence analysis of pBUS1 indicated that this clone is identical or related to High Mobility Group protein I(Y), a non-histone, nuclear protein. From recent studies it appeared that this protein might be implicated in replication and/or transcription processes, and is induced in fast proliferating/undifferentiated cells. The overexpression of HMG-I(Y) correlates rather with metastatic ability than with growth rate, hence it may serve as valuable marker to identify progressionally advanced prostate cancer cells.

INTRODUCTION

Among cancer related deaths in the American male population, prostate cancer is the second leading cause (i.e., 11 %). Moreover, prostate cancer accounts for 21 % of all newly diagnosed cancers in that group (highest incidence) (1). The majority of patients presents clinically with localized disease (Stage A-C). Patients with truly localized disease (i.e., no capsular penetration, stage A-B), are amenable to curative radical surgery. However, a considerable fraction of this group clinically progress to a metastatic state. This can be explained only by the fact that at the time of surgery, dissemination of tumor cells had already occurred resulting in microscopic metastases. Identification of patients at risk for having such micrometastases is likely to be of great significance, since experimental therapeutical studies revealed that small disseminated lesions are still amenable to curative chemotherapy, whereas they are not later in their clinical progression (2). Clearly, adjuvant chemotherapy for all stage A-B patients is unacceptable since the majority of patients would be overtreated.

Prediction of the aggressiveness of an individual tumor can be achieved by histological examination of the primary tumor. Whereas classical pathological grading, according to Gleason (3), is not able to discriminate tumors that do progress clinically from those that do not, analysis of nuclear morphometrical characteristics seems to be more promising (4). Another approach is to identify molecular characteristics specific for aggressive tumor cells. Thus far no markers are available that meet the demands mentioned above.

A method that is useful to isolate and characterize molecular markers for progressionally advanced cancer cells is comparison of steady-state mRNA levels by differential or subtraction hybridization analyses. Earlier studies using the well characterized rat prostatic cancer Dunning R-3327 model system, revealed that fibronectin is down-modulated upon progression from anaplastic non-metastasizing rat prostate tumors to metastasizing tumors (5). So far, there are no clinical implications of these findings for the early steps in the progression of prostate cancer, since well differentiated tumors also have a low expression of fibronectin. Furthermore, loss of a molecular characteristic is less suitable for diagnostic purposes. In the present study, we used the same technique to compare the most benign tumor from the Dunning R-3327 rat prostatic cancer model system, the H-tumor, with the metastatic MATLyLu tumor by differential hybridization analysis,

screening, in this case, for up-regulation of genes.

The cDNA clones that detected differentially expressed genes were evaluated for their relation to the aggressive phenotype by screening ten Dunning R-3327 sublines. Finally, the cDNA clones that met the selection criteria were sequenced and the resulting nucleotide sequences were compared to nucleotide databases to search for homology with known genes.

MATERIALS AND METHODS

Dunning R-3327 rat prostatic tumors

The parental tumor from which all rat prostatic tumor sublines were derived is the original R-3327 tumor described by Dunning (6). To denote differences in the history and characteristics of the various R-3327 tumors passaged at several institutes, the tumor lines are denoted by different letters. The G subline was developed by Dunning and is poorly differentiated but androgen responsive (7). The G subline was generously provided by Dr. Alice Claflin (University of Miami). The slow-growing, well-differentiated subline obtained from Dr. Arthur Bogden has been serially passaged at The Johns Hopkins University (Baltimore, Md) and was termed R-3327-H.

The H subline is a well-differentiated, androgen-responsive tumor. The H tumor was shown to be a heterogeneous tumor composed of both androgen-dependent and -independent cells (8). By growing the H tumor in castrated rats it appeared to be possible to select *in vivo* for the androgen-independent subpopulation; the resulting slow-growing, well-differentiated line was termed HIS. By continuous passaging of HIS, random tumor progression gave rise to a moderately fast-growing, well-differentiated tumor, HIM, and an even faster growing, moderately-differentiated subline, HIF. The sublines AT1 and AT2 arose from the Dunning R-3327-H tumor passaged at Johns Hopkins (7). All these lines arose within one passage and are anaplastic, hormone-independently growing tumors with a low metastatic ability (7), i.e., less than 10% of the animals inoculated with tumor sublines develop distant metastases. MatLyLu (9) and MatLu (10) both arose from the AT1 tumor. AT3 arose from the HIF line. In rats, these last three sublines grow as anaplastic tumors with a high metastatic ability, i.e., more than 90 % of the animals inoculated with these tumor sublines develop distant metastases (7). The characteristics of the

Dunning sublines used in this study are shown in Table 1.

Each of the tumor sublines described above is routinely passaged by inoculating male inbred Copenhagen (Cop) rats (Harlan Sprague-Dawley, Indianapolis, In) subcutaneously in the flank with a 25 mg trocar piece of the respective tumor subline as described before (8). Tumor samples were harvested when the respective tumors were growing exponentially at a tumor volume of 1-2 cc, frozen in liquid nitrogen and stored at -80 ^{o}C.

mRNA isolation

Total RNA from the Dunning tumors was isolated using the lithium-chloride/urea procedure as described by Auffray and Rougeon (11). Poly-A^+-RNA was purified by selection on an oligo-dT-cellulose column (12).

Construction of cDNA library

For the construction of the cDNA library, a strategy of adaptor ligation was used. According to Haymerle *et al.* (13) unphosphorylated adaptor oligonucleotides are ligated onto both vector and insert DNA. Upon removal of unligated adaptors and phosphorylation of the 5'-OH termini, the cDNA is ligated in the vector.

Briefly, 10 μg of poly-A^+-RNA isolated from the MatLyLu-tumor was oligo-dT_{12-18} primed and cDNA synthesis was performed according to Gubler and Hofman (14). To the blunt-ended cDNA, a 3-fold molar excess of *Bam*HI-cut

Table 1: *In vivo* biological characteristics of Dunning R-3327 rat prostatic cancer sublines.

Subline	Histology	Doubling time (in days)	Androgen responsive	Metastatic ability*
H	Well-differentiated	22 ± 5	Yes	Low
HIS	Well-differentiated	24 ± 5	No	Low
HIM	Well-differentiated	9.0 ± 0.8	No	Low
HIF	Moderately-differentiated	4.8 ± 1.8	No	Low
G	Poorly-differentiated	4.0 ± 0.2	Yes	Low
MATLu	Anaplastic	2.7 ± 0.2	No	High (lungs)**
AT-1	Anaplastic	2.5 ± 0.2	No	Low
AT-2	Anaplastic	2.5 ± 0.2	No	Low to moderate (lungs)
AT-3	Anaplastic	1.8 ± 0.2	No	High (lymph nodes & lungs)
MATLyLu	Anaplastic	1.5 ± 0.1	No	High (lymph nodes & lungs)

* Low metastatic ability, <5% of s.c. inoculated rats develop distant metastases; moderate ability, >5%, <20%; high metastatic ability, >75% develop distant metastases.
** Organs in the parentheses are the site of the distant metastases for the individual sublines.

pUC18 was added and the mixture was ethanol precipitated. After dissolving the vector and cDNA, a 100-fold molar excess of unphosphorylated adaptor oligonucleotides (*Bam*HI/blunt end (Boehringer)) was added. The sample was heated to 65 °C for 5 min and then allowed to cool down to room temperature in 10 min. After overnight ligation (T4-DNA-ligase) at 12 °C, the sample was heated to 65 °C for 5 min and loaded on a Biogel A-50 column to remove the non-ligated adaptors from the vector and cDNA. After eluting the column with 10 mM Tris-HCl/pH7.5-1 mM EDTA, the fractions containing the vector and cDNA were pooled, precipitated and phosphorylated. Finally, the vector and the cDNA were ligated overnight and transformed to competent *Escherichia coli* (strain DH5α) cells.

Differential screening of the cDNA library

Approximately 10,000 colonies containing inserts were plated on nitrocellulose filters and 4 replicas were made. The replica filters were lysed according to Sambrook *et al.* (15). Hybridization was performed according to Hanahan and Meselson (16) in 40% formamid at 42 °C for 60 h. For the differential screening, probes representative for the H-tumor and the MatLyLu-tumor mRNA populations were prepared as follows: 1.5 μg oligo-$dT_{12\text{-}18}$ was annealed to 1 μg of poly-A^+-RNA by incubation at 68 °C for 5 min and quenching on ice. First strand synthesis was then performed for 60 min at 37 °C in 50 mM Tris-HCl (pH=8.3), 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 100 μg/ml BSA, 0.6 mM of unlabeled dATP, dGTP, dTTP, 0.06 mM of unlabeled dCTP and 10 μCi of α-^{32}P-dCTP (>3000 Ci/mM) using 500 units of reverse transcriptase (BRL). Alkaline hydrolysis of the remaining RNA was performed for 30 min at 60 °C in 10 mM EDTA, 0.3 % SDS and 160 mM NaOH. After addition of HAc and Tris-HCl (pH=7.5) to the final concentration of 140 mM and 60 mM respectively, the sample is run on a Sephadex G-50 spin column to remove the excess of unincorporated nucleotides. The eluate is ethanol precipitated and this first strand cDNA is used as a template for a random prime labeling reaction (17) in presence of 50 μCi α-^{32}P-dCTP (>3000 Ci/mM). The specific activity of the cDNA probes thus obtained was 0.5-1.0x10^8 dpm/μg template RNA which is at least 10-fold higher than can be achieved in a reverse-transcriptase-labeling. For the hybridization of the filters (2 replicas with probe derived from the H tumor, 2 replicas with probe derived from the MatLyLu tumor), 1x10^6 dpm/ml hybridization solution was used.

Northern blot analysis

Ten micrograms of total RNA was glyoxylated, size fractioned on 1 % agarose gels and transferred to Hybond-N (Amersham).

Probes were made of DNA, obtained from small scale plasmid isolations or of purified inserts of cDNA clones, by random prime labeling reactions (17).

Hybridizations were performed according to Church and Gilbert (18); the membranes were preincubated in hybridization buffer (7 % SDS, 1 % BSA, 0.5 M sodium-phosphate-buffer/pH=7.4, 1 mM EDTA, 100 μg/ml salmon sperm DNA) for 1-4 hours at 65 °C. The radioactively labeled probe was added to a maximum of $1x10^6$ dpm/ml and the membranes were hybridized overnight at 65 °C. Filters were then washed to high stringency (i.e., buffers containing 1 % SDS, 1 mM EDTA and decreasing concentrations of sodium-phosphate-buffer; 0.5 M, 0.1 M, 0.05 M. Each wash step was performed at 65 °C for 30 min). Dehybridization was performed in 0.1 x Denhardt's solution, 5 mM Tris-HCl/pH=7.4, 2 mM EDTA at 65 °C.

DNA sequence analysis and computer analysis

DNA fragments were ligated into the polylinker region of M13mp8-19. All of the DNA sequences were determined using the dideoxy sequencing method as described by Sanger *et al.* (19). The gel readings were recorded and edited using IntelliGenetics computer software (release 5.35). Computer comparison studies were performed with the EMBL (release 22) and Genbank (release 60) nucleotide sequence databases (20).

RESULTS

Differential hybridization reveals 3 MatLyLu-specific cDNA clones

A cDNA library was constructed from the anaplastic, hormone-independent, metastasizing tumor MatLyLu. The library had a complexity of 10,000 recombinant clones, i.e., with a probability of 99 %, a mRNA expressed at a relative abundancy of 0.05 % will be represented in this library (21). After amplification of the library, approximately 10,000 colonies were plated and 4 replica filters were prepared for *in situ* colony hybridization. Sets of 2 replica filters were hybridized with cDNA probes, using as template poly-A^+-RNA of the MatLyLu-tumor and the more benign H tumor (well-differentiated, hormone-sensitive, non-metastasizing). After 60 h of

hybridization, the filters were washed and exposed for autoradiography for 3 days (see Fig. 1). Extensive comparison of the resulting autoradiographs revealed 18 clones that seemed to be differentially expressed, i.e., a signal was evident on both replicas hybridized with the cDNA probe derived from the MatLylu tumor and no or a very weak signal was detected on any of the replicas hybridized with the probe derived from the H tumor. Upon long exposure (14 days, 2 intensifying screens, Kodak XAR5) 8 additional cDNA clones appeared to detect differentially expressed mRNAs.

The secondary screening of these 26 cDNA clones was a Northern assay on a panel of three Dunning tumors, in which cDNA-containing plasmids were radioactively labeled and hybridized on blots containing 10 μg of total RNA of the H, AT2 (anaplastic, hormone-independent, non-metastasizing) and MatLyLu tumor. The expression patterns of the most significant clones are shown in Fig. 2. From this secondary screening it appeared that the hybridization patterns could be divided in three groups; no significant difference in the three tumors (i.e., false positives); increase in expression towards MatLyLu, whereas the increase in expression was less

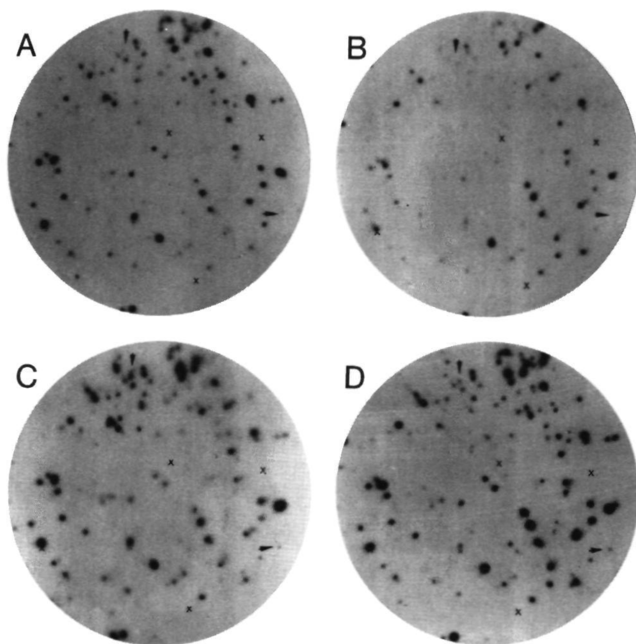


Figure 1: Differential screening of a cDNA library of the hormone-independent, anaplastic, metastasizing MatLyLu-tumor. Replicas of the original plating were hybridized to ^{32}P-labeled cDNA probes prepared from poly-A^{+}-RNA from the H-tumor (hormone-dependent, well-differentiated, non-metastasizing) (A + B) or the MatLyLu tumor (C + D). Arrow heads indicate cDNA clones whose RNAs are more abundant in the MatLylu tumor.

than 3-fold (e.g., Fig. 2; pBUS4, 6, 8, 17, 22); and increase in expression towards MatLyLu more than 5-fold (e.g., Fig. 2; pBUS1, 10, 14, 19, 24, 25, 30). The cDNA clones from the last group were analyzed on Northern blots containing 10 μg of total RNA of normal rat prostate and ten Dunning sublines representing the different stages of tumor progression (see Table 1). pBUS10, 14, 24 and 25 showed no consistent relation between the mRNA levels and progression of prostate cancer whereas the expression patterns detected with pBUS1, 19 and 30 did correlate with progression related parameters (i.e., growth rate, hormone dependency, histology and metastatic ability).

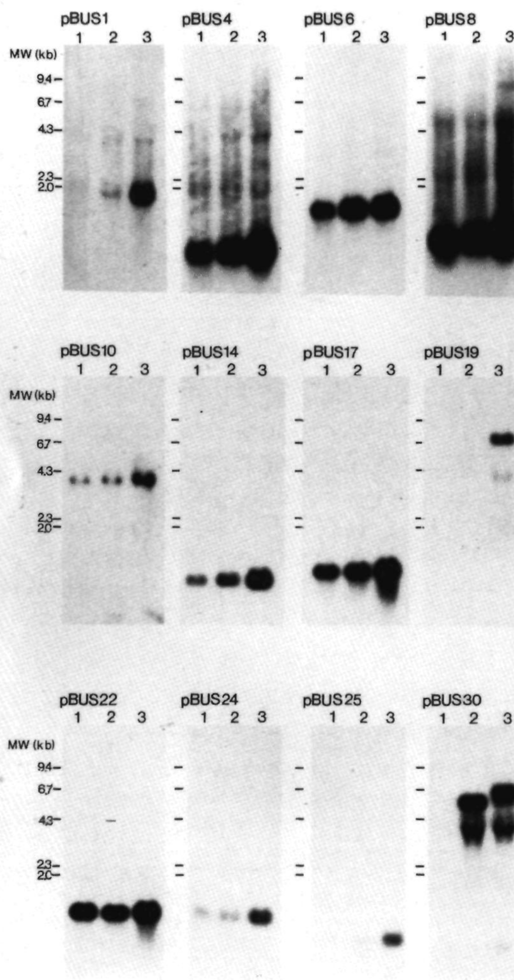


Figure 2: Northern blot analysis of 12 cDNA clones isolated upon differential hybridization. Ten μg of total RNA from the H tumor (lane 1), the AT-2 tumor (lane 2), and the MatLyLu tumor (lane 3) was loaded per lane. ^{32}P-labeled DNA probes were derived from small scale plasmid isolations of the cDNA clones.

Expression pattern of pBUS1

pBUS1 detects a single transcript of 1.8 kb (Fig. 3A). The expression level is high in all metastatic Dunning sublines tested; i.e., pBUS1 transcripts are at least 10-fold more abundant in the metastasizing tumors AT3, MatLu, and MatLyLu than in the anaplastic, non-metastasizing tumors AT1 and AT2 (concluded from densitometrical scannings of longer exposures of the same autoradiograph as shown in Fig. 3A). Moreover, in the hormone-responsive G and H sublines, as well as, in the lines HIS, HIM, and HIF that arose from the H tumor through castration-induced selection, no detectable levels of pBUS1 transcripts were found. Also in normal prostate tissue no pBUS1 mRNA was detectable. (Even upon longer exposure no signals were detected in normal prostate, G, H, HIS, HIM, or HIF.)

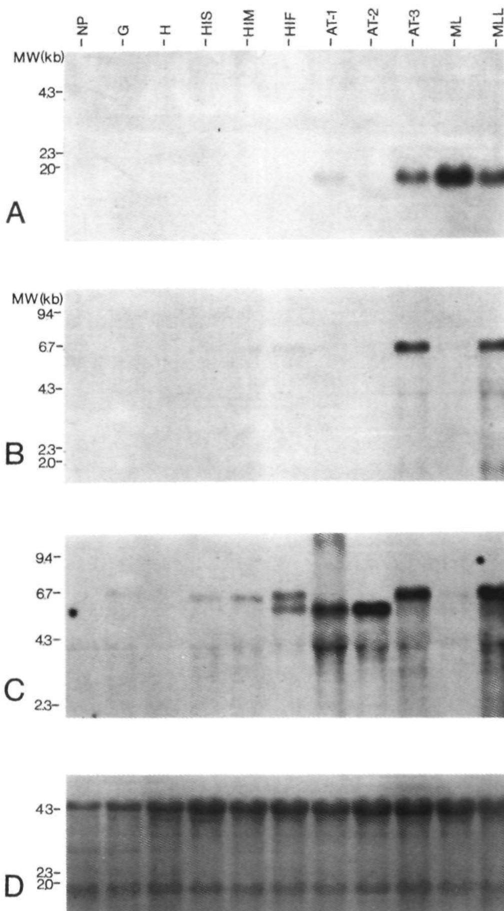


Figure 3: Northern blot analysis of pBUS1 (A), pBUS19 (B), and pBUS30 (C). Ten μg of total RNA of normal prostate (NP) and 10 Dunning tumors was loaded per lane. ^{32}P-labeled DNA probes were derived from purified inserts of the cDNA clones. rRNA was used as an internal control for the amounts of RNA loaded (D).

Thus it appears that pBUS1 expression is highly correlated with the metastatic phenotype, rather than with growth rate [e.g., MatLu has a doubling time of 2.7 days, while AT1 and AT2, which show a much lower expression, have a doubling time of 2.5 days (see Table 1)].

Expression patterns of pBUS19 and pBUS30

Northern analyses using pBUS19 and pBUS30 as a molecular probe revealed expression patterns that at some points are strikingly similar, yet at other aspects differ significantly (Fig. 3B and C). Both probes detect a 7.0 kb transcript, abundantly expressed in the metastasizing AT3 and MatLyLu tumors while MatLu, a tumor line that metastasizes exclusively to lungs, contains no detectable transcripts. The 7.0 kb pBUS19/30 mRNA is expressed at much lower levels in G, HIS, HIM, and HIF (longer exposure, not shown in Fig. 3B, revealed a very low expression of the transcript). It should be noted that in G, HIS and HIM the transcript might be slightly smaller than 7.0 kb (see fig. 3C). The relative intensities of the transcript using either probe were similar (based on densitometrical scanning; data not shown). However, pBUS30 detected an additional transcript of 6.0 kb exclusively found in AT1, AT2 and HIF. The expression of this transcript is higher in AT1 and AT2 than in the HIF tumor. Considering the data on the 7.0 kb transcript we conclude that pBUS19 and PBUS30 might be related cDNA clones. (For both Fig. 3B and C, the band seen at approximately 4.3 kb is probably due to background hybridization).

DNA Sequence analyses of pBUS1, pBUS19 and pBUS30

To obtain further information on the cDNA clones, the cDNA inserts were subcloned in M13mp8-19 and the nucleotide sequence was determined using the dideoxy-sequencing method. Computer-assisted comparison of the resulting nucleotide sequences with the EMBL and Genbank nucleotide sequence databases should reveal homology with known sequences.

