Toll-like receptor 5 and 8 in hepatocellular carcinoma

Running head: TLR5 and TLR8 in HCC

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Summary

Background

Toll-like receptors (TLRs) are components of innate immunity, but also have a role in carcinogenesis. The prognostic value of TLR5 and TLR8 tumor expression was examined in contrast with known risk markers Ki67 and p53.

Methods:

All HCC patients from Oulu University Hospital with available representative tumor sample were included in this study (n=182). TLR5, TLR8, Ki67 and p53 expression were investigated by immunohistochemistry. The relation between patient survival and TLR, Ki67 and p53 expression was calculated with Cox regression adjusted for confounding factors.

Results:

TLR5 cytoplasm intensity was associated with 5-year overall (strong 0.0% vs weak 23.4%, $p<0.001$) and disease-specific (strong 0.0% vs weak 34.9%, $p<0.001$) survival. TLR5 nuclei percentage was associated with poor 5-year disease-specific survival (high 16.3% vs low 31.5%, $p=0.022$). In adjusted analysis, strong TLR5 cytoplasm intensity was an independent risk factor for poor 5-year overall (adjusted HR 1.88, 95% CI 1.26-2.81) and disease-specific (adjusted HR 2.00, 95% CI 1.27-3.15) survival. High Ki67 and p53 expression associated with 5-year overall- and disease-specific survival. TLR8 was not associated with patient survival.

This study suggests that TLR5 expression is independently prognostic in HCC with similar point estimate as previously known p53.

Keywords: Toll-like receptors, TLR5, TLR8, hepatocellular carcinoma, Ki67, p53

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cause of cancer-related death worldwide.(1) Despite some progress, HCC remains as a major cause of death often detected at inoperable stage.(2) New biomarkers to identify patients who could benefit from more aggressive treatment are needed. Toll-like receptors (TLRs) are a family of pattern-recognition receptors. The stimulation of TLRs initiate a production of cytokines necessary for the development of effective host defense mechanisms, which are not limited only to the induction of inflammatory response, but influence also the adaptive immunity.(3) TLR5 is a bacterial flagellin recognizing receptor, which mobilizes nuclear factor-κB and tumor necrosis factor-α production upon stimulation. (4) TLR5 is localized in cell surface.(5) TLR5 expression has been detected in various cancer types (6–11), but not in human HCC. Previously, increased TLR5 cytoplasm expression was reported in nasopharyngeal carcinoma (12), gastric dysplasia (9), breast cancer (13) and in squamous cell carcinoma of the tongue (11). In nasopharyngeal carcinoma, nuclear membrane expression of TLR5 has been also detected.(12) In esophageal cancer, TLR5 nuclear expression was associated with higher tumor stage, although the biological mechanism explaining this localization is unclear.(14) TLR5 functions in tumor development and has anti-tumoral effects. TLR8 is located intracellularly (5), it recognizes single-stranded RNA and is involved in the recognition of viral and bacterial pathogens resulting in the activation of various proinflammatory cytokines.(15,16) TLR8 expression has been detected in various cancers but not in HCC.(17–20) Cytoplasmic TLR8 expression is associated with tumor cell survival and chemoresistance in lung cancer.(20) Anti-tumoral effects of TLR8 have been previously suggested in HCC.(21)

The aim of this study was to investigate the prognostic role of TLR5 and TLR8 cytoplasmic and nuclear expression in HCC, and compared with previously known risk factors Ki67 and p53.(22,23)

Materials and methods

Study design

This study was a retrospective cohort study in a single tertiary care hospital in Northern Finland. The Oulu University Hospital cohort has been described in previous study.(24) A total of 273 histologically confirmed HCC patients were treated in Oulu University Hospital between January 1983 and March 12, 2018. Of these, the final series consisted of 182 patients with available representative tissue material. Patient survival data was acquired form Statistics Finland. The Oulu University Hospital Ethics Committee approved the study and the need to obtain informed consent from the study patients was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA Dnro 10832/06.01.03.01/2014).

Data collection

The patients were originally identified from the archives using ICD-10 code C22.0& indicating hepatocellular carcinoma. Diagnoses from each patient were confirmed by histological examination. Clinical data was collected from Oulu University Hospitals' patient records. Diagnostic Hematoxylin and Eosin-stained (HE) histological samples were retrieved from the pathological archives. The 8th edition of TNM classification was used in staging.

