Original research

Downstream neighbor of SON (DONSON) is associated with unfavorable survival across diverse cancers with oncogenic properties in clear cell renal cell carcinoma

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A B S T R A C T

A precise stratification of our patients is essential and can support clinicians to determine the right therapy. The aim of this study was to identify clinically relevant genes using The Cancer Genome Atlas (TCGA) datasets. A comprehensive pan-cancer analysis of 30 distinct tumor entities (N = 9022) identified the largely unknown gene Downstream neighbor of SON (DONSON) to be particularly associated with unfavorable overall survival in clear cell renal cell carcinoma (KIRC). This prognostic potential of DONSON was validated in an independent KIRC cohort via quantitative real-time PCR (n = 152). Further, DONSON protein expression was evaluated via immunohistochemical staining followed by quantitative image analysis using the image analysis software QuPath on a renal cancer tissue microarray (n = 270).

Interestingly, DONSON overexpression was preferentially associated with poor survival in 9 of the 30 entities, suggesting tumor-independent oncogenic properties of this largely unknown gene. A particularly strong association of DONSON to an aggressive phenotype was evident in KIRC and proved to be a strong independent predictor of unfavorable overall survival in two additional cohorts on the mRNA and protein level. In our KIRC cell culture model, we observed a substantial attenuation of proliferative activity and migration capacity of the KIRC cells Caki1 and 769p. Able overall survival in two additional cohorts on the mRNA and protein level. In our KIRC cell culture model, we observed a substantial attenuation of proliferative activity and migration capacity of the KIRC cells Caki1 and 769p.

In conclusion, we identified DONSON as a robust biomarker for risk stratification in KIRC in three independent cohorts and provide evidence that DONSON is linked to a malignant phenotype in the KIRC cell culture model.

Introduction

Renal cell carcinoma (RCC) is the most prevalent malignant kidney lesion in adults, with continuously increasing incidence [1,2]. The major histological subtypes in descending order include clear cell RCC in about 80% (KIRC), followed by papillary RCC (KIRP) and chromophobe (KICH) [3]. In metastatic RCC, the clinical implementation of multi-tyrosine kinase inhibitors (TKI) and immune checkpoint inhibitors (ICI), led to an improved clinical outcome [4–8]. However, many patients do not respond or ultimately develop resistance under these treatment regimens. Thus, the identification of diagnostic and prognostic biomarkers, based on molecular tumor characteristics, is essential for predicting tumor aggressiveness and improved therapeutic management tailored to individualized therapy approaches.

Publicly available genomic databases, such as The Cancer Genome Atlas (TCGA) have become essential tools in cancer research [9–11]. In a systematic multivariate Cox regression analysis, the relatively unknown Downstream neighbor of SON (DONSON) was found as a particularly strong predictor for unfavorable overall survival in the KIRC, which prompted us to examine this gene more closely [12,13]. DONSON has previously been identified as a replisome component, responsible for fork stabilization during genomic replication [14]. Overall, DONSON plays a crucial role in maintaining genomic integrity, cell cycle progression, and efficient replication [15]. Regarding cancer research, not much was known about DONSON until very recently.

A MicroRNA (miRNA) passenger strand, miR101-5p, acting tumor suppressive in RCC, was found to directly regulate the DONSON expression. Ecopic expression of miR101-5p, as well as a siRNA-mediated DONSON-knockdown, attenuated the malignant features of RCC cells [16].

However, the implications of DONSON in KIRC pathogenesis and tumor progression, as well as its value as a promising biomarker for patient stratification, are not yet fully characterized.
Fig. 1. A, Heatmap of the prognostic value of DONSON in 30 different tumor entities using log-rank tests after median dichotomization ($N = 9022$); standard TCGA study abbreviations were used; red/OE = high expression, blue/UE = low expression. Tumor abbreviations: ACC = adrenocortical carcinoma, BLCA = bladder urothelial carcinoma, BRCA = breast invasive carcinoma, CHOL = cholangiocarcinoma, CESC = cervical squamous cell carcinoma and endocervical adenocarcinoma, COAD = colon adenocarcinoma, DLBL = lymphoid neoplasm diffuse large B-cell lymphoma, ESCA = esophageal carcinoma, GBM = glioblastoma multiforme, HNSC = head and neck squamous cell carcinoma, KICH = kidney chromophobe carcinoma, KIRC = kidney renal clear cell carcinoma, KIRP = kidney renal papillary carcinoma, LAML = acute myeloid leukemia, LGG = brain lower grade glioma, LIHC = liver hepatocellular carcinoma, LUAD = lung adenocarcinoma, LUSC = lung squamous cell carcinoma, MESO = mesothelioma, OV = ovarian serous cystadenocarcinoma, PAAD = pancreatic adenocarcinoma, PCPG = pheochromocytoma and paraganglioma, PRAD = prostate adenocarcinoma, SARC = sarcoma, SKMC = skin cutaneous melanoma, STAD = stomach adenocarcinoma, TGCT = testicular germ cell tumors, THCA = thyroid carcinoma, THYM = thymoma, UCEC = uterine corpus endometrial carcinoma. B, Significant associations between DONSON expression and unfavorable overall survival across the TCGA cohorts are depicted as Kaplan-Meier estimate curves. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Methods

