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Vimentin 3 Expression in Prostate Cancer Cells

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Abstract. *Background/Aim: Vimentin3 (Vim3) was recently described as a tumour marker for the direct discrimination between benign and malignant kidney tumours. Here, we examined its expression in prostate cancer (PCa) cell lines and the regulation of its expression by endothelin receptors. Materials and Methods: Prostate cancer cell lines (PC3, DU145, LNCap) were incubated with endothelin 1 (ET-1), BQ123 [endothelin A receptor (ETAR) antagonist], BQ788 [endothelin B receptor (ETBR) antagonist], BQ123+ET-1, BQ788+ET-1 for 24 h and a scratch assay was performed. Cell extracts were analysed by western blotting and qRT-PCR. Results: ET-1 induced Vim3 overexpression. Blocking the ETBR in the different prostate cancer cell lines yielded a higher migration rate, whereby Vim3 expression was significantly increased. Conclusion: Vim3 concentration increases in cell lines without a functional ETBR and may be used as a marker for PCas where ETBR is frequently methylated.*

Endothelin-1 (ET-1) was first described in 1988 as a vasoconstrictor peptide (1), and is currently also known as a multifunctional protein in cancer. The ET-1 axis, including the endothelin A receptor (ETAR) and the endothelin B receptor (ETBR), has been found to be frequently involved in the development of several different solid tumours, *e.g.* breast, ovarian, and prostate (2). The binding of ET-1 to the ETAR induces a survival pathway. It can result in cell proliferation, escape from apoptosis, angiogenesis, invasion and metastasis formation (3, 4). The binding to the ETBR results in the clearance of ET-1 and apoptosis 3. In 1996, Nelson *et al.* reported that the ETBR is frequently down-regulated in some prostate cancers (PCa) and that this down-regulation is a sign of a more aggressive behaviour of the tumour (5). The down-regulation of ETBR is due to promoter methylation (3). Elevated ET-1 levels in prostate cancer patients have been associated with higher metastatic potential (6). Furthermore, the levels of ET-1 in cancer cells correlate with tumour progression (3).

A clear correlation between the overexpression of ET-1 and the production of a truncated Vimentin version, called Vim3, was recently described (7). Due to this truncation of Vimentin, cells lose their normal cell structure, as full-length Vimentin (VimFL) is important for the arrangement of cell organelles and the anchorage of cells (8). Truncated Vimentin (Vim3) lacks the C-terminal end and therefore, the normal Vimentin function is lost. Furthermore, it is known that prostate cancer patients with increased Vimentin levels have a higher potential for developing metastases (9). Nevertheless, due to the use of several different commercially available antibodies, either directed against the full-length version or both versions of Vimentin, many of the results from formerly performed experiments are questionable (10). It has previously been shown that overexpression of Vim3 correlates with increased metastatic potential of breast cancer cells (11). Since the different PCa cell line have a different metastatic potential, we examined the expression of Vim3 in these cell lines and the expression of Vim3 and their behavior of after blockage of the ETBR.

Materials and Methods

Cell culture and treatment. DU145 cells were cultured in DMEM (Life Technology GmbH, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS) (PAN Biotech GmbH, Aidenbach, Germany) and 1% Penicillin/Streptomycin (Invitrogen, Karlsruhe, Germany) at 37˚C and 5% CO_2. PC3 and LN-Cap cells were cultured in RPMI (Life Technology GmbH) supplemented as mentioned above. After serum starvation for 24 h, cells were treated with