Sample evaluation

The samples originally used for clinical decision-making, were retrieved and used in the present study. At first, multiple sections from each patient were viewed with light microscope. A representative slide with visible tumor component was selected and digitized using Aperio AT2 (Leica Biosystems, Wetzlar, Germany). Gastrointestinal pathologist (V-M.P) re-evaluated and

confirmed the diagnoses of all included patients. All cases were re-graded (25) by gastrointenstinal pathologist (V-M.P).

Tissue microarray

Tissue microarrays (TMAs) were constructed using method that has been described earlier.(26) At first, the most representative areas with visible tumor cells were selected from the HE-stained slides. Gastrointestinal pathologist (V-M.P) confirmed the chosen areas. TMAs were constructed with Galileo CK4500 tissue microarray platform. Tissue cores with diameter of 1,0 mm were taken from the tumor, using the chosen scanned slides as a guideline. One core was taken per sample block.

Immunohistochemistry

Immunohistochemistry was performed on tissue cores, which were selected on the basis of HE-staining as representative for tumor tissue. Antigen retrieval was performed by exposure to high temperature in Tris-EDTA buffer for 15 min (pH 9.0). The used kit was Dako REAL EnVision Peroxidase/DAB+, Rabbit/Mouse, REF K5007. Immunostaining was performed manually with mouse antibodies against TLR5 (NBP2-24787, Novus Biologicals, Littleton, USA) at a dilution of 1:75 (Dilution solution (Dako REAL antibody Diluent REF S2022)), overnight in refrigerator (+8 °), TLR8 (NBP-2-24917, Novus Biologicals, Littleton, USA) at a dilution of 1:850 (Dilution solution (Dako REAL antibody Diluent REF S2022)), 60 minutes in room temperature, Ki-67 (Bond, Leiga REF PAO230, Leica Biosystems Newcastle Ltd, UK) without dilution, 60 minutes in room temperature, p53 (DAKO monoclonal mouse clone DO-7, Envision kit, DAKO, Glostrup, Denmark), at a dilution of 1:400 (Dilution solution (Dako REAL antibody Diluent REF S2022)), 30 minutes in room temperature. For detection of the first antibody binding, we used Dako REAL EnVision Peroxidase/DAB+, Rabbit/Mouse, REF K5007 (Dako, Copenhagen, Denmark). The

reaction was visualized by Dako REAL™ DAB+ Chromogen. As negative control, we used omission of the primary antibody and replacement of the primary antibody with non-specific mouse primary antibody isotype. Isotype control (Invitrogen FEF 086599, USA).

Histological analysis

All histological analysis was performed independently by two investigators (V.K. and N.K.) blinded to the clinical data. The assessment of cytoplasm intensity was evaluated using 4-point scale from 0 (negative) to 1 (weak), 2 (moderate) and 3 (strong) according to most prevalent positive expression score. The extent of staining was estimated from 0 to 100% to express the percentage of positive cytoplasm and nuclei. Thus, all values are means of intensities and percentages from two investigators. For statistical evaluation, each stain (TLR5, TLR8, Ki67 and p53) were dichotomized by median value into two groups. Ki67 was evaluated by using QuPath 0.2.1 software (27) to detect positively stained Ki67 cells, which has shown a great reproducibility in breast cancer (28). Cut-offs were as follows: TLR5 cytoplasm intensity <=1.0, TLR5 nuclei percentage <=95.0, TLR5 cytoplasm percentage <100.0, TLR8 cytoplasm intensity <=2.0, TLR8 nuclei percentage <=27.5, TLR8 cytoplasm percentage <100.0, Ki67 positive cells <=8.0 and p53 nuclei percentage <=10.0. To exclude possible bias related to sample staining intensity, TLR5 and TLR8 cytoplasm intensities were compared between surgical resection samples and core needle biopsies with Mann-Whitney U- test. Significant difference between groups was observed ($p<0.001$). Since technical reason related to smaller staining area could not be excluded, given treatment was adjusted to exclude possible bias. Examples of TLR5 and TLR8, immunohistochemical staining are presented in Figure 1, Examples of Ki67 and p53 immunohistochemical staining are presented in Supplementary Figure 1.

Outcomes

Primary outcomes were 5-year overall- and disease-specific survival. This was defined as death from any cause (overall survival) or HCC (disease-specific survival) during the interval between the date of treatment and the end of 5-year follow-up.