TCGA data

The UCSC Xena browser (http://xena.ucsc.edu) was used to download TCGA transcriptome sequencing data (Log2 transformed RNA-Seq v2) for DONSON in 30 different tumor types (see Fig. 1A, N = 9022, including KIRC N = 532, plus normal adjacent kidney tissue (NAT) N = 72) [12].

RCC cohorts of the University Hospital Bonn (UHB)

Via the Biobank at the University Hospital Bonn fresh-frozen or formalin-fixed paraffin-embedded paraffin (FFPE) RCC tissue samples were assembled as a KIRC cDNA cohort and KIRC tissue microarray as described previously [17,18]. All patients provided written informed consent before the specimens were collected in accordance with the declaration of Helsinki.

RNA isolation and real-time PCR

50 mg of cryo-preserved tissue was homogenized, and the total RNA was isolated using the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) as described previously [17,18] (KIRC N = 103, NAT N = 20). The DNA elimination was achieved by treatment of the RNA with DNase (DNA-free Kit, Ambion).

The RNA of the cell lines was isolated from pellets using the Total RNA Purification Mini Spin Column Kit (Genaxxon bioscience GmbH, Ulm, DE). The RNA quantity and quality were determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Reverse transcription of approximately 1 μg total RNA was performed using the PrimeScript RT Reagent Kit with gDNA Eraser. The DONSON-knockdown validation PCR experiments were carried out following genomic DNA elimination by treating 1 μg of total RNA with gDNA Eraser for 2 min at 42 °C and subsequent reverse transcription with the same PrimeScript RT Reagent Kit. 5 ng of the resulting cDNA was used for real-time PCR (1 × SYBR Premix Ex Taq II with ROX Plus and 10 pmol/μl PCR primers; all reagents: Takara Bio, Saint-Germain-en-Laye, France).

The following primer sequences were used: DONSON forward: 5′-GTCGCC AGCATTTGAGGGAAC-3′; and reverse: 5′-GGCCTGCCTGGAAGGTACAA-G-3′; β-Actin forward: 5′-CAACCGGAGGAGATGA-3′ and reverse: 5′-CACG AGGCCGTACAGGATAG-3′. The primer annealing temperature was 60 °C for both primer pairs.

Western Blot

For the Western Blot of 8 paired NAT and KIRC samples, approximately 50 mg tissue was homogenized in a Precellys 24 homogenizer (Peqlab, Erlangen, Germany) with 400 μl Cell Lysis Buffer (Cell Signaling, Cambridge, United Kingdom) including protease inhibitor (Complete Mini EDTA-free, Roche, Basel, Switzerland). Each 30 μg protein per lane was separated on a NuPAGE 4–12% Gel in an XCell4 SureLock electrophoresis system (Life Technologies, Carlsbad, CA, USA).

For the Western Blot confirming the DONSON-knockdowns, cells were collected 72 h post-transfection and centrifuged to cell pellets. These were washed with buffered saline (Gibco DPBS, Thermo Fisher Scientific, Waltham, MA, USA) and homogenized in 50 μl of the same cell lysis buffer with a protease inhibitor as stated above. The protein concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) while the CAKI1 cell line was cultured in McCoy’s 5A medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), both supplemented with 10% fetal bovine serum (FBS Superior, Biochrom GmbH, Berlin, Germany), 0.4% penicillin/streptomycin and only the RPMI1640 medium supplemented with 1% glutamine (Thermo Fisher Scientific, Inc., Darmstadt, Germany). All experiments were performed with mycoplasma-free cells, which have been authenticated before the study began.

