NOT ALL NOTCH IS CREATED EQUAL: THE ONCOGENIC ROLE OF NOTCH2 IN BLADDER CANCER AND ITS IMPLICATIONS FOR TARGETED THERAPY

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Translational Relevance

While molecular targeted therapy has revolutionized the care of patients with many other cancers, few advances have been made in bladder cancer (BC). Although prior studies reported that NOTCH is a tumor suppressor in BC, we hypothesized, based on NOTCH2 genomic gain and NOTCH1/3 genomic deletion in The Cancer Genome Atlas (TCGA) dataset, that NOTCH2 signaling contributes to BC progression. We have discovered that NOTCH2 activation correlates with adverse disease parameters and worse prognosis, and have demonstrated that NOTCH2 acts as an oncogene that promotes BC growth and metastasis through EMT, cell cycle progression and maintenance of stemness in vitro and in vivo. Furthermore, we have shown that silencing NOTCH2 inhibits orthotopic xenograft growth and lymph node metastasis. These results provide pre-clinical proof of principle that NOTCH2 inhibition by a NOTCH2 inactivating antibody may be a rational treatment for muscle invasive BC. (142 word)
Abstract

Purpose: Recent molecular analyses of bladder cancer (BC) open the door to significant advances in targeted therapies. NOTCH has been identified as a tumor suppressor in BC, but prior reports have focused on NOTCH1. Here we hypothesized that NOTCH2 is an oncogene suitable for therapeutic targeting in BC.

Experimental design: We studied genomic aberrations of NOTCH, compared survival and tumor progression according to NOTCH2 expression levels, and studied NOTCH2 function in vitro and vivo.

Results: We report a high rate of NOTCH2 copy number gain in BC. High NOTCH2 expression was identified especially in the basal subtype and in mesenchymal tumors. NOTCH2 activation correlated with adverse disease parameters and worse prognosis by immunohistochemistry. Forced over-expression of the intracellular domain of NOTCH2 (N2ICD) induced cell growth and invasion by cell cycle progression, maintenance of stemness and epithelial-to-mesenchymal transition (EMT). These effects were abrogated by silencing of CSL, indicating that the effects were mediated through the canonical NOTCH signaling pathway. In an orthotopic xenograft model, forced over-expression of N2ICD increased growth, invasion and metastasis. To explore the potential for therapeutic targeting of NOTCH2, we first silenced the receptor with shRNA and subsequently treated with a specific inhibitory antibody. Both interventions decreased cell growth, invasion and metastasis in vitro and in the orthotopic xenograft model.

Conclusions: We have demonstrated that NOTCH2 acts as an oncogene that promotes BC growth and metastasis through EMT, cell cycle progression and...
maintenance of stemness. Inhibition of NOTCH2 is a rational novel treatment strategy for invasive BC.

(250 words)
Introduction

While molecular targeted therapy has achieved breakthroughs in the management of many other cancers, few advances have been made in muscle invasive bladder cancer (MIBC). The recent molecular characterization of MIBC and the classification into luminal and basal subtypes has provided a framework that promises to facilitate the development of novel therapeutics in MIBC (1-5). In this context we have focused on Notch signaling as critical pathway in MIBC.

NOTCH is a family of transmembrane receptors (NOTCH1, 2, 3 and 4) that regulate differentiation, proliferation and invasion. When the NOTCH ligands DLL and JAG bind to the receptor, they trigger a conformational change in the receptor that enables cleavage of the receptor first by TNF-α converting enzyme and then by gamma secretase. In the canonical NOTCH pathway, the NOTCH-intracellular domain (NICD) translocates into the nucleus where it interacts with CSL to modulate expression of target genes (6-8).

Aberrant signaling through each of the NOTCH receptors has been linked to numerous cancers, and the NOTCH pathway represents a compelling target for new drug development, although disparate roles for the different NOTCH receptors are an important consideration in therapeutic design (6, 9-11). Up until recently, little has been know about NOTCH in bladder cancer. Based on expression patterns of NOTCH2 in MIBC cell lines and our prior studies into the mechanisms of epithelial-to-mesenchymal transition (EMT) in MIBC, we hypothesized that NOTCH2 is an oncogene that drives MIBC progression. We showed in this study that NOTCH2 activation correlated with adverse disease parameters and worse prognosis in our own patients and The Cancer...
Genome Atlas (TCGA) dataset (2). With forced over-expression and silencing of NOTCH2 in cell line models we have been able to demonstrate that NOTCH2 promotes cell proliferation and invasion through cell cycle progression, dedifferentiation and EMT. Furthermore, we have demonstrated the proof of concept that these findings can be translated into a therapeutic strategy for patients with bladder cancer by studying the effects of a NOTCH2 inactivating antibody, NRR2Mab. Our results are particularly timely as they build on three recent reports suggesting that NOTCH is a tumor suppressor in MIBC (12-14).
Materials and Methods