Statistical analysis

$\chi 2$-test was used to obtain p-values when comparing categorical variables. The threshold for significance was set at $P < 0.05$. Mann-Whitney U was used to compare differences between two independent groups with continuous variables. Cohen's kappa was calculated to analyze interobserver agreement.(29) If interobserver difference was less than one point in intensity or less than 30% in the proportion of positive cells, the value was counted as an equal. Kaplan-Meier method was used to compare survival between groups and log-rank test was used to analyze statistical differences between groups. Cox regression model was used to perform multivariable analysis between groups with the following covariates: sex (female/male), age (continuous), comorbidities (Charlson Comorbidity Index 0-1, 2 or higher), cirrhosis (no/yes), Child-Pugh points (A, B or C), year of operation/diagnosis (1983-2005, 2006-2018), tumor differentiation grade (1-2, 3), stage (1, 2 or higher, according to the 8th edition of the UICC/AJCC TNM categories) and given treatment (surgery/local ablation/TACE/palliative treatment). Hazard ratios (HR) with 95% confidence intervals (CI) were provided. Statistical analysis was performed with IBM SPSS statistics 24.0 (IBM Corp., Armonk, NY).

Results

Patients

In 182 HCC patients, median age was 71.1 years (IQR 64.0-79.7) with male dominance (72.2%). Thirty-six (19.3%) patients underwent surgery, 18 (9.6%) local ablation, 32 (17.1%) received angiological treatment and 101 (54.0%) palliative treatment. Median tumor size was 65.0 mm (IQR 40.0-100.0). Eighty (42.8%) patients had tumor stage I and 104 (55.6%) tumor stage II or higher. Median follow-up time was 0.8 years (IQR 0.2-2.0). Overall 5-year survival of the patients was 14.4% and disease-specific survival 22.9%. Baseline characteristics are presented in Table 1.

TLR5 staining and correlation with clinicopathological variables in hepatocellular carcinoma

Cohen's Kappa value for TLR5 cytoplasm intensity was 0.984, TLR5 nuclei percentage 0.840 and TLR5 cytoplasm percentage 0.939. Cytoplasmic TLR5 staining was unreliable with 6 patients and they were excluded. TLR5 expression was not found on cell membranes. TLR5 cytoplasm intensity was associated with tumor unifocality (p=0.003). TLR5 nuclei percentage was associated with local recidives (p=0.021). TLR5 cytoplasm percentage was associated with tumor unifocality (p=0.048). Baseline characteristics and correlation with clinicopathological variables of TLR5 expression are presented in Supplementary Table 1.

TLR8 staining and correlation with clinicopathological variables in hepatocellular carcinoma

Cohen's Kappa value for TLR8 cytoplasm intensity was 0.973, for TLR8 nuclei percentage 0.788 and for TLR8 cytoplasm percentage 0.781. Cytoplasmic TLR8 staining was unreliable with 7 patients and were excluded. Because only in few cases the cytoplasmic percentage was under 100%, it was not used in statistical testing. TLR8 expression was not found on cell membranes. TLR8 cytoplasm intensity was associated with AFP (p=0.034). TLR8 nuclei percentage was associated with tumor unifocality (p=0.008), tumor stage (p=0.014) and tumor recurrence (p=0.040). Baseline characteristics and correlation with clinicopathological variables of TLR8 expression are presented in Supplementary Table 2.

Outcomes

TLR5, 5-year survival

Overall- and disease-specific 5-year survival, TLR5 cytoplasm intensity, percentage of positive nuclei and cytoplasm percentage

5-year overall survival in strong and weak TLR5 cytoplasm intensity group was 0.0% and 23.8%, p<0.001 (Figure 2A**),** in high and low TLR5 nuclei percentage group 11.7% and 19.0%, p=0.121 (Figure 2C), and in high and low TLR5 cytoplasm percentage group was 11.3% and 22.4%, p=0.018, respectively. In similar order, disease-specific 5-year survivals were 0.0% and 34.9%, p<0.001 (Figure 2B), 16.3% and 31.5%, p=0.022 (Figure 2D) and 18.8% and 32.2%, p=0.038, respectively.