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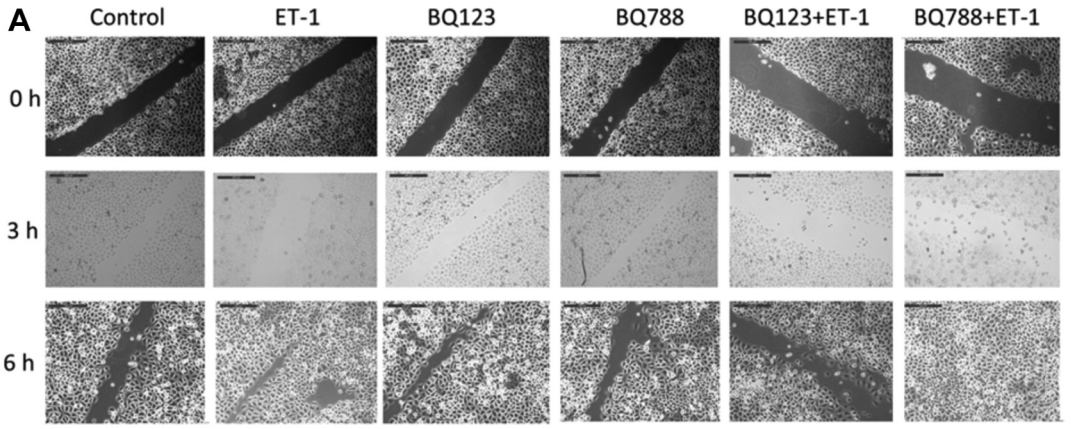
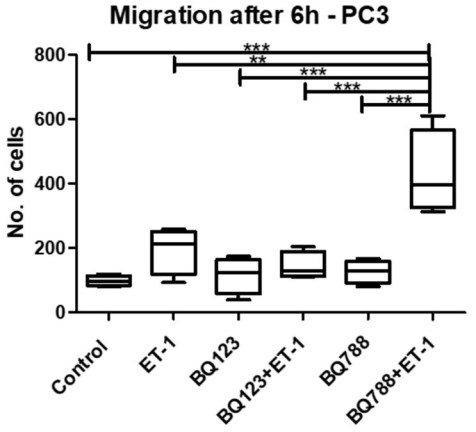
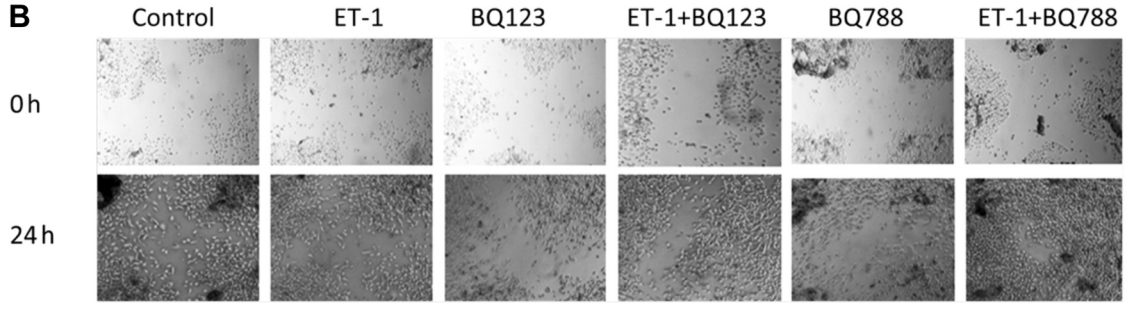
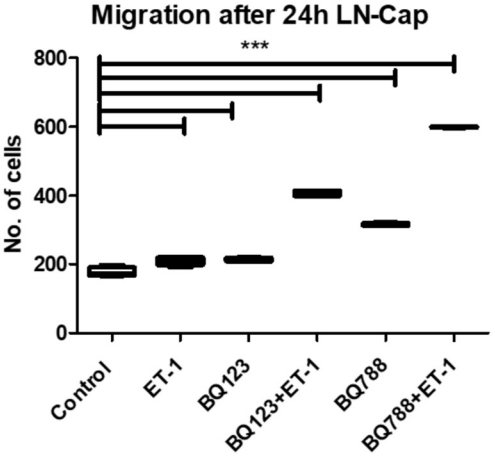
Key Words: Prostate cancer, Vimentin 3, Endothelin-1.





