

USP22 Promotes Therapeutic Resistance to Prostate Cancer by Deubiquitinating Myc Leading to Increased Myc Regulated Oncogenic Cell Transformation

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Abstract: USP22 deubiquitinating subunit serving as an oncogenic driver of prostate cancer by regulating cell proliferation and DNA repair. USP22's negative relationship with prostate cancer is well established, but its mechanism of action is not well defined. Here we show that USP22 is progressively overexpressed with prostate cancer progression. USP22 controls androgen receptor signaling and promotes the expression of various genes coregulated by androgen receptor and MYC. This reprogramming also was found to induce transition to therapeutic resistance. Additionally, inhibiting USP22 was found to be beneficial and improved prostate cancer prognosis. Taken together, we demonstrate the promise of USP22 as a therapeutic target for prostate cancer.

1 INTRODUCTION

Prostate cancer is the second most deathly and common type of cancer in developed countries. More than 209,900 American men were diagnosed with prostate cancer in 1997 (Mazhar, 2002). The main risk factor is age. Prostate cancer is very rare under the age of 40, but the risk of developing prostate cancer exponentially increases with age. Environmental factors and race are other risk factors as well (Prostate cancer - Symptoms and causes, 2021). This includes diet and radiation exposure. Compared to men of other races, African Americans have a greater chance of developing prostate cancer. An analysis showed that exposure to radiation was linked to cases of prostate cancer. Prostate cancer is caused by at least eight genetic mutation events. Most cases are due to the loss of tumor suppressive genes. P53 and p21 are examples of genes that are mutated in prostate cancer. Obesity is another risk factor, as people who are obese may have a higher risk of prostate cancer than those who have a healthy weight.

Several treatments have been developed to help treat prostate cancer. Combined androgen blockade eliminates all sources of androgen. Studies have

shown a 2.3% increase in survival with the use of flutamide (Mazhar, 2002).

Intermittent androgen blockade involves medical castration and discontinuation of the GnRH agonist, which delays the development of hormone resistance. Androgen blockade uses finasteride and antiandrogens (Mazhar, 2002). Finasteride is a reductase inhibitor, meaning that it prevents testosterone converting to dihydrotestosterone. Dihydrotestosterone has a higher affinity for the androgen receptor as well.

Increasing evidence has shown the deregulation of the UPS22 deubiquitinase, a protease that cleaves ubiquitin from proteins to cancer development. USP22 is a promoter of the tumor phenotype by modulating nuclear receptor, a ligand-regulated transcription factor, and oncogenic signaling, networks of signaling pathways that interact with each other to control the growth and progression of a tumor (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013). USP22 uses deubiquitination to modulate protein function and promote cell proliferation. USP22 is required for Myc function and potentiates Myc-mediated oncogenic cell transformation in some cancers. This enhances the expression of target genes co-regulated by AR and Myc, a regulator proto-oncogene that codes for transcription factors. USP22 included the transition to therapeutic resistance (Schrecengost,

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Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013). USP22 has been found to be a critical effector of tumor progression that drives lethal phenotypes. This makes the enzyme a promising therapeutic target to treat advanced disease. The deregulation of USP22 induces androgen-independent AR recruitment to target gene loci and supports cell growth without androgens. The depletion of USP22 down-regulates AR protein levels and evades AR activity in ADT-sensitive, a hormone therapy that stops testosterone from being produced, and CRPC cells, cells that express low levels of the androgen receptor (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013). It was thought that USP22 may control fundamental oncogenic signaling pathways that are implicit to prostate cancer initiation and progression. USP22 expression promotes the activation of target genes that are coordinated by AR and Myc. These are both maintained without androgens and AR antagonists.

The predicted outcome is that USP22 promotes therapeutic resistance to prostate cancer by deubiquitinating Myc leading to increased Myc regulated oncogenic cell transformation.