Cox regression analysis, TLR5 cytoplasm intensity, percentage of positive nuclei and cytoplasm percentage

In univariable analysis, strong TLR5 cytoplasm intensity was associated with increased risk for 5-year overall mortality (HR 2.36, 95% CI 1.65-3.38) and for 5-year disease-specific mortality (HR 2.48, 95% CI 1.66-3.71) (Table 2). In multivariable analysis adjusted for confounding factors, strong TLR5 cytoplasm intensity remained as a risk for 5-year overall mortality (HR 1.88, 95% CI 1.26-2.81) and 5-year disease-specific mortality (HR 2.00, 95% CI 1.27-3.15) (Table 2). In univariable analysis, high TLR5 nuclei percentage was associated with increased risk for disease-specific mortality (HR 1.56, 95% CI 1.06-2.28), but not in adjusted analysis (Table 2). In

univariable analysis high TLR5 cytoplasm percentage was associated with increased risk for 5-year overall (HR 1.53, 95% CI 1.07-2.18) and disease-specific mortality (HR 1.52, 95% CI 1.02-2.27) but not in adjusted analysis.

TLR8, 5-year survival

Overall- and disease-specific 5-year survival, TLR8 cytoplasm intensity staining and percentage of positive nuclei

5-year overall survival in strong and weak TLR8 cytoplasm intensity group was 10.3% and 20.0%, p=0.354, in high and low nuclei percentage group 9.9% and 20.9%, p=0.157, respectively. Disease-specific 5-year survivals were 17.0% and 31.2%, p=0.182 and 16.8% and 32.0%, p= 0.058, respectively. Multivariable analysis was not performed in TLR8 due to non-significant differences in crude survival between groups.

Ki67 and p53 in HCC

Ki67 and p53 staining and correlation with clinicopathological variables in hepatocellular carcinoma

Cohen's Kappa value for p53 nuclei percentage was 0.979. Ki67 nuclei percentage was associated with histological tumor grade (p=0.001). P53 nuclei percentage was associated with tumor size (p=0.044), tumor stage (p=0.023) and AFP (p=0.008). Baseline characteristics of Ki67 and p53 expression are presented in Supplementary Table 3.

Ki67 and p53, 5-year survival

Overall- and disease-specific 5-year survival, Ki67 and p53

5-year overall survival in high and low Ki67 nuclei percentage group was 11.2% and 17.4%, p=0.010 (Supplementary Figure 2A), in high and low p53 nuclei percentage group 6.5% and 23.3%, p<0.001 (Supplementary Figure 2C) respectively. Disease-specific 5-year survivals were 15.5% and 29.9%, p=0.001 (Supplementary Figure 2B) and 10.6% and 35.3%, p<0.001 (Supplementary Figure 2D), respectively.

Ki67 and p53 nuclei percentage, cox regression analysis

In univariable analysis high Ki67 was associated with increased mortality for 5-year overall -and disease-specific survival, but not in adjusted analysis (Table 3). High p53 was associated with increased risk for 5-year overall -and disease-specific mortality in univariable and adjusted analysis (Table 3).

Discussion

In this study, we show for the first time that TLR5 expression is a predictor of poor prognosis in HCC. TLR8 was not associated with patient survival. TLR5 predicted mortality with similar point estimate as previously well shown p53 in HCC.

The strengths of this study are homogenous study population and single geographical area where the diagnosis and treatment occurred in same hospital minimizing the selection bias. Full access to patient records was available. Good interobserver repeatability was seen throughout the study. One possible limitation of the study may be the heterogenous staining of the tumor that we were unable to observe using only TMA. Since the technical reason related to smaller staining sample could not be excluded, given treatment was adjusted to exclude possible bias. Our group has previous experience in TLR research, and the utilization of antibodies and immunohistochemical stainings are well validated.

To include the effect of given treatment, we included surgery, local ablation, TACE or palliative separately in adjusted model. However, treatment strongly overlaps with other covariates such as stage, cirrhosis and Child-Pugh index, but to avoid false positive results, this adjusted model was used as the primary analysis despite the possibility of overadjustment. Despite the strong adjustment, TLR5 cytoplasm expression remained prognostic. A single institution study causes limitations to number of patients. The long follow-up period of 35 years (1983-2018) may cause confounding due to the improvements in HCC treatment and staging over the years. Nevertheless, all patient charts were reviewed and limitations were taken into account by adjusting with relevant confounding factors. Patients' histological samples were re-graded to match with present system.