Control vs ET-1	ns
Control vs BQ123	ns
Control vs BQ123+ET-1	ns
Control vs BQ788	ns
Control vs BQ788+ET-1	***
ET-1 vs BQ123	ns
ET-1 vs BQ123+ET-1	ns
ET-1 vs BQ788	ns
ET-1 vs BQ788+ET-1	**
BQ123 vs BQ123+ET-1	ns
BQ123 vs BQ788	ns
BQ123 vs BQ788+ET-1	***
BQ123+ET-1 vs BQ788	ns
BQ123+ET-1 vs BQ788+ET-1	***
BQ788 vs BQ788+ET-1	***





Control vs ET-1	***
Control vs BQ123	**
Control vs BQ123 + ET-1	***
Control vs BQ788	***
Control vs BQ788 + ET-1	***
ET-1 vs BQ123	ns
ET-1 vs BQ123 + ET-1	***
ET-1 vs BQ788	*
ET-1 vs BQ788 + ET-1	***
BQ123 vs BQ123 + ET-1	***
BQ123 vs BQ788	**
BQ123 vs BQ788 + ET-1	***
BQ123 + ET-1 vs BQ788	***
BQ123 + ET-1 vs BQ788 + ET-1	***
BQ788 vs BQ788 + ET-1	***

Figure 1. *Continued*

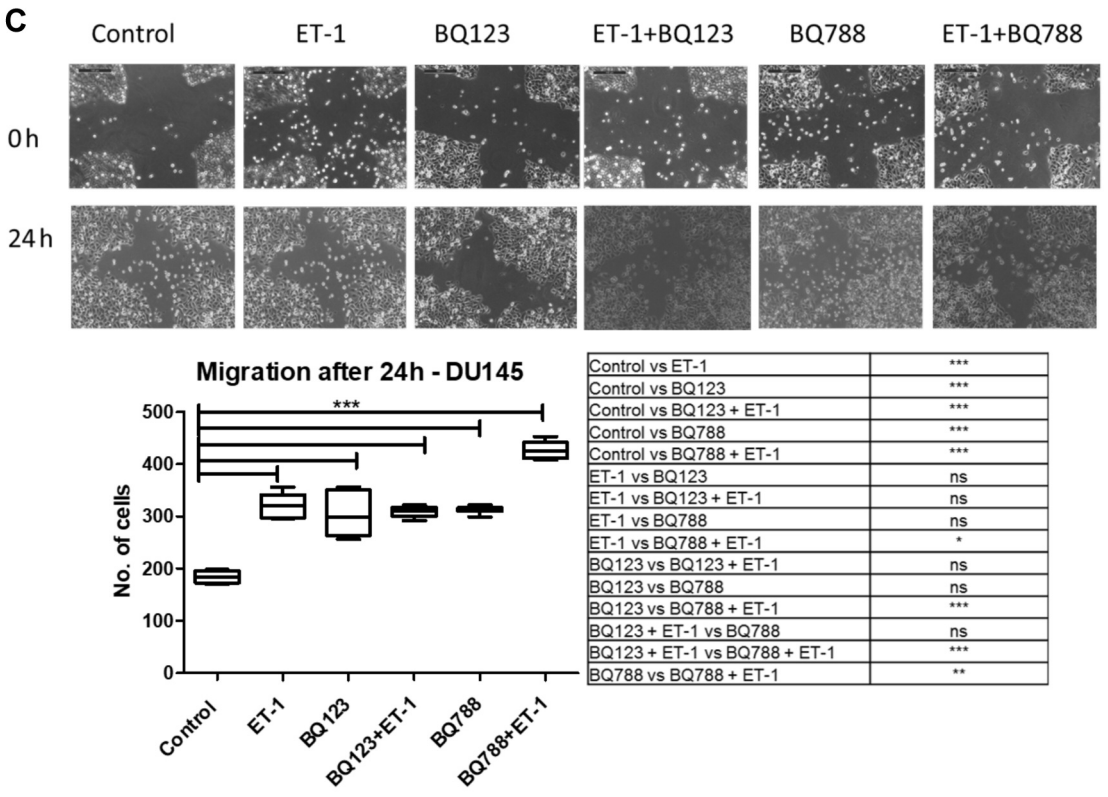


Figure 1. *Migration of PCa cells under treatment. (A) Scratch Assay in PC3 after ET-1 stimulation and receptor blockage for 3 and 6 h. Increased migration after ETBR block (BQ788) and ET-1 treatment for 6 h. (B) Scratch Assay of LNCap cells after 24 h. Here the same results can be seen. (C) Scratch Assay of DU145 cells after 24 h.*

Endothelin-1 (50 nM, Sigma, Deisenhofen, Germany), BQ123 (ETAR antagonist, 100 nM, Sigma), BQ788 (ETBR antagonist, 100 μM, Sigma) for 3, 6 and 24 h depending on the experimental setting.

Western blot. Total protein extraction and Western Blots were performed as already published (11). All blots were analysed with the INTAS Chemostar (Intas Science Imaging, Göttingen, Germany).