To investigate whether pBUS19 and pBUS30 are indeed related, we first compared the nucleotide sequences of these two cDNA clones. No homology was found between pBUS19 and 30. Furthermore, upon screening the nucleotide sequence databases, no homology to any of the known sequences could be found for pBUS30 (cDNA insert 0.5 kbp compared to a mRNA size of 6.0 and 7.0 kb). For pBUS19 (cDNA insert 1.4 kbp), however, the computer comparison showed the presence of parts of two rat-specific repetitive elements, RAL6 and RAL10 (22). As

outlined in Fig. 4, pBUS19 contains 289 bp of the RAL6 element (89 % homology) and 91 bp of the RAL10 element (92 % homology). The RAL elements are very homologous to one another and belong to a family of long terminal repeat-like sequences. It should be noted that pBUS19 and pBUS30 contain only small portions of the entire mRNAs of 6.0 and 7.0 kb. Since both cDNA clones contain poly-A-tails, it is very likely that they represent the 3' non-coding (and probably less conserved) sequences of the transcripts. Hence evidence for the putative relation between pBUS19 and pBUS30 might come from sequence analysis of the upstream regions, not yet cloned.

For pBUS1 (cDNA insert 0.9 kbp) a high homology to the mouse (83 %)(23) and human (71 %)(24) HMG-I(Y) cDNA sequence was found (HMG-I: High Mobility Group protein I / HMG-Y is an isoform). A schematic representation of the alignment of the sequence data is shown in Fig. 5. pBUS1 contains 0.9 kbp of the 3'-end of the murine HMG-I(Y) cDNA which is 1.7 kbp. As illustrated in Fig. 5, only a small part of the HMG-I(Y) cDNA is coding. pBUS1 contains only non-


```
         820       830       840       850       860       870
pBUS19   GAATG AAAGAAAATTATATAAATTCTAGGTTTAAAAATATAAAAATTAAAAAAATAAAC
RNRAL6   GACTGAAAAGAAAATTATACGAATTCTAGGTTTATAAATATAAAAATT AAAAAGTAAAC
            490       500       510       520       530

            880       890       900       910       920       930
pBUS19   CCCAAAAGGCCACAGACCCAGGGCTAAGCCTGCAGCCAAGACTAGCAGGCCATAAAGATA
RNRAL6   CCCAAAAGGCCACAGACCCAGGGCTAAGCCTGCAGCCAAGACTAGCAGGCCATAAAGATA
       540       550       560       570       580       590

            940       950       960       970       980       990
pBUS19   AAGGAGCACAGGAAACACTGTTCAGGCAGGACTGACAAGCCAT AAAAAAAGGAATGCAG
RNRAL6   AAGGAGCACAGGAAACACTGTTCAGGCAGGACTGACAAGCCATAAAAAAAAGGAATGCAG
       600       610       620       630       640       650

              1000      1010      1020      1030      1040
pBUS19   GAAC  CAGCCTGAGTTAATGAGACTGATTCATGGGACGTCTGGCAGGAAGACATCTCCC
RNRAL6   GAACCTCAGCCTGAGTTAATGGGACTGATTCATGGGACGTCTGGCAGGAAGACATCCCCC
       660       670       680       690       700       710

         1050      1060      1070      1080      1090      1100
pBUS19   C  CAGCTCACTCAGGGCCATATTTCAACTAGGTGTCCTCCGGCCCCTGATAAGCCCCTG
RNRAL6   CCGCAGCTCACTCAGGGCCA                TCCTCCAGACCCTGATAAGCCCCT
       720       730                       740       750       760

         1110
pBUS19   ACTTCT
RNRAL6   ACTTGT

          1240      1250      1260      1270      1280      1290
pBUS19    CCTCTGTCTCCTGCGTGGGATGCGTGTCGGCCCGGAGATCTCTGTAATAGGTCTCCGTAA
RNRAL10   CCTCTGTCTCCTGCTTGAGAAACGTGTCAGCGCGCAGATCTCTGTAATAGGTCTCCGTAA
          870       880       890       900       910       920

          1300      1310      1320
pBUS19    TAAACCTCGCCTTTGCTTATTACATCCAAAA
RNRAL10   TAAACCTCGCCTTTGCTTATTACATCCAAAA
          930       940       950
```

Figure 4: Schematic representation of the alignment between pBUS19 and the RAL elements. In the lower part sequence alignments are shown.

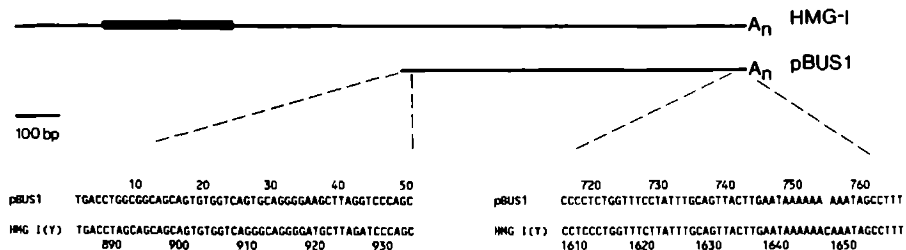


Figure 5. Schematic representation of the alignment between pBUS1 and HMG-I(Y). The box represents the coding sequences of HMG-I(Y).

coding sequences (3'-UTR) in which, nevertheless, a high homology to mouse and human sequences is found, hence it is very likely that pBUS1 is the rat homolog of HMG-I(Y).

DISCUSSION

Many molecular alterations appear to be associated with the process of tumor progression. Molecular studies on the development and progression of cancer have indicated several groups of genes that might be implicated in this process. These groups comprise oncogenes, tumor suppressor genes, genes encoding growth factors, growth factor receptors, transcription factors, extracellular matrix proteins, and cell adhesion molecules. The expression of oncogenes has been studied in both human primary prostate tumors (25, 26), as well as in Dunning sublines (27). Even though the studies on primary tumors suggested a correlation with *ras* and *myc* expression and tumor progression, studies on the Dunning system did not corroborate these findings. Our own studies (data not published) confirm these last results.

The objective of the study described here was to identify genes that are specifically expressed or overexpressed in metastasizing prostatic tumors, using the technique of differential hybridization. In a previous experiment, this technique was successfully applied to compare the steady-state mRNA populations of the AT1 and MatLyLu Dunning sublines (5). In order to be able to identify transcripts that are expressed at lower abundancy and to increase the specificity, we improved the technique of differential hybridization. Screening of 4 replicas decreased the number

of "false positives" and the alternative strategy to label the mRNA populations increased the sensitivity of the experiment: the threshold level for abundancy of mRNAs that can be detected, was decreased from 0.1-0.5 % to 0.05 % (estimation). Thus, comparison of the mRNA populations of the most benign Dunning tumor, the H tumor, and the most progressionally advanced Dunning subline MatLyLu, revealed 26 cDNA clones that are overexpressed in the MatLyLu tumor. After selection by Northern blot screening, three clones appeared to detect mRNAs the expression of which correlate well with the metastatic phenotype. Other clones showed only a slight increase in expression during tumor progression.

Two of the differentially expressed cDNA clones that met all selection criteria, pBUS19 and pBUS30, showed RNA expression patterns that share some characteristics: a high expression of a 7.0 kb transcript in the metastasizing tumors AT3 and MatLyLu, while MatLu, which exclusively metastasizes to lungs, shows no transcript; and the appearance after longer exposure of the autoradiographs, of this transcript in the more benign G, HIS, HIM, and HIF tumors (the size of the transcript in the G, HIS and HIM sublines seems to be slightly smaller than 7.0 kb). However, the cDNA clones differ by the fact that pBUS30 detects an additional transcript of 6.0 kb that is expressed in HIF and at much higher level in the anaplastic tumors AT1 and AT2. To show a possible relationship between pBUS19 and pBUS30, the nucleotide sequences of the cDNA clones were determined and compared. Even though both clones contain a poly-A-tail, no homology could be found. (Further studies are necessary to establish or exclude an overlap of the two clones, possibly due to alternative splicing). The DNA sequences were also used for computer-assisted screening of nucleotide sequence databases. For pBUS30 no homology was found with any of the known sequences. Computer comparison of pBUS19, however, revealed the presence of parts of repetitive RAL elements. Although it is reported that these elements can be specifically expressed in rat tumors (22), in our rat tumor model system we see that only two of ten tumors show expression of this transcript. Southern blot analysis of human chromosomal DNA revealed that no RAL homologous elements are present in the human genome (data not shown). Furthermore, to our knowledge, specific expression of repetitive elements in human malignancies has not been shown yet.

The expression of pBUS1 correlates well with metastatic behavior: a transcript of 1.8 kb is expressed at very high levels in the three tested metastasizing tumors, the expression in the anaplastic, non-metastasizing tumors is at least 10-fold lower

while in the more benign tumors no transcripts are detected. DNA sequence and computer analysis revealed that pBUS1 has a high homology with human (71 %) and murine (83 %) HMG-I(Y) cDNA. HMG-I belongs to the high-mobility-group proteins which are non-histone, chromatin binding (23). HMG-Y is an isoform of HMG-I, lacking 33 nt in the coding sequences. The HMG-I and -Y proteins appear to be members of an isoform family of proteins (all of whose members have not yet been fully characterized) that are probably derived by alternative splicing of a common precursor mRNA (28). Nucleotide sequence data of HMG-I(Y) reported thus far indicate that the 3'-untranslated region (3'-UTR) of all of the potential isoforms are on a single exon, i.e., are transcribed from one gene. Thus from the nucleotide sequence analysis of only the 3'-UTR it is not possible to determine which of the isoforms of the HMG-I family, pBUS1 represents. Previous studies report on the possible involvement of HMG-I(Y) in metaphase chromatin condensation (29), in heterochromatin nucleosome phasing (30), in nuclear matrix-DNA interactions (31) or in the 3'-end processing of genes (32, 33). p16, which is most likely a member of the HMG-I isoform family, is implicated in the regulation of the rRNA gene expression (34). Initial reports mention that HMG-I(Y) transcripts are most abundant in fast proliferating, undifferentiated cells (23). Moreover, Gianotti *et al.* (35, 36) suggest that HMG-I(Y) expression is rather related to a highly malignant phenotype than to neoplastic transformation. Comparative analysis of HMG-I(Y) expression in the Dunning system, in which degree of differentiation, growth rate, and metastatic capacity are represented in the various lines, revealed that, whereas the correlation between expression of HMG-I(Y) and growth rate/differentiation was low, there is a clear correlation with metastatic capacity, i.e., the highly malignant phenotype. Likewise, AT1, AT2, AT3, MatLu, and MatLyLu, are all anaplastic tumors; nonetheless, the more malignant sublines (i.e., metastatic) have significant higher levels of HMG-I(Y)-related transcripts. Hence our data support those of Giancotti *et al.* (35,36). To substantiate whether there is a functional relation between the expression of HMG-I(Y) and acquisition of the metastatic phenotype, the effects of modulation of the HMG-I expression in the Dunning lines (by cDNA mediated transfection) will be studied.

ACKNOWLEDGMENTS

We would like to thank J. van Groningen for excellent technical assistance. We also would like to thank Dr. R. Reeves for critically reading the manuscript. These studies were supported by the Dutch Cancer Foundation (NUKC 90-01) and the Royal Dutch Academy of Arts and Sciences.

REFERENCES

1 Silverberg, E., Boring, C.C., and Squires, T.S. Cancer Statistics 1990. CA, 40: 9-26, 1990.
2 Henry, J.M., and Isaacs, J.T. Relationship between tumor size and the curability of metastatic prostatic cancer by surgery alone or in combination with adjuvant chemotherapy. J. Urol., 139: 1119-1123, 1988.
3 Gleason, D.F. Classification of prostatic carcinoma. Cancer Chemother. Rep., 50: 125-128, 1966.
4 Partin, A.W., Walsh, A.C., Pitcock, R.V., Mohler, J.L., Epstein, J.I., and Coffey, D.S. A comparison of nuclear morphometry and gleason grade as a predictor of prognosis in stage A2 prostate cancer: a critical analysis. J. Urol., 142: 1254-1258, 1989.
5 Schalken, J.A., Ebeling, S.B., Isaacs, J.T., Treiger, B., Bussemakers, M.J.G., De Jong, M.E.M., and Van de Ven, W.J.M. Differential hybridization reveals down-modulation of fibronectin mRNA in metastasizing prostatic cancer cells. Cancer Res., 48: 2042-2046, 1988.
6 Dunning, W.F. Prostate cancer in rat. Natl. Cancer Inst. Monogr., 12: 351-369, 1963.
7 Isaacs, J.T. Development and characterization of available animal model system for the study of prostatic cancer. In: D.S. Coffey, M. Bruchovsky, W.H. Gardner, M.I. Resnick, and I. Pekarr (eds.), Current Concepts and Approaches to the study of Prostatic Cancer, pp. 513-576. New York: Alan R. Liss, 1987.
8 Isaacs, J.T., and Coffey, D.S. Adaptation *versus* selection as the mechanism responsible for the relapse of prostatic cancer to androgen ablation therapy as studied in the Dunning R-3327-H adenocarcinoma. Cancer Res., 41: 5070-5075, 1981.
9 Isaacs, J.T., Yu, G., and Coffey, D.S. The characterization of a newly identified, highly metastatic variant of the Dunning R-3327 rat prostatic adenocarcinoma system: the MAT-LyLu tumor. Invest. Urol., 19: 20-23, 1981.
10 Lazan, D., Heston, W.D., Kadman, D., and Fair, W. Inhibition of the R-3327 MAT-Lu prostatic tumor by diethylstilbestrol and 1,2-bis(3,5-dioxopiperazin-1-yl)propane. Cancer Res., 42: 1390-1394, 1982.
11 Auffray, C., and Rougeon, F. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem., 107: 303-314, 1980.
12 Aviv, H, and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. Proc. Natl. Acad. Sci. USA, 69: 1408-1412, 1972.
13 Haymerle, H., Herz, J., Bressan, G.M., Frank, R., and Stanley, K.K. Efficient construction of cDNA libraries in plasmid expression vectors using an adaptor strategy. Nucl. Acids Res., 14: 8615-8624, 1986.
14 Gubler, U., and Hofman, B.J. A simple and very efficient method for generating cDNA libraries. Gene, 25: 263-269, 1983.
15 Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular cloning: A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989.
16 Hanahan, D., and Meselson, M. Plasmid screening at high colony density. Gene, 10: 63-67, 1980.
17 Feinberg, A.P., and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., 132: 6-13, 1983.

18 Church, G.M., and Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA, 81: 1991-1995, 1984.
19 Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol., 143: 161-178, 1980.
20 Devereux, J., Haeberli, P., and Smithies, O. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res., 12: 387-395, 1984.
21 Williams, J.G. The preparation and screening of a cDNA clone bank. In: R. Williamson (ed.), Genetic engineering, pp. 1. London, Academic Press, 1981.
22 Suzuki, N., Fuyiyoshi, T., Maehara, Y., Takahashi, K., Yamamoto, M., and Endo, H. A new family of LTR-like sequences abundantly expressed in rat tumors. Nucl. Acids Res., 14: 9271-9289, 1986.
23 Johnson, K.R., Lehn, D.A., Elton, T.S., Barr, P.J., and Reeves, R. Complete murine cDNA sequence, genomic structure, and tissue expression of the high mobility group protein HMG-I (Y). J. Biol. Chem., 263: 18338-18342, 1988.
24 Eckner, R., and Birnstiel, M.L. Cloning of cDNAs coding for the human HMG-I and HMG-Y proteins: both are capable of binding to octamer sequence motif. Nucl. Acids Res., 17: 5947-5959, 1989.
25 Viola, M.V., Fromowitz, F., Ovarez, S., Deb, S., Finkel, G., Lundy, J., Hand, P., Thor, A. and Schlom, J. Expression of *ras* oncogene p21 in prostate cancer. N. Eng. J. Med., 314: 133-137, 1986.
26 Fleming, W.H., Hamel, A., MacDonald, R., Ramsey, E., Pettigrew, N.M., Johnston, B., Dodd, J. G., and Matusik, R.J. Expression of the c-*myc* proto-oncogene in human prostatic carcinoma and benign prostatic hyperplasia. Cancer Res., 46: 1535-1538, 1986.
27 Cooke, D.B., Quarmby, V.E., Mickey, D.D., Isaacs, J.T., and French, F.S. Oncogene expression in prostatic cancer: Dunning R-3327 rat dorsal prostatic adenocarcinoma system. Prostate, 13: 263-272, 1988.
28 Johnson, K.R., Lehn, D.A., and Reeves, R. Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y. Mol. Cell. Biol., 9: 2114-2123, 1989.
29 Lund, T., Holtlund, J., Fredriksen, M., and Laland, S.G. On the presence of two new high mobility group like proteins in Hela S3 cells. FEBS Lett., 152: 163-167, 1983.
30 Solomon, M.J., Strauss, F., and Varshavsky, A. A mammalian high mobility group protein recognizes any stretch of six A.T base pairs in duplex DNA. Proc. Natl. Acad. Sci. USA, 83: 1276-1280, 1986.
31 Elton, T.S., Nissen, M.S., and Reeves, R. Specific A.T DNA sequence binding of RP-HPLC purified HMG-I. Biochem. Biophys. Res. Commun., 143: 260-265, 1987.
32 Reeves, R., Elton, T.S., Nissen, M.S., Lehn, D., and Johnson, K.R. Posttranscriptional gene regulation and specific binding of the non-histon protein HMG-I by the 3'-untranslated region of bovine interleukin-2 cDNA. Proc. Natl. Acad. Sci. USA, 84: 6531-6535, 1987.
33 Russnak, R.H., Candido, E.P.M., and Astell, C.R. Interaction of the mouse chromosomal protein HMG-I with the 3' ends of genes *in vitro*. J. Biol. Chem., 263: 6392-6399, 1988.
34 Yang-Yen, H.-F., and Rothblum, L.I. Purification and characterization of a high-mobility-group-like DNA-binding protein that stimulates rRNA synthesis *in vitro*. Mol. Cell. Biol., 8: 3406-3414, 1988.
35 Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M.T., Di Fiori, P.P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicholas, R.H., Wright, C.A., and Goodwin, G.H. Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-*ras* and v-*mos* oncogenes and by cotransfection with c-*myc* and polyoma middle T genes. EMBO J., 6: 1981-1987, 1987.
36 Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco, A., and Goodwin, G.H. Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. Exp. Cell Res., 184: 538-545, 1989.

CHAPTER III

INCREASED EXPRESSION OF RETROVIRAL SEQUENCES IN PROGRESSIONALLY ADVANCED RAT PROSTATIC TUMORS

Marion J.G. Bussemakers, Adrie van Bokhoven, Jan J.M. van Groningen (1), Frans M.J. Debruyne, Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands, (1) Department of Biochemistry, University of Nijmegen, The Netherlands

Biochemical Biophysical Research Communications 182: 318-324, 1992

SUMMARY

Differential hybridization analysis revealed two cDNA clones, pBUS19 and pBUS30, to be overexpressed in progressionally advanced rat prostatic tumors. Northern blot analysis suggested the clones to be related although no homology in nucleotide sequence could be shown. Isolation and characterization of a pBUS19-related clone, pJG116, and computer-assisted database comparison showed that all three clones could be mapped within a rat-specific endogenous retrovirus. The importance of overexpression of retroviral sequences in advanced prostatic cancer remains unclear.

INTRODUCTION

In our studies to identify genes that are activated during the proces of prostate tumor progression, we applied the technique of differential hybridization analysis to compare the steady-state mRNA levels of two different tumor stages. Since no well defined human prostate cancer progression model system is available, two sublines of the established Dunning R-3327 rat prostatic cancer model system (1) were used. The serially transplantable Dunning R-3327 sublines represent the various stages of prostate tumor progression. Comparing the steady-state mRNA levels of the most benign Dunning R-3327-H tumor (hormone-dependent, well-differentiated, non-metastasizing), and the aggressive MatLyLu tumor (hormone-independent, anaplastic, metastasizing), three clones that are overexpressed in the metastatic tumors, were isolated (2). Two cDNA clones, pBUS19 and pBUS30, showed a striking homology in the mRNA expression patterns: a 7.0 kb transcript was highly expressed in the anaplastic, metastasizing (to lymph and lung) tumors AT3 and MatLyLu, but not in MatLu (metastasizes exclusively to lung). pBUS30 showed the expression of additional transcripts in the anaplastic, non-metastasizing tumors and, at much lower levels, in the well- and moderately-differentiated tumors. Nucleotide sequence analysis and computer-assisted comparison revealed no homology between the clones, although both clones contained a poly-A-tail. Screening of computer databases showed no homology of pBUS30 to any of the known sequences whereas pBUS19 appeared to contain sequences homologous to a retroviral LTR-like repeat, termed RAL-element (3). This RAL-element was reported to belong to a new family of LTR-like sequences abundantly expressed in rat tumors but rarely in normal tissues. In order to gain more information on the possible role of the repetitive RAL-elements in the progression of rat prostatic cancer, and to elucidate a possible relationship between pBUS19 and pBUS30, a MatLyLu cDNA library was screened using pBUS19 and pBUS30 as a probe to isolate additional cDNA clones that can be used for further characterization of the RAL-elements.

MATERIALS AND METHODS

Dunning R-3327 sublines

The phylogeny and the characteristics of the Dunning R-3327 rat prostatic cancer model system have already been described extensively (1, 2) and are summarized in Table 1.

Screening of cDNA library

Fifty thousand colonies of a cDNA library of the Dunning MatLyLu tumor (2) were screened according to Sambrook *et al.* (4), using either the 1.5 kb cDNA insert of pBUS19 or the 0.5 kb cDNA insert of pBUS30 as a probe.

Northern blot analysis

Total RNA was isolated using the lithium chloride/urea method as described by Auffray and Rougeon (5). Ten microgram of total RNA was glyoxylated, separated on an agarose gel by electrophoresis and transferred to Hybond-N^+ (Amersham). Hybridizations were performed as described before (2).

Nucleotide sequence analysis

DNA fragments were ligated into the polylinker region of M13mp8-19. All of the DNA sequences were determined on both strands using the dideoxy sequencing

Table 1: *In vivo* biological characteristics of Dunning R-3327 rat prostatic cancer sublines.