2 METHOD

Chromatin Tethering Assays

The cells were harvested from a 10 cm plate and suspended in a 100 μ L CSK buffer with protease inhibitor (McCann, Vasilevskaya, Poudel Neupane, Shafi, McNair, Dylgjeri, et al. 2019). After removing μ L of the suspension, the suspension leftover was diluted with CSK buffer to a volume of 1 mL. This was incubated in ice for 20 minutes. The chromatin-tethered assay was pelleted and extracted in 1 mL of the CSK buffer for 10 minutes. This was repeated and sample buffer was added. After the sample was boiled for 5 minutes, western blot analysis can begin.

Western Blotting

CRISPR was used to knock out an expression vector to overexpress USP22 in LNCaP prostate cancer cells. Ubiquitination of Myc will be measured using western blot for Myc and looking for Ubiquitin mediated ladders with a GAPDH western blot loading control (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013). Antibodies used to detect proteins were AR-N20, USP22, Myc, GAPDH, and β -actin. LNCaP cells were infected with shRNA-containing lentivirus and mixed with puromycin for 5 days (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013).

Immunohistochemistry

TMA's were stained to search for USP22 using USP22 polyclonal antibody (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013). The antibody was diluted 1:150 with detection using LEICA polyvision+. The unstained 5 μ m sections were taken out of paraffin TMA blocks (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek et al., 2013). It was incubated with the primary antibody at room temperature for 45 minutes.

Ubiquitination

Myc deubiquitination will be measured in vitro using ubiquitinated Myc as a substrate. Recombinant Myc will first be ubiquitinated by either Skp2, Fbw7, and Huw1 and then tested for deubiquitination by adding recombinant USP22.

Gene expression analysis

Cells were treated as listed on the packaging and RNA was isolated using TRIzol (McCann, Vasilevskaya, Poudel Neupane, Shafi, McNair, Dylgjeri et al. 2019). LNCaP cells are analyzed by qPCR using the manufacturer's instructions.

Cell growth assays

The cell culture was made up of LNCaP and C4-2 cells. They were both maintained in IMEM and supplemented with 5% FBS. A separate culture of 22Rv1 cells were maintained in DMEM and supplemented with 10% FBS. Media was supplemented with 2 mmol/L L-glutamine and 100 units/mL penicillin-streptomycin (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek et al., 2013). To ensure that the cell lines were stable, the LNCaP and C4-2 cells were transduced using lentivirus and went through three rounds of selection with their corresponding antibiotic. The cells were plated at equal densities and the cell number was measured using trypan blue exclusion with a hemocytometer (McCann, Vasilevskaya, Poudel Neupane, Shafi, McNair, Dylgjeri et al. 2019).

USP22 depletion

USP22 was transfected with Dharmafect and incubated for 72 hours for siRNA-mediated depletion. shRNA sequences that targeted USP22 were annealed, cloned, and packaged into virus for inducible USP22 depletion (Schrecengost, Dean, Goodwin, Schiewer, Urban, Stanek, et al., 2013).

3 RESULT

Table 1 shows USP22 and ubiquitination and Table 2 shows USP22 and apoptosis. Exhaustive lists of possible results are listed in the two tables below. Refer to notes.

Table 1: USP22 and Ubiquitination.

Possible Results	PR 1A	PR 2A	PR 3A	PR 4A	PR 5A	PR 6A
Knockout USP22 Increases Myc Ubiquitin Western Blot	+	-	-	-	-	-
Overexpress USP22 in HCC Decreases Myc Ubiquitin Western Blot	+	+	-	+	-	-
USP22 Decreases Ubiquitination Induced by Skp2	+	+	+	+	-	-
USP22 Decreases Ubiquitination Induced by Fbw7	+	+	+	-	+	-
USP22 Decreases Ubiquitination Induced by Huwe1	+	+	+	-	+	-
USP22 Decreases Myc Ubiquitination Caused by SKP2	+	+	+	-	+	-

Note: “+” represents a result corresponding to the possible result and “-” represents a result contradicting the possible result.

