

HIGH LEVEL EXPRESSION OF STAG1/PMEPA1 IN AN ANDROGEN- INDEPENDENT PROSTATE CANCER PC3 SUBCLONE

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Abstract: In this paper, we describe the isolation and characterization of two PC3 subclones. One subclone, mr, showed an epithelial phenotype, the other, M1, showed a sarcomatous morphology. Transplanted into nude mice, mr developed tumors at a dramatically faster rate than M1. Comparing the two subclones, differentially expressed genes were identified, including E-cadherin, IL-8 and STAG1/PMEPA1. These genes were expressed at higher levels in mr than in M1.

Key words: STAG1/PMEPA1, IL-8, E-cadherin, Prostate cancer cell line

INTRODUCTION

Prostate cancer cell lines have been established from prostate and metastatic lesions of prostate cancer patients. LNCaP, PC3 and DU145 are the three primary cell lines used in prostate cancer research. They were respectively established from lymph node, bone and brain metastases of prostate cancer [1]. Many sublines have been isolated from these parental lines using a variety of techniques including nude mouse metastasis, *in vitro* culture selection (androgen

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Abbreviations used: DHT – 5 α -dihydrotestosterone; IL-8 – interleukin-8; PSA – prostate specific antigen; STAG1 – solid tumor associated gene 1; STR – short tandem repeat

depletion), Boyden chamber migration, and long-term culture. During prostate cancer growth and metastasis, changes have been observed in the expression patterns of several genes. One such change is a decreased expression of the androgen-regulated gene STAG1/PMEPA1 [7]. STAG1/PMEPA1 is a recently identified androgen-regulated protein of high homology to ubiquitin ligase-binding protein. Here, we describe new subclones which show unique gene expression profiles and tumor growth behavior.

MATERIALS AND METHODS

Cells and culture conditions

The human prostate cancer cell lines PC3, LNCaP and DU145 were cultured in RPMI 1640 (Sigma) with 10% fetal bovine serum (Gibco/Invitrogen) at 37°C in an atmosphere of 5% CO₂ in air. The PC3 cells were suspended at a concentration of 20 cells/ml in a 10-cm dish and cultured for several weeks as above. Based on microscopic observation, the clones which showed distinct cell morphologies were transferred to separate dishes. The mr and M1 clones retained their morphological features for at least 35 passages.

Polymorphic analysis of short tandem repeat (STR) loci

Two STR loci were PCR amplified from the genomic DNA of each PC3 clone and the two prostate cancer cell lines. Their locus names and GeneBank Accession of UniSTS numbers (in parentheses) are DS5818 (G08446) and D7S820 (G08616). The PCR products were separated with a GeneGel Excel 12.5/24 Kit using a GenePhor Electrophoresis Unit, and stained with a DNA Silver Staining Kit (GE Healthcare).

Reverse transcription PCR, differential display

Total RNA was isolated from prostate cancer cell lines using an RNA extraction kit according to the manufacturer's protocol (QIAGEN). First-strand cDNA synthesis was performed using the superscript II RNase Reverse Transcriptase kit (Invitrogen). The primer sequences for PSA were 5' TAGTAAA CTTGGAACCTTGAAATGACCAG 3' and 5' AATAACACAGACAACCCA TCCTATCTGTGT 3'; for STAG1/PMEPA1 were 5' CGTACCTGCAGCACGA GAT 3' and 5' CCTGGCACTATCCATCAGGT 3'; for IL-8 were 5' AAACCACCGGAAGGAACCATCTCACT 3' and 5' CCTCTTCAAAA CTTCTCCACAACCCT 3'; and for E-cadherin were 5' TGCCCAGAAAATG AAAAAGG 3' and 5' GTGTATGTGGCAATGCGTTC 3'.

Tumorigenesis in nude mice

Groups of six BALB/c nu/nu mice were inoculated subcutaneously with 1×10^7 mr or M1 cells. Tumor volumes were measured every week after cell injection. The mice were sacrificed after 5 weeks, and the tumors were excised, fixed in 10% formalin/PBS, and embedded in paraffin blocks.