Cell lines and Antibodies. A panel of human bladder cancer cell lines was provided by the Pathology Core of the Bladder Cancer SPORE at MD Anderson Cancer Center. Cell lines were authenticated by DNA fingerprinting using the AmpFISTR Amplification or AmpFISTR Profiler PCR Amplification protocols (Life Technologies). Antibodies against NOTCH 1, 2 and 3 were purchased from Cell Signaling Technology (Danvers, MA). A full list of antibodies used is shown in Supplementary Table 1.

Bioinformatics analysis of The Cancer Genome Atlas. We analyzed the published dataset of The Cancer Genome Atlas (TCGA) which included 131 patients (2). The dataset included 128 tumors for copy number and 129 patients for RNA-sequence.

Western blot and Quantitative PCR. Protein and RNA extraction, Western blot and quantitative real time PCR (qRT-PCR) were performed as previously described (15, 16) and as outlined in Supplementary Material and Methods. Primary antibodies are shown in Supplementary Table 1. qRT-PCR amplification of cDNA was carried out using primers listed in Supplementary Table 2.

Immunohistochemistry and Immunofluorescence. Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as previously described (16, 17). MIBC tissue microarrays (TMA) containing 156 human bladder cancer (BC) samples treated by radical cystectomy were used for IHC, and the patient characteristics shown in supplemental Table 3. Detailed IHC and IF information is shown in Supplementary Material and Methods. Three NOTCH2 staining patterns were defined. Any sample with >5% of nuclei staining for NOTCH2 was defined as having nuclear staining. All other
samples were defined as membranous or cytoplasmic based on the predominant staining pattern, and no significant overlap was observed between these three. TMAs were scored as follows: 0 = no staining, 1 = faint or focal stain, 2 = convincing intensity in a minority of cells, and 3 = convincing intensity in a majority of cells.

**Cell proliferation in adherent culture and cell cycle.** Cell growth in adherent culture was measured using the crystal violet assay. Cell-cycle analysis was performed by propidium iodide staining and flow cytometry as described previously(15, 17). Detailed information is described in Supplementary Material and Methods.

**Cell proliferation in anchorage independent conditions.** Cells were seeded in a 24 well plate coated with poly-HEMA (Sigma Aldrich, St. Louis, MO) and evaluated by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) on day 4 as described previously(18). For soft agar colony formation assays, 10,000 cells were seeded in 6 cm dishes in 0.35% agar/media with 10% FBS on top of a bed of 0.7% agar in the same medium. Cultures were incubated for 21 days and then colonies counted. Each assay was repeated in triplicate.

**RNA interference.** To knockdown endogenous *NOTCH2*, *CSL* and *SNAIL*, RNA interference was performed. siRNA oligonucleotides purchased from Life Technologies (Carlsbad, CA) are listed in Supplementary Table 4. Transfection was performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s protocol.

**Invasion assays.** Invasion assays were performed as previously described(16) using Matrigel™ coated invasion chambers (BD Biosciences, San Jose, CA) according to
manufacturer’s instructions. Detailed information is provided in Supplementary Material and Methods.

**Stable NOTCH2 intracellular domain overexpression and stable NOTCH2 knockdown.** The cDNA construct containing the NOTCH2 intracellular domain (ICD) was obtained by amplifying NOTCH2 from nucleotide 5115 to the stop codon, and cloning into pLenti4-To-V5 using the Gateway system (Life Technologies). Gateway PCR and Sequencing Primers are listed in Supplementary Table 5. NOTCH2 ICD lentivirus was generated in HEK293T cells using the Profection Mammalian Transfection System (Promega) and Virapower Lentiviral Expression plasmids (Life Technologies). Resultant virus was used to infect RT4V6 and RT112 with 6μg/mL polybrene for 24 hours. Overexpressing cells were selected in 200μg/mL Zeocin. Empty vector transduced cells were used as a negative control (mock cells). For stable knockdown, NOTCH2 targeting or control shRNA lentiviral particles (Santa Cruz Biotechnologies, Dallas, TX) were used according to manufacturer’s instructions.

**Orthotopic bladder cancer xenograft model.** All animal work was approved by the Institutional Review Board of the University of British Columbia (Vancouver, BC, Canada). Procedures were performed as previously described(15) and as outlined in Supplementary Material and Methods. Bioluminescence was used to quantify tumor burden and was measured on the Xenogen IVIS Spectrum imaging system (Perkin Elmer, Waltham, MA).