Toll-like receptor 5 recognizes bacterial flagellin from both Gram-negative and positive bacteria, and the activation of TLR5 mobilizes nuclear factor kappa B (NF- κB) and stimulates tumor necrosis factor- α (TNF-α) production.(4) TLR5 seems to be differently involved in tumor development depending on tissue or cell origin. Previously, association between TLR5 and cancer progression has been observed in squamous cell carcinoma of the tongue (11) , cervical neoplasia,(10) gastric dysplasia and carcinoma,(6,9) esophageal dysplasia (30) and colon

carcinogenesis (8). In breast cancer TLR5 expression has been observed, but in contrary, TLR5 activation to flagellin resulted in tumor suppressive activation.(13)

In mouse model study of human colon cancer, lack of MyD88 or TLR5 expression enhanced tumor growth and inhibited tumor necrosis indicating anti-tumoral activity of TLR5.(7) In a *in vivo* study by Kasurinen et al.(31) high TLR5 expression predicted better outcome compared to low TLR5 expression in gastric cancer. In current study high TLR5 expression was connected with poor survival, compared to low TLR5 expression. One explanation could be a special type of colon and gastric cancer microbiome compared to HCC.(32–34) Fusobacterium nucleatum and Helicobacter pylori are known colon and gastric cancers promoting microbial species and they induce innate immune response partly through TLR5 signaling via NF- κB dependent manner.(35,36) Low TLR5 activation might lead to impaired recognition of carcinogenetic species such as F. Nucleatum and H.pylori. This would allow harmful species colonizing colon and gastric tissues and modulating tumor microenvironment to further promote its growth. In addition, metagenomic analysis have shown enrichment of *Bacteroides* and *Ruminococcus* in HCC. In xenograft model HCC incidence increased more in wild-type mice compared to TLR5 knock down mice after modulating intestinal environment more favorable for *Bacteroides* and *Ruminococcus* with diet.(37) TLR5 nuclei expression was associated with increased local recidives, the mechanisms responsible for this and nuclear localization are currently unknown. *In silico* modeling suggest that though TLR5 has potential sequences indicating nuclear localization, the probability is relatively low (NucPred Score 0.34).(38) Previously, TLR5 nuclear expression was seen in esophageal cancer where it was associated with lymph node metastases.(14) Ruuskanen et al. noticed that if TLR5 cytoplasm expression was strong in nasopharyngeal carcinoma, the nuclear membrane expression was similarly strongly expressed.(12) Their observations suggest that activation of TLRs in abnormal locations may be related to carcinogenetic processes.(12) Pimentel-Nunes et al. reported similar findings in gastric carcinogenesis.(39) More studies are needed to understand the biology behind

abnormal localization of TLR5 and the possible carcinogenic functions that the abnormal location may generate.

TLR8 recognizes viral or bacterial single-stranded RNA promoting innate immune system responses.(40) Positive correlation between expression level of TLR8 and Bcl-2 or VEGF was found in cervical cancer samples, which correlated with poor prognosis.(19) High TLR8 expression has been observed also in various other cancers.(17,18,20)

In our study, TLR8 was not associated with survival, but we noticed that patients with high TLR8 nuclei percentage had multifocal tumors more often. Also, high TLR8 nuclei percentage was associated with tumor stage and tumor recurrence, these findings have not been reported before. Interestingly, we documented high nuclear TLR8 expression in 50% of patients. Using NucPred tool (38), with score 0.86 it is likely that TLR8 protein translocates into nucleus. Similar findings have been observed in esophageal cancer (14). It was speculated that viral infections and carcinogenic alterations may possibly affect TLR8 trafficking.(14) More studies are needed to understand these mechanisms.

Antigen Ki67 is a nuclear protein, which is present during active phases of the cell cycle, but is absent in resting cells.(36) The expression of Ki67 is associated with tumor cell proliferation and growth, and commonly used in pathological examination as a proliferation marker.(36) The prognostic value of Ki67 has been observed in HCC as in other cancers.(41,42) In HCC expression of Ki67 has been linked to poor survival and tumor node metastasis and tumor recurrence. (23,43,44) In our study, high Ki67 nuclei percentage was associated with poor survival, but in multivariable analysis the prognostic impact did not remain.

P53 is a tumor suppressor, stimulation of which initiates cell cycle arrest, apoptosis and senescence in response to cellular stress.(45) In HCC, both viruses and chemicals are associated with the

etiology of p53 mutations during the molecular pathogenesis of HCC.(22) Activation of p53 family is a central event in tumor progression, DNA-damage response, chemosensitivity and prognosis in HCC.(46) A comprehensive systematic review and meta-analysis showed high p53 association for worse overall survival in HCC patients, compared with patients with low/undetectable p53 expression,(47) which is in line with the current study as high p53 nuclei percentage was an independent prognostic predictor for poor survival.