Western blotting

Prostate cancer cells were lysed in buffer (10 mM Tris-HCl, pH 8.0, 1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail [NAKARAI]) and sonicated. The lysates were cleared by centrifugation at 20,000 X g for 5 min. Protein samples (30 µg) were separated by SDS-PAGE, and transferred to Hybond-ECL (GE Healthcare). The membranes were probed with anti-E-cadherin (1:2,500 dilution, BD Biosciences, BD-610404), followed by Protein G HRP (BIO-RAD). Specific signals were visualized using the ECL detection system (GE Healthcare).

Immunohistochemistry

4-µm thick sections were cut from the paraffin-embedded blocks of the tumors from the transplanted mice. The sections were incubated in 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 100°C. The VENTANA EX system (VENTANA) was used to stain the sections with anti TMEPAI (STAG1/PMEPA1) antibody (1:100 dilution, Abnova, H00056937-M01).

RESULTS AND DISCUSSION

Two of the isolated PC3 subcellular clones were designated mr and M1. Upon reaching confluence *in vitro*, these clones were found to have distinct morphologies (Fig. 1A). The mr clone showed polygonal cobblestone-like morphology. The M1 cells were spindle-shaped with engrailed edges and large nuclei.

Since many of the subclones claimed as derivatives from the original lines were eventually determined to be contaminated by other cell lines, DNA profiling, in the form of polymorphic short tandem repeat (STR) analysis was performed. STRs consist of various numbers of 3-7 basepair repeats distributed throughout the human genome, providing a convenient tool for personal identification [8]. The PCR products of mr, M1 and PC3 had a single (identical) band, whereas the products of LNCaP and DU145 showed double bands and their mobility was slightly different (Fig. 1B). Prostate-specific antigen (PSA) is the most commonly used molecular marker for prostate cancer. PSA expression is detected in LNCaP cells but not in PC3 or DU145 cells [1]. Thus, PSA expression is a reliable marker for detecting LNCaP cells. As shown in Fig. 1C, PSA expression was only detected in the LNCaP cell line, indicating that cross contamination of the cell lines was unlikely.

Generally, cell lines used in the laboratory are comprised of heterogeneous populations. Studies with flow cytometry revealed that PC3 cells could be divided into E-cadherin positive and negative cells [2]. However, after several passages, sorted cells returned to their original heterogeneous gene expression pattern. This might be an inherent property of PC3 cells. Unlike the reversion seen in PC3, the mr and M1 clones maintained their cell morphology for at least 35 passages. Furthermore, following transplantation to nude mice, their original

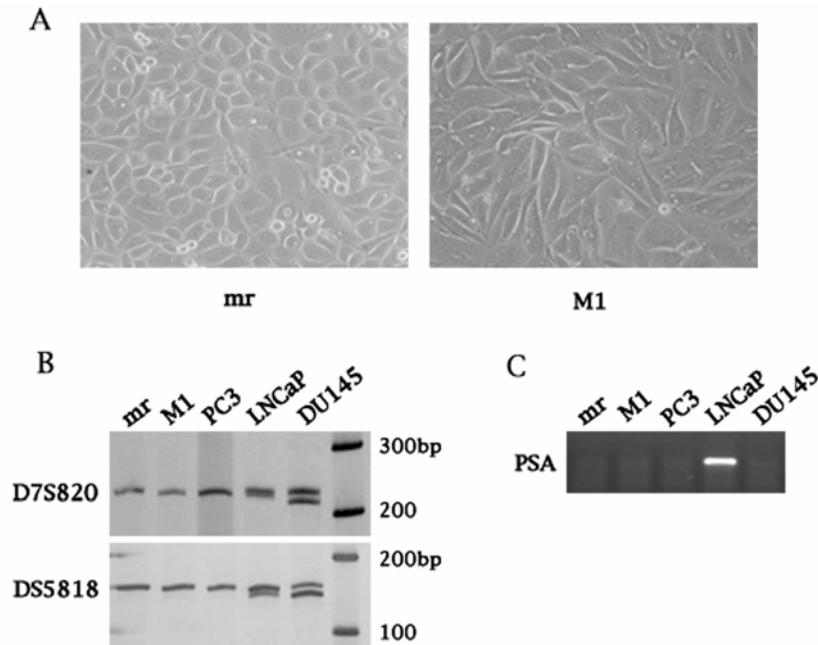


Fig. 1. Characteristics of isolated subclones. (A) The cellular morphologies of mr and M1 at confluence. (B) STR analysis of subclones (mr and M1), and parental PC3, LNCaP and DU145 cell lines. The single PCR band patterns of both STR loci are identical in mr, M1 and PC3, whereas double banding is observed in LNCaP and DU145. (C) Consistent PSA expression in the LNCaP cell line.