**NRR2Mab treatment.** The NOTCH2 targeting antibody (NRR2Mab) and the human isotype control antibody (IgG) were provided by Genentech (San Francisco, CA).
vitro, cells were treated with 10μg/mL NRR2Mab or IgG, split every 2-3 days and retreated as previously described(10). Cells were sub-cultured after 4-5 days for cell growth, invasion, Western blot and IF; after 7-8 days for qRT-PCR. In cell growth or invasion, drugs were added at day 0 and media was not changed until the end of the experiment. In vivo, 10 or 20 mg/kg was administered intraperitoneally twice weekly starting on day 5 after tumor inoculation.

**Statistics.** Statistical analysis was done via chi-square test, unpaired t-test, ANOVA and Wilcoxon test. Significance was defined as p<0.05.
Results

Analysis in patient MIBC tissue suggests oncogenic role of NOTCH2

We examined genetic aberrations in NOTCH1, 2 and 3 in The Cancer Genome Atlas (TCGA) dataset(2). NOTCH2 demonstrated genomic gain, while NOTCH1 and NOTCH3 were frequently deleted (Fig.1A left panel). Similar results were obtained in other datasets(19) (Fig. 1A middle and right panel). In the same TCGA datasets, patient tumors with high NOTCH2 mRNA expression showed significantly worse prognosis than patients with low and moderate NOTCH2 expression (Fig.1B). Tumors expressing high NOTCH2 were predominantly of the basal subtype and showed higher EMT and higher stem cell marker expression (Fig.1C).

NOTCH2 expression was detected by IHC in 111 of 156 (72%) BC tumors on TMA (Fig.2A). Non-malignant urothelium showed cell membrane staining, but nuclear and cytoplasmic staining were observed in BC. Nuclear NOTCH2, which is indicative of activated NOTCH2 signaling, was detected more frequently in G3 and pT3/4 tumors compared to G2 and pTis/a/1/2 tumors, respectively (Fig.2B). Patients with nuclear NOTCH2 staining showed worse prognosis than patients with membrane and cytoplasmic staining (Fig.2C), although NOTCH2 expression pattern was not an independent predictor on multivariable analysis (data not shown).

The expression of NOTCH receptors was assessed in a panel of 12 MIBC cell lines and compared to the expression of EMT markers (Fig.2D, 2E). The results of Western blot and qRT-PCR revealed that NOTCH2, especially cleaved NOTCH2, was highly expressed in MIBC cells with a mesenchymal phenotype, and NOTCH1 and NOTCH3
were highly expressed in cells with an epithelial phenotype. Together these results suggest that NOTCH2 is an oncogene that drives tumor progression.

**Forced over-expression of NOTCH2 intracellular domain promoted bladder cancer progression through increased proliferation and invasion.**

To assess NOTCH2 function in MIBC, we transduced 2 cell lines, RT4V6 and RT112, with an epithelial phenotype and low constitutive NOTCH2 expression with a lentiviral construct coding for the NOTCH2 intracellular domain (N2ICD). These N2ICD over-expressing cell lines (RT4V6-N2ICD and RT112-N2ICD) showed higher cleaved NOTCH2 expression than mock transduced cells (Fig. 3A). Both N2ICD cells showed nuclear staining of NOTCH2, while both mock transduced cells did not, suggesting N2ICD translocation to the nucleus (Fig. 3B). The N2ICD cells showed a more spindle-like morphology (Fig. 3C). Correspondingly, both N2ICD cells showed higher invasive ability compared to mock cells (Fig. 3D, S1A), and increased expression of the mesenchymal markers VIMENTIN, N-CADHERIN, FIBRONECTIN, ZEB1, SNAIL and SLUG, with concomitant decreased expression of E-CADHERIN, an epithelial marker, in Western blotting (Fig. 3A). Similar results were obtained in the cDNA microarray analysis comparing RT4V6-N2ICD to mock cells (Fig. S1B).

Previous studies have linked activated NOTCH signaling to aberrant cell cycle progression in several cancers (6, 20). In our analysis, both N2ICD cells showed higher cell proliferation than mock cells in adherent culture (AH) (Fig. 3E), as well as increased expression of phosphorylated RB (pRB) and SKP2, and decreased expression of p21 and p27 (Fig. 3F). Transduction of RT4V6 and RT112 with N2ICD increased the
percentage of cells in S phase and decreased the percentage in G0/G1 phase, which suggests cell cycle progression through the G1/S phase transition (Fig. 3G, S1C).

Since NOTCH regulates stem cell activity in several malignancies (21, 22), we compared cell growth in anchorage independent conditions (AI) and stem cell marker expression between N2ICD over-expressing and mock cells. Both N2ICD over-expressing cell lines showed higher growth in AI conditions, as well as higher expression of THY1, NES, NANO2 and SOX2 (Fig. 3H, 3I, S1D).