Antisense locked nucleic acids (LNA) GapmeR-mediated knockdown

Transfections in the cell lines were performed using a quantity of 10 μl/well for 6-well plates and 0.5 μl/well for 96-well plates with a final concentration of 10 pmol/μl Antisense LNA GapmeR (QIAGEN, Hilden, DE) and FuGENE HD-Transfection reagent (#E2311, Promega Corporation, Madison, WI, USA) in a ratio of 1:1 according to the manufacturer’s instructions. The following DONSON GapmeR sequence was used (5′-3′): A+C+C+A+G+T+C+A+C+T+C+A+T+A+G+A. As a non-targeting negative control, the following GapmeR sequence was used: A+C+C+A+G+T+C+A+C+T+C+G+C+C. Knockdowns were performed at least three times in each cell line.

Cell proliferation and cytotoxicity assays

Cell viability was measured at 48-, 72- and 96-h post-transfection according to the manufacturer’s protocol (EZ4U; Biomedica Group, Vienna, Austria). The absorbance of a viability-dependent derivative as a surrogate for cell proliferation was measured using a microplate reader. Each experiment was performed in triplicates.

Migration assays

5000 cells were plated in the upper chamber of the migration inserts (VWR, Darmstadt, Germany) containing 2% FCS medium, whereas the lower chamber was filled with a medium containing 10% FCS for chemotactic attraction. After 24 h of incubation, cells were fixed with 4%
formaldehyde and stained with hematoxylin. Membranes were scanned, and cells were counted automatically by nucleus detection using the QuPath software [21]. Each experiment was performed in triplicates.

Statistical analysis

SPSS Statistics v25, Microsoft Excel, and GraphPad Prism Version 8.2.1 were used for statistics. Group comparisons were performed using the nonparametric Mann-Whitney-U or Kruskal-Wallis test. Uni-/multivariate Cox regression analyses [TNM; Age] and Kaplan Meier estimates were performed to evaluate the prognostic value of the DONSON [22,23].

Results

Pan-cancer analyses of DONSON using TCGA datasets

To comprehensively investigate the general prognostic potential of DONSON, we performed a systematic pan-cancer survival analysis for 30 different tumor entities of TCGA (N = 9022; Fig. 1A + B). Interestingly, DONSON overexpression was preferentially associated with poor survival in 9 of the 30 entities, suggesting tumor-independent oncogenic properties for this gene (Fig. 1A + B). Especially in KIRC, adrenocortical carcinoma (ACC) and mesothelioma (MESO) DONSON was strongly associated with an unfavorable OS (p < 0.0001). It has to be mentioned that DONSON overexpression was also associated with unfavorable OS in other frequently occurring tumor entities, which is of particular interest as most of these tumors do not share similar phenotypes or phylogenetic features. Of note, DONSON has not been investigated in all of these cancers so far. As the second most common subtype of RCC, KIRP also showed an association between an aggressive phenotype and enhanced DONSON expression (p = 0.002). In multivariate Cox regression analyses, DONSON was found to be the strongest independent predictor of unfavorable OS in KIRC compared to all other investigated entities (Hazard Ratio (HR) 2.3, 95% CI; 1.7–3.1; p < 0.0001, Table 1). This strong association prompted us to investigate the role of DONSON in KIRC more comprehensively. By comparing the DONSON mRNA expression in KIRC to normal adjacent renal tissue (NAT), we observed a significant upregulation in the tumor samples (Fig. 2A). Advanced pathological T-stages, lymphonodal, and distant metastatic spread, known as the critical steps during RCC progression, were also

| Table 1 | Multivariate Cox regression analyses in the evaluated KIRC cohorts regarding overall survival (OS). |
|----------|----------------------------------|
| Clinical-pathological parameters | p value | Hazard ratio [95% CI low/high] |
| **KIRC TCGA cohort (RNAseq)** | | |
| DONSON mRNA | < 0.0001 | 2.27 [1.68; 3.07] |
| T-stage | 0.001 | 1.03 [1.01; 1.05] |
| N-stage | 0.07 | 1.27 [0.98; 1.65] |
| M-stage | 0.54 | 1.24 [0.62; 2.50] |
| Age | < 0.0001 | 2.89 [1.76; 4.75] |
| **KIRC cDNA cohort (qPCR)** | | |
| DONSON mRNA | 0.04 | 2.36 [1.04; 5.37] |
| T-stage | 0.54 | 1.16 [0.73; 1.84] |
| N-stage | 0.72 | 0.76 [0.18; 3.29] |
| M-stage | 0.01 | 3.46 [1.32; 9.08] |
| Age | 0.02 | 1.06 [1.01; 1.12] |
| **KIRC TMA cohort (IHC)** | | |
| DONSON protein | 0.05 | 2.95 [1.00; 8.69] |
| T-stage | 0.21 | 1.45 [0.81; 2.59] |
| N-stage | 0.61 | 1.10 [0.75; 1.60] |
| M-stage | 0.43 | 1.21 [0.75; 1.96] |
| Age | 0.60 | 1.01 [0.97; 1.06] |