Scratch-assay. PCa cell lines were grown in 100 mm tissue culture dishes until they reached a confluency of 80%. Cells were serum-starved for 24 h and a scratch was performed with a white pipette tip. After serum starvation, cells were treated for 24 h with either ET-1, ETAR block, ETBR block or different combinations. Pictures were obtained after 3, 6, and 24 h. For DU145 and LNCap cells, pictures were acquired after 24 h.

Reverse transcriptase polymerase chain reaction (RT-PCR). The cDNA was obtained from 250 ng RNA using random primers and SuperScript III according to the manufacturer's protocol (Invitrogen, Darmstadt, Germany). The RT-PCR was performed as previously described (10, 11).

Quantitative real-time PCR (qRT-PCR). One μl of the cDNA (transcribed from 250 ng RNA) was used for real-time PCR analysis. The experimental settings were as previously described (10, 11). All samples were normalised to β-actin. Relative

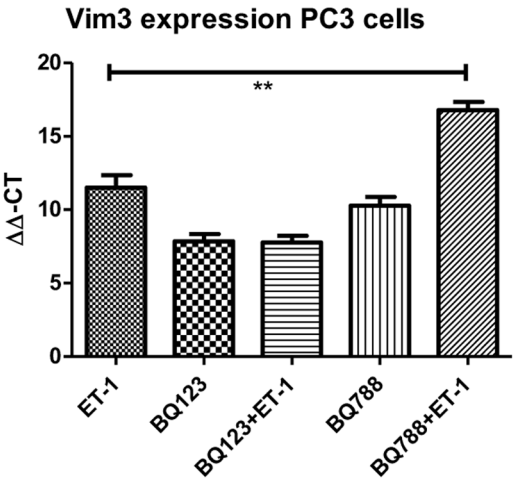


Figure 2. *qRT-PCR for Vim3 and VimFL levels in PC3 after ET-1 and receptor block treatment for 24 h. Significant increase was observed after BQ788 (ETBR block) and ET-1 treatment.*

fluorescence was calculated using the ΔΔCT method as outlined in User Bulletin 2 (PE Applied Biosystems, Forster City, CA, USA). Untreated cells were used as controls. The statistical significance of