Subline	Histology	Doubling time (in days)	Androgen responsive	Metastatic ability*
H	Well-differentiated	22 ± 5	Yes	Low
HIS	Well-differentiated	24 ± 5	No	Low
HIM	Well-differentiated	9.0 ± 0.8	No	Low
HIF	Moderately-differentiated	4.8 ± 1.8	No	Low
G	Poorly-differentiated	4.0 ± 0.2	Yes	Low
MATLu	Anaplastic	2.7 ± 0.2	No	High (lungs)**
AT-1	Anaplastic	2.5 ± 0.2	No	Low
AT-2	Anaplastic	2.5 ± 0.2	No	Low to moderate (lungs)
AT-3	Anaplastic	1.8 ± 0.2	No	High (lymph nodes & lungs)
MATLyLu	Anaplastic	1.5 ± 0.1	No	High (lymph nodes & lungs)

* Low metastatic ability, <5% of s.c. inoculated rats develop distant metastases; moderate ability, >5%, <20%; high metastatic ability, >75% develop distant metastases.
** Organs in the parentheses are the site of the distant metastases for the individual sublines.

method as described by Sanger *et al.* (6). The gel readings were recorded and edited using IntelliGenetics computer software (release 5.35). Computer comparison studies were performed with the EMBL (release 28) and Genbank (release 68) nucleotide sequence databases (7).

RESULTS

Screening of the cDNA library

About fifty thousand clones of the MatLyLu cDNA library were screened using either the 1.5 kb *Bam*HI cDNA insert of pBUS19 or the 0.5 kb *Bam*HI cDNA insert of pBUS30 as a probe. pBUS30 did not detect any related cDNA clones. Of 132 pBUS19-positive clones, 24 were randomly chosen and DNA was isolated. *Bam*HI and *Eco*RI/*Hin*dIII restriction digestions revealed that we had isolated 13 different cDNA clones. pJG116 was selected for further characterization since it contained the largest insert (2.7 kb), possibly containing an overlap with pBUS30. To ascertain that pJG116 was indeed related to pBUS19, the insert of pJG116 was used for Northern blot analysis. Interestingly, a hybridization pattern similar to the one found for pBUS30 was seen (see Fig. 1); in addition to the 7.0 kb transcript in the anaplastic, metastasizing (to lymph and lung) tumors AT3 and MatLyLu (as found for pBUS19), a 6.0 kb transcript was seen in the anaplastic, non-metastasizing tumors AT1 and AT2, whereas in the more benign Dunning sublines (G, H, HIS, HIM) a low expression of a 6.7 kb transcript was seen. The poorly-differentiated, hormone-independent HIF tumor expressed transcripts of 6.0 and 7.0 kb. This suggested that indeed pJG116 may contain sequences also present in pBUS30 and thus may help unravel the relation between the different clones. When pJG116 was used for a Southern blot analysis, a smear identical to that seen for pBUS19 and pBUS30, was found in the Dunning sublines whereas no signal was detected in human liver (data not shown), confirming the species specificity of the repetitive sequences.

Nucleotide sequence analysis

In order to determine the nucleotide sequence of pJG116, a restriction map was constructed. The map of the 3' end of pJG116 was almost identical to that determined for pBUS19, confirming the expected correlation with pBUS19 and showing that pJG116 was a 5'-end extended clone of pBUS19. Next, the nucleotide

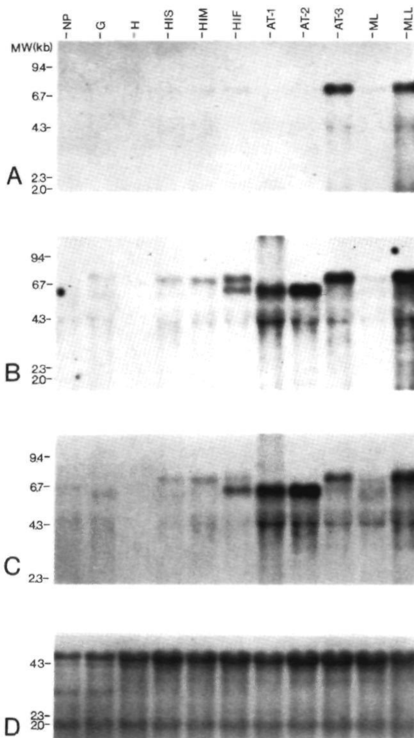


Figure 1: Northern blot analysis of pBUS19 (A), pBUS30 (B) and pJG116 (C). Ten μg of total RNA of normal prostate and 10 Dunning tumors were loaded per lane. rRNA was used as an internal control for the amount of RNA loaded (D).

sequence of pJG116 was determined. Computer-assisted analysis of the nucleotide sequences of pBUS19, pBUS30 and pJG116 showed that pBUS19 and pJG116 shared a high homology (94 %). An overlap between pJG116 and pBUS30 could not be found. In order to find out what was contained in the additional sequences of pJG116 and to perform an update search for pBUS19 and pBUS30, nucleotide sequences of the three clones were used for computer-assisted database comparison with all known sequences. This revealed the relationship between all clones: pBUS19 and pJG116 showed homology with the RAL-elements and new retroviral sequences that had become available (8) made it possible to link those two clones to pBUS30. As shown in Figure 2, pBUS19 and pJG116 are located at the 3' end of a 7.3 kb rat endogenous retrovirus which contains the RAL-elements: pBUS19 has a homology of 90.7 % over 1010 nt (of 1303 nt determined), pJG116 has a homology of 91.3 % over 2300 nt (of 2684 nt determined). pBUS30 is also contained within the endogenous retrovirus: 87 % homology over 466 nt (of 466 nt determined). This comparison also showed that the assumed poly-A-tail of pBUS30 is due to an

internal A-stretch in the retrovirus, probably allowing oligo-dT-priming. This explains why no evident poly-A-addition-signal could be found in pBUS30. The fact that pBUS30 is not located at the 3'-end but is primed at an internal A-stretch may also explain why no additional cDNA clones were isolated.

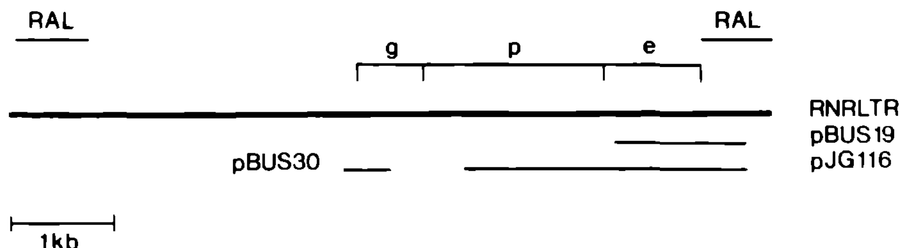


Figure 2: Schematic presentation of the location of pBUS19, pJG116 and pBUS30 compared to the endogenous retrovirus (RNRLTR) as described by Nakamuta *et al.* (8). Also the gag (g), pol (p) and env (e) homologous regions (as indicated by Nakamuta *et al.* (8)) are shown as are the positions of the RAL elements. [Sequence data have been deposited with the EMBL/Genbank Data libraries under accession nos. X62950 (pBUS19), X62951 (pBUS30), and X62952 (pJG116)].

DISCUSSION

The isolation of pJG116 and an update database screening revealed the relation of the three cDNA clones pBUS19, pBUS30 and pJG116. All three cDNA clones contain parts of a rat endogenous retrovirus of 7.3 kb (RNRLTR, (8)), which was shown to be abundantly expressed in rat hepatic tumors whereas no expression was detected in normal liver. In rat prostatic tumors, we also see an overexpression of the retroviral sequences when compared to normal prostate, but instead of a smear pattern on Northern blot as found by Suzuki *et al.* (3), we detect transcripts of discrete sizes. The transcript in the anaplastic, metastasizing tumors AT3 and MatLyLu are approximately 7.0 kb and may represent a putative full-length transcript of the retroviral sequences (7.3 kb). The fact that in the anaplastic, non-metastasizing tumors AT1 and AT2 a transcript of 6.0 kb and in the more benign Dunning tumors a transcript of 6.7 kb is found, raises the question as to whether the transcripts are derived from different retroviral sequences, whether there is a change of transcription-start-point during tumor progression or if we are dealing with specific splice-products? Since pBUS19 and pJG116 are not 100 % identical, this suggest that there are several, highly homologous, retroviral sequences in the genome

of the rat, and they might give rise to different transcripts in different tumors or tumor stages.

The role of the induced expression of the endogenous retroviral sequences in rat prostatic tumors remains to be established. Endogenous retroviral-like sequences in eukaryotic cells have been extensively studied (9) and it was shown that usually the expression of the retroviral sequences is restricted to specific stages of embryonic development or to specific tissues (10). However, also the specific enhancement of expression of repetitive sequences has been reported: the repetitive sequences are activated during several processes including differentiation (11), tumorigenesis (12) or due to induction by exogenous factors (13). On the other hand, also loss of expression of endogenous proviruses in tumors has been described (14). The involvement of deregulation of the expression of endogenous retroviral sequences has been shown in a variety of tumors and transformed cells (15-20), and the altered pattern of expression of retroviral transcripts may provide markers for the detection of neoplastic disease (20). On a possible functional/structural relation between the expression of endogenous retroviral sequences and prostate tumor progression, one can only speculate. It is very well possible that we are dealing with an epiphenomenon in that the endogenous retroviral-like sequences are activated non-specifically. In cancer research the activation of cellular genes by promoter/enhancer insertion of retroviral sequences is a known mechanism but although the correlation is striking, i.e., in the anaplastic lines an overexpression is found, we did not find evidence for fusion of RAL-elements with endogenous genes. In contrast, Liu and Abraham (21), studying differential gene expression in human prostatic cancer cell lines, identified a cDNA containing human endogenous retroviral sequences spliced to human calbindin. The long terminal repeat (LTR) of this retroviral sequence was suggested to possibly activate the calbindin gene. The relation between overexpression of rètroviral sequences and progression of rat prostatic cancer, however, remains elusive.

REFERENCES

1 Isaacs, J.T. and Hukku, B. Nonrandom involvement of chromosome 4 in the progression of rat prostatic cancer. Prostate, 13: 165-188, 1988.

2 Bussemakers, M.J.G., Van de Ven, W.J.M., Debruyne, F.M.J. and Schalken, J.A. Identification of high mobility group protein I (Y) as potential progression marker for prostate cancer by differential hybridization analysis. Cancer Res., 51: 606-611, 1991.

3 Suzuki, N., Fujiyoshi, T., Maehara, Y., Takahashi, K., Yamamoto, M. and Endo, H. A new family of LTR-like sequences abundantly expressed in rat tumors. Nucl. Acids Res., 14: 9271-9289, 1986.

4 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

5 Auffray, C., and Rougeon, F. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem., 107: 303-314, 1980.

6 Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol., 143: 161-178, 1980.

7 Devereux, J., Haeberli, P., and Smithies, O. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res., 12: 387-395, 1984.

8 Nakamuta, M., Furuich, M., Takahashi, K., Suzuki, N., Endo, H. and Yamamoto, M. Isolation and characterization of a family of rat endogenous retroviral sequences. Virus Genes, 3: 69-83, 1989.

9 Scott, M.R.D., Westphal, K.-H., and Rigby, P.W.J. Activation of mouse genes in transformed cells. Cell, 34: 557-567, 1983.

10 Kelly, F. and Condamine, H. Tumor viruses and early mouse embryos. Biochim. Biophys. Acta, 651: 105-141, 1982.

11 Khochbin, S., Grunwald., D., Bergeret, E. and Lawrence J.-J. Modulation of b2 containing small RNAs during induced differentiation of murine erythroleukemia cells. Biochem. Biophys. Res. Comm., 175: 91-97, 1991.

12 Tomita, N., Horii, A., Doi, S., Yokouchi, H., Ogawa, M., Mori, T. and Matsubara, K. Transcription of human endogenous retroviral long terminal repeat (LTR) sequence in a lung cancer cell line. Biochem. Biophys. Res. Comm., 166: 1-10, 1990.

13 Cho, K.-O., Skarnes, W.C., Minsk B., Palmieri, S., Jackson-Grusby, L. and Wagner, J.A. Nerve growth factor regulates gene expression by several distinct mechanisms. Mol. Cell. Biol., 9: 135-143, 1989.

14 Cohen, M., Kato, N. and Larsson, E. ERV3 human endogenous provirus mRNAs are expressed in normal and malignant tissues and cells, but not in choriocarcinoma tumor cells. J. Cell. Biochem., 36: 121-1282, 1988.

15 Kohnoe, S., Maehara, Y. and Endo, H. A systematic survey of repetitive sequences abundantly expressed in rat tumors. Biochim. Biophys. Acta, 909: 107-114, 1987.

16 Melber, K., Krieg, P., Fürstenberger, G. and Marks, F. Molecular cloning of sequences activated during multi-stage carcinogenesis in mouse skin. Carcinogenesis, 7: 317-322, 1986.

17 Dragani, T.A., Manenti, G., Della Porta, G., Gattoni-Celli, S. and Weinstein, I.B. Expression of retroviral sequences and oncogenes in murine hepatocellular tumors. Cancer Res., 46: 1915-1919, 1986.

18 Courtney, M.G., Schmidt, L.J. and Getz, M.J. Organization and expression of endogenous virus-like (VL30) DNA sequences in nontransformed and chemically transformed mouse embryo cells in culture. Cancer Res., 42: 569-576, 1982.

19 Rabson, A.B., Steele, P.E., Garon, C.F. and Martin, M.A. mRNA transcripts related to full-length endogenous retroviral DNA in human cells. Nature, 306: 604-607, 1983.

20 Gattoni-Celli, S., Kirsch, K., Kalled, S. and Isselbacher, K.J. Expression of type C-related endogenous retroviral sequences in human colon tumors and colon cancer cell lines. Proc. Natl. Acad. Sci. USA, 83: 6127-6131, 1986.

21 Liu, A.Y., and Abraham, B.A. Subtractive cloning of a hybrid endogenous retrovirus and calbindin gene in the prostate cell line PC3. Cancer Res., 51: 4107-4110, 1991.

CHAPTER IV

DIFFERENTIAL EXPRESSION OF VIMENTIN IN RAT PROSTATIC TUMORS

Marion J.G. Bussemakers, Gerald W.C.T. Verhaegh (1), Adrie van Bokhoven,
Frans M.J. Debruyne and Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands,
(1) Department of Biochemistry, University of Nijmegen, The Netherlands

Biochemical Biophysical Research Communications 182: 1254-1259, 1992

SUMMARY

Differential hybridization analysis was used to identify molecular differences between a relative benign and a highly aggressive rat prostatic tumor derived from the Dunning R-3327-H adenocarcinoma. From the several differentially expressed mRNAs identified, we here report the characterization of pBUS51 which encodes a transcript highly expressed in all anaplastic Dunning tumors. Only a very low expression was detectable in normal rat prostate or in the differentiated tumors of the Dunning system. Nucleotide sequence analysis and computer-assisted database comparison revealed that pBUS51 was highly homologous to vimentin and therefore likely the rat homolog of this protein.

INTRODUCTION

In cancer development, the progression of normal cells through premalignancy to malignancy can be characterized by several changes, biochemically as well as morphologically. These changes may result from alterations in cellular gene expression (1-3). By using the technique of differential or subtraction hybridization analysis, cDNA clones can be identified that represent genes whose expression is up- or down-regulated during carcinogenesis (4, 5). In order to isolate cDNAs that are overexpressed in progressionally advanced prostatic cancers, we compared the steady-state mRNA populations of two tumors from the well characterized Dunning R-3327 rat prostatic cancer model system (6): the most benign Dunning subline, the hormone-dependent, well-differentiated, non-metastasizing H-tumor was compared to the most aggressive subline, the hormone-independent, anaplastic, metastasizing MatLyLu-tumor. In our first experiment we identified several differentially expressed cDNAs from which pBUS1 (highly homologous to the High Mobility Group protein I (Y)) was shown to be a good candidate progression marker for prostate cancer (7). To isolate additional progression markers we performed a second differential screening. Amongst the cDNA clones selected for their overexpression in the malignant MatLyLu tumor, pBUS51 showed the most interesting expression pattern: a high mRNA expression in the anaplastic tumors was seen when compared to the low levels in normal rat prostate and the more benign sublines of the Dunning model system. When studying the expression of pBUS51 in a *de novo* arisen model system (derived from the Dunning-H tumor), the correlation of pBUS51 with tumor progression was confirmed, showing that the overexpression of pBUS51 is not merely a tumor-transplantation artefact. Nucleotide sequence analysis revealed pBUS51 to be the rat homolog of vimentin. Upon isolation of an additional cDNA clone (pGV40), we determined the complete coding sequence of rat vimentin which is highly homologous to mouse and human vimentin.

MATERIALS AND METHODS

Dunning R-3327 sublines

The phylogeny and the characteristics of the Dunning R-3327 rat prostatic cancer model system have already been described extensively (6, 7).

Differential screening of cDNA library

A cDNA library constructed from the hormone-independent, anaplastic, metastasizing Dunning tumor MatLyLu (7) was used for differential hybridization analysis. cDNA probes were made, representing the poly-A^{+}-RNA population of the MatLyLu tumor and the more benign Dunning H tumor (hormone-dependent, well-differentiated, non-metastasizing) and hybridization was performed as described before (7). Screening of the cDNA library in order to isolate a full-length rat vimentin cDNA clone, was performed according to Sambrook *et al.* (8).

Northern blot analysis

Total RNA was isolated using the lithium chloride/urea method as described by Auffray and Rougeon (9). Ten microgram of total RNA was glyoxylated, separated on an agarose gel by electrophoresis and transferred to Hybond-N^{+} (Amersham). Hybridizations were performed as described before (7).

Nucleotide sequence analysis

DNA fragments were ligated into the polylinker region of M13mp8-19. All of the DNA sequences were determined on both strands using the dideoxy sequencing method as described by Sanger *et al.* (10). The gel readings were recorded and edited using IntelliGenetics computer software (release 5.35). Computer comparison studies were performed with the EMBL (release 28) and Genbank (release 68) nucleotide sequence databases (11).

RESULTS AND DISCUSSION

Differential hybridization analysis

Comparison of the autoradiographs resulting from the differential screening revealed 14 cDNA clones representing genes overexpressed in the aggressive MatLyLu tumor. Northern blot analysis was performed on normal rat prostate and ten Dunning sublines to establish a possible relation of expression of these 14 cDNA clones to prostatic tumor progression. pBUS51 showed the most interesting expression pattern (see Figure 1): pBUS51 is highly expressed in all hormone-independent, anaplastic tumors (i.e., AT1, AT2, AT3, MatLu and MatLyLu) whereas only a very low expression is found in normal prostate and the well or moderately

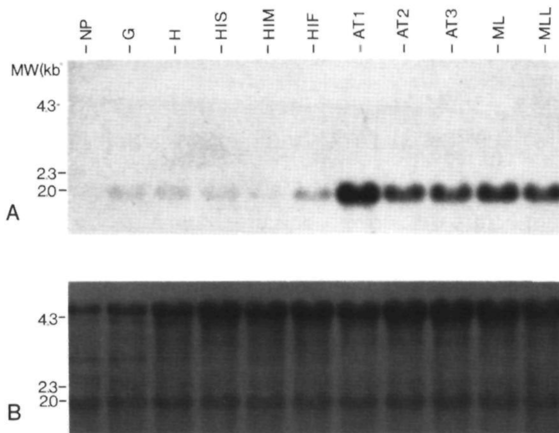


Figure 1: Northern blot analysis of pBUS51 (A). Ten μg of total RNA of normal prostate and 10 Dunning tumors were loaded per lane. rRNA was used as an internal control for the amount of RNA loaded (B).

differentiated Dunning sublines (i.e., H, HIS, HIM, HIF, G) suggesting pBUS51 to correlate with a hormone-independent, anaplastic phenotype. Next the expression of pBUS51 was studied in a newly developed model system. Upon transplantation of the Dunning H tumor, a highly metastatic but still differentiated tumor, AT6p0 arose [see chapter V]. AT6p0 shows pBUS51 expression at the same level as does the H tumor (data not shown). A lung metastases of the AT6p0 showed elevated levels of pBUS51 and upon further transplantation of both the original AT6p0 and the lung metastases, anaplastic tumors arose, expressing pBUS51 at high levels. This confirms a correlation of pBUS51 with tumor progression, or more specific, degree of differentiation, and proves that the overexpression of pBUS51 is not merely due to tumor transplantation.

DNA sequence analysis of pBUS51

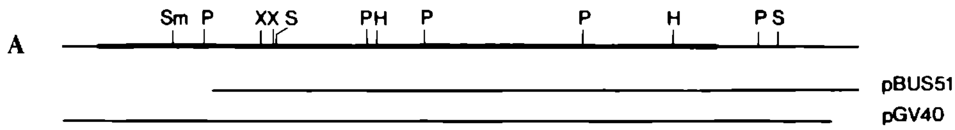
To obtain further information on pBUS51, its 1.5 kb insert was subcloned in M13mp8-19 and the nucleotide sequence was determined. Computer-assisted comparison of the resulting nucleotide sequences with the EMBL and Genbank nucleotide sequence databases revealed that pBUS51 is highly homologous to vimentin as characterized in different species (12-14). pBUS51 lacked approximately 400 bp at the 5'-end when compared to mouse vimentin cDNA. We rescreened the cDNA library and isolated an additional rat vimentin cDNA clone, pGV40, with a 1.8 kb insert. Nucleotide sequence analysis showed that pGV40 contains the complete coding sequence of rat vimentin but, unfortunately, lacks (compared to

mouse) 55 bp at the 5-'end (5' non-coding sequences) and 52 bp at the 3'-end. In Figure 2 a partial restriction map and the nucleotide sequence of rat vimentin cDNA are shown as is the location of pBUS51 and pGV40. Also the deduced amino acid sequence is indicated. The nucleotide sequences of the two overlapping clones comprise 1796 bp, not including the poly-A-tail. There is an open reading frame encoding a protein of 466 amino acids and a 3'-end non-coding region of 315 bp. The nucleotide sequence of rat vimentin thus obtained, shares a high homology with mouse (94 %) (12), hamster (92 %) (13) and human (88 %) (14) vimentin cDNA. At the protein level, an even higher homology is predicted: 99 % with mouse, 99 % with hamster and 96 % with human vimentin.