Fig. 2. Association of DONSON with pathological parameters and survival using the KIRC TCGA dataset A: DONSON expression in KIRC vs normal adjacent kidney tissue (NAT). Association of DONSON expression with TNM-Staging (B–D). E, Survival analysis following the dichotomization of the whole (E) as well as only the localized KIRC (F) cohort based on the median DONSON mRNA expression.
strongly associated with increased DONSON mRNA expression (Fig. 2B–D). Significantly reduced OS was seen in the group of DONSON overexpressing tumors (Fig. 2E). Of note, also in the group of localized KIRC (N = 443, Fig. 2F), DONSON showed a strong association with shortened OS, which may be a hint for the value of DONSON for risk stratification in the non-metastatic setting.

DONSON mRNA expression in an independent KIRC cDNA cohort

In accordance with the TCGA results, we detected significantly increased DONSON mRNA expression in KIRC compared to NAT (Fig. 3A). Further, DONSON overexpressing KIRC was associated with a significantly reduced overall (OS), cancer-specific (CSS) and progression-free survival (PFS) (Fig. 3B–D). After adjusting the co-variates TNM stage and age at initial diagnosis, we found that DONSON represents an independent risk factor for reduced OS (HR 2.4, 95% CI; 1.0–5.4; p = 0.04, Table 1) and CSS (HR 3.0, 95% CI; 1.1–8.8; p = 0.03, Table 1) in our KIRC cDNA cohort. Hence, we were able to validate the prognostic potential of DONSON in an independent RCC cohort on the transcriptional level.

DONSON protein expression on RCC TMA

We have further investigated a clinically annotated RCC tissue microarray (TMA) by immunohistochemical staining against DONSON to test its potential as a prognostic biomarker at the protein level. We observed preferentially cytoplasmatic staining against DONSON, which is in line with the staining pattern of DONSON in the RCC cohort of The Human Protein Atlas (HPA, www.proteinatlas.org; [24]). In the non-cancerous renal cortex, the proximal tubules exhibited the strongest DONSON expression (Fig. 4A). DONSON protein expression was again higher in KIRC than in NAT (Fig. 4B) in accordance with the transcriptional upregulation in the TCGA and cDNA cohorts. Further, we performed Western Blots for DONSON on 8 paired formalin-fixed paraffin-embedded paraffin (FFPE) KIRC and NAT tissue samples. Six of the eight matched specimens presented noticeably higher DONSON expression compared to NAT, and only one sample vice versa (Fig. 4C).

In KICH, DONSON expression was low, whereas the KIRP subtype showed the strongest DONSON expression across the RCC subtypes (Fig. 4A + D). The differential DONSON expression between KICH, KIRC, and KIRP was similar at the transcriptional level using the TCGA datasets. KICH showed the lowest DONSON expression levels, followed by KIRC, whereby KIRP exhibited the strongest DONSON expression (Fig. 4D + E). Interestingly, KIRC exhibited a heterogeneous DONSON expression throughout the cohort, with a negative as well as a DONSON-overexpressing subgroup (Fig. 4A). Consistent with DONSON’s prognostic value seen on the transcriptional level, this DONSON-overexpressing subgroup showed a significantly reduced progression-free (PFS) and overall survival (OS) (Fig. 4E + F). Of particular interest is that DONSON overexpression on the protein level was again shown to be an independent predictor of both unfavorable PFS (HR 3.1, 95% CI; 1.2–8.3; p = 0.02, Table 1) and OS (HR 2.9, 95% CI; 1.0–8.7;
p = 0.05, Table 1) measured by a multivariate Cox regression co-adjusting the TNM stage and age at initial diagnosis. In metastatic KIRC, DONSON expression was enhanced, although lacking a statistical significance, which might be due to the low sample size (Suppl. Fig. 1A, \( M1 \) = 15, \( p = 0.15 \)). DONSON overexpression was associated with high-grade KIRC histopathology, nonetheless only a trend towards statistical significance was seen (Suppl. Fig. 1B, \( p = 0.12 \)).

