Implications of NOTCH3 expression and signaling for cholangiocarcinogenesis

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Abstract P-90

ABSTRACT

The NOTCH pathway is an evolutionary conserved signaling pathway with a pivotal role for physiological liver function and homeostasis. Aberrant activation of NOTCH signaling is a potential driver of liver cancer development and progression, particularly of the cholangiocarcinoma (CCA) subtype. Among the NOTCH receptors, especially NOTCH1–3 are implicated in CCA formation[1,2]. However, the individual contribution of NOTCH receptors to biliary carcinogenesis remains unresolved and compared to the well-studied NOTCH1 receptor, the role of NOTCH3 is poorly characterized. Similarly, the expression of NOTCH receptors in benign precursor lesions including biliary intraepithelial neoplasia (BilIN) or intraductal papillary neoplasms of the bile duct (IPNB) was not yet investigated. Here, we elucidated the function of the atypical NOTCH3 receptor during cholangio-carcinogenesis.

Using patient material of human non-neoplastic, corresponding precursor lesion and invasive CCA, a significant upregulation of NOTCH3 was observed. *In vitro* analyses showed that both NOTCH1 and NOTCH3 inhibited cell viability and colony formation. The examination of downstream target genes further revealed similar and distinct effects of NOTCH1 and NOTCH3 on differentiation and epithelial-mesenchymal transition (EMT) genes. *In vivo* experiments demonstrated that similarly to the potent oncogene NOTCH1[3], NOTCH3 together with AKT induced CCA development in mice *in vivo*.

Taken together, our data suggest that particularly NOTCH1 and NOTCH3 signaling might be implicated in CCA development. Investigating the differences of both receptors on downstream signaling and tumor features is subject of our current research.

NOTCH3 is upregulated during human CCA development

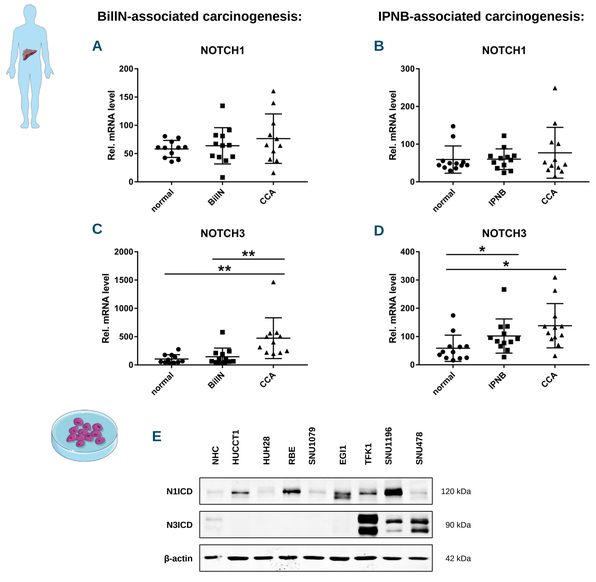

Figure 1: Expression and activity of NOTCH receptors in the course of human cholangiocarcinogesis. The mRNA levels of NOTCH1 **(A+B)** and NOTCH3 **(C+D)** in normal bile duct epithelium, corresponding precursor lesions (BilIN, left or IPNB, right) and invasive CCA were determined by Nanostring analysis in a matched design using a well-characterized human CCA cohort. **(E)** The abundance of cleaved active intracellular domains of NOTCH1 (N1ICD) and NOTCH3 (N3ICD) in multiple human CCA cell lines were analyzed by Western blot compared to the immortalized cholangiocyte cell line NHC. Statistical difference was evaluated by Student's t-test with * p<0.05 and ** p<0.01.

Ectopic NOTCH1 and NOTCH3 suppress tumor cell growth *in vitro*

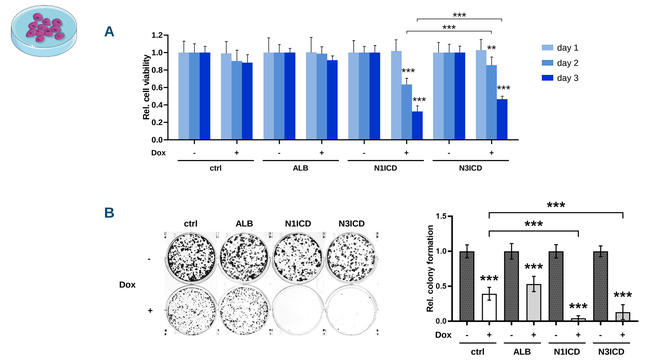

Figure 2: Cell biological behavior after NOTCH receptor overexpression. Following induction of N1ICD or N3ICD overexpression in HUCCT1 cells by 0.5 µg/ml doxycycline (Dox), cell viability **(A)** and colony formation capacity **(B)** were analyzed. Data are presented as mean ± standard deviation of 3 independent experiments. Statistical difference was evaluated by Student's t-test compared to the respective sample without Dox or as indicated with * p<0.05, ** p<0.01 and *** p<0.001.