<u>Human primary prostate tumors</u>

Since the prostate is known to be of epithelial origin, one would expect only the expression of cytokeratins, the intermediate filaments specifically expressed in epithelial tissues. The different classes of intermediate filaments are generally expressed only in cells of a specific origin, and also individual tumors usually express only a single class of intermediate filaments (15, 16). Most times, the expression of the specific intermediate filament is retained after neoplastic transformation, offering the abililty to use these proteins as markers for the origin of a tumor (15, 16). However, an increasing number of reports are published, describing the coexpression of two classes of intermediate filaments in neoplasms (17). The overexpression of vimentin in rat prostate tumors suggests a coexpression of two classes of intermediate filaments. Coexpression of cytokeratins and vimentin has already been reported for normal human tissues and for several epithelial tumors (17). Also for benign and malignant prostatic epithelium the coexpression of vimentin and cytokeratins has been shown (18). However, more detailed studies will be necessary to investigate the potential relation of overexpression of vimentin with human prostate tumor progression. Especially immunohistological studies have to be performed to confirm that vimentin is present in the epithelial cells of prostatic tumors and that coexpression within cells occurs.

Figure 2: (A) Partial restriction map of rat vimentin cDNA. H, *Hin*dII; P, *Pst*I; S, *Sst*I; Sm, *Sma*I; X, *Xho*I. The bar indicates the vimentin-encoding region. (B) Nucleotide sequence and deduced amino acid sequence (in one-letter-code) of rat vimentin. The signal for polyadenylation (AATAAA) is underlined, the asterisk indicates the stopcodon. [Sequence data have been deposited with the EMBL/Genbank Data Libraries under accession no. X62953]



B

```
-50                                                                          -1
GTTCACAGCCACTGCGCCCTCGCTCTCTTCTTGCAGATCTTGCAGCCGCAGCAAGCCAGGCCACCTCGTCCTTCGAAGCC

1
ATG TCC ACC AGG TCC GTG TCC TCG TCC TCC TAC CGC AGG ATG TTC GGT GGC TCC GGC ACA TCG AGC CGG CCC AGC
 M   S   T   R   S   V   S   S   S   S   Y   R   R   M   F   G   G   S   G   T   S   S   R   P   S
TCC AAC CGG AGC TAT GTG ACC ACA TCC ACC CGC ACC TAC AGC CTA GGC AGC GCG CTG CGC CCC AGC ACT AGC CGC
 S   N   R   S   Y   V   T   T   S   T   R   T   Y   S   L   G   S   A   L   R   P   S   T   S   R
AGC CTC TAT TCC TCG TCC CCG GGT GGC GCC TAT GTG ACC CGG TCC TCC GCC GTG CGC CTG CGG AGC AGC ATG CCC
 S   L   Y   S   S   S   P   G   G   A   Y   V   T   R   S   S   A   V   R   L   R   S   S   M   P
GGC GTG CGG CTG CTG CAG GAC TCC GTG GAC TTC TCG CTG GCC GAC GCC ATC AAC ACC GAG TTC AAG AAC ACC CGC
 G   V   R   L   L   Q   D   S   V   D   F   S   L   A   D   A   I   N   T   E   F   K   N   T   R
ACC AAC GAG AAG GTG GAA TTG CAG GAG CTG AAT GAC CGC TTC GCC AAC TAC ATC GAC AAG GTG CGC TTC CTC GAG
 T   N   E   K   V   E   L   Q   E   L   N   D   R   F   A   N   Y   I   D   K   V   R   F   L   E
CAG CAG AAC AAA ATC CTG CTG GCC GAG CTC GAG CAG CTT AAG GGC CAG GGC AAG TCG CGC CTG GGC GAC CTC TAC
 Q   Q   N   K   I   L   L   A   E   L   E   Q   L   K   G   Q   G   K   S   R   L   G   D   L   Y
GAG GAG GAG ATG AGG GAG TTG CGC CGG CAG GTG GAT CAG CTC ACC AAT GAC AAG GCC CGT GTC GAG GTG GAG AGG
 E   E   E   M   R   E   L   R   R   Q   V   D   Q   L   T   N   D   K   A   R   V   E   V   E   R
GAC AAC CTG GCC GAG GAC ATC ATG CGG CTG CGA GAA AAA TTG CAG GAG GAG ATG CTC CAG AGG GAG GAA GCC GAG
 D   N   L   A   E   D   I   M   R   L   R   E   K   L   Q   E   E   M   L   Q   R   E   E   A   E
AGC ACC CTG CAG TCA TTC AGA CAG GAT GTT GAC AAT GCT TCT CTG GCA CGT CTT GAC CTT GAA CGT AAA GTG GAA
 S   T   L   Q   S   F   R   Q   D   V   D   N   A   S   L   A   R   L   D   L   E   R   K   V   E
TCC TTG CAG GAA GAA ATT GCC TTT TTG AAG AAG CTG CAC GAT GAA GAG ATC CAG GAG CTG CAG GCC CAG ATT CAG
 S   L   Q   E   E   I   A   F   L   K   K   L   H   D   E   E   I   Q   E   L   Q   A   Q   I   Q
GAA CAG CAT GTC CAG ATC GAT GTG GAC GTT TCC AAG CCT GAC CTC ACC GCT GCC CTG CGT GAT GTC CGC CAG CAG
 E   Q   H   V   Q   I   D   V   D   V   S   K   P   D   L   T   A   A   L   R   D   V   R   Q   Q
TAT GAA AGT GTG GCT GCC AAG AAC CTC CAG GAG GCC GAG GAA TGG TAC AAG TCC AAG TTT GCT GAC CTC TCT GAG
 Y   E   S   V   A   A   K   N   L   Q   E   A   E   E   W   Y   K   S   K   F   A   D   L   S   E
GCT GCC AAC CGG AAC AAC GAT GCC CTG CGC CAG GCA AAG CAG GAG TCA AAC GAA TAC CGG AGA CAG GTG CAG TCA
 A   A   N   R   N   N   D   A   L   R   Q   A   K   Q   E   S   N   E   Y   R   R   Q   V   Q   S
CTC ACC TGC GAA GTG GAT GCC CTT AAA GGC ACT AAT GAG TCC CTG GAG CGC CAG ATG CGT GAA ATG GAA GAG AAT
 L   T   C   E   V   D   A   L   K   G   T   N   E   S   L   E   R   Q   M   R   E   M   E   E   N
TTT GCC CTT GAA GCT GCT AAC TAC CAG GAC ACT ATT GGC CGC CTG CAG GAT GAG ATC CAG AAC ATG AAG GAA GAG
 F   A   L   E   A   A   N   Y   Q   D   T   I   G   R   L   Q   D   E   I   Q   N   M   K   E   E
ATG GCT CGC CAC CTT CGT GAA TAC CAG GAC CTG CTC AAT GTA AAG ATG GCT CTT GAC ATT GAG ATC GCC ACC TAC
 M   A   R   H   L   R   E   Y   Q   D   L   L   N   V   K   M   A   L   D   I   E   I   A   T   Y
AGG AAG CTG CTG GAA GGG GAG GAG AGC AGG ATT TCT CTG CCT CTT CCA AAC TTT TCT TCC CTG AAC CTG AGA GAA
 R   K   L   L   E   G   E   E   S   R   I   S   L   P   L   P   N   F   S   S   L   N   L   R   E
ACT AAC CTG GAG TCA CTT CCT CTG GTT GAC ACC CAC TCC AAA AGA ACA CTC CTG ATT AAG ACG GTT GAA ACC AGA
 T   N   L   E   S   L   P   L   V   D   T   H   S   K   R   T   L   L   I   K   T   V   E   T   R
GAC GGA CAG GTG ATC AAT GAG ACT TCT CAG CAC CAC GAT GAC CTT GAA TAA AAACTGCACAGGCTCAGTGCAACGGCGCAGT
 D   G   Q   V   I   N   E   T   S   Q   H   H   D   D   L   E   *
ACCAGCAAGAAGGAAAAAAAAATCGTATCTTAGAAAAAAGAGCTTTCAAGTGCCTTTACTGCAGTTTTCAGGAGCGCAAGATAGATCTGGGATAGAAAC
GAGCTCAGCACATAACAACTGACACCCCCAAAAGGCGTAGAAAAGGTTTACAAAATAATCTAGTTTTACGAAGAAATCTTGTGCTAGAATACTTTTTAA
AGTATTTTTGAATACCATTAAAACTGCTTTCTTTTTCCAGAAAATATCTGACCAACTTGTTACTGCTTCAATAAAACTTCAGAAAT(A)n
```

REFERENCES

1 Scott, M.R.D., Westphal, K.-H., and Rigby, P.W.J. Activation of mouse genes in transformed cells. Cell, 34: 557-567, 1983.

2 Yamamoto, M., Maehara, Y., Takahashi, K., and Endo, H. Cloning of sequences expressed specifically in tumors of rat. Proc. Natl. Acad. Sci. USA, 80: 7524-7527, 1983.

3 Matrisian, L.M., Glaichenhaus, N., Gesnel, M.-C., and Breathnach, R. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. EMBO J., 4: 1435-1440, 1986.

4 Steeg, P.S., Bevilacqua, G., Kopper, L., Thorgeirsson, U.P., Talmadge, J.E., Liotta, L.A., and Sobel, M.E. Evidence for a novel gene associated with low tumor metastatic potential. J. Natl. Cancer Inst., 80: 200-204, 1988.

5 Dear, T.N., Ramshaw, I.A., and Kefford, R.F. Differential expression of a novel gene WDNM1 in nonmetastatic rat mammary adenocarcinoma cells. Cancer Res., 48: 5203-5209, 1988.

6 Isaacs, J.T., and Hukku, B. Nonrandom involvement of chromosome 4 in the progression of rat prostatic cancer. Prostate, 13: 165-188, 1988.

7 Bussemakers, M.J.G., Van de Ven, W.J.M., Debruyne, F.M.J., and Schalken, J.A. Identification of high mobility group protein I(Y) as potential marker for prostate cancer by differential hybridization analysis. Cancer Res., 51: 606-611, 1991.

8 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

9 Auffray, C., and Rougeon, F. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem., 107: 303-314, 1980.

10 Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. Cloning in single stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol., 143: 161-178, 1980.

11 Devereux, J., Haeberli, P., and Smithies, O. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res., 12: 387-395, 1984.

12 Hennekes, H., Kühn, S., and Traub, P. Coding sequence and flanking regions of the mouse vimentin gene. Mol. Gen. Genet., 221: 33-36, 1990.

13 Bloemendal, H., Quax, W., Quax-Jeuken, Y., Dodemont, H., Ramaekers, F., Dunia, I., and Benedetti, L. Organization and expression of the vimentin gene. Mol. Biol. Rep., 9: 115-118, 1983.

14 Ferrari, S., Battini, R., Kaczmarek, L., Rittling, S., Calabretta, B., De Riel, J.K., Philiponis, V., Wei, J.F., and Baserga, R. Coding sequence and growth regulation of the vimentin gene. Mol. Cell. Biol., 6: 3614-3620., 1986.

15 Ramaekers, F.C.S., Puts, J.J.G., Moesker, O., Kant, A., Huysmans, A., Haag, D., Jap, P.H.K., Herman, C.J., and Vooijs, G.P. Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview. Histochem. J., 15: 691-713, 1983.

16 Nagle, R.B. Intermediate filaments: Efficacy in surgical pathological diagnosis. Am. J. Clin. Pathol., 91: S14-S18, 1989.

17 Coggi, G., Dell'Orto, P., Braidotti, P., Coggi, A., and Viale, G. Coexpression of intermediate filaments in normal and neoplastic human tissues: a reappraisal. Ultrastruct. Pathol., 13: 501-514, 1989.

18 Leong, A.S.-Y., Gilham, P., and Milios, J. Cytokeratin and vimentin intermediate filament proteins in benign and neoplastic prostatic epithelium. Histopathology, 13: 435-442, 1988.

CHAPTER V

DECREASED EXPRESSION OF E-CADHERIN IN THE PROGRESSION OF RAT PROSTATIC CANCER

Marion J.G. Bussemakers, Reindert J.A. van Moorselaar, Laurence A. Giroldi, Tomohiko Ichikawa (1), John T. Isaacs (1), Masatoshi Takeichi (2), Frans M.J. Debruyne, Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands, (1) Oncology Center, Johns Hopkins Hospital, Baltimore, MD, USA, (2) Department of Biophysics, Kyoto University, Sakyo-ku, Kyoto, Japan.

Cancer Research 52: May 1992, In Press

SUMMARY

Cadherins represent a family of Ca^{2+}-dependent cell adhesion molecules involved in homotypic, homophilic cell-cell interactions. Recent studies have shown that the cadherins can play a role in invasive and metastatic behaviour. Using the established Dunning R-3327 model system of serially transplantable rat prostate cancers, the expression of E- and P-cadherin in rat prostatic cancers was studied. Analysis within this system demonstrated that whereas E-cadherin was expressed in the normal rat prostate and the well- or moderately-differentiated, non-invasive Dunning tumors, no expression, neither at the mRNA nor at the protein level, could be detected in the invasive sublines. Since not all invasive Dunning tumors studied have metastatic ability, these results suggest that a decreased expression of E-cadherin is correlated with invasive behaviour rather than with metastatic ability.

Recently, genetic instability occurred in an animal bearing the well differentiated, androgen-responsive, slow growing, non-metastatic Dunning R-3327-H rat prostate cancer resulting in the progression to an anaplastic, androgen-independent, fast growing, highly metastatic state. This spontaneously arising tumor, termed the AT6 subline, in its original host was heterogeneously composed of both a well differentiated and an anaplastic population of cancer cells in which areas of squamous cell differentiation were occasionally observed. The original animal bearing this heterogeneous AT6 cancer developed multiple metastases, the lung metastases being heterogeneously composed of anaplastic and squamous cell populations. Cytogenetic analysis demonstrated that the lung metastases were derived from a specific subpopulation of cancer cells present in the original AT6 primary tumor. Immunohistochemical studies demonstrated that only the area of lung metastases displaying squamous morphology were positive for E-cadherin. In contrast, the anaplastic areas of the lung metastases and the metastases in other organs were E-cadherin negative. By the first passage of the AT6 tumor only the anaplastic cells were present and no detectable E-cadherin mRNA or protein was found in the primary tumor and metastatic deposits. These results suggest that a decreased expression of E-cadherin is associated with the progression of prostatic cancer.

INTRODUCTION

In the progression of cancer to an invasive and/or metastatic state, changes in cell-cell and cell-substratum interactions occur, thus indicating a critical involvement of cell adhesion molecules in tumor progression. Since adhesion molecules play a role in cell attachment, cellular motility and intercellular communication, uncontrolled expression of these molecules may lead to changes in cellular adhesion and increased motility, processes that may result in metastatic behaviour of cells. Several groups of adhesion molecules have been described comprising the integrins, adhesion molecules belonging to the immunoglobulin superfamily, the LEC-CAMs and the cadherins (1, 2). Recent studies showing a functional relation between E-cadherin and invasion render the Ca^{2+}-dependent cadherins of particular interest in the study of cancer progression.

Three cadherins have been cloned and extensively characterized in different species, namely N-, P-, and E-cadherin (2, 3). Recently, other subclasses of cadherins were isolated, suggesting that this family of adhesion molecules is much larger, comprising at least ten different cadherins (4-8).

In embryos, each of the well characterized cadherins has a specific spatiotemporal pattern of expression. The association or separation of cell layers is tightly correlated with the differential expression of cadherins and it is proposed that the selective adhesion of cells through homophilic interaction of the expressed cadherins is a key phenomenon in morphogenesis (3). Indeed, *in vitro* experiments have shown that the expression of recombinant cadherins mediates cell sorting in model systems (9, 10). In particular, when L-cells (which have virtually no endogenous cadherin activity) are transfected with E- or P-cadherin cDNAs, the E- and P-transfectants are able to segregate from each other when mixed.

In adult organisms, each cadherin displays a characteristic tissue distribution pattern although expression is not tissue-specific. N-cadherin is predominantly expressed in neural tissues and cells of mesodermal origin, yet it is also detected in local regions of certain epithelia. E-cadherin expression is generally restricted to epithelial tissues. The expression of P-cadherin has been less widely studied. In mice, it is expressed in certain epithelia and in the mesothelium (11). Of particular interest is the comparative study of E- and P-cadherin expression in human epithelial tissues by Shimoyama *et al.* (12). They show that while E-cadherin is expressed in virtually all epithelia, expression of P-cadherin is restricted to the basal or lower layers of

stratified epithelia (including the prostate glandular epithelium) where both antigens are expressed. This study and others dealing with an analysis of E- and P-cadherin expression in relation to cancers suggest that expression of P-cadherin may be correlated with maintenance of the proliferative compartment of certain epithelia while E-cadherin expression is associated with general differentiation features of epithelia (12-14).

Disfunction in the regulation of expression of the cadherins might very well be involved in cancer development and progression. In order to gain insight into the role of cadherins in the progression of prostate cancer, the expression of E- and P-cadherin was evaluated in the Dunning R-3327 system of serially transplantable rat prostatic cancers.

MATERIALS AND METHODS

Dunning R-3327 rat prostatic tumors

The geneaology, characteristics and maintenance of the Dunning sublines used in this study were as described before (15, 16). The parental tumor from which all rat prostatic tumor sublines were derived, is the original R-3327 tumor described by Dr. Dunning. The original tumor was passaged at different institutes, the tumor lines developing being termed after the institute. The G subline was developed by Dr. Dunning and is poorly differentiated, but androgen responsive. The R-3327-H is a slow-growing, well-differentiated, androgen responsive subline serially passaged at the Johns Hopkins University (Baltimore, Md). Furthermore, the H-tumor is heterogeneously composed of both androgen-dependent and -independent cells. By growing the H tumor in castrated rats it appeared to be possible to select *in vivo* for the androgen-independent subpopulation; the resulting slow-growing, well-differentiated line was termed HIS. By continuous passaging of HIS, random tumor progression gave rise to a moderately fast-growing, well-differentiated tumor HIM, and an even faster growing, moderately-differentiated subline HIF. The sublines AT1 and AT2 arose from the Dunning R-3327-H tumor passaged at Johns Hopkins. All these lines arose within one passage and are anaplastic, androgen-independently growing tumors with a low metastatic ability, i.e., less than 10% of the animals inoculated with tumor sublines develop distant metastases. MatLyLu and MatLu both arose from the AT1 tumor. AT3 arose from the HIF line. In rats, these last three sublines grow as

anaplastic tumors with a high metastatic ability, i.e., more than 90 % of the animals inoculated with these tumor sublines develop distant metastases. The characteristics of the Dunning sublines used in this study are summarized in Table 1.

Tumor transplantation

Primary tumors and tumors from subsequent passages were excised from tumor-bearing animals. After removal of normal and necrotic tissue, the tumor tissue was cut into 20-mg pieces. An 0.5 cm incision was made in the right flank of recipient animals, and after separating the subcutaneous tissue by blunt dissection, the tumor fragment was placed subcutaneously (s.c.). The incision was closed using skin clips (7.5 x 1.75 mm; agrave, Michel Instruvet, Amerongen, The Netherlands). In this way a tumor take of 100 % was achieved. Animals were sacrificed when the tumors were growing exponentially. Tumors were snap frozen in liquid nitrogen and stored for further analysis.

Cytogenetic analysis

Single cell suspensions were made by mincing tumors with scissors in RPMI 1640 containing 10 % fetal bovine serum (FBS) and colcemid (0.02 μg/ml) (all obtained from Life Technologies Inc.). After incubation at 37 ^{o}C for 10 min the

Table 1 : *In vivo* biological characteristics of Dunning R-3327 rat prostatic cancer sublines.

Subline	Histology	Doubling time (in days)	Androgen responsive	Invasive*	Metastatic ability**
H	Well-differentiated	22 ± 5	Yes	No	Low
HIS	Well-differentiated	24 ± 5	No	No	Low
HIM	Well-differentiated	9.0 ± 0.8	No	No	Low
HIF	Moderately-differentiated	4.8 ± 1.8	No	No	Low
G	Poorly-differentiated	4.0 ± 0.2	Yes	Yes	Low
AT-1	Anaplastic	2.5 ± 0.2	No	Yes	Low
AT-2	Anaplastic	2.5 ± 0.2	No	Yes	Low to moderate (lungs)***
AT-3	Anaplastic	1.8 ± 0.2	No	Yes	High (lymph nodes & lungs)
MATLu	Anaplastic	2.7 ± 0.2	No	Yes	High (lungs)
MATLyLu	Anaplastic	1.5 ± 0.1	No	Yes	High (lymph nodes & lungs)

* Invasiveness was histologically scored by a pathologist
** Low metastatic ability, <5% of s.c. inoculated rats develop distant metastases; moderate ability, >5%, <20%; high metastatic ability, >75% develop distant metastases.
*** Organs in the parentheses are the site of the distant metastases for the individual sublines.

medium was replaced with 0.075 M KCl hypotonic solution containing colcemid (0.02 μg/ml) which was prewarmed to 37 °C . The suspension was incubated at 37 °C for 25 min and fixed with methanol:acetic acid (3:1). Chromosomal slides were prepared by dropping the cell suspension onto clean slides in a humid box. Thirty to 50 metaphases were counted for each tumor. Chromosomes were banded using the trypsin-Giemsa technique (17) and arranged according to the scheme of Satoh *et al.* (18). At least five G-banded metaphases were karyotyped for each tumor.