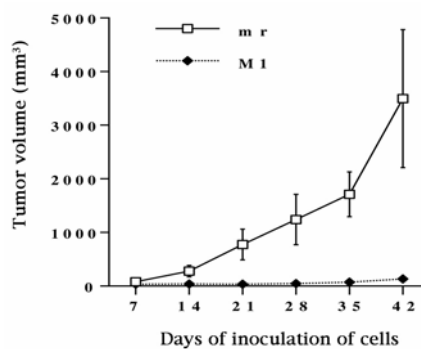


Fig. 2. Tumor growth in nude mice after subcutaneous transplantation.

morphology was retained. We have not yet investigated genome-wide gene expression profiles for the two subclones, and it is possible that some of the genes responsible for controlling cell morphology are invariant.

The two clones were analyzed for their *in vitro* cell proliferation rate. The mr clone showed a higher proliferation rate than M1, but the difference was not robust (data not shown). By contrast, the proliferation rates in nude mice differed significantly (Fig. 2). The mr clone generated tumors very rapidly, and the central area of the tumors eventually became necrotic. M1 produced tumors slowly and necrosis was not seen. Neither of the clones generated metastatic lesions during the period of observation.

When the tumor sections were examined using light microscopy (Fig. 3), the mr cells, which had oval nuclei with condensed chromatin, were found to be arranged in cords. The M1 cells had abundant cytoplasm and large nuclei with distinct nucleoli, and did not show cord-like architecture. Rather, they made contacts without specific structure formation. Both clones showed comparable degrees of local invasion into muscle tissue.

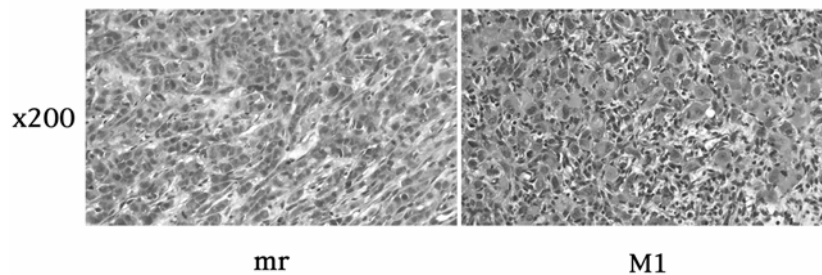


Fig. 3. The histology of the tumors developed in nude mice. HE staining of the specimens from each tumor transplant. An epithelial phenotype of trabecular arrangement is seen in the mr tumor. A relatively scattered array of cells compose the M1 tumor.

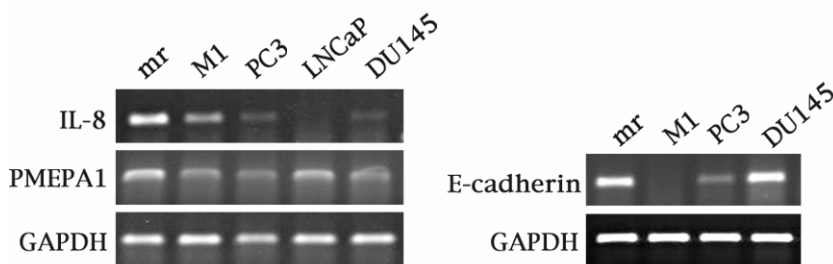


Fig. 4. RT-PCR analysis of differentially expressed genes. IL-8 and STAG1/PMEPA1 are identified by differential display.

In order to characterize the cellular differences between the two clones, molecular profiles of gene expression were compared by differential display. After sequencing and reconfirmation of the differentially expressed gene by RT-PCR, two genes, IL-8 and STAG1/PMEPA1, were identified as candidates (Figs 4 and 5).