Table 2: USP22 and Apoptosis.

Possible Results	PR 1B	PR 2B
USP22 Correlates with Cell Apoptosis Acceleration	+	-

Note: “+” represents increased level of apoptosis and “-” represents decreased level of apoptosis.

3.1 Explanation of Tables

Possible Result 1A

Knockout USP22 increases Myc ubiquitin western blot and USP22 decreases Myc ubiquitination caused by SKP2. This is the expected result that aligns with the hypothesis presented.

Possible Result 2A

Knockout USP22 decreases Myc ubiquitin western blot and USP22 decreases Myc ubiquitination caused by SKP2.

Possible Result 3A

Knockout USP22 decreases Myc ubiquitin western blot, overexpress USP22 in HCC increases Myc ubiquitin western blot, and USP22 decreases Myc

ubiquitination caused by SKP2.

Possible Result 4A

Knockout USP22 decreases Myc ubiquitin western blot, overexpress USP22 in HCC decreases Myc ubiquitin western blot, USP22 increases ubiquitination induced by Huwe1, and USP22 increases Myc ubiquitination caused by SKP2.

Possible Result 5A

Knockout USP22 decreases Myc ubiquitin western blot, overexpress USP22 in HCC increases Myc ubiquitin western blot, USP22 increases ubiquitination induced by Skp2, and USP22 decreases Myc ubiquitination caused by SKP2.

Possible Result 6A

Knockout USP22 decreases Myc ubiquitin western

blot and USP22 increases Myc ubiquitination caused by SKP2. This is the unexpected result that contradicts with the hypothesis presented.

Possible Result 1B

USP22 correlates with cell killing. This is the expected result that aligns with the hypothesis.

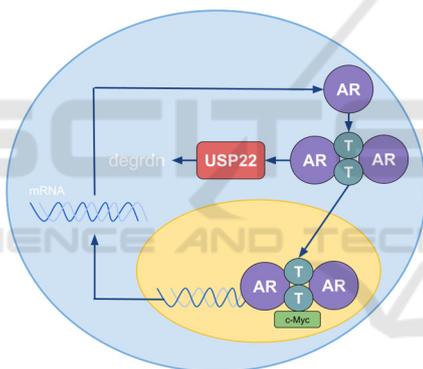
Possible Result 2B

USP22 does not correlate with cell killing. This is the unexpected result that contradicts the hypothesis.

3.2 Controls

Positive control is to add a known deubiquitinase for Myc. The positive control should be implemented to make sure USP22 is active. The negative control is a control treatment such as a scrambled targeting vector and an overexpression of an irrelevant protein.

4 DISCUSSION



Note: AR corresponds with androgen receptor. T corresponds with testosterone. USP22 leads to the degradation of the intracellular hormones (Michmerhuizen, Spratt, Pierce, Speers 2020). c-Myc acts as the co-regulator of the transcription inside the nucleus.

Figure 1.

Because USP22 regulates transcriptional activation of target substrates, which in turn promotes oncogenic phenotypes, knocking out USP22 will decrease Myc ubiquitination rates (Chan, Lee, Wang, Lin. 2010). Since Myc ubiquitin is responsible for transcription regulation, decreasing the ubiquitination rate is expected for a Skp2 deficient cell. Skp2 deficiency inhibits cancer development, as it prevents aerobic glycolysis and Akt activation (Chan, Lee, Wang, Lin, 2010). Since

Akt activation causes a chain reaction of Glut 1 expression, it promotes cancer development. Inhibiting Akt activation will prevent cancer initiation and slow its growth. Therefore, possible result 1A is the most likely result and aligns with the presented hypothesis. Possible Result 6A contradicts the current understanding of the ubiquitin signaling pathway. This result is unlikely to happen on the LNCaP cell line. This result may occur in variations of mutations and genetic disorders in a large group of people. Possible result 1A is consistent with previous studies investigating USP22's effect on malignant cells. Since USP22 promotes therapeutic resistance to prostate cancer, there is an acceleration in cell apoptosis. The relation between USP22 and Skp2 should be investigated to find a more specific ubiquitin pathway. Therefore, possible result 6 is unlikely and contradicts with the presented hypothesis.