Northern blot analysis

Total RNA was isolated according to Auffray and Rougeon (19). Ten μg of total RNA were glyoxylated, size fractioned on 1 % agarose gels and transferred to Hybond-N^+ (Amersham). Hybridizations were performed as described before (16). Probes were radioactively labeled by nick-translation. As probes were used a 600 bp *Eco*RI/*Ava*I fragment of the mouse E-cadherin cDNA (20) and the 3.2 kb *Eco*RI fragment, comprising the full length mouse P-cadherin cDNA (21).

Immunohistochemistry

Paraffin embedded sections were deparaffinized with xylene, and rehydrated in ethanol (sequentially 100 % and 70 %). Frozen sections were fixed for 15 min in 3 % paraformaldehyde. After this, both protocols are similar. After rinsing with PBS (2 x 10 min), quenching in 50 mM NH_4Cl in PBS for 10 minutes and again rinsing with PBS (2 x 10 min), cells were permeabilized in 0.2 % Triton X100 in PBS for 5 min. Following rinsing with PBS (2 x 10 min) and treatment with PBS/5 % BSA for 30 min, the sections were incubated overnight at 4 °C with a polyclonal rabbit antiserum raised against the purified 84 kDa fragment of rat E-cadherin (kindly provided by Dr. R. Kemler). The antibody was diluted 1 : 90 in PBS/BSA. After washes with PBS (2 x 10 min), biotinylated secondary antibody (biotinylated anti-rabbit immunoglobulin from donkey / Amersham - diluted 1 : 200 in PBS/1 % BSA) was applied for 30 min. Following rinsing in PBS as before, sections were incubated with Avidine-biotine complexes (Vectastain / Brunschwig) for 45 min. After rinsing in PBS (2 x 10 min) the sections were treated with diaminobenzidine solution (diluted 1 : 10 in PBS/0.65 % imidazol). Following rinsing with water for 5 min, sections were incubated with 0.5 % $CuSO_4$ (in 0.9 % NaCl) for 5 min, rinsed with water for 5 min and finally counterstained with hematoxylin.

RESULTS

Expression of E- and P-cadherin in established Dunning R-3327 rat prostatic cancer sublines

The Dunning R-3327 model system consists of series of distinct rat prostatic cancers each related to the parental R-3327 tumor (15). The tumor lines differ widely in their biological characteristics (i.e. hormone dependency, growth rate, differentiation and metastatic ability) (see Table 1). Steady-state mRNA levels of E-cadherin and P-cadherin were determined in these tumor lines (Fig. 1 and 2). In a Northern assay using a mouse E-cadherin probe (20), all non-invasive lines with some degree of differentiation, had levels of E-cadherin mRNA similar to those found in the normal prostate (i.e., H subline) or even higher (i.e., HIS, HIM, HIF sublines). The poorly differentiated G line, the anaplastic non-metastasizing tumors AT1 and AT2, and the anaplastic metastasizing tumors AT3, MatLu and MatLyLu (all invasive tumor lines) did not express E-cadherin at detectable levels. It thus appeared that E-cadherin was not expressed in the invasive Dunning tumor lines.

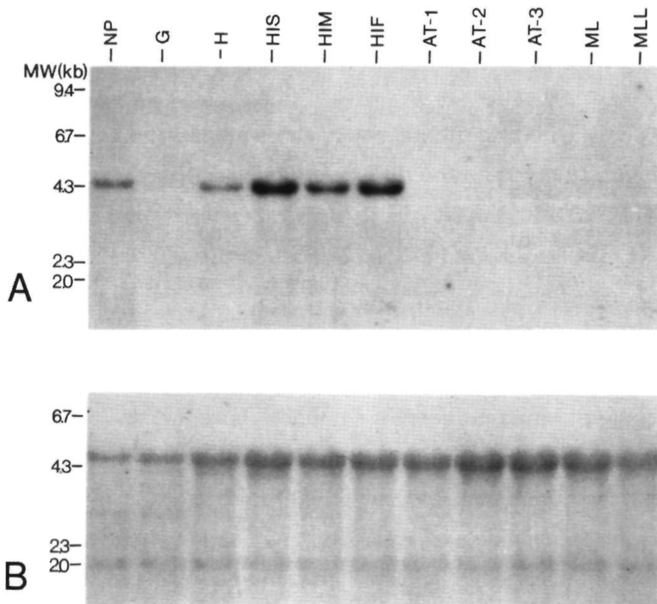


Figure 1: Northern blot analysis of E-cadherin (A). Ten μg of total RNA of normal prostate (NP) and 10 Dunning tumors were loaded per lane: H, HIS, HIM and HIF are non-invasive; G, AT-1 and AT-2 are invasive and non-metastatic; AT-3, MatLu and MatLyLu are invasive and metastatic. rRNA was used as an internal control for the amounts of RNA loaded (B). Lambda DNA cut with *Hind*III was used as a molecular marker.

Similar studies using P-cadherin as a probe (21) showed expression of this gene in all E-cadherin expressing lines. In addition, AT1 and AT2 (low metastatic sublines) and MatLu (metastatic line) also expressed P-cadherin. The G subline did not express P-cadherin (Fig. 2).

The E-cadherin expression pattern was also studied immunohistochemically. Using a polyclonal antibody against rat E-cadherin, sections of the different Dunning sublines were stained. In the (well-) differentiated tumors the antibody clearly reacts with the cell membrane (Figure 5A, B) whereas in the sections of the anaplastic tumors no staining is found (comparable to Figure 5C, D), thereby confirming our results obtained by Northern analysis (see Table 2).

<u>Spontaneous progression of the well-differentiated Dunning R-3327-H subline to the anaplastic, metastatic AT6 subline is associated with specific chromosomal changes</u>

The Dunning R-3327 sublines have been passaged through many transplant generations. This serial transplantation may result in molecular changes which are not critically associated with the particular phenotype of any subline. The fact that decreased E-cadherin expression occurs, without exception, in the invasive sublines makes it rather unlikely that it is a trivial phenomenon. To test this more directly, advantage was taken from the fact that during a recent passage of the well-differentiated, androgen-responsive, H subline, a spontaneous progression took place in a tumor-bearing animal, resulting in the development of an androgen-independent, anaplastic subline that was highly metastatic in its initial passage. This new tumor is termed the AT6 subline. The AT6 tumor in its original host (AT6p0) was highly metastatic to lymph nodes, lung, adrenals, and liver, yet its doubling time (i.e., 4 days) was longer than the other established Dunning R-3327 metastatic sublines (i.e., approximately 2 days for AT3, MatLu and MatLyLu). Both the primary tumor and a lung metastasis from the original passage were serially transplanted and both gave rise to highly metastatic tumors.

Karyotype analyses were performed on the parental AT6 tumor in its first passage and its lung metastases. In the primary tumor, there was a marked heterogeneity in the chromosomal pattern. Three subpopulations could be discriminated on basis of karyotype termed "A", "B", and "C" (Table 3). Relative proportions of the "A" through" C" populations were 24%: 50%: 24% respectively). The karyotypes all revealed an additional copy of chromosome 4 and deletions on the long arm of chromosome 3 (del(3)(q32q36)) and the short arm of chromosome

15 (del(15)(p14)). Karyotype "A" could be discriminated on basis of an additional copy of chromosome 12, and "C" on basis of the presence of a marker chromosome. All karyotypes were different from the parental H subline which has a normal karyotype (15).

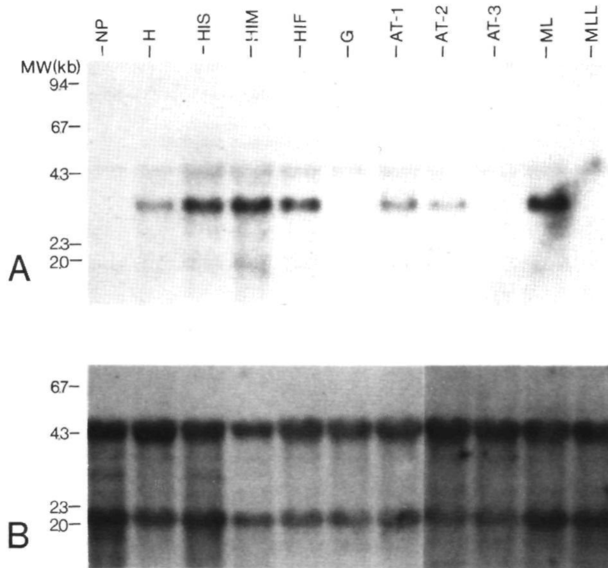


Figure 2: Northern blot analysis of P-cadherin (A). Ten μg of total RNA of normal prostate (NP) and 10 Dunning tumors were loaded per lane: H, HIS, HIM and HIF are non-invasive; G, AT-1 and AT-2 are invasive and non-metastatic; AT-3, MatLu and MatLyLu are invasive and metastatic. rRNA was used as an internal control for the amounts of RNA loaded (B). Lambda DNA cut with *Hin*dIII was used as a molecular marker.

Table 2: Evaluation of E-cadherin expression in the Dunning R-3327 rat prostatic cancer sublines

Subline	Histological invasive	mRNA*	protein**
H	No	+	+
HIS	No	+	+
HIM	No	+	+
HIF	No	+	+
G	Yes	-	-
AT-1	Yes	-	-
AT-2	Yes	-	-
AT-3	Yes	-	-
MATLu	Yes	-	-
MATLyLu	Yes	-	-

* E-cadherin mRNA expression evaluated by Northern blot analysis.
** Immunohistochemical evaluation using a polyclonal anti-E-cadherin antibody

Table 3: Karyotype analyses of the AT6p0 tumor

karyotype A	44 XY	+4, +12, del (3)(q32q36), del 15(p14)
karyotype B	43 XY	+4, del (3)(q32q36), del 15(p14)
karyotype C	43 XY	+4, del (3)(q32q36), del 15(p14), +mar

Interestingly, in the lung metastases only the population with karyotype "A" could be found. Thus, chromosomal analyses revealed that a selective enrichment of this subpopulation occured. We established a cell line from the lung metastasis (AT6-Lu), and in the first ten passages it retained its parental karyotype "A". Furthermore, injection of $1x10^6$ cells gave rise to highly metastatic tumors (lungs, 100 %; lymph nodes and kidney, 40-60%; occasionally liver metastases).

Expression of E- and P-cadherin in the newly arising metastatic Dunning R-3327 AT6 subline

Studying the E-cadherin expression in the AT6 tumor, it was demonstrated that in the initial host, the metastatic AT6 tumor had detectable levels of E-cadherin mRNA although these levels were lower than in normal prostate or in the parental Dunning R3327-H-tumor (see Fig. 3). In the first passage, termed AT6p1, however,

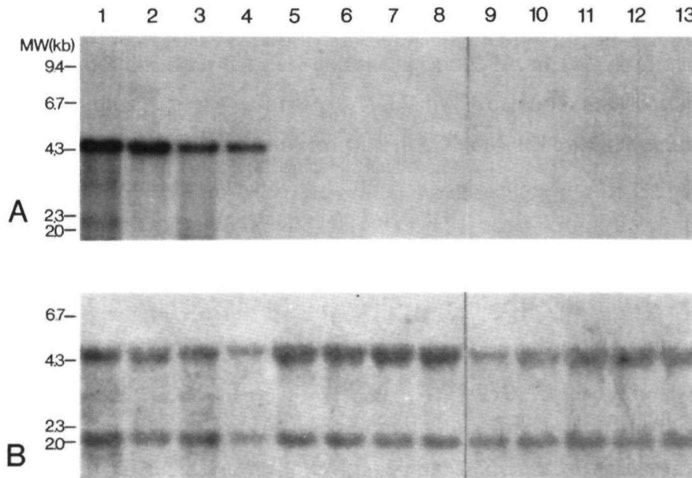


Figure 3: Northern blot analysis of E-cadherin (A). Ten μg of total RNA was loaded per lane. 1) normal prostate; 2) Dunning-H tumor; 3) AT6p0; 4) lung metastasis derived from AT6p0; 5-8) further transplant generations of the lung metastasis; 9-13) further transplant generations of the original AT6p0 (AT6p1-AT6p5). rRNA was used as an internal control for the amounts of RNA loaded (B). Lambda DNA cut with *Hin*dIII was used as a molecular marker.

no E-cadherin transcripts were detected (Fig. 3, lane 9). The AT6 lung metastases in the initial host also expressed E-cadherin (Figure 3, lane 4) whereas upon further transplantation of the lung metastases, E-cadherin expression was completely lost (Fig. 3, lanes 5-8). P-cadherin expression parallels this pattern, by showing expression in normal prostate, H tumor, and AT6p0, and no detectable levels of expression in the AT6 after its first passage (AT6p1 and further) (data not shown).

In order to gain more insight in the process that led to a decreased expression of E-cadherin, histological- (Fig. 4) and immunohistochemical (Fig. 5) analyses of the initial passages of AT6 and the metastases were performed. In the original host, AT6 was heterogeneously composed of well-differentiated cells [derived from the original H tumor (Fig. 4A)] and an anaplastic subpopulation (Fig. 4B) in which also squamous cell differentiation was evident (Fig. 4C). In the metastases only anaplastic tumor cells were found with exception of the lung metastases, in which the anaplastic cell populations (Fig. 4D) were accompanied by cell clusters with squamous cell differentiation (Fig. 4E). In some cases within one tumor cell focus, both morphotypes were evident (Fig. 4F). Immunohistochemical analyses of the lung metastases revealed that the anaplastic cells were negative for E-cadherin (Fig. 5C, D), whereas cells with the squamous morphotype stained with E-cadherin antibodies (Fig. 5E, F). The heterogeneous expression of E-cadherin may in fact explain the reduced abundance of E-cadherin mRNA in these tumors. In the subsequent passages, both the primary AT6 tumor as well as its metastases were homogeneously composed of anaplastic tumor cells, that did not express E-cadherin either at the mRNA or at the protein level.

DISCUSSION

The important role of cadherins in invasive and metastatic behaviour of cancer cells and the fact that cadherins are expressed in normal prostate, led us to study the expression of E- and P-cadherin in prostatic cancer using the Dunning model system. Comparisons within the established Dunning sublines showed a clear expression of E-cadherin in normal prostate and the sublines with some degree of differentiation and low invasive potential (H, HIS, HIM, HIF) whereas no transcripts were found in poorly differentiated (G), anaplastic, non-metastasizing (AT1 and AT2), and anaplastic, metastasizing (AT3, MatLu, MatLyLu) sublines, all having a

high invasive potential, indicating a correlation between decreased expression of E-cadherin and invasion rather than metastasis. These findings were confirmed at the protein level where a membrane staining in the (well-)differentiated, non-invasive Dunning sublines is seen and no reaction at all in the poorly-differentiated or

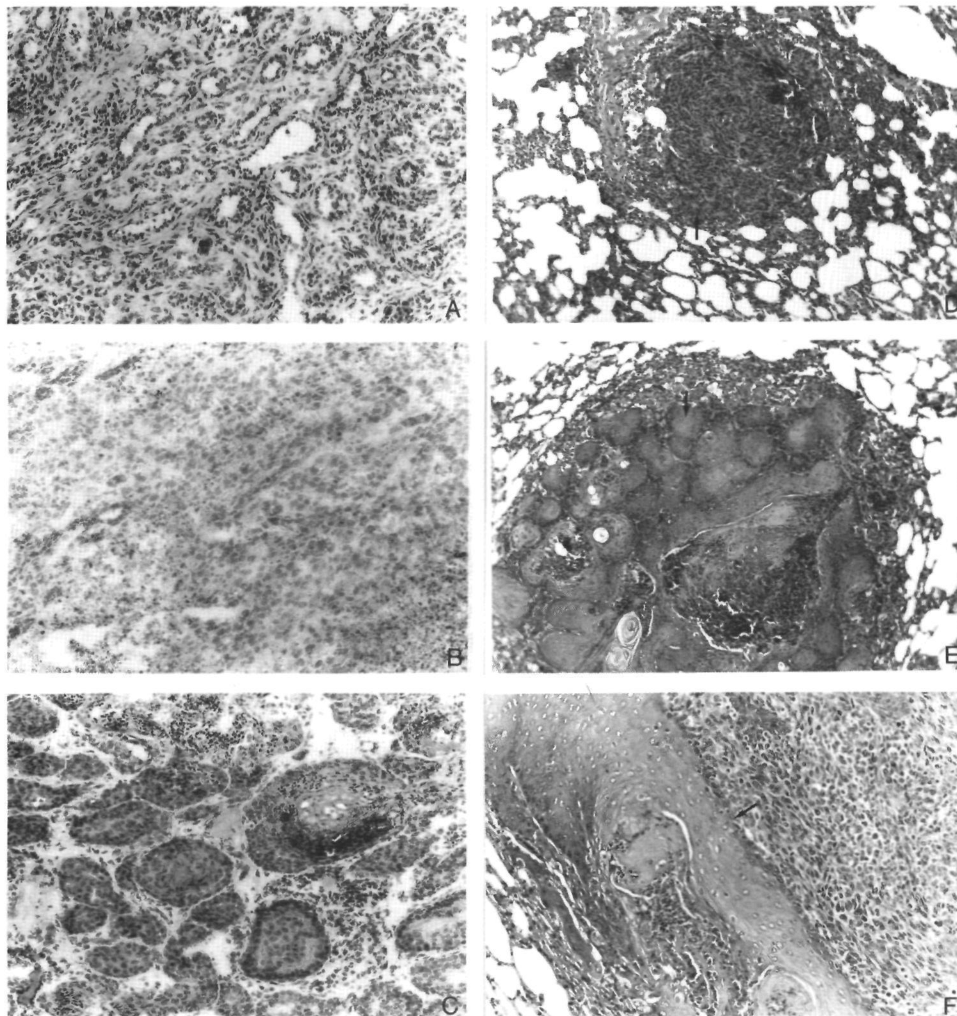


Figure 4: Histological evaluation of AT6 in its original host, primary tumor (A-C) and lung metastasis (D-F). A: subpopulation of well differentiated cells (400x). B: anaplastic subpopulation (400x). C: area with squamous cell differentiation (400x). D: lung metastasis comprised of anaplastic cells (indicated by arrow) (100x). E: squamous cell differentiation in lung metastasis (indicated by arrow) (100x) and F: mixed type morphology in lung metastasis (indicated by arrow) (400x).

anaplastic, invasive sublines. A correlation of E-cadherin loss with invasion is also suggested by work from Frixen *et al.* (14) who analyzed the expression of E-cadherin at both the mRNA and protein level in 25 human cell lines derived from bladder, colon, breast, lung and pancreatic carcinomas. They showed that the differentiation

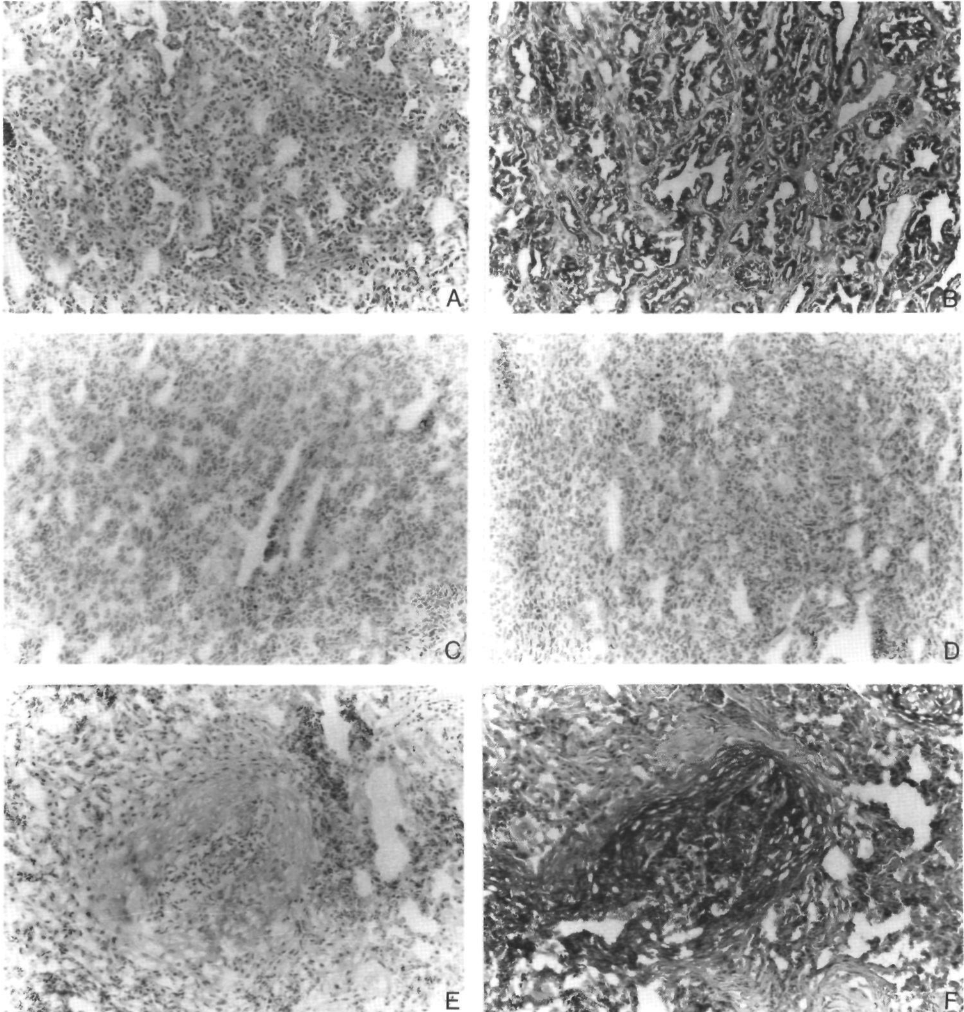


Figure 5: Immunohistochemical evaluation of E-cadherin expression in the lung metastasis of AT6 in its original host, using a polyclonal antibody against E-cadherin on frozen sections. A, C, E: incubation with preimmune serum. B, D, F: incubation with antibody. As positive control the parental Dunning-R-3327-H tumor was included (A, B). AT6 anaplastic cell populations (C, D) and squamous cell populations (E, F).

state of the cell lines (judged by their morphotype) did not perfectly correlate with E-cadherin expression but that there was an inverse relationship between the level of cadherin expression and the extent of invasion in a collagen gel.