We validated the effects of NOTCH2 overexpression by the orthotopic xenograft model. RT4V6-N2ICD tumors grew more rapidly than RT4V6-mock tumors by bioluminescent imaging, which was confirmed tumor volume determination at the time of necropsy (Fig. 3J). To evaluate for regional extension of tumor, we measured bioluminescence in the mice after removal of the tumor-containing bladder, and observed that mice inoculated with RT4V6-N2ICD cells showed significantly higher bioluminescence than mice inoculated with mock cells (Fig.3K). IHC analysis of the xenografts confirmed strong nuclear NOTCH2 expression in the RT4V6-N2ICD tumors and low expression in the mock tumors. The RT4V6-N2ICD tumors differed from the mock tumors also by high N-CADHERIN and VIMENTIN expression and very low E-CADHERIN expression, indicating an increase in EMT characteristics (Fig.4A, 4B). Furthermore, we could confirm that RT4V6-N2ICD tumors had a higher percentage of Ki67 positive cells than mock tumors (Fig.4A, 4B). Together these results suggest that NOTCH2 promotes tumor progression through cell proliferation and invasion.
NOTCH2-induced cell invasion and growth were blocked by downstream inhibition of canonical NOTCH2 signaling.

To verify that the effects observed with forced over-expression of N2ICD were dependent on the canonical NOTCH pathway, we first assessed the expression of the main NOTCH downstream genes, HES and HEY family(8). Both N2ICD-transduced cell lines showed higher HEY1, HEY2 and HEYL but unaltered HES1 expression compared to the corresponding mock cells (Fig. 4C, S1B, S1E). HEY1 is the most extensively studied member of the HEY family of genes and is known to accumulate in the nucleus upon activation of NOTCH (23, 24). We confirmed by IHC in the orthoptoic xenografts that RT4V6-N2ICD tumors, which stained strongly for nuclear NOTCH2, also expressed nuclear HEY1, while the mock tumors expressed cytoplasmic HEY1 (Fig. 4A right panel). In order to verify that this activation of the HEY genes was regulated through CSL, we examined the effects of CSL silencing in N2ICD cells by RNA interference. Silencing CSL in RT4V6-N2ICD cells with two different siRNA resulted in decreased expression of the HEY genes compared to cells transfected with scramble siRNA (Fig. 4D, 4E). These results suggest that NOTCH2 in MIBC activates the canonical NOTCH pathway.

We verified that the effects of N2ICD on invasion were also dependent on signaling through CSL. RT4V6-N2ICD cells transfected with CSL siRNA were less invasive than the same cells treated with negative control siRNA (Fig. 4F, S2A). As a possible explanation for the effects on invasion, we investigated whether silencing CSL down-regulated the transcription factors SNAIL and SLUG, which are both suppressors of E-CADHERIN expression and are both known to be directly regulated by CSL (8, 25-29).
Indeed we observed lower expression levels of SNAIL and SLUG compared to control transfected cells (Fig. 4D, 4E). We dissected the pathway one step further by silencing SNAIL in RT4V6-N2ICD with specific siRNA. Silencing SNAIL decreased the invasive ability of these cells, which was associated with increased E-CADHERIN compared to cells transfected with negative control siRNA (Fig. S2B, S2C). Similarly we verified that the effects of N2ICD over-expression on cell growth were regulated through CSL, focusing also on genes regulated directly by CSL, including p21 and SKP2, as well as p27 which is regulated by SKP2 (27, 29, 30). RT4V6-N2ICD cells transfected with siRNA targeting CSL demonstrated a lower level of proliferation in both AI and AH conditions compared to negative control transfected cells (Fig. 4G, 4H). These effects on proliferation were associated with increased expression of the cell cycle regulators p21 and p27, decreased expression of SKP2 and decreased expression of stem cell markers THY1 and NES (Fig. 4D, 4E).

Together these results demonstrated that N2ICD-induced cell growth and invasion was mediated through the canonical NOTCH2 pathway.