In vitro studies, an analysis took place, examining a range of concentrations of USP22/the E3 ligases/Myc. This increased confidence that a specific effect would be uncovered. It also should be noted that the experiment was performed three times to discern significant changes. Previous studies report that USP22 knockdown decreased *in vitro* survival of cancerous cells compared to the controls. Doing an analysis of the western blot of the control and the knockout USP22 cancer cells show that USP22 modulates the Myc FOXO1 and YAP signaling pathways (Liu, Liu, Zhao, Zhu, Wang, Liu, 2019). Injection of knockout USP22 cancer cells into mice generated smaller tumors than did the control cells. This previous study overall shows that USP22 regulates the growth and progression of cancerous cells through the Myc dependent FOXO1 and YAP signaling pathways (Liu, Liu, Zhao, Zhu, Wang, Liu, 2019). Previous studies have also shown that overexpression of USP22 is associated with enhanced angiogenesis. However, USP22 knockout suppressed *in vitro* proliferation. It also impaired non-homologous DNA damage repair capacity and enhanced irradiation-induced apoptosis (Zhang, Yang, Wang, Sun, Guo, Nelson 2019). USP22 is vital for castration-resistant AR expression, cell proliferation, and tumor growth.

As prostate cancer progresses, namely, transitioning from hormone therapy-resistant to castration therapy-resistant, USP22 protein expression increases (McCann, Vasilevska, Poudel Neupane, Shafi, McNair, Dylgjeri 2019). This is because USP22 modulates AR stability and activity. When AR is overexpressed, the prostate cancer is able to progress to castration level of

androgen (Fujita, Nonomura, 2019). The prostate cancer cells can then progress to castration-resistant prostate cancer if AR is further amplified under androgen deprivation circumstances. The fact that USP22 modulates the AR plays a part in this progression. Even after new AR-targeted therapy is developed, castration-resistant prostate cancer will eventually gain resistance again. This provides a motivation to find out more about the mechanisms in which USP22 controls AR. There are multiple possible findings that could answer this question. USP22 upregulation occurs in most progressions of prostate cancer and is correlated to poor outcomes (McCann, Vasilevskaya, Poudel Neupane, Shafi, McNair, Dylgjeri, 2019). This USP22 alteration is associated with pro-proliferative oncogenes, including AR and Myc. This shows that knockout of USP22 decreases the proliferation of cells in prostate cancer. USP22 is enough to cause hyperproliferative prostate cancer, which demonstrates that USP22 alone can induce pro-tumorigenic phenotypes in previously normal tissue. All of this supports the fact that USP22 is a critical driver of the oncogenic phenotype in prostate cancer.

5 CONCLUSION

Although USP22 is a deubiquitinating enzyme that has been linked to carcinogenesis, not much is known about its function and regulation in both cancerous and non-cancerous tissue. Further studies investigating the specific regulation mechanism of USP22 should be done for a more thorough understanding of the role of USP22. These further studies will solidify USP22's role in cancer development. Since not many studies of USP22 have been done on mammals, preclinical testing on mice should also be done to improve the therapeutic method of a ubiquitin pathway.

USP22 is a critical effector that modulates AR levels, AR-Myc coordination, and the progression of prostate cancer to castration-resistant prostate cancer (Schreengost, Dean, Goodwin, Schiewer, Urban, Stanek 2013). Since USP22 plays such a major role in this progression, therapies that target USP22 would be highly beneficial to prevent the initiation and progression of prostate cancer.

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