For P-cadherin no obvious relation of expression with differentiation or invasion is seen in the Dunning model system. The coexpression of E- and P-cadherin in rat prostatic tumors is probably associated with the basal cell layer of the prostatic epithelium; in earlier studies it was shown that in the tumor lines HIS, HIM, and HIF, which arose through castration-induced selection of androgen-independent cells (15, 22), there is a specific enrichment of tumor cells with a cytokeratin expression pattern specific for the basal cell population. Since this enrichment for a basal cell population is paralleled by an increase of E- and P-cadherin expression, this can indicate that indeed this coexpression is specific for hyperproliferative basal layers of stratified epithelium as was already suggested by Shimoyama *et al.* (12).

The spontaneously developed AT6 subline offered the ability to study changes in cadherin expression at the time when a more aggressive tumor phenotype arose. In this AT6 subline a decrease of E-cadherin mRNA expression was observed in its initial host in both the primary tumor and the lung metastases, suggesting that a decreased expression of E-cadherin is associated with the progression from an androgen-responsive, non-metastasizing tumor to an androgen-independent, metastasizing tumor. The processes that led to the lower E-cadherin mRNA levels in the AT6 tumor were partly elucidated by detailed histological and immuno-histochemical analyses using E-cadherin antibodies. As expected, the primary AT6 tumor was heterogeneously composed of well differentiated, E-cadherin-positive areas, presumably originating from the H tumor, and anaplastic, E-cadherin-negative cell populations in which also a squamous cell differentiation was found. In human prostate cancer squamous differentiation is extremely rare. In the Dunning R-3327-H rat prostate cancer, however, we occasionally observed squamous differentiation following androgen withdrawal (unpublished observation).

In metastases one would expect selective outgrowth of the metastatic subpopulation. Indeed the karyotype analyses of AT6 indicated that the lung metastases were derived from a subpopulation in the primary tumor. Nevertheless, the histology of the metastases in the original host revealed two morphotypes, i.e., anaplastic and squamous cells. The squamous cell morphology was never found in the absence of anaplastic cells, whereas the anaplastic cells were frequently found in the absence of squamous cell morphology. Furthermore, after the first transplant

generations, the squamous differentiation was completely absent. Therefore, the squamous populations in the original AT6 tumor are most likely derived from the anaplastic cells. The development of squamous morphology appears to be associated with reexpression of E-cadherin, thus explaining the lower levels of E-cadherin mRNA in mixed morphology AT6 primary tumors and metastases in the original host.

The temporary decreased expression of E-cadherin suggests a role for E-cadherin in the development of invasiveness. This is in agreement with other observations that heterogeneous expression of E-cadherin is related to invasive behaviour. Mareel *et al.* (23) showed that upon injection of *ras* transformed cells with an epithelial morphotype into nude mice, primary tumors developed which were heterogeneous with respect to cadherin expression, i.e., E-cadherin-positive, well-differentiated, epithelial structures as well as E-cadherin-negative, undifferentiated areas were present in the primary tumor and in metastases. Other interesting data have been obtained by Hashimoto *et al.* (24) working with ovarian tumor cell lines with low or high metastatic ability. While the low metastatic cells homogeneously expressed E-cadherin, the highly metastatic cell line contained both E-cadherin-negative and E-cadherin-positive cells, a phenomenon persisting even upon subcloning. This shows that expression of E-cadherin can become unstable and that this may in fact be causally related to the metastatic phenotype. Unstable expression of adhesion molecules seems to be a common event in cancer progression, since mot only is it found for E-cadherin in gastric tumors (13), hepatocellular carcinoma (25) and prostatic tumors (our results) but also unstable expression of the integrin VLA-4 is found in metastatic melanomas (26).

Our findings suggest that E-cadherin may have an invasion/metastasis suppressor function also in prostate cancer development. Interestingly, restriction fragment length polymorphism (RFLP) analyses of 28 human prostate cancer specimen have shown a frequent loss of heterozygosity of the chromosomes 10q and 16q (27). The region involved on chromosome 16q comprises the E-cadherin locus (28). Since loss of heterozygosity is considered to be the hallmark of tumor suppressor gene inactivation, it is tempting to speculate that E-cadherin is a candidate for being an invasion/metastasis suppressor gene. Frequent deletions involving the same chromosomal region have also been detected in hepatocellular carcinomas (29-31), in breast cancer (32) and neuroectodermal tumors of the central nervous system (33) suggesting common pathological mechanisms in these malignancies. Whether there is

a relationship between allelic loss of E-cadherin and the deregulation of its normal function remains yet to be established.

ACKNOWLEDGMENTS

We greatly acknowledge Dr. D.J. Ruiter for reviewing the histopathological analyses. We would like to thank Dr. R. Kemler for kindly providing the polyclonal E-cadherin antibody. We also would like to thank Tilly Aalders, Adrie van Bokhoven and Anja de Ruijter for excellent technical assistance. These studies were supported by the Dutch Cancer Foundation (NUKC90-01) and the Royal Dutch Academy of Arts and Sciences (J.A.S.).

REFERENCES

1 Albelda, S.M., and Buck, C.A. Integrins and other cell adhesion molecules. FASEB J., 4: 2868-2880, 1990
2 Takeichi, M. Cadherins. a molecular family important in selective cell-cell adhesion. Annu. Rev. Biochem., 59 237-252, 1990.
3 Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator Science, 251: 1451-1455, 1991
4 Suzuki, S., Sano, K., and Tanihara, H. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. Cell Regul, 2. 261-270, 1991.
5 Napolitana, E.W., Venstrom, K., Wheeler, E.F., and Reichardt, L.F. Molecular cloning and characterization of B-cadherin, a novel chick cadherin. J Cell Biol, 113: 893-905, 1991.
6 Ranscht, B., and Bronner-Fraser, M. T-cadherin expression alternates with migrating neural crest cells in the trunk of the avian embryo Development, 111. 15-22, 1991
7 Ginsberg, D., DeSimone, D., and Geiger, B. Expression of a novel cadherin (EP-cadherin) in unfertilized eggs and early *Xenopus* embryos Development, 111. 315-325, 1991.
8 Angres, B., Muller, A.H.J., Kellermann, J., and Hausen, P. Differential expression of two cadherins in *Xenopus laevis*. Development, 111: 829-844, 1991.
9 Nose, A., Nagafuchi, A., and Takeichi, M. Expressed recombinant cadherins mediate cell sorting in model systems. Cell, 54: 993-1001, 1988.
10 Friedlander, D.R., Mège, R.-M., Cunningham, B.A., and Edelman, G.M. Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces Proc Natl Acad. Sci. USA, 86. 7043-7047, 1989.
11 Takeichi, M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development, 102: 639-655, 1988.
12 Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M., and Abe, O Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Res., 49 2128-2133, 1989.
13 Shimoyama, Y., and Hirohashi, S. Expression of E- and P-cadherin in gastric carcinomas. Cancer Res., 51. 2185-2192, 1991.
14 Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Löchner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells J Cell Biol., 113: 173-185, 1991.

15 Isaacs, J.T., and Hukku, B. Nonrandom involvement of chromosome 4 in the progression of rat prostatic cancer. Prostate, 13: 165-188, 1988.

16 Bussemakers, M.J.G., Van de Ven, W.J.M., Debruyne, F.M.J., and Schalken, J.A. Identification of High Mobility Group Protein I(Y) as potential progression marker for prostate cancer by differential hybridization analysis. Cancer Res., 51: 606-611, 1991.

17 Seabright, M. A rapid banding technique for human chromosomes. Lancet, 2: 971-972, 1971.

18 Satoh, H., Yoshida, M.C., and Sasaki, M. High resolution chromosome banding in the Norway rat, *Rattus norvegicus*. Cytogenet. Cell Genet., 50: 151-154, 1989.

19 Auffray, C., and Rougeon, F. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem., 107: 303-314, 1980.

20 Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yashuda, K., and Takeichi, M. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. Nature, 329: 341-343, 1987.

21 Nose, A., Nagafuchi, A., and Takeichi, M. Isolation of placental cadherin cDNA: identification of a novel gene family of cell-cell adhesion molecules. EMBO J., 6: 3655-3661, 1987.

22 Verhagen, A.P.M., Aalders, T.W., Ramaekers, F.C.S., Debruyne, F.M.J., and Schalken, J.A. Differential expression of keratins in the basal- and luminal compartment of rat prostatic epithelium during degeneration and regeneration. Prostate, 13: 25-38, 1988.

23 Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C., and Van Roy, F.M. Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. Int. J. Cancer, 47: 922-928, 1991.

24 Hashimoto, M., Niwa, O., Nitta, Y., Takeichi, M., and Yokoro, K.A.F. Unstable expression of E-cadherin adhesion molecules in metastatic ovarian tumor cells. Jpn. J. Cancer Res., 80: 459-463, 1989.

25 Shimoyama, Y., and Hirohashi, S. Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. Cancer Lett., 57: 131-135, 1991.

26 Martin-Padura, I., Motarini, R., Lauri, D., Bernasconi, S., Sanchez-Madrid, F., Parmiani, G., Mantovani, A., Anichini, A., and Dejana, E. Heterogeneity in human melanoma cell adhesion to cytokine activated endothelial cells correlates with VLA-4 expression. Cancer Res., 51: 2239-2241, 1991.

27 Carter, B.S., Ewing, C.M., Ward, W.S., Treiger, B.F., Aalders, T.W., Schalken, J.A., Epstein, J.I., and Isaacs, W.B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc. Natl. Acad. Sci. USA, 87: 8751-8755, 1991.

28 Mansouri, A., Spurr, N., Goodfellow, P.N., and Kemler, R. Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. Differentiation, 38: 67-71, 1988.

29 Zhang, W., Hirohashi, S., Tsuda, H., Shimosato, Y., Yokota, J. Terada, M., and Sugimura, T. Frequent loss of heterozygosity on chromosomes 16 and 4 in human hepatocellular carcinoma. Jpn. J. Cancer Res., 81: 108-111, 1990.

30 Tsuda, H., Zhang, W., Shimosato, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T., and Hirohashi, S. Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA, 87: 6791-6794, 1990.

31 Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Immamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., Nakagama, H., Harada, H., Yagura, M., Matsubara, K., and Nakamura, Y. Allelotype study of primary hepatocellular carcinoma. Cancer Res., 51: 89-93, 1991.

32 Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G., and Nakamura, Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res., 50: 7184-7189, 1990.

33 Thomas, G. A., and Raffel, C. Loss of heterozygosity on 6q, 16q, and 17p in human central nervous system primitive neuroectodermal tumors. Cancer Res., 51: 639-643, 1991.

CHAPTER VI

DECREASED EXPRESSION OF E-CADHERIN IN HIGH GRADE PROSTATE CANCER

Rainy Umbas, Marion J.G. Bussemakers, William B. Isaacs (1), Tilly W. Aalders, Bob S. Carter (1), H. Ewout Schaafsma (2), Herbert F.M. Karthaus (3), Frans M.J. Debruyne and Jack A. Schalken

Urological Research Laboratory, University Hospital Nijmegen, The Netherlands,
(1) Brady Urological Institute, Johns Hopkins Hospital, Baltimore MD, USA,
(2) Department of Pathology, University Hospital Nijmegen, The Netherlands
(3) Department of Urology, Canisius Wilhelmina Hospital,
Nijmegen, The Netherlands

Submitted

SUMMARY

E-cadherin is a Ca^{2+}-dependent cell adhesion molecule thought to play an important role in normal growth and development, via mediation of homotypic, homophilic cell-cell interactions. Recent studies suggest that E-cadherin may be involved in neoplastic progression as a suppressor of invasion and/or metastasis. We have previously demonstrated that the invasive phenotype of rat prostatic cancer cells is associated with decreased expression of E-cadherin [see chapter V]. This is of particular interest since the locus to which the human E-cadherin gene is mapped (16q21-23), is frequently involved in allelic loss in prostate cancer.

To further characterize a potentially important role of E-cadherin as an invasion/metastasis suppressor gene in the progression of prostate cancer we analyzed E-cadherin expression at the protein and mRNA level in non-malignant and malignant specimens of human prostatic tissue. In 40 of 84 tumors, immunohistochemical studies showed reduced E-cadherin staining when compared to non-malignant prostate which uniformly stained strongly positive. In this study a significant correlation was found between decreasing expression of E-cadherin and increasing Gleason score of the tumors. Decreased levels of E-cadherin were also detected in 6 of 8 metastatic deposits of prostate cancer. These results indicate that in most, but not all prostate cancers, tumor progression is accompanied by decreasing levels of E-cadherin protein. Interestingly, in 16 primary tumors in which mRNA levels were simultaneously assayed, there was no significant correlation between E-cadherin mRNA expression and diminished protein staining. These results suggest that the observed decrease in E-cadherin staining intensity cannot easily be explained by transcriptional inhibition of the E-cadherin gene and therefore other mechanisms should be considered.

INTRODUCTION

Cadherins are cell surface glycoproteins involved in homophilic-homotypic Ca^{2+}-dependent cell-cell adhesion (1). These molecules play an essential role in embryonic development and morphogenesis and in maintenance of the normal structure and function of adult tissues (2, 3).

Thus far, more than ten subclasses of cadherins have been identified (4-9). Until now, three of them, E-, N-, and P-cadherin, have been well characterized and shown to share a common basic structure and mediate cell-cell binding in a homophilic and subclass-specific manner (10). E-cadherin is also known as uvomorulin (11), L-CAM (12), cell-CAM 120/80 (13) or Arc-1 (14). Its expression is restricted to epithelial tissues. Only a few epithelia such as that at proximal tubules, in which clear boundaries between cells cannot be seen at the light microscopic level, do not express E-cadherin (15, 16). Although E-cadherin can be detected in almost all human epithelial cells, its expression levels are different in various anatomic sites and may be influenced by maturation or malignant transformation of cells (17).

In a study on a variety of human cancer cell lines, Frixen *et al.* found that non-invasive cell lines expressed E-cadherin whereas invasive carcinoma cell lines had lost E-cadherin expression. Invasiveness of these latter cells could be prevented by transfection with E-cadherin cDNA (18). Recent studies showed that a highly un-differentiated liver carcinoma lacked E-cadherin expression (19) as did poorly-differentiated squamous cell carcinomas of the head and neck (20) while only a few of undifferentiated gastric carcinomas did not express E-cadherin immuno-histochemically (21). Our previous study using the Dunning R-3327 rat prostatic cancer model system revealed expression of E-cadherin mRNA in the well- and poorly-differentiated sublines with low invasive potential, while all established lines with high invasive potential had no detectable levels of E-cadherin transcripts. These findings are confirmed at the protein level. In tumor lines derived from a *de novo* arisen metastatic subline (AT6p0) E-cadherin expression was also decreased (22). Another relevant finding is the fact that in human prostate cancer, the chromosomal segment most frequently implicated in allelic loss (16q22-24) harbors the E-cadherin gene, which supports an invasion/metastasis suppressor function for E-cadherin (23, 24). To further substantiate a possible important role of E-cadherin in prostate cancer development, we evaluated the expression of E-cadherin in human prostatic cancer tissues by immunohistochemical and Northern blot analysis.

MATERIALS AND METHODS

Surgical Specimens

We used snap frozen tissues of 84 human prostatic carcinoma specimens as well as 8 metastatic lesions in lymph nodes [7] and testis [1]. Twentythree non-malignant prostate specimens were also included in this investigation. All of these tissues were obtained at the time of surgery. Serial sections from the frozen tissues were cut on a cryostat at 4-6 μm and air dried. The sections were stored at -20 ^oC until use.

Antibodies

L-CAM (Uvomorulin) monoclonal (Euro-diagnostics BV, The Netherlands), a monoclonal antibody against E-cadherin was used for immunohistochemical staining. In addition, some sections were stained with another monoclonal antibody against E-cadherin, HECD-1 (a kind gift from Dr. S. Hirohashi, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed at room temperature. The sections were fixed with acetone for 10 min and air dried. Preincubations were done with normal rabbit serum diluted 1:10 with PBS for 30 min. The sections were subsequently incubated with primary mAb diluted 1:10 for 1 h. After rinsing with PBS the sections were incubated with rabbit anti-mouse immunoglobulin peroxydase (Dakopatts A/S, Denmark/ diluted 1:100) for 30 min, and rinsed again with PBS before staining with diaminobenzidine 0.6 mg/ml in 0.65% imidazol/PBS containing 25 μl H_2O_2 for 5 min. Following brief rinsing with water, sections were incubated with 0.5% $CuSO_4$ (in 0.9% NaCl) for 5 min. After rinsing briefly with water, the sections were counter-stained with hematoxylin, dehydrated and mounted. Primary antibody and rabbit anti-mouse immunoglobulin peroxydase were diluted with 1% BSA/PBS.

Immunohistochemical criteria of E-cadherin expression

Staining was scored positive (+) if >90% of the tumor cells stained with an intensity comparable to non-malignant prostate. If 10-90% and <10% of the cancer cells were positively stained, it was scored as heterogeneous and negative (-) respectively. In cases with two different patterns of Gleason grade, the staining score was determined toward the poorest grade.

Histopathological grading

One section from each serial sections for the immunohistochemical analysis was stained with hematoxylin and eosin to determine the histopathological grading using the Gleason score (25). The tumor specimens score ranged from 4 to 10, while two of the metastatic tissues were determined as Gleason 4, and six as Gleason 8.

Northern blot analysis

For the Northern blot analysis tumor material was step sectioned. Samples containing >70 % of cancer cells were used for isolation of total RNA according to Auffray and Rougeon (26). Ten μg of total RNA was glyoxylated and loaded on a 1 % agarose gel. After capillary transfer onto Hybond-N^+ (Amersham), RNA was fixed by UV-irradiation and blots were hybridized with a human E-cadherin probe pV962 (27), kindly provided by Dr. R. Kemler (Freiburg, FRG). For quantification of the levels of expression of E-cadherin, autoradiographs were scanned using an LKB 2202 Ultrascan laser densitometer connected with an LKB 2202 recording integrator. After quantification of the autoradiographs, the amount of rRNA was used as a reference to correct the quantity of RNA as described before (28).

RESULTS

E-cadherin expression in non-malignant and malignant prostatic tissue: immunohistochemical evaluation

E-cadherin in non-malignant prostate

Samples of non-malignant prostatic tissue either obtained after cystoprostatectomy or radical prostatectomy were examined for E-cadherin expression immunohistochemically. E-cadherin was strongly expressed in all epithelial cells with a typical diversity between the basal and luminal epithelial layer. The staining was localized on the membrane, particularly at areas of cell-cell contact, the basal cells reacting more strongly than the luminal cells (Fig. 1).

E-cadherin in prostatic carcinoma

84 prostatic carcinomas which consisted of 8 Gleason 4, 4 Gleason 5, 15 Gleason 6, 14 Gleason 7, 26 Gleason 8, 8 Gleason 9 and 9 Gleason 10 were

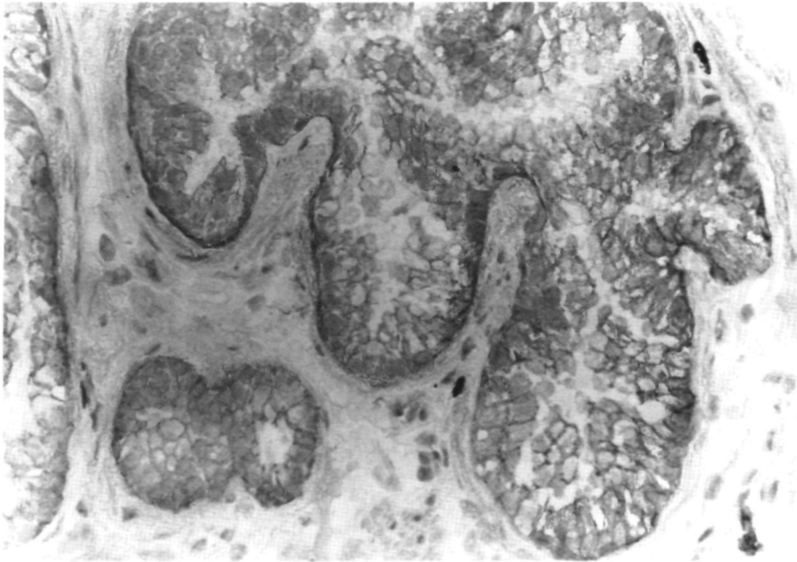


Figure 1: Immunohistochemical detection of E-cadherin expression in non-malignant human prostate tissue using an anti-L-CAM (Uvomorulin) monoclonal antibody (400x).

examined immunohistochemically using an anti-L-CAM (Uvomorulin) mAb. E-cadherin was strongly and uniformly expressed in all of the Gleason 4 (Fig. 2A, B) and 5 (Fig. 2C, D) tumor samples at the cell-to-cell borders (Table 1). The staining pattern and intensity were similar to those observed in non-malignant prostatic tissue.