**NOTCH2 silencing inhibited tumor growth and metastasis, suggesting NOTCH2 as a novel therapeutic target.**

In order to explore NOTCH2 inhibition as a potential therapeutic paradigm, we established NOTCH2 stable knockdown cell lines using bladder cancer cells with a mesenchymal phenotype and high N2ICD expression (UM-UC3 and UM-UC13). Effective silencing of NOTCH2 in these cells was shown on Western blot, and a corresponding decrease in HEY1 expression was shown by qRT-PCR (Fig. 5A, S3A).
Silencing NOTCH2 in UM-UC3 and UM-UC13 cells significantly decreased their invasive ability compared to cells transduced with control shRNA (Fig. 5B, S3B), and this correlated with decreased expression of the mesenchymal markers (Fig. 5C, S3D). We obtained similar results in both cell lines using siRNA targeting NOTCH2 (Fig. S3C). Growth under AI conditions and colony formation in soft agar were both reduced compared to control shRNA cells (Fig. 5D, 5E). These findings were associated with decreased THY1 and NANOG expression in UM-UC3 (NES and SOX2 were not detectable at baseline), and decreased THY1 and NES expression in UM-UC13 (NANOG and SOX2 were not detectable at baseline) (Fig. 5F, S3E). These effects were consistent with the observations made in N2ICD over-expressing cells.

In orthotopic xenografts, stable silencing of NOTCH2 significantly inhibited tumor growth in UM-UC3 and UM-UC13 compared to control shRNA cell lines (Fig. 5G). The xenografts were shown by qRT-PCR to have lower expression of NOTCH2 and HEY1, as well as decreased expression of mesenchymal and stem cell markers (Fig. 5H). Together experiments with stable silencing of NOTCH2 suggest that NOTCH2 inhibition blocks MIBC progression.

For potential clinical translation, we tested the effects of a NOTCH2 inactivating antibody, NRR2Mab, which has been shown to have little cross-reactivity with the other NOTCH receptors (10). In vitro, treatment with NRR2Mab significantly inhibited cell invasion and growth in UM-UC3 and UM-UC13 compared to control IgG treatment (Fig. 6A, 6B, S4B), and this was associated with decreased expression of HEY1, mesenchymal and stem cell markers (Fig. 6C, 6D, S4A, S4C, S4D).
In the UM-UC13 orthotopic xenograft model, NRR2Mab treatments were found to significantly inhibit tumor growth compared to control IgG (Fig. 6E, 6F). All mice treated by control IgG had gross para-aortic and/or iliac lymph node metastases, but only 7/13 (54%) and 5/14 (36%) mice treated with 10mg/kg and 20mg/kg NRR2Mab, respectively, developed gross lymph node metastases. NRR2Mab treatment decreased the size of lymph node metastases compared to control IgG in those mice that harboured nodal metastases (Fig. 6G). Mice treated with NRR2Mab showed no adverse effects and maintained their body weight compared to the control groups (Fig. S4F). With qRT-PCR we demonstrated that NRR2Mab treatment inhibited the expression of HEY1, mesenchymal and stem cell markers compared to control IgG (Fig. 6H), which is highly consistent with the results observed with silencing of NOTCH2. These results suggest that NRR2Mab inhibits bladder cancer growth and metastasis through inhibition of the NOTCH2 signaling pathway.
Discussion

Up until recently, little was known about NOTCH signaling in MIBC(31). However, two groups have now independently described frequent copy number loss of \textit{NOTCH1} in MIBC, and frequent mutation of \textit{NOTCH1}, 2 and 3 (12, 13). The mutations are in regions similar to those described previously for skin cancer(32), and are thought likely to be inactivating based on damage predictions and basic functional analysis in cell culture. However, these are non-recurrent mutations and their true importance is uncertain. Both research groups generated transgenic models of bladder-specific loss of a component of the gamma secretase complex, with resultant promotion of bladder tumourigenesis either spontaneously or after treatment with carcinogen(12, 13). These models are problematic because gamma secretase affects many pathways other than just NOTCH signaling. They are further limited by their dependence on a luminal (and not basal) promoter. Nonetheless, this work establishes the critical role of NOTCH signaling in bladder cancer, and especially highlights the tumour suppressor role of \textit{NOTCH1}(14).

However, we believe that the functional relevance of \textit{NOTCH2} mutations has not been adequately studied, and we in fact propose in contradiction to these published reports that \textit{NOTCH2} is an oncogene in bladder cancer. \textit{NOTCH}1 and 2 have been shown to have antagonistic effects in several solid cancers(6, 11), including embryonal brain tumors like medulloblastoma(33). Interestingly, this report on brain tumours was the first for any cancer to describe \textit{NOTCH2} copy number gains (15% of cases). The common context for the disparities between \textit{NOTCH}1 and 2 may relate to normal development in the specific organ (e.g. cerebellum), during which \textit{NOTCH2} is predominantly expressed.
in proliferating progenitors and NOTCH1 in post-mitotic differentiating cells. These findings highlight that molecular context is critical for understanding NOTCH pathway aberrations, and the molecular subtyping of MIBC into luminal and basal subtypes provides the necessary framework for investigating NOTCH in MIBC(3, 4).