The results of this study have clinical and research-related implications. This is the first study to show association with TLR5 and poor prognosis in HCC. The mechanism underlying is not yet fully understood.
Replication studies are needed in the future to examine the prognostic role of TLR5 and TLR8 in HCC. Optimal cut-offs need to be determined in future, in order to use TLR in daily work. Based on this study, TLR5 is a useful biomarker with good interobserver agreement.

Conclusion

This study suggests that TLR5 expression is independently prognostic in HCC.

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Statement of Ethics

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Conflicts of interest/Competing interests: Authors declare that they have no conflicts of interest.

Availability of data and material (data transparency): Anonymized data is available from the corresponding author upon request. Sharing the data will require additional ethical approval.

Code availability (software application or custom code): Not applicable.

Authors' contributions: Investigation: V.K, N.K, V-M.P and O.H. Formal analysis: All researchers. Conceptualization: All researchers. Methodology: All researchers. Writing – original draft: V.K. Writing – review & editing: All researchers. Resources: O.H. Supervision: H.H, V-M.P and O.H.

Ethics approval and consent to participate: The study was approved by the Oulu University Hospital Ethics Committee and the hospital district (committee’s reference number 81/2008). The need to obtain informed consent from the study patients was waived by the Finnish National Authority for Medicolegal Affairs (VALVIRA, reference number 10832/06.01.03.01/2014). The study was performed in accordance with the declaration of Helsinki.

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Table 1. Baseline characteristics of HCC patients.

Variable	**N (%)**
Age, median (IQR)	71.1 (64.0-79.7)
BMI kg/m^2 (median, IQR)	26.8 (24.1-30.9)
Male, n (%)	135 (72.2%)
Treatment	
Surgery	36 (19.3%)
Local ablation (RF, Laser, PEI)	18 (9.6%)
Angiological treatment (TACE)	32 (17.1%)
Palliative/Best supportive treatment	101 (54.0%)
Year of treatment	
1983-2005	50 (26.7%)
2006-2018	137 (73.3%)
Postoperative chemo or radiotherapy	48 (25.7%)
ASG	

No complication	129 (69.0%)
Minor complication	32 (17.1%)
Major complication	26 (13.9%)
Alcohol consumption	
History of alcohol consumption	56 (29.9%)
No/Missing	131 (70.1%)
Liver cirrhosis	66 (35.3%)
Charlson Comorbidity Index	
0-1	80 (42.8%)
2 or higher	107 (57.2%)
Child-Pugh classification	
Child-Pugh A	114 (61.0%)
Child-Pugh B	32 (17.1%)
Child-Pugh C	7 (3.7%)
Missing	34 (18.2%)

WHO performance status	
Grade 1	52 (27.8%)
Grade 2	64 (34.2%)
Grade 3	55 (29.4%)
Grade 4 or higher	16 (8.6%)
AFP, median (IQR)	8.0 (4.0-107.0)
Tumor size (mm), median, (IQR)	65.0 (40.0-100.0)
Unifocal tumor	98 (52.4%)
Tumor stage	
Stage I	80 (42.8%)
Stage II or higher	104 (55.6%)
Histological tumor grade	

Grade 1 or 2	157 (84.0%)
Grade 3	29 (15.5%)
Vascular invasion	
Yes	11 (5.9%)
No	22 (11.8%)
Tumor recurrence	57 (30.5%)
Local recidive	25 (13.4%)

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Table 2. Overall- and disease-specific mortality of TLR5. Hazard ratios (HR) with 95% confidence intervals (CI) of mortality comparing patients with HCC treated in Oulu University Hospital 1983-2018.

	Weak TLR5 cytoplasm (n=96) HR (95% CI)	**Strong TLR5 cytoplasm (n=80) HR (95% CI)**	**Low TLR5 nuclei (n=101) HR (95% CI)**	**High TLR5 nuclei (n=78) HR (95% CI)**
5-year overall mortality				
Crude	1 (reference)	2.36 (1.65-3.38)	1 (reference)	1.31 (0.93-1.84)
Adjusted model [a]	1 (reference)	1.88 (1.26-2.81)	1 (reference)	0.73 (0.50-1-07)
5-year disease specific mortality				
Crude	1 (reference)	2.48 (1.66-3.71)	1 (reference)	1.56 (1.06-2.28)
Adjusted model [a]	1 (reference)	2.00 (1.27-3.15)	1 (reference)	0.88 (0.57-1.33)

[a] Adjustment for age (continuous), sex (female/male), Charlson Comorbidity Index (0-1, 2 or higher), stage (1, 2 or higher), cirrhosis (no/yes), year of surgery/diagnosis (1983-2005, 2006-2018), Child-Pugh index (A, B or C), Tumor grade (1-2, 3), Treatment (Surgery, Local ablation, TACE, Palliative treatment).