In high grade prostate cancer, E-cadherin expression evaluated immunohistochemically could discriminate three groups of cancers (Table 1): In the Gleason 6 cases, 13 tumors (87%) stained strongly positive as did 11 (79%) and 8 tumors (31%) of Gleason 7 and 8 respectively. This strong staining is similar to that found in non-malignant prostate and low grade prostate cancers. Interestingly, however, 2 tumors (13%) of Gleason 6, 3 tumors (21%) of Gleason 7 and 13 tumors (50%) of Gleason 8 showed a staining pattern that was significantly different. We termed this pattern heterogeneous (Fig. 2E) and 5 (19%) cases of Gleason 8 had no detectable staining with the antibody at all (Fig. 2F). In the group of Gleason 9 and 10 cancers, we never found a strong expression of E-cadherin. In 4 (50%) cases of Gleason 9 and 5 (55%) cases of Gleason 10 a heterogeneous staining was found (Fig. 2G). Four (50%) and 4 (45%) cases of Gleason 9 and 10 respectively, did not express E-cadherin at all (Fig. 2H).

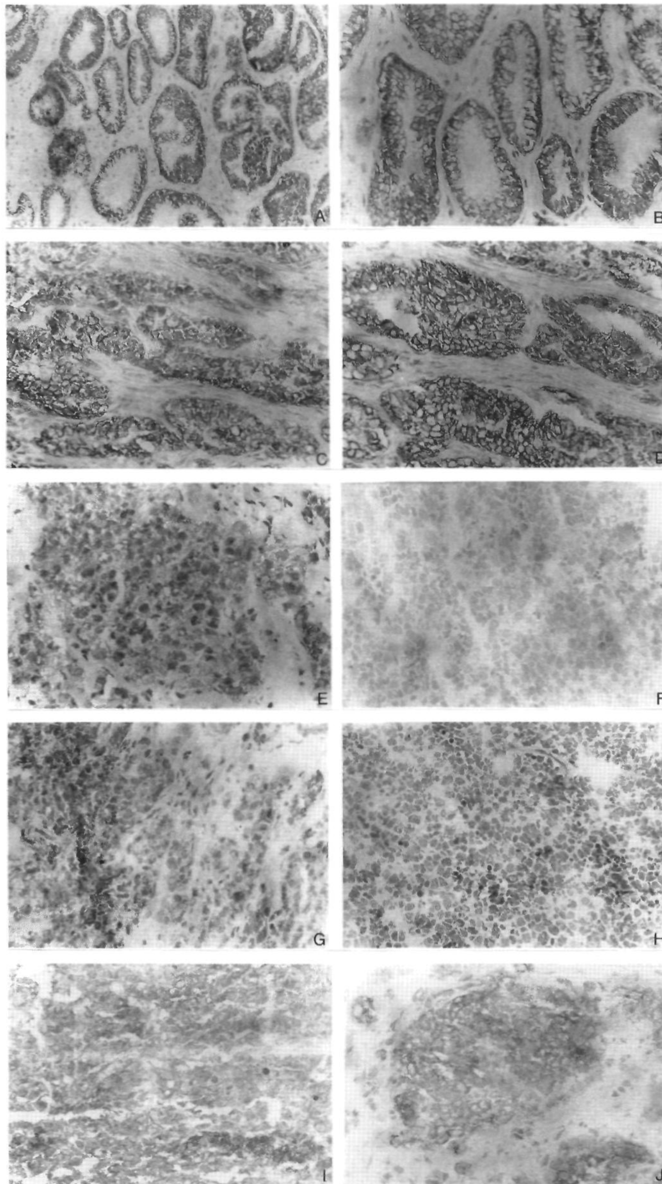


Figure 2: Immunohistochemical detection of E-cadherin expression in prostate cancer (A - H) and metastatic tissue (I - J) using an anti-L-CAM (Uvomorulin) monoclonal antibody. A, B: Gleason score 4 tumor (200x, 400x); C, D: Gleason score 5 tumor (200x, 400x) showing positive and homogeneous staining; E, F: Heterogeneous and negative staining of the Gleason score 8 tumor (400x); G, H: Heterogeneous and negative staining of the Gleason score 10 tumor (400x); I: Metastatic deposit in lymph node consisting of moderately differentiated prostatic adenocarcinoma cells showing heterogeneous staining (400x); J: Metastatic deposit in testicle consisting of moderately differentiated prostatic adenocarcinoma cells showing heterogeneous staining (400x).

Table 1: E-cadherin staining in primary and metastatic lesions of prostate tumors.

Primary tumors *Gleason score*	E-cadherin expression* +	heterogeneous	-	total
4	8			8
5	4			4
6	13 (87%)	2 (13%)		15
7	11 (79%)	3 (21%)		14
8	8 (31%)	13 (50%)	5 (19%)	26
9		4 (50%)	4 (50%)	8
10		5 (55%)	4 (45%)	9
Overall	44 (52%)	27 (32%)	13 (16%)	84
Metastatic lesions	2 (25%)	5 (62%)	1 (12%)	8

* Immunohistochemical criteria of E-cadherin expression in prostate cancer: when more than 90%, 90-10% and <10% of the cancer cells were positively stained, it was regarded as +, heterogeneous and -, respectively.

E-cadherin in prostatic cancer metastatic tissue

Since it is difficult to obtain prostate cancer metastases we could study only a limited number of metastases that originated from prostate cancers. Six of eight of the prostatic cancer metastatic tissues showed either no or a heterogeneous staining with anti-E-cadherin antibodies (Table 1; Fig. 2I, J). Those with heterogeneous staining showed a low-intensity, diffuse staining pattern in which the cell borders were only barely marked. The two remaining metastatic deposits consisted of well differentiated prostatic adenocarcinoma cells which stained strongly for E-cadherin.

E-cadherin mRNA in prostate cancer.

The immunohistochemical findings using anti-E-cadherin antibodies clearly indicate a decreased expression of membrane-bound E-cadherin in high grade prostate cancers. The mechanisms that lead to this decreased expression are unknown. To gain insight in the level at which decrease of E-cadherin is caused, we studied steady-state mRNA levels of E-cadherin in human prostate cancers. We used a Northern assay on pathologically scored (step sectioned) material. From 16 cancers that were scored immunohistochemically, mRNA was thus analyzed. The results are

summarized in Table 2. Surprisingly, it appeared that there was no relation between steady-state E-cadherin mRNA levels and the immunohistochemically scored E-cadherin expression. In some cases of high grade cancers, there was even a higher level of E-cadherin transcripts than in non-malignant tissues. Moreover, there was no statistical significant difference of E-cadherin mRNA levels in low grade *versus* high grade cancers, also no obvious difference in E-cadherin mRNA levels between the non-malignant and malignant prostatic tissues was found.

Table 2: Steady-state E-cadherin mRNA level of human non-malignant and malignant prostatic tissues.

No.	Gleason	Protein score*	mRNA level
1	N**	+	3,8
2	4	+	2,1
3	4	+	14,8
4	5	+	6,6
5	6	+	10,5
6	6	+	11,2
7	6	+	2,3
8	6	+	6,4
9	8	heterogeneous	7,8
10	8	heterogeneous	6,5
11	8	heterogeneous	1,8
12	8	+	5,5
13	8	+	3,6
14	8	-	2,7
15	8	-	4,2
16	10	-	18,8

* Immunohistochemical criteria of E-cadherin expression in prostate cancer: when more than 90%, 90-10% and <10% of the cancer cells were positively stained, it was regarded as +, heterogeneous and -, respectively.
** non-malignant prostate tissue.

DISCUSSION

E-cadherin is thought to play an important role in normal growth and development. Alternatively, when deregulated, it can contribute to the invasive potential, and therefore, E-cadherin can be considered as a candidate invasion/metastasis suppressor. Moreover, the fact that chromosome locus 16q22, to which the E-cadherin gene is mapped, is frequently involved in allelic loss in prostatic cancer, prompted us to examine the expression of E-cadherin in non-

malignant and malignant human prostate tissues. In this study we evaluated E-cadherin expression immunohistochemically and by Northern blot analysis. Using anti-E-cadherin antibodies, we found that all of the non-malignant prostatic tissues stained positively.

All of the well-differentiated (Gleason score 4) and 28 of 33 (85%) moderately-differentiated (Gleason score 5-7) prostatic cancers stained with similar intensity as found in non-malignant prostatic tissue. The poorly-differentiated prostatic cancer tissues (Gleason score 8 and 9) showed decreased expression of E-cadherin. Only 8 of 34 (24%) showed a strong staining while 50% showed heterogeneous and 26% showed no detectable staining with the antibody. Moreover, none of the anaplastic prostatic tissues (Gleason score 10) had a strong E-cadherin expression. Five of 9 (55%) stained heterogeneously and the other 45% did not express E-cadherin. From this we can conclude that as in the Dunning R-3327 rat prostatic cancer model system (22), E-cadherin expression is decreased in high grade malignant lesions of the prostate. Also for several other human cancers, a decreased expression of E-cadherin was reported (19-21).

In a previous study we showed that during progression of rat prostate cancer the decrease in E-cadherin expression resulted from unstable expression rather than from a complete shut off of expression. The Northern analysis presented here clearly shows that a complete shut off of transcription is not evident in human prostate cancers. Sometimes E-cadherin mRNA expression levels are even elevated in the high grade tumors studied. Several mechanisms could explain these findings, e.g., a subpopulation of E-cadherin-negative cells could in fact be present but an abundance of E-cadherin-expressing cells might mask this population. Alternatively, the lack of membrane-bound E-cadherin could be caused by posttranscriptional mechanisms, including either decreased translational efficiency or lack of proper post-transcriptional modification. Presence of non-functional E-cadherin could also be explained by mutations in the gene. Especially in light of the fact that the E-cadherin locus is frequently lost in prostate cancer (23, 24) it is tempting to speculate that the last mechanism causes the decreased expression. Structural studies on E-cadherin mutations are hampered by the fact the nucleotide sequence from the human E-cadherin cDNA is not yet known.

In conclusion, the fact that E-cadherin can function as an invasion/metastasis suppressor experimentally (18, 20, 29-33), is encoded for by a genomic region (chromosome 16q21-23) demonstrating loss of heterozygosity in prostate cancers (23,

24), and, as shown in this study, has reduced protein expression in high grade prostate cancers, make this molecule a candidate invasion/metastasis suppressor in prostate cancer. Although we have demonstrated that the reduced expression of E-cadherin is not due to complete transcriptional inhibition, further studies are ongoing in order to elucidate the specific mechanisms of reduced E-cadherin expression in high grade prostate cancers.

ACKNOWLEDGMENTS

The authors would like to thank Dr. S. Hirohashi (Tokyo, Japan) for the kind gift of the monoclonal antibody against E-cadherin, HECD-1, and Dr. R. Kemler (Freiburg, FRG) for kindly providing the human E-cadherin probe pV962. We acknowledge Anja de Ruijter for excellent technical assistance.

REFERENCES

1 Takeichi, M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development, 102: 639-655, 1988.

2 Edelman, G.M. Expression of cell adhesion molecules during embryogenesis and regeneration. Exp. Cell Res., 161: 1-16, 1985.

3 Edelman, G M. Cell adhesion molecules in the regulation of animal form and tissue pattern. Ann Rev. Cell Biol., 2: 81-116, 1986.

4 Angres, B, Müller, A.H.J., Kellermann, J., and Hausen, P. Differential expression of two cadherins in *Xenopus laevis*. Development, 111: 829-844, 1991.

5 Donalies, M., Cramer, M., Ringwald, M., and Starzinski-Powitz, A. Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. Proc. Natl. Acad. Sci. USA, 88: 8024-8028, 1991.

6 Ginsberg, D., DeSimone, D., and Geiger, B. Expression of a novel cadherin (EP-cadherin) in unfertilized eggs and early *Xenopus* embryos Development, 111: 315-325, 1991.

7 Napolitano, E W, Venstrom, K., Wheeler, E.F., and Reichardt, L.F. Molecular cloning and characterization of B-cadherin, a novel chick cadherin. J. Cell Biol, 113: 893-905, 1991.

8 Ranscht, B., and Bronner-Fraser, M. T-cadherin expression alternates with migrating neural crest cells in the trunk of the avian embryo. Development, 111: 15-22, 1991.

9 Suzuki, S., Sano, K., and Tanihara, H. Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. Cell Regulation, 2: 261-270, 1991.

10 Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator Science, 251· 1451-1455, 1991

11 Hyafil, F, Babinet, C., and Jacob, F. Cell-cell interactions in early embryogenesis: a molecular approach to the role of calcium Cell, 26: 447-454, 1981.

12 Gallin, W J., Edelman, G M, and Cunningham, B A. Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells Proc Natl. Acad. Sci. USA, 80 1038-1042, 1983.

13 Damsky, C.H., Richa, J., Solter, D., Knudsen, K., and Buck, C.A. Identification and purification of a cell surface glycoprotein mediating intercellular adhesion in embryonic and adult tissue. Cell, 34: 455-466, 1983.

14 Imhof, B A., Vollmers, H P., Goodman, S.L., and Birchmeier, W Cell-cell interaction and polarity of epithelial cells: specific perturbation using a monoclonal antibody Cell, 35: 667-675, 1983
15 Behrens, J., Birchmeier, W., Goodman, S L. and Imhof, B.A. Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-Arc-1. mechanistic aspects and identification of the antigen as a component related to uvomorulin. J. Cell Biol., 101: 1307-1315, 1985.
16 Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M , Shimosato, Y , Takeichi, M , and Abe, O. Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Res., 49: 2128-2133, 1989.
17 Eidelman, S., Damsky, C.H., Wheelock, M.J., and Damjanov, I. Expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in normal human tissues and tumors. Am. J. Pathol., 135: 101-110, 1989.
18 Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Löchner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol., 113: 173-185, 1991.
19 Shimoyama, Y., and Hirohashi, S Cadherin intercellular adhesion molecule in hepatocellular carcinomas. loss of E-cadherin expression in an undifferentiated carcinoma. Cancer Lett., 57: 131-135, 1991
20 Schipper, J H , Frixen, U.H., Behrens, J , Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Res., 51: 6328-6337, 1991.
21 Shimoyama, Y., and Hirohashi, S. Expression of E- and P-cadherin in gastric carcinomas. Cancer Res., 51: 2185-2192, 1991.
22 Bussemakers, M.J.G., Van Moorselaar, R J A., Giroldi, L A., Ichikawa, T., Isaacs, J.T., Takeichi, M , Debruyne, F M.J., and Schalken, J.A. Decreased expression of E-cadherin in the progression of rat prostatic cancer. Cancer Res , 52: In Press, 1992
23 Carter, B S., Ewing, C.M., Ward, W S., Treiger, B.F., Aalders, T W., Schalken, J.A., Epstein, J.I., and Isaacs, W B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc. Natl. Acad. Sci USA, 87: 8751-8755, 1990.
24 Bergerheim, U.S., Kunimi, K., Collins, V P., and Ekman, P. Deletion mapping of chromosomes 8, 10, and 16 in human prostatic carcinoma. Genes Chromosome Cancer, 3: 215-220, 1991.
25 Gleason, D.F. Histologic grading and clinical staging of prostatic carcinoma. In: M. Tannembaum (ed), Urologic Pathology: The Prostate, pp 171-197. Philadelphia: Lea & Febiger, 1977
26 Auffray, C., and Rougeon, F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem., 107 303-314, 1980.
27 Mansouri, A., Spurr, N., Goodfellow, P.N., and Kemler, R. Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. Differentiation, 38: 67-71, 1988.
28 Bussemakers, M.J G., Isaacs, J.T., Debruyne, F.M.J., Van de Ven, W.J.M., and Schalken, J.A. Oncogene expression in prostate cancer. World J Urol., 9. 58-63, 1991
29 Hashimoto, M , Niwa, O, Nitta Y., Takeichi, M., and Yokoro, K. Unstable expression of E-cadherin adhesion molecules in metastatic ovarian tumor cells. Jpn. J. Cancer Res , 80: 459 -463, 1989.
30 Behrens, J., Mareel, M.M., Van Roy, F M., and Birchmeier, W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. J. Cell Biol. 108: 2435-2447, 1989.
31 Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C., and Van Roy, F M Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice Int. J Cancer, 47: 922-928, 1991.
32 Vleminckx, K., Vakaet Jr., L., Mareel, M., Fiers, W., and Van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell, 66: 107-119, 1991.
33 Chen, W., and Obrink, B. Cell-cell contacts mediated by E-cadherin (Uvomorulin) restrict invasive behavior of L-cells. J. Cell Biol., 114· 319-327, 1991

CHAPTER VII

SUMMARY

SAMENVATTING

SUMMARY

As described in **Chapter I**, prostate cancer is an increasing medical problem, yet little is known about the mechanisms involved in the onset and progression of this disease. In order to gain insight in the genetic changes associated with development of cancer in this gland and to contribute to a more accurate diagnosis of patients with prostate cancer, we aimed at the identification of molecular markers associated with the process of prostate tumor development, that might be useful in predicting the aggressiveness of individual prostatic lesions. To achieve this goal, two approaches were used, i.e., differential hybridization analysis as a "direct approach" and the studies on the expression of a known gene (E-cadherin) with potential relevance for carcinogenesis as an "indirect approach".

Chapter II describes the technique of differential hybridization analysis and its application to compare steady-state mRNA levels of a relative benign and a highly malignant subline of the Dunning R-3327 rat prostatic cancer model system. Three cDNA clones overexpressed in the highly malignant tumor were isolated; pBUS1 was shown to be specifically overexpressed in the anaplastic, metastatic sublines of the Dunning model system, whereas no mRNA was detected in the relatively benign tumors. Nucleotide sequence analysis and computer-assisted database-comparison revealed pBUS1 to be highly homologous to the High Mobility Group protein I(Y). HMG-I(Y) is a non-histon, chromatin-binding protein that is implicated in transcription and/or replication processes. Furthermore, HMG-I(Y) was reported to be highly abundant in fast-proliferating, undifferentiated cells. pBUS19 was highly expressed in two of three metastatic Dunning sublines studied and nucleotide sequence analysis showed pBUS19 to contain parts of RAL-elements which are rat-specific repetitive elements. pBUS30 detected transcripts of the same size as pBUS19 in the two metastatic sublines but also showed additional bands in the anaplastic, non-metastasizing tumors and, at a lower expression level, in some hormone-independent, moderately-differentiated, non-metastasizing tumors. Nucleotide sequence analysis showed no homology between pBUS30 and pBUS19 and no homology of pBUS30 with any of the known sequences was found.

Chapter III reports on the attempt made to reveal the possible relation between pBUS19 and pBUS30. Using pBUS19 as a probe, a cDNA-library was screened and pJG116 was isolated. Northern blot analysis showed pJG116 to have an expression

pattern similar to pBUS30, however, nucleotide sequence analysis of pJG116 could not confirm any relation between the clones. Computer-assisted database-comparison finally revealed their relationship; additional nucleotide sequence data on the RAL-elements had become available, indicating the elements to be part of a 7.3 kb rat-specific endogenous retrovirus and it was shown that pBUS19, pJG116 and pBUS30 contained parts of this endogenous retrovirus. Since the retroviral sequences are rat-specific, it is unlikely that the cDNA clones are useful for human diagnosis.

Chapter IV describes the results of a second differential hybridization analysis and in particular the identification of vimentin as being overexpressed in anaplastic rat prostatic tumors. Furthermore, the complete coding sequence of rat vimentin was determined and was shown to be highly conserved between rat, mouse, hamster and human. The overexpression of vimentin in prostatic tumors is puzzling since the prostate is of epithelial origin and the exclusive expression of cytokeratins would be expected. Vimentin is normally expressed in tissues of mesenchymal origin and in cells in tissue culture. Although most times the expression of the specific intermediate filaments is retained upon neoplastic transformation, an increasing number of studies dealing with coexpression of two classes of intermediate filaments in neoplasms, are reported. Also for prostate cancer a study on the coexpression of vimentin and cytokeratins was reported. However, more detailed (immunohisto-chemical) studies have to be performed to confirm that coexpression of vimentin and cytokeratins within epithelial cells of prostatic tumors occurs and to establish the potential role of vimentin overexpression in human prostatic tumors.

In the progression of cancer, acquisition of metastatic ability is an important event and several genes might be implicated in this process. Considering the many reports on a potential role of the Ca^{2+}-dependent cell adhesion molecule E-cadherin in invasion suppression and the fact that the E-cadherin gene is mapped to the human chromosomal segment 16q21-23 which is frequently deleted in prostatic tumors, it is tempting to speculate that E-cadherin is a potential invasion/metastasis suppressor gene implicated in prostate cancer development. In order to test this hypothesis, we decided to study the expression of E-cadherin in both our animal model system as well as in human prostatic tumors.

Chapter V deals with the expression of E-cadherin in rat prostatic tumors. In the established Dunning R-3327 model system E-cadherin mRNA was expressed in the well- and moderately differentiated tumors with low invasive potential whereas

no expression was seen in the anaplastic and metastasizing tumors with a high invasive potential. These results were confirmed at the protein level: using an E-cadherin polyclonal antibody, clear staining of the non-invasive Dunning sublines was seen whereas the invasive lines were negative. During a recent passage of the androgen-dependent, well-differentiated, non-metastasizing Dunning-H tumor, a spontaneous progression to an androgen-independent, highly metastatic tumor took place. This new tumor was termed AT6. In the original passage of the AT6 tumor (AT6p0) and in a lung metastasis derived from this tumor, E-cadherin was expressed although at lower levels than in the H-tumor. Histological studies demonstrated AT6p0 to be composed of well-differentiated areas and an anaplastic cell population in which sometimes squamous cell differentiation was seen. The lung metastasis was shown to be composed of clusters of anaplastic cells accompanied by squamous cell populations. Immunohistochemical studies on the lung metastasis revealed the anaplastic tumor cells to be E-cadherin-negative whereas the squamous cells were E-cadherin-positive. Upon further transplantation of both the original tumor and the lung metastasis, anaplastic, metastatic tumors arose, no longer expressing E-cadherin at either the mRNA or protein level. These results suggest a decreased expression of E-cadherin to be associated with the progression of prostate cancer to an invasive phenotype.