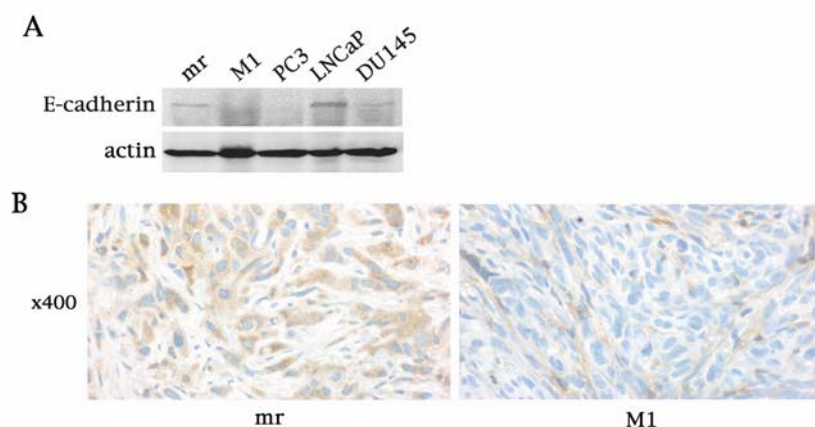


Fig. 5. E-cadherin and STAG1/PMEPA1 protein expression. (A) Western blot analysis of E-cadherin in the prostate cancer cell lines. (B) The STAG1/PMEPA1 immunohistochemistry of the tumors from the mice. The protein localization is observed in the cytoplasm of the mr but not the M1 tumor. Note that the stromal staining in the M1 tumor is non-specific binding of the primary antibody immunized in mouse.

The distinctly different proliferation rates of mr and M1 in nude mice could be explained by the differential IL-8 expression. IL-8 has been shown to participate in angiogenesis, tumorigenesis and metastasis of prostate cancer cells in the nude mouse implantation model [3, 4]. IL-8-neutralizing antibody suppressed tumor growth in nude mice and in the rat angiogenesis model [9]. Furthermore, clinical study of prostate cancer biopsy tissue demonstrated that IL-8 and its receptor expression patterns correlate with the proliferation index, gleason score and microvessel density [10].

STAG1/PMEPA1 was originally identified as an androgen-responsive gene in the LNCaP cell line. A subcellular protein localization is observed in the cytoplasm [7, 11]. STAG1/PMEPA1 up-regulation is induced by TGF beta [11], EGF, IGF, PDGF [12], and androgen in non-cancer and cancer cell lines. Although the expression of growth factors and their receptors has not been examined in mr and M1, we suggest that IL-8 induction might be triggered by TGF beta stimulation [13]. TGF beta signaling might be the common pathway for IL-8 and STAG1/PMEPA1 up-regulation in the mr clone. Little is known about the function of STAG1/PMEPA1 in prostate cancer. Cell proliferation is inhibited by STAG1/PMEPA1 over-expression in prostate cancer cell lines. STAG1/PMEPA1 down-regulation is correlated with human prostate cancer progression [7]. However, the mr clone expresses a high level of STAG1/PMEPA1 with rapid growth in nude mice. In some colon, breast and ovarian cancer cell lines and tissue, over-expression of STAG1/PMEPA1 is observed. However, not all breast and ovarian cell lines display elevated STAG1/PMEPA1 expression. Each cell line might have a difference in its

response to growth factors. The other possibility is a functional difference between the two STAG1/PMEPA1 variants. When the LNCaP cell line is treated with DHT, the heterologous expression pattern is changed to the single variant type [11]. The role of STAG1/PMEPA1 in the context of cellular proliferation of different cell lines should be elucidated.

Previously, a PC3 subline with a scattered spindle-shaped phenotype was observed to lack E-cadherin expression [14]. The spindle sarcomatous morphology of M1 prompted us to examine E-cadherin expression in our sublines. In analogy with the previous subline, E-cadherin expression was lacking in M1, while mr showed higher E-cadherin levels than either M1 or the parental PC3 cells (Figs 4 and 5). The higher growth rates in mice and the elevated E-cadherin expression levels for the mr clone are incompatible with data from surgically resected prostate cancer. That is, aberrant or low expression of E-cadherin is correlated with local extension, metastasis, a higher gleason score and a poor survival rate [5, 6]. Although many clinical prostate cancers exhibit high IL-8 and low E-cadherin expression, not all prostate cancer is as relevant. We found one prostate cancer among thirty-four cases presenting high IL-8 and low E-cadherin expression compared to noncancerous prostate tissues (data not shown). We propose that a better understanding of the cancer biology of individual patients could be achieved through genomic and biological examinations of these clones.

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