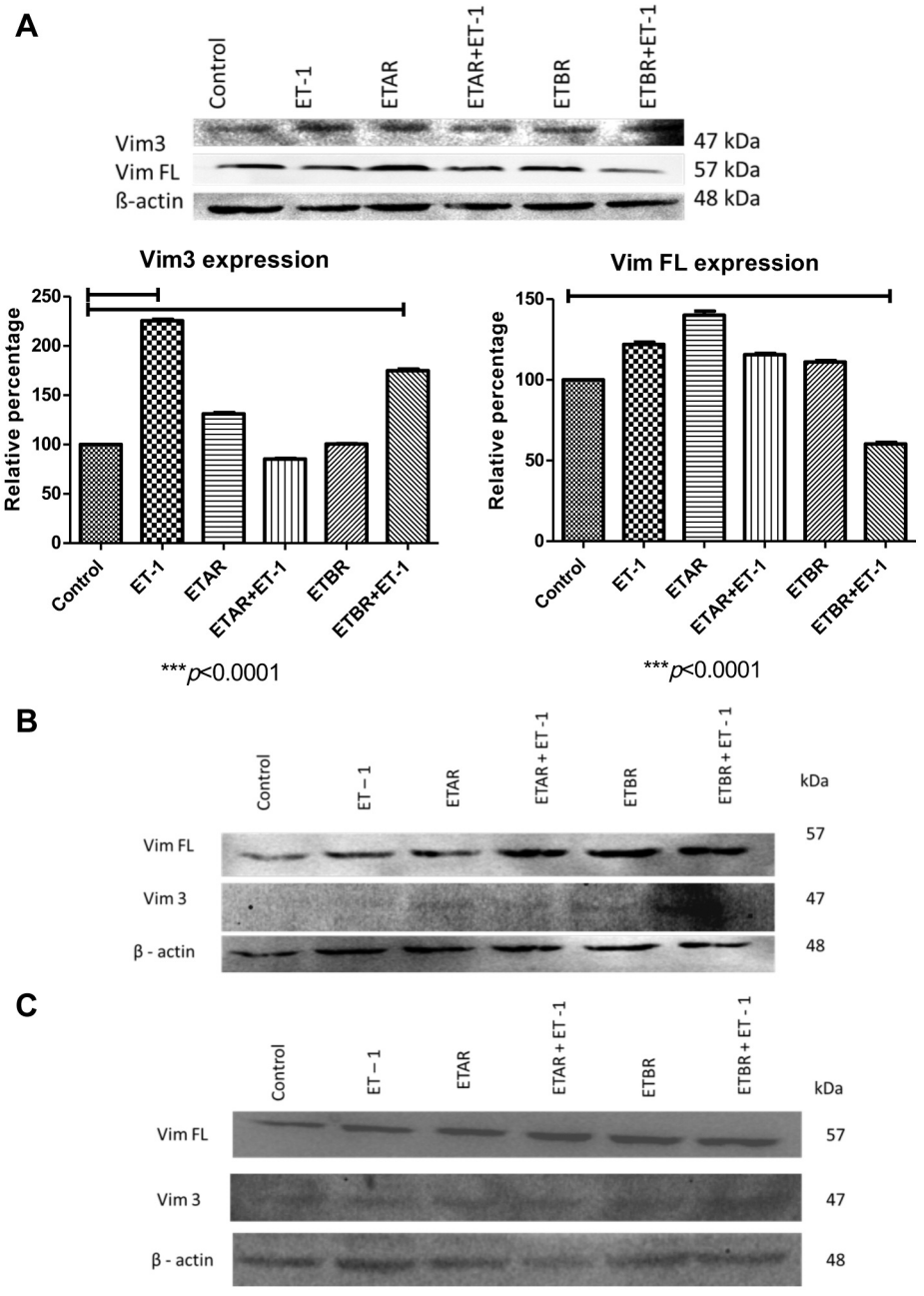


Figure 3. *Vim3 and VimFL expression in prostate cancer cell lines. (A) Western Blot for Vim3, Vim FL, and β-actin in all PCas. (A) in PC3 cells, (B) in LNCap and (C) in DU145.*

qRT-PCR values after different treatments was assessed by multiple analyses of variance (ANOVA).