The frequent rate of NOTCH2 copy number gain in MIBC, the close association with the basal subtype, and the correlation of NOTCH2 to overall survival in the TCGA dataset and our own TMA underscore the clinical relevance of this gene. Furthermore we have been able to delineate the downstream effectors of NOTCH2 activity in pre-clinical models of MIBC. We have demonstrated that N2ICD drives EMT, as previously reported in other contexts (26, 34), and it does so through the canonical pathway and is dependent on SNAIL and SLUG. Effects on proliferation were dependent on changes in the cell cycle regulators p21, p27 and SKP2 through the canonical NOTCH pathway (27, 29, 30).

The NOTCH2 inactivating antibody NRR2Mab offers a potentially clinically relevant agent for translation of these findings into patient care. This antibody inhibited BC growth and metastasis in vivo with no signs of toxicity. NOTCH pathway inhibitors in the form of GSIs are currently in early clinical development for the treatment of solid tumors and leukemia (35). GSIs, however, inhibit not only all four NOTCH receptors, but also multiple other pathways, and they have been associated with significant gastrointestinal toxicity (9, 36). The disparate roles for NOTCH1 and NOTCH3 versus NOTCH2 in MIBC would suggest that GSIs are not suitable for MIBC therapy. NRR2Mab is specific to NOTCH2 and therefore circumvents these limitations to clinical translation (10).
results provide pre-clinical proof of principle that NOTCH2 inhibition by NRR2Mab is a rational therapy for MIBC.

In Conclusion, NOTCH2 expression correlates with basal phenotype and worse prognosis in MIBC, and we have demonstrated with genetic and pharmacologic manipulation \textit{in vitro} and \textit{in vivo} that NOTCH2 over-expression promotes tumor growth, invasion and metastasis, and inhibition of NOTCH2 blocks MIBC progression. Using NRR2Mab, a selective NOTCH2 inhibitor, we have established the pre-clinical proof of principle for targeting NOTCH2 as a novel therapeutic paradigm for MIBC.

\textbf{Acknowledgments}

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Reference


**Figure legends**

**Fig. 1:** NOTCH2 is genomically gained and NOTCH2 expression correlates with basal phenotype and worse prognosis.  

A, Genomic aberrations detected in NOTCH1, 2 and 3 in published TCGA bladder cancer dataset, the provisional TCGA dataset (n=408) and in a series of other 109 high grade bladder cancers profiled by MSK-IMPACT at Memorial Sloane Kettering Cancer Center (MSKCC). NOTCH2 tends to be genomically gained, while NOTCH1 and 3 tend to be deleted.  

B, Waterfall plot showing expression of NOTCH2 across the published TCGA cohort (left panel). When the TCGA cohort is classified according to high (25 patients with highest expression) and low (25 patients with lowest expression) NOTCH2 RNA expression by RNA sequencing, high NOTCH2 correlates with lower overall survival as displayed in this Kaplan-Meier plot (right panel).  

C, The expression of a range of markers in TCGA tumors expressing high NOTCH2 compared to those with low NOTCH2, showing that high NOTCH2 expression is associated with a basal, mesenchymal phenotype and higher stem cell marker expression. * p<0.05; ** p<0.01.

**Fig. 2:** NOTCH2 expression correlates with mesenchymal phenotype, advanced stage and poor prognosis in immunohistochemical (IHC) study.  

A, Three patterns of NOTCH2 staining in bladder cancer.  

B, Comparison of staining patterns with respect to histological grade, pT stage and tumor site (primary versus lymph nodes metastasis) showed nuclear NOTCH2 staining was detected significantly more frequently in advanced stage.  

C, Nuclear NOTCH2 staining correlates to poorer survival rates after cystectomy.  

D and E, Bladder cancer cell lines are listed from epithelial to mesenchymal.
phenotype. The results of qRT-PCR(D) and Western blot(E) revealed that NOTCH2 expression, especially cleaved NOTCH2, correlates with mesenchymal phenotype, while NOTCH1 and 3 expression correlates with epithelial phenotype. FL=full length. CL=cleaved. *P < 0.05. ** P = 0.0613