NOTCH1 and NOTCH3 regulate differentiation and EMT genes

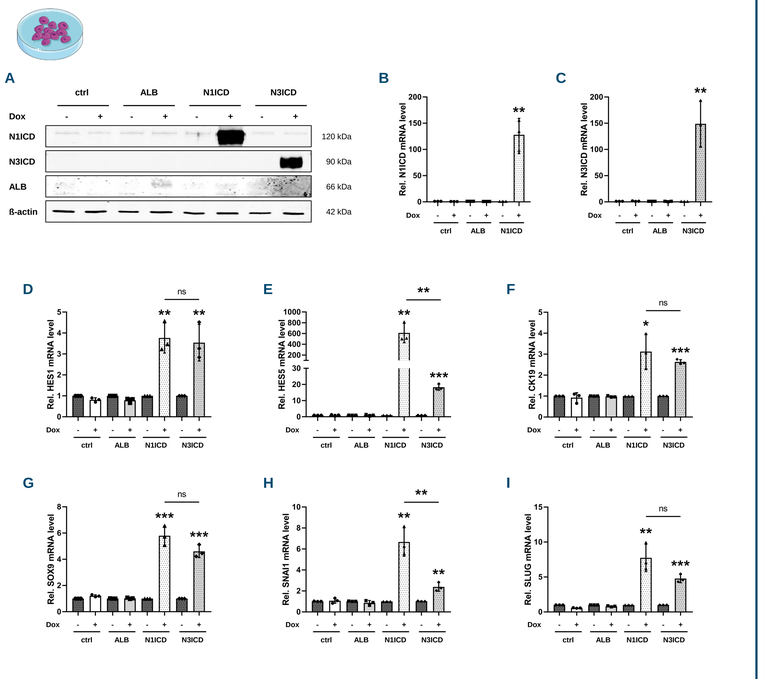

Figure 3: Effects of NOTCH receptor signaling on downstream target genes. Following treatment of inducible HUCCT1 cells by 0.5 µg/ml doxycycline (Dox), overexpression of N1ICD or N3ICD was verified on protein level by Western blot **(A)** and on mRNA level by qRT-PCR **(B+C)**. Furthermore, expression of the recognized downstream targets HES1 **(D)** and HES5 **(E)**, the cholangiocyte differentiation markers CK19 **(F)** and SOX9 **(G)** and the EMT markers SNAI1 **(H)** and SLUG **(I)** was determined by qRT-PCR. Data are presented as mean ± standard deviation of 3 independent experiments. Statistical difference was evaluated by Student's t-test compared to the respective sample without Dox or as indicated with ns (not significant) p>0.5, * p<0.05, ** p<0.01 and *** p<0.001.

NOTCH3 promotes AKT-associated liver carcinogenesis *in vivo*

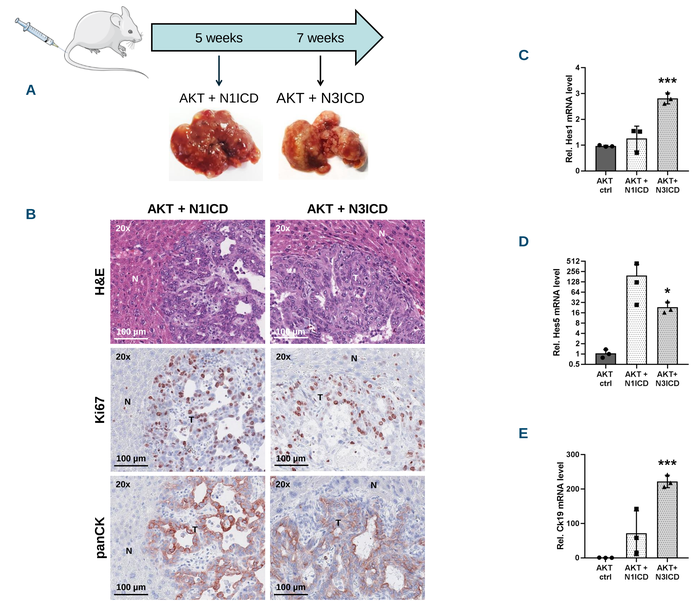

Figure 4: Effect of NOTCH receptor signaling on AKT-dependent liver tumor development. Hydrodynamic tail vein injection into 8 week old FVB/N mice was performed to stably express myrAKT, N1ICD or N3ICD in mouse hepatocytes. Livers were isolated 5 weeks or 7 weeks post injection for N1ICD or N3ICD, respectively **(A)**. Furthermore, H&E and immunohistochemical stainings **(B)** were evaluated. In addition, mRNA levels from isolated mouse liver tissues transduced with myrAKT (N=3) or myrAKT/N1ICD (N=3) or myrAKT/N3ICD (N=3) were analyzed by qRT-PCR of Hes1 **(C)**, Hes5 **(D)** and Ck19 **(E)**. Statistical difference was evaluated by Student's t-test with * p<0.05, ** p<0.01 and *** p<0.001.

CONCLUSIONS

- **NOTCH3 is upregulated during cholangiocarcinogenesis in CCA patients and expressed in a subset of human CCA cell lines**
- **Ectopic expression of N1ICD and N3ICD similarly inhibit cell viability and colony formation of CCA cells *in vitro***
- **NOTCH1 and NOTCH3 have overlapping target genes involved in cell differentiation and epithelial-mesenchymal transition**
- **NOTCH3 drives AKT-dependent tumorigenesis in mice**

REFERENCES

1 Guest, Rachel V., et al. "Notch3 drives development and progression of cholangiocarcinoma." Proceedings of the National Academy of Sciences 113.43 (2016): 12250-12255.
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3 Fan, Biao, et al. "Cholangiocarcinomas can originate from hepatocytes in mice." The Journal of clinical investigation 122.8 (2012): 2911-2915.