Table 3. Overall- and disease-specific mortality of Ki67 and P53. Hazard ratios (HR) with 95% confidence intervals (CI) of mortality comparing patients with HCC treated in Oulu University Hospital 1983-2018.

	Low Ki67 nuclei (n=97) HR (95% CI)	**High Ki67 nuclei (n=85) HR (95% CI)**	**Low p53 nuclei (n=96) HR (95% CI)**	**High p53 nuclei (n=83) HR (95% CI)**
5-year overall mortality				
Crude	1 (reference)	1.55 (1.11-2.17)	1 (reference)	2.29 (1.62-3.23)
Adjusted model [a]	1 (reference)	1.21 (0.84-1.74)	1 (reference)	1.83 (1.28-2.62)
5-year disease specific mortality				
Crude	1 (reference)	1.85 (1.26-2.71)	1 (reference)	2.48 (1.68-3.66)
Adjusted model [a]	1 (reference)	1.41 (0.93-2.14)	1 (reference)	1.97 (1.31-2.96)

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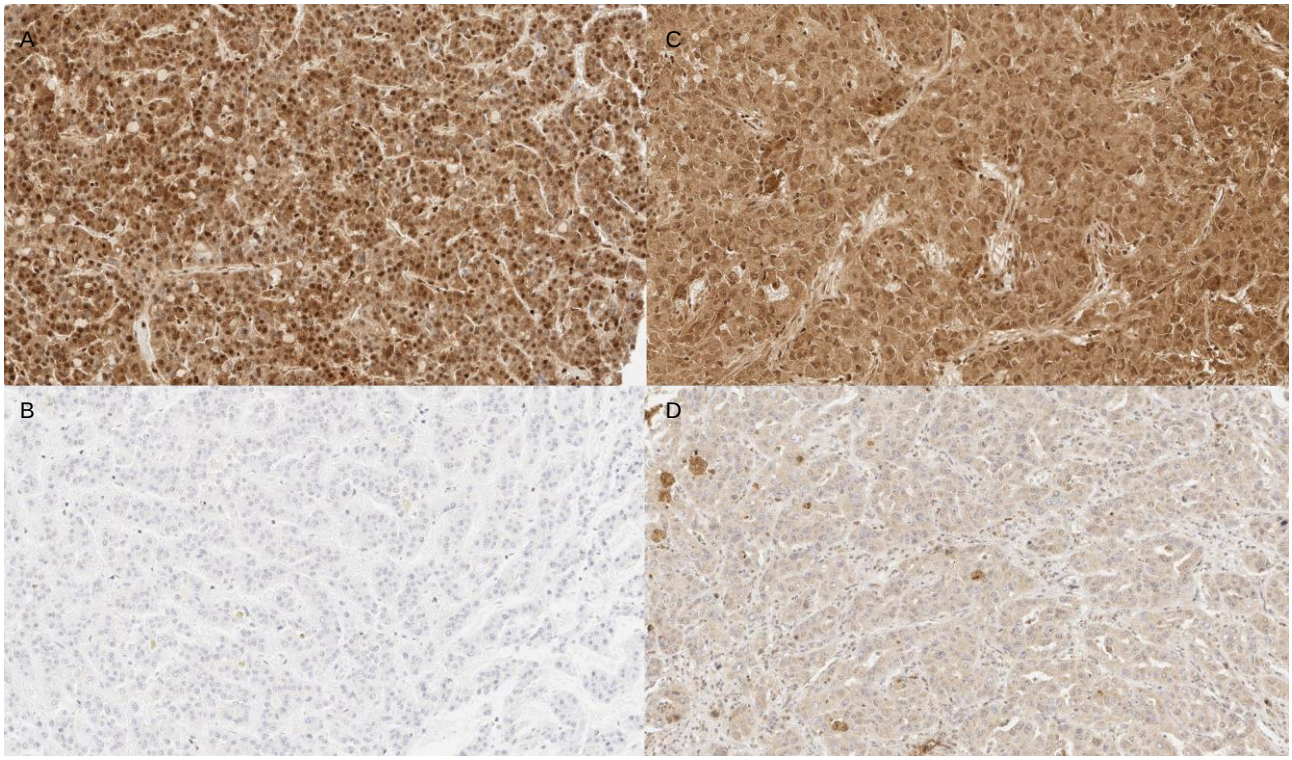
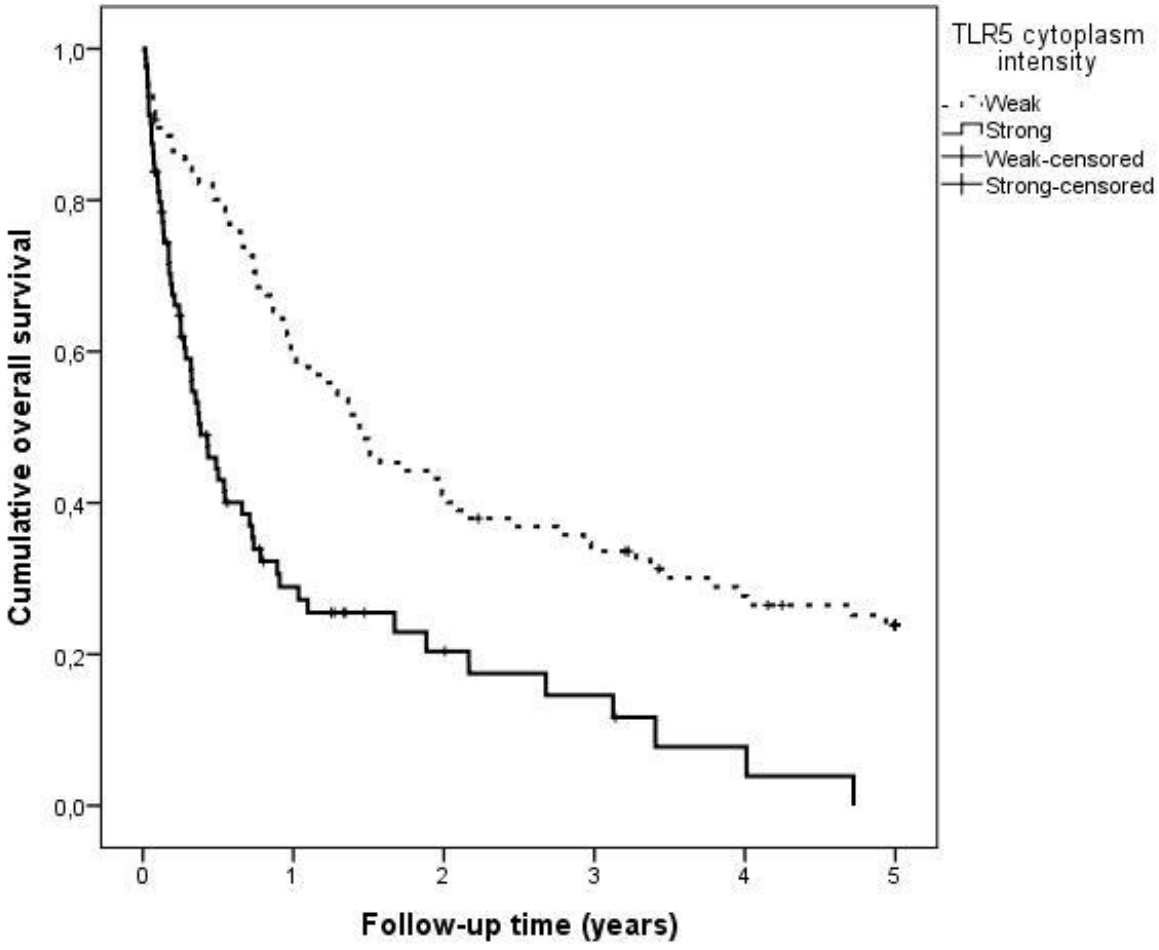
Figure Legends

Figure 1. Examples of TLR5 and TLR8 immunohistochemical staining. Immunohistochemical staining showing (A) strong TLR5 cytoplasm intensity and high nuclei percentage, (B) weak TLR5 cytoplasm intensity and low nuclei percentage, (C) strong TLR8 cytoplasm intensity and high nuclei percentage and (D) weak TLR8 cytoplasm intensity.



549 Figure 2A. Overall 5-year survival, TLR5 cytoplasm intensity



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