Fig. 3: NOTCH2 overexpression promotes cell invasion and growth by epithelial-to-mesenchymal transition (EMT) and cell cycle progression in vitro and in vivo. A, Cleaved NOTCH2 and EMT marker expression in NOTCH2 intracellular domain (N2ICD)-overexpressing cells and mock transduced cells by Western blot. B, Immunofluorescence staining for expression and subcellular localization of NOTCH2 (red) in the nucleus (blue). C, N2ICD cells show more spindle-like morphology. D, N2ICD cells show increased invasion compared to control in a Boyden chamber assay. E, N2ICD cells show increased cell growth in adherent (AH) culture. F, The expression of cell cycle related proteins indicates that N2ICD promotes cell cycle progression. G, Cell cycle analysis shows decreased G0/G1 and increased S phase in RT4V6-N2ICD cells. H, Growth of N2ICD cells is increased in anchorage independent (AI) conditions. I, Stem cell marker expression is increased in RT4V6 cells overexpressing N2ICD. NOTCH2 overexpression increases tumor growth and metastasis in an orthotopic xenograft model. J, RT4V6-N2ICD cells show increased tumor growth compared to mock cells as monitored by bioluminescence (left) and by tumor volume at the time of necropsy (right). K, Mice inoculated with RT4V6-N2ICD cells showed higher residual bioluminescence after bladder removal compared to mice inoculated with mock cells. *P<0.01, **P<0.05.
Fig. 4: Canonical NOTCH2 pathway drives cell growth and invasion.  
A, IHC analysis of NOTCH2, EMT markers and Ki67 in xenograft tissue (left panel, reduced from X40). RT4V6-N2ICD tumors showed strong nuclear NOTCH2 and HEY1 staining while mock tumors showed cell surface and cytoplasmic staining (right panel, reduced from X200).  
B, Scoring comparison of NOTCH2, EMT marker expression and Ki67 positivity between RT4V6-N2ICD and mock tumors.  
C, Western blot analysis of the expression of common NOTCH target genes. CSL-silenced RT4V6-N2ICD cells show: 
D, decreased expression of HEY family genes, SKP2, and mesenchymal and stem cell markers by qRT-PCR;  
E, increased expression of p21 and p27 and decreased expression of SNAIL and SLUG by Western blot;  
F, decreased cell invasion; and,  
G, decreased cell proliferation in adherent (AH); and,  
H, decreased cell proliferation in anchorage independent (AI) culture.  * P < 0.01.

Fig. 5: Stable NOTCH2 knockdown inhibits bladder cancer progression.  
A, Stable NOTCH2 knockdown in UM-UC3 and UM-UC13 results in decreased NOTCH2 expression by Western blot. NOTCH2-knockdown cells show:  
B, decreased invasion in Boyden chamber assay;  
C, decreased expression of mesenchymal markers by qRT-PCR (UM-UC3);  
D, decreased cell growth in anchorage independent (AI) culture;  
E, decreased colony formation in soft agar;  
F, decreased expression of THY1 and NANOG (UM-UC3); and,  
G, decreased xenograft growth in orthotopic bladder cancer model by bioluminescence (UM-UC3 and UM-UC13).  
H, qRT-PCR of UM-UC3 xenografts shows
decreased expression of NOTCH2, HEY1, and mesenchymal and stem cell markers. * $P < 0.01$, ** $P < 0.05$.

Fig. 6: NRR2Mab treatment inhibits tumor growth and metastasis in an orthotopic bladder cancer xenograft model. NRR2Mab decreases: A, cell invasion of UM-UC3 and UM-UC13; B, cell growth of UM-UC3 and UM-UC13 in anchorage independent (AI) culture; C, expression of mesenchymal markers in UM-UC13; D, expression of THY1 and NES in UM-UC13. E and F, NRR2Mab treatment (10mg/kg and 20mg/kg) inhibits tumor growth compared to control IgG treatment (20mg/kg) in UM-UC13 as monitored by bioluminescence and tumor volume at the time of necropsy. G, NRR2Mab treatment decreases the average size of lymph node metastases. H, qRT-PCR analysis of xenografts treated with NRR2Mab shows decreased expression of HEY1, as well as mesenchymal and stem cell markers. * $P < 0.01$, ** $P < 0.05$. 
Fig. 1

A

TCGA

Patients with genomic aberration (n=128)

TCGA provisional

Patients with genomic aberration (n=108)

MKSCC, Eur Urol.

Patients with genomic aberration (n=109)

- amplification
- gain
- missense mutation
- truncating mutation
- heterozygous loss
- homozygous deletion

B

RNA-seq derived Notch2 expression (thousands)

Individual patient tumors (TCGA; n=129)

Patient tumors with high NOTCH2 expression (n=25)

Patient tumors with low NOTCH2 expression (n=25)

Overall survival

Low NOTCH2 expression

Rest of cohort

High NOTCH2 expression

Months

C

RNA-seq derived mRNA expression (Log_{10} transformed)

FGR3, GATA3, UPK4, KRT14, KRT5, KRT8

Luminal

Basal

CDH1, CDH2, SNAI1, SNAI2, ZEB1, VIM

Mesenchymal

THY1, NES, SOX2

Stem cell
Fig. 2

A

Cell membrane staining

Cytoplasmic staining

Nuclear staining

B

No staining  Nuclear staining  Cytoplasmic staining  Membrane staining

% of staining patterns

Grade 2  Grade 3  pTis/a/1/2  pT3/4  Primary tumor  LN metastasis

C

Probability of survival (%)