In **Chapter VI** we further substantiated this hypothesis by studying the expression of E-cadherin in human prostatic tumors. Immunohistochemical studies on non-malignant prostate specimens showed a strong expression of E-cadherin in all epithelial cells, the staining being localized at the membranes. In low grade tumors E-cadherin was also strongly expressed, the staining pattern and intensity being similar as observed in the non-malignant samples. In high grade tumors often only weak, heterogeneous or no detectable staining was found. Also in undifferentiated metastatic tissue only a weak heterogeneous or no E-cadherin expression was detected. Interestingly, when studying the mRNA expression levels of E-cadherin in some of the tumor specimens by Northern blot analysis, it was shown that high grade tumors did express E-cadherin mRNA so the decreased protein expression could not easily be explained by a decreased expression of the mRNA. In conclusion, decreased levels of E-cadherin protein expression in high grade tumors suggest prostate tumor progression to be accompanied by decreased expression of E-cadherin, however, the mechanism that causes this decrease remain elusive. In order to gain insight in these mechanisms, we are now focussing on the isolation and

characterization of the human E-cadherin cDNA to search for point mutations, and the human E-cadherin gene promoter to study gene regulation.

Summarizing, two candidates for the determination of the biological aggressiveness of prostatic tumors have resulted from the studies described in this thesis, i.e., HMG-I(Y) and E-cadherin. The upregulation of HMG-I(Y) in metastatic tumors suggests the involvement of HMG-I(Y) in the acquistion of a highly malignant phenotype. Overexpression of HMG-I(Y) in (parts of) human prostate tumors may, therefore, be a bad prognosticator. The downregulation of E-cadherin correlates with an invasive phenotype of rat prostatic tumors and decreased expression of E-cadherin in (parts of) the human primary tumors may indicate the more aggressive subpopulation of cells within a tumor. Although the differential expression of HMG-I(Y) and E-cadherin in prostate cancer, are promising findings, their predictive value remains to be established.

SAMENVATTING

Zoals beschreven in **Hoofdstuk I** is er, ondanks het feit dat prostaatkanker een toenemend medisch probleem vormt, weinig bekend over de mechanismen die betrokken zijn bij het ontstaan en de progressie van deze ziekte. Om inzicht te krijgen in de genetische veranderingen die geassocieerd zijn met de ontwikkeling van kanker in deze klier, hebben we ons gericht op de identificatie van moleculaire markers die betrokken zijn bij het proces van prostaattumor-ontwikkeling en die mogelijk bruikbaar zijn bij het voorspellen van de aggressiviteit van prostaattumoren. Om dit doel te bereiken is gekozen voor twee benaderingswijzen nl. differentiële hybridisatie-analyse als een "directe aanpak" en het bestuderen van de expressie van een bekend gen (E-cadherine) dat mogelijk een rol speelt in de carcinogenese, als een "indirecte aanpak".

Hoofdstuk II beschrijft de techniek van differentiële hybridisatie-analyse en de toepassing ervan om steady-state mRNA-niveaus te vergelijken van een relatief goedaardige en een uiterst kwaadaardige sublijn van het Dunning R-3327 ratte-prostaatkanker-modelsysteem. Er werden drie cDNA-klonen geïsoleerd die hoger tot expressie kwamen in de uiterst kwaadaardige tumoren: pBUS1 bleek specifiek verhoogd tot expressie te komen in de anaplastische, metastaserende sublijnen van het Dunning modelsysteem, terwijl er geen mRNA aangetoond kon worden in de relatief goedaardige tumoren. Nucleotidevolgorde-bepaling en databank-vergelijkingen met behulp van de computer, lieten zien dat pBUS1 zeer homoloog was met het High Mobility Group protein I(Y) [Hoge-Mobiliteits-Groep-eiwit I (Y)]. HMG-I(Y) is een niet-histon, chromatine-bindend eiwit dat mogelijk betrokken is bij transcriptie-en/of replicatie-processen. Verder was eerder gerapporteerd dat HMG-I(Y) in grote hoeveelheden voorkomt in snel prolifererende, ongedifferentieerde cellen. pBUS19 kwam hoog tot expressie in twee van de drie bestudeerde, metastaserende Dunning sublijnen en nucleotidevolgorde-bepaling toonde aan dat pBUS19 gedeeltes van RAL-elementen, welke rat-specifieke repetitieve elementen zijn, bevatte. pBUS30 detecteerde transcripten van dezelfde grootte als pBUS19 in de metastaserende tumoren maar toonde verder additionele banden in de anaplastische, niet-metastaserende tumoren en, op een lager expressie-niveau, in enkele hormoon-onafhankelijke, matig gedifferentieerde, niet-metastaserende tumoren. Nucleotide-volgorde-analyse kon geen homologie aantonen tussen pBUS30 en pBUS19 en ook

werd geen homologie van pBUS30 met een van alle tot nu toe bekende sequenties gevonden.

Hoofdstuk III rapporteert over de poging die werd ondernomen om de mogelijke relatie tussen pBUS19 en pBUS30 te ontrafelen. pBUS19 werd gebruikt als probe om een cDNA-bibliotheek te screenen waarbij pJG116 werd geïsoleerd. Northern blot-analyse toonde dat pJG116 een expressie-patroon had vergelijkbaar met dat van pBUS30, echter nucleotidevolgorde-bepaling van pJG116 kon geen enkele relatie tussen beide klonen bevestigen. Databank-vergelijkingen met behulp van de computer, onthulden uiteindelijk hun relatie: additionele nucleotidevolgorde-gegevens over de RAL-elementen waren beschikbaar gekomen en toonden aan dat de elementen onderdeel waren van een 7300 base-paren groot rat-specifiek endogeen retrovirus en het kon worden getoond dat pBUS19, pJG116 en pBUS30 gedeelten van dit endogene retrovirus bevatten. Aangezien de retrovirale sequenties rat-specifiek zijn, is het niet te verwachten dat de cDNA-klonen bruikbaar zijn voor humane diagnose-stelling.

Hoofdstuk IV beschrijft de resultaten van een tweede differentiële hybridisatie analyse en in het bijzonder de identificatie van vimentine als een eiwit dat verhoogd tot expressie komt in anaplastische ratte-prostaattumoren. Verder werd de complete coderende nucleotidevolgorde van ratte-vimentine bepaald en werd getoond dat deze sequentie zeer sterk geconserveerd is tussen rat, muis, hamster en mens. De overexpressie van vimentine in prostaattumoren is verwarrend gezien het feit dat de prostaat van epitheliale oorsprong is en eigenlijk de exclusieve expressie van cytokeratines verwacht zou worden. Vimentine komt normaal tot expressie in weefsels van mesenchymale oorsprong en in cellen in weefselkweek. Alhoewel de expressie van de specifieke intermediaire filamenten meestal behouden blijft na neoplastische transformatie, verschijnt er een toenemend aantal publicaties die co-expressie van 2 klassen van intermediaire filamenten in neoplasmata beschrijven. Ook voor prostaatkanker is er een studie over de co-expressie van vimentine en cytokeratines gepubliceerd. Er zullen echter meer gedetailleerde (immunohisto-chemische) studies moet worden uitgevoerd, om te bevestigen dat co-expressie van vimentine en cytokeratines in epitheliale cellen van prostaattumoren voorkomt en om een mogelijke rol van vimentine-overexpressie in humane prostaattumoren vast te stellen.

In de progressie van kanker is het verkrijgen van metastaserende capaciteit een belangrijke gebeurtenis waarbij verschillende genen betrokken zouden kunnen zijn. Gezien de vele publicaties over de mogelijke rol van het Ca^{2+}-afhankelijke cel-adhesiemolecuul E-cadherine in invasie-suppressie en het feit dat het E-cadherine-gen gelocaliseerd is op het humane chromosomale segment 16q21-23 hetgeen frequent gedeleteerd is in prostaattumoren, is het verleidelijk te speculeren dat E-cadherine een potentiëel invasie/metastase-suppressor-gen is, betrokken bij prostaattumor-ontwikkeling. Om deze hypothese te testen hebben we besloten de expressie van E-cadherine in zowel ons diermodelsysteem alsook in humane prostaattumoren te bestuderen.

Hoofdstuk V behandelt de expressie van E-cadherine in ratte-prostaattumoren. In het gevestigde Dunning R-3327 modelsysteem bleek E-cadherine tot expressie te komen in de goed- en matig-gedifferentieerde tumoren met lage invasieve capaciteit terwijl geen expressie kon worden aangetoond in de anaplastische en metastaserende tumoren met hoge invasieve potentie. Deze resultaten werden bevestigd op eiwit-niveau: gebruikmakend van een E-cadherine polyklonaal antilichaam, werd een sterke aankleuring van de niet-invasieve Dunning sublijnen waargenomen terwijl de invasieve lijnen negatief waren. Tijdens een recente passage van de androgeen-afhankelijke, goed gedifferentiëerde, niet metastaserende Dunning H-tumor, trad een spontane progressie naar een androgeen-onafhankelijke, metastaserende tumor op. De nieuw ontstane tumor werd AT6 genoemd. In de originele passage van de AT6 tumor (AT6p0) en in een daarvan afgeleide longmetastase, kwam E-cadherine tot expressie, alhoewel in geringere hoeveelheden dan in de H-tumor. Met behulp van histologische studies werd aangetoond dat AT6p0 samengesteld was uit goed-gedifferentiëerde gebieden en een anaplastische celpopulatie waarin af en toe squameuze celdifferentiatie zichtbaar was. De longmetastase bleek te zijn samengesteld uit clusters van anaplastische cellen die vergezeld werden door squameuze celpopulaties. Immunohistochemische studies aan de longmetastase lieten zien dat de anaplastiche tumorcellen E-cadherine-negatief waren terwijl de squameuze cellen positief kleurden met het E-cadherine-antilichaam. Na het verder doorplanten van zowel de originele tumor als de longmetastase, ontstonden anaplastische, metastaserende tumoren die geen E-cadherine meer tot expressie brachten, noch op mRNA-, noch op eiwitniveau. Deze resultaten suggereren dat een verminderde expressie van E-cadherine geassocieerd zou zijn met de progressie van prostaatkanker naar een invasief fenotype.

In **Hoofdstuk VI** hebben we deze hypothese verder getest door de expressie van E-cadherine in humane prostaattumoren te bestuderen. Immunohistochemische studies aan niet-kwaadaardige prostaatweefsels toonden een sterke expressie van E-cadherine in alle epitheliale cellen waarbij de kleuring vooral aan de membraan was gelocaliseerd. In laag-gradige tumoren kwam E-cadherine ook sterk tot expressie waarbij het kleuringspatroon en -intensiteit hetzelfde waren als in de niet-kwaadaardige weefsels. In hoog-gradige tumoren echter werd vaak slechts een zwakke, heterogene of zelfs geen kleuring gevonden. Ook in ongedifferentiëerd, metastaserend weefsel werd vaak slechts een zwakke, heterogene of helemaal geen kleuring gedetecteerd. Bij het bestuderen van de mRNA expressie niveaus van E-cadherine in sommige tumorsamples door middel van Northern blot analyse, was het interessant te zien dat hoog-gradige tumoren E-cadherine mRNA tot expressie brachten zodat de verlaagde eiwit-expressie niet eenvoudigweg kon worden verklaard door een verlaagde expressie van het mRNA. Concluderend kan men stellen dat de verlaagde niveaus van E-cadherine eiwit-expressie in hoog-gradige tumoren suggereren dat prostaattumor-progressie gepaard gaat met een verlaagde expressie van E-cadherine, waarbij het mechanisme dat deze verlaging veroorzaakt, vooralsnog onbekend blijft. Om inzicht te krijgen in deze mechanismen, richten we momenteel onze aandacht op de isolatie en karakterisatie van het humane E-cadherine cDNA om te zoeken naar puntmutaties, en van de humane E-cadherine promoter om gen-regulatie te bestuderen.

Samenvattend kan men stellen dat de studies beschreven in dit proefschrift hebben geresulteerd in de identificatie van twee kandidaten voor het bepalen van de biologische aggressiviteit van prostaattumoren, te weten HMG-I(Y) en E-cadherine. De verhoogde expressie van HMG-I(Y) in metastaserende tumoren suggereert de betrokkenheid van HMG-I(Y) in het verkrijgen van een uiterst maligne phenotype. Overexpressie van HMG-I(Y) in (gedeelten van) humane prostaattumoren zou daarom beschouwd kunnen worden als een slecht voorteken. Een verminderde expressie van E-cadherine correleert met een invasief phenotype in ratte-prostaattumoren en een afname van de expressie van E-cadherine in (gedeelten van) humane primaire tumoren zou een indicatie kunnen zijn voor een meer aggressieve subpopulatie van cellen binnen een tumor. Alhoewel de differentiële expressie van HMG-I(Y) en E-cadherine in prostaattumoren veelbelovende vindingen zijn, moet hun voorspellende waarde nog worden bepaald.

LIST OF PUBLICATIONS

Roebroek, A.J.M., Schalken J.A., Bussemakers, M.J.G., Van Heerikhuizen, H., Onnekink, C., Debruyne, F.M.J., Bloemers, H.P.J., and Van de Ven, W.J.M. Characterization of human *c-fes/fps* reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. Molec. Biol. Rep. 11: 117-125, 1986.

Karthaus, H.F.M., Bussemakers, M.J.G., Schalken J.A., Kurth, K.H., Feitz, W.F.J., Debruyne, F.M.J., Bloemers, H.P.J., and Van de Ven W.J.M. Expression of proto-oncogenes in xenografts of human renal cell carcinomas. Urol. Res. 15: 349-353, 1987.

Schalken, J.A., Ebeling, S.B., Isaacs, J.T., Treiger, B., Bussemakers, M.J.G., De Jong, M.E.M., and Van de Ven , W.J.M. Down modulation of fibronectin mRNA in metastasizing rat prostatic cancer cells revealed by differential hybridization. Cancer Res., 48, 2042-2046, 1988.

Martens, G.J.M., Bussemakers, M.J.G., Ayoubi, T.A., and Jenks, B.G. The novel pituitary polypeptide 7B2 is a highly conserved protein coexpressed with proopiomelanocortin. Eur. J. Biochem. 181: 75-79, 1989.

Martens, G.J.M., Groenen P.J.T.A., Braks, A.A.M., and Bussemakers, M.J.G. Expression of two growth hormone genes in the *Xenopus* pituitary gland. Nucleic Acids Res. 17: 3974, 1989.

Schalken, J.A., Bussemakers, M.J.G., and Debruyne, F.M.J. Oncogene expression in prostate cancer. In: Prostate cancer and Testis cancer. EORTC Monograph 7 (D. Newling, W. Jones, eds.) 97-105, 1990.

Schalken, J.A., and Bussemakers, M.J.G. Differential hybridization analysis as a tool to study prostatic cancer metastasis. In: Molecular and cellular biology of prostate cancer. (J.P. Karr, D.S. Coffey, R.G. Smith, and D.J. Tindall Eds.) Plenum Press, New York, 1991. pp355-362.

Bussemakers, M.J.G., Isaacs, J.T., Debruyne, F.M.J., Van de Ven, W.J.M., and Schalken, J.A. Oncogene expression in prostate cancer. World J. Urol., 9, 58-63, 1991.

Bussemakers, M.J.G., Van de Ven, W.J.M., Debruyne, F.M.J., and Schalken, J.A. Identification of HMG-I(Y) as potential progression marker for prostate cancer by differential hybridization analysis. Cancer Res., 51, 606-611, 1991.

Deen, P.M.T., Terwel, D, Bussemakers, M.J.G., Roubos E.W., and Martens, G.J.M. Structural analysis of the entire proopiomelanocorin gene B of *Xenopus laevis*. Eur. J. Biochem., 201, 129-137, 1991.

Bussemakers, M.J.G., Van Bokhoven, A., Van Groningen, J.J.M. Debruyne, F.M.J., and Schalken, J.A. Increased expression of retroviral sequences in progressionally advanced rat prostatic tumors. Biochem. Biophys. Res. Comm., 182, 318-324, 1992.

Bussemakers, M.J.G., Verhaegh, G.W.C.T., Van Bokhoven, Debruyne, F.M.J., and Schalken, J.A. Differential expression of vimentin in rat prostatic tumors. Biochem. Biophys. Res. Comm., 182, 1254-1259, 1992.

Bussemakers, M.J.G., Van Moorselaar, R.J.A., Giroldi, L.A., Ichikawa, T., Isaacs, J.T., Takeichi, M., Debruyne, F.M.J., and Schalken, J.A. Decreased expression of E-cadherin in the progression of rat prostate cancer. Cancer Res., 52, In press/ May 1992.

Bussemakers, M.J.G., Debruyne, F.M.J., and Schalken, J.A. Genetic steps associated with prostate cancer development. In: Oncogenes and Molecular Genetics of Urological Tumors (C.A. Olssen, M. Marberger Eds.) SIU Monograph, 1992, Churchill Livingstone, Edinburgh, UK. pp - In Press.

Deen, P.M.T., Bussemakers, M.J.G., Terwel, D., Roubos, E.W., and Martens, G.J.M. Comparative structural analysis of the transcriptionally active proopiomelanocortin genes A and B of *Xenopus laevis*. Molecular Biology and Evolution, 1992, In Press

Van Moorselaar, R.J.A., Hendriks, B.Th., Bussemakers, M.J.G., Van Stratum, P., Borm, G., Debruyne, F.M.J., and Schalken, J.A. Combination treatment with tumor necrosis factor alpha and DNA topoisomerase-II-targeted drugs in a rat renal cell carcinoma model system. Submitted.

Umbas, R., Bussemakers, M.J.G., Isaacs, W.B., Aalders, T.W., Carter, B.S., Debruyne, F.M.J., and Schalken, J.A. Decreased expression of E-cadherin in high grade prostate cancer. Submitted.

DANKWOORD

Na het wetenschappelijke gedeelte van het proefschrift wil ik graag nog een aantal mensen bedanken die ieder op eigen wijze een steentje hebben bijgedragen aan het tot stand komen van dit proefschrift. Allereerst wil ik de medewerkers van de Afdeling Biochemie NW, waar het grootste gedeelte van het werk beschreven in dit proefschrift werd uitgevoerd, bedanken voor de prettige werksfeer en met name de mensen van de groep Molonc en Bloemers bedanken voor het aanhoren van alle gezeur over mislukte proefjes. Ook wil ik de studenten die ik heb mogen begeleiden tijdens hun biochemie-stage, te weten Erwin van Ballegoy, Gerald Verhaegh, Leo Perdon, Stefan Mees en Jeroen Geerdink bedanken voor hun bijdragen aan het onderzoek en voor het bijschaven van mijn didactische vaardigheden (voor zover aanwezig). Verder wil ik de medewerkers van het Urologisch Research Laboratorium bedanken voor hun collegialiteit en voor het leren leven met mijn vele decibellen. Tenslotte wil ik een aantal mensen bij naam noemen, hopende daarbij niemand te kort te doen;

Jan van Groningen, bedankt voor de practische ondersteuning in mijn derde jaar en voor het stug doorzetten bij een klonering die maar niet wou lukken.

Els van Genne, bedankt voor het (gewild maar vaak ook ongewild) aanhoren van alle frustraties en voor onze frequente besprekingen van de toestand in de wereld en dan met name op het lab. Ik hoop dat je werk er niet te zeer onder heeft geleden.

Adrie van Bokhoven, jij was de laatste twee jaar mijn onmisbare rechterhand, linkerhand en mijn meedenkbrein en iemand waar ik nogal wat klusjes op kon (en nog steeds kan) afschuiven. Bedankt voor alle werk verricht "ter mijne meerdere eer en glorie".

Astrid Freriksen, zonder jou (en de PTT-telecom) zou deze promotie me wellicht veel zwaarder zijn gevallen: bedankt voor onze wekelijkse telefoontjes en de morele opkikkers wanneer dat weer eens hard nodig was.

En last but not least, Jack Schalken, bedankt voor van alles en nog wat.

Marion Bussemakers werd 19 september 1964 geboren te Helden-Panningen. Na haar VWO-opleiding aan het Bouwens van der Boye-College te Helden-Panningen werd in september 1982 begonnen met de studie Biologie aan de Katholieke Universiteit te Nijmegen. Het doctoraal Biologie behaalde zij in augustus 1987 met als bijvak Biochemie (Prof. dr. H.P.J. Bloemers / o.l.v. dr. J.A. Schalken) en als hoofdvak Moleculaire Biologie (Prof. dr. J.J.G. Schoenmakers / o.l.v. dr. G.J.M. Martens). Voor het onderzoek verricht tijdens de studie werd haar in juni 1988 een DSM prijs toegekend.

Van september 1987 tot augustus 1991 was de schrijfster dezes werkzaam op de afdeling Biochemie NW (Prof. dr. H.P.J. Bloemers) in opdracht van de afdeling Urologie (Prof. dr. F.M.J. Debruyne / dr. J.A. Schalken) van de Katholieke Universiteit te Nijmegen. In deze tijd werd het grootste gedeelte van het werk, beschreven in dit proefschrift, uitgevoerd. Tijdens deze periode was zij verscheidene malen betrokken bij het derde-jaars scheikunde practicum. In april/mei 1988 nam zij deel aan de EMBO-cursus "Subtraction Hybridizations: cloning and identification of specific transcripts" te Heidelberg (Duitsland). Verder werden in het kader van de wetenschappelijke vorming verscheidene congressen bezocht waaronder de American Association for Cancer Research (1989, 1990), de American Urological Association (1990) en de European Society for Urological Oncology and Endocrinology (1989, 1991).

Sinds augustus 1991 is zij werkzaam in het Urologisch Research Laboratorium (Prof. dr. F.M.J. Debruyne / dr. J.A. Schalken) van het Academisch Ziekenhuis te Nijmegen waar de zoektocht naar genetische veranderingen betrokken bij het ontstaan en de progressie van prostaatkanker wordt voortgezet.