Statistics. All experiments were performed in triplicates. The statistical analysis was performed with the GraphPrism 5 (Graphpad software, La Jolla, CA, USA). A paired T-test with one-way ANOVA was performed. The alpha–value was 0.05 and the results are visualized as boxplots (***p<0.0001, **p<0.001, *p<0.01).

Results

As recently described, ET-1 plays a role in the expression of Vim3 (12). Therefore, the PCa cell lines (PC3, DU145 and LNCap) were stimulated with ET-1, BQ123 (ETAR antagonist), BQ788 (ETBR antagonist) or in combination. A Scratch Assay was performed. The highest migration rate

was found in PC3 cells after 6 h treatment with ET-1 and BQ788 (Figure 1A, upper part). In LNCap (Figure 1B) and DU145 (Figure 1C) highest migration was found after 24 h treatment with ET-1 and BQ788. Boxplot analysis was performed. Cells migrating into the specific area of the gap were counted.

The expression of Vim3 and VimFL was determined *via* qRT-PCR after different stimulations (Figure 2). Figure 2 shows that Vim3 mRNA expression was significantly increased after ET-1 and BQ788 treatment. VimFL mRNA expression did not show any significant differences.

In Western Blot a significant increase in Vim3 levels after ET-1 treatment as well as ET-1 and BQ788 (Figure 3) was detectable. Similar results were obtained for all three prostate cancer cell lines. Interestingly, Vimentin full-length shows a reversal of results after ET-1 and ETBR treatment (Figure 3).

Discussion

The presented results indicate that the migration rate of PCa cells (DU145, LNCap and PC3) is influenced by the presence of ET-1 and the blockage of the two ET-1 receptors (Figure 1). The highest migration rate was found in case of PC3 cells treated for 6 h with ET-1 and ETBR. This result agrees with the literature, since this cell line is described as more aggressive (13). Furthermore, even the LNCap cell line, which is hormone sensitive, seems to be influenced by blocking the ETBR/ET-1 pathway. According to these results it seems that this pathway operates in both castration-naïve and castration resistant prostate cancer. Since we hypothesized that Vim3 is a malignancy marker, we analysed the mRNA as well as the protein levels in the different cell lines. In comparison to the VimFL, significant differences were detected, indicating that Vim3 could be a malignancy marker (Figures 2 and 3). This can be explained by the fact that Vimentin is associated with increased malignancy and is highly expressed in poorly differentiated prostate cancers (14). The differentiation is quite difficult since different antibodies are available which detect either only VimFL or both VimFL and Vim3 (10). Nevertheless, the newly designed Vim3 monoclonal antibody only detects the truncated version as described (10). Based on our results, it can be hypothesized that Vim3 plays a major role in malignancy. VimFL levels did not change significantly at the mRNA and protein level. Therefore, only Vim3 seems to be associated with increased migration and proliferation of prostate cancer cells, especially after an inhibition of ETBR. These results are in agreement with those in the literature, since in most prostate cancer patients with aggressive tumours ETBR is down-regulated (3, 15), concluding that the ET-1 signalling pathway is predominantly exerted *via* ETAR. Interestingly, all three cell lines showed the same overexpression of the newly identified malignancy marker Vim3 (Figure 3) protein after treatment with ET-1 and ETBR.

We concluded that Vim3 may be responsible for the increased metastatic behaviour of cancer cells. Vim3 expression can be detected in cells after ETBR block which is frequently the case in patients with high grade PCa. In these patients, ETBR is methylated and as a result ET-1 signalling is mediated by ETAR.

Conclusion

Vim3 levels and proliferation are significantly increased in prostate cancer cell lines after ETBR block, indicating that Vim3 can be a possible marker for the detection of aggressive PCas.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

BK, AS: experimental part, cell culture; MvB: wrote the manuscript with the support of AH, JF, IH, BK, HG, AS; MvB and AH: designed the study and directed the project; all other Authors performed the analysis, designed the figures and discussed the results.

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