Days after cystectomy

No staining  Other staining

D

Notch1

mRNA expression level

Notch2

mRNA expression level

Notch3

mRNA expression level

Epithelial  Mesenchymal

E

FL NOTCH1  CL NOTCH1  FL NOTCH2  CL NOTCH2  FL NOTCH3  CL NOTCH3  E-CADHERIN  ZEB1  VIMENTIN  ACTB  9416  UM-UC1  UM-UC5  UM-UC14  253JBV  T24  UM-UC3  UM-UC13  Epithelial  Mesenchymal
**Fig. 4**

(A) Immunostaining of NOTCH2, E-CADHERIN, VIMENTIN, N-CADHERIN, and Ki67 in Mock and N2ICD cells. 

(B) Bar graph showing expression score of NOTCH2, E-CADHERIN, VIMENTIN, and N-CADHERIN in Mock and N2ICD cells.

(C) Western blot analysis of HES1, HEY1, HEY2, and ACTB in RT4V6 and RT112 cells.

(D) Graph showing relative expression (fold change) of CSL, HEY1, HEY2, HEYL, SNAI1, SNAI2, SKP2, THY1, and NES in RT4V6 N2ICD cells.

(E) Western blot analysis of CSL, p27, p21, SNAIL, SLUG, and VINCulin in Control siRNA, CSL siRNA1, and CSL siRNA2.

(F) Bar graph showing relative invasion (fold change) in RT4V6 N2ICD cells.

(G) Graph showing relative growth (fold change) in AH culture (RT4V6 N2ICD).

(H) Graph showing relative growth (fold change) in Al culture.
Fig. 5

A. Western blot analysis showing the expression of NOTCH2 in UM-UC3 and UM-UC13 cells treated with control and NOTCH2 shRNA.

B. Relative invasion (fold change) of UM-UC3 and UM-UC13 cells treated with control and NOTCH2 shRNA.

C. Relative expression (fold change) of VIM, CDH2, FN1, ZEB1, SNAI1, and SNAI2 in UM-UC3 cells treated with control and NOTCH2 shRNA.

D. Relative growth (fold change) of UM-UC3 and UM-UC13 cells treated with control and NOTCH2 shRNA.

E. Number of colonies in UM-UC3 and UM-UC13 cells treated with control and NOTCH2 shRNA.

F. Relative expression (fold change) of THY1 and NANOG in UM-UC3 cells treated with control and NOTCH2 shRNA.

G. Bioluminescence (photons/sec) in UM-UC3 and UM-UC13 xenografts treated with control and NOTCH2 shRNA.

H. Relative expression (fold change) of NOTCH2, HEY1, VIM, CDH2, FN1, ZEB1, SNAI1, and SNAI2 in UM-UC3 xenograft tumor treated with control and NOTCH2 shRNA.
Fig. 6 A

Relative invasion (fold change)

![Graph showing relative invasion (fold change) for UM-UC3 and UM-UC13.]

B

Relative growth (fold change)

![Graph showing relative growth (fold change) for UM-UC3 and UM-UC13.]

C

UM-UC13

Relative expression

![Graph showing relative expression for VIM, CDH2, FN1, ZEB1, SNAI1, SNAI2, THY1, and NES.]

D

UM-UC13

![Graph showing relative expression for THY1 and NES.]

E

UM-UC13 xenograft

Bioluminescence (photons/sec)

![Graph showing bioluminescence (photons/sec) for UM-UC13 xenograft treated with Control IgG (20mg/kg), NRR2Mab (10mg/kg), and NRR2Mab (20mg/kg).]

F

Tumor volume (mm³)

![Bar graph showing tumor volume (mm³) for Control IgG, NRR2Mab (10mg/kg), and NRR2Mab (20mg/kg).]

G

Size of lymph node metastasis (mm²)

![Bar graph showing size of lymph node metastasis (mm²) for Control IgG (20mg/kg) and NRR2Mab (20mg/kg).]

H

Relative expression (fold change)

![Bar graph showing relative expression (fold change) for HEY1, FN1, ZEB1, SNAI1, SNAI2, THY1, and NES.]
# Clinical Cancer Research

**NOT ALL NOTCH IS CREATED EQUAL: THE ONCOGENIC ROLE OF NOTCH2 IN BLADDER CANCER AND ITS IMPLICATIONS FOR TARGETED THERAPY**

Tetsutaro Hayashi, Kilian M Gust, Alexander W Wyatt, et al.

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