In total, DONSON as an independent predictor of unfavorable OS on both transcriptional and translational levels in three distinct RCC cohorts represents a promising and robust prognostic biomarker for risk stratification of our KIRC patients.

**Functional in vitro analyses of DONSON**

In order to evaluate the role DONSON plays in tumor progression and metastasis, we conducted in-vitro investigations in established RCC cell culture models. All evaluated RCC cell lines - ACHN, Caki1 and 769p - express DONSON protein in similar amounts (Fig. 5A). We induced specific DONSON-knockdowns via transfection of antisense oligonucleotides (Antisense LNA-GapmeR system) and established efficient knockdowns assessed by qRT-PCR (Fig. 5B). Western Blot analyses also confirmed efficient knockdowns on the translational level in all three evaluated cell lines (Fig. 5C). Thereafter, we investigated the effect of specific DONSON depletion on the proliferative activity of the RCC cells compared to negative control. We observed inhibition of cell proliferation in a time-dependent manner over a time course of 96 h post-transfection with significantly impaired proliferation observed for Caki1 and 769p (Fig. 5D). Moreover, in Caki1 and 769p, depletion of DONSON led to significantly attenuated migration capacities measured by Boyden Chamber Migration assays (Fig. 5E + F), whereas only a trend was seen in the ACHN cell lines without statistical significance reached (\( p = 0.19 \)).

We concluded that DONSON expression in the KIRC cell culture model is necessary to maintain the malignant potential in vitro. Thus, DONSON could serve as an interesting therapeutic target.

**Discussion**

In this study, in a comprehensive pan-cancer analysis, we were able to associate the largely unknown gene DONSON with unfavorable OS in a variety of solid tumors (Fig. 1). DONSON, therefore, seems to be cancer-type independently associated with aggressive phenotype and thus is a highly promising gene for further basic and oncological research. The strongest
The association between poor survival and DONSON overexpression was found in KIRC, which was consequently selected for further analyses. Thus, the robust expression data of the KIRC TCGA cohort could be validated in two further independent RCC cohorts on the transcriptional and translational level, which highlights its robustness as a promising biomarker [22].

We hypothesized that genes that show a strong correlation to unfavorable survival, and therefore to particularly aggressive tumors, represent interesting targets for further research. Thus, we have investigated DONSON's functional role in established RCC cell culture models. In our cell culture model, the depletion of DONSON caused a decrease in the proliferative activity and an attenuation of the migration capacities of CAKI1 and 769p KIRC cells. Recently, the tumor-suppressive miR-101-5p was shown to negatively regulate DONSON and several replisome genes in KIRC. Expression of miR-101-5p, as well as siRNA-mediated DONSON knockdown, caused cell cycle arrest, apoptosis, and impaired motility in the RCC cell lines 766O and A498 [16]. Our results are, therefore, in line with this previously published study.

Chemotherapeutic agents play an essential role in the therapy of many tumors. These drugs especially interfere with metabolic processes associated with cell growth or cell division. Tumor cells have an increased cell division rate as well as limited DNA damage repair capacity, which is why they are more sensitive to these drugs than healthy cells. DONSON could be an interesting targeted therapeutic target as it plays a vital role in both cellular processes, replication, and maintaining genome stability [14,15]. It is an essential gene for stable replication forks during S-phase and thus for proliferation itself and plays a pivotal role in maintaining genomic integrity and stability. It could, therefore, serve as a promising target in the therapy of various tumors (Fig. 1), where DONSON inhibition could restrict proliferation while negatively affecting the genomic integrity of the tumor cell, which ultimately could lead to tumor shrinkage. Our functional investigations already underline this hypothesis as DONSON-depletion caused growth restriction in a time-dependent manner in our RCC cell culture model. This is in line with previous literature, where DONSON-depletion also induced apoptosis [16]. Nevertheless, it should be mentioned that DONSON's potential as a therapeutic target requires further investigation. However, our study, which associates DONSON with an aggressive phenotype in multiple tumor types, could be a starting point for further basic and oncological research on DONSON and may open the avenue for DONSON being an attractive therapeutic target.

**Conclusion**

In total, we were able to associate the widely unknown gene DONSON with poor overall survival in various solid tumors. In particular, we identify DONSON as an interesting biomarker for risk stratification in KIRC in three independent cohorts and provide evidence that DONSON is linked to a malignant phenotype in the RCC cell culture model.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100844.

**Authorship**

Niklas Klümper: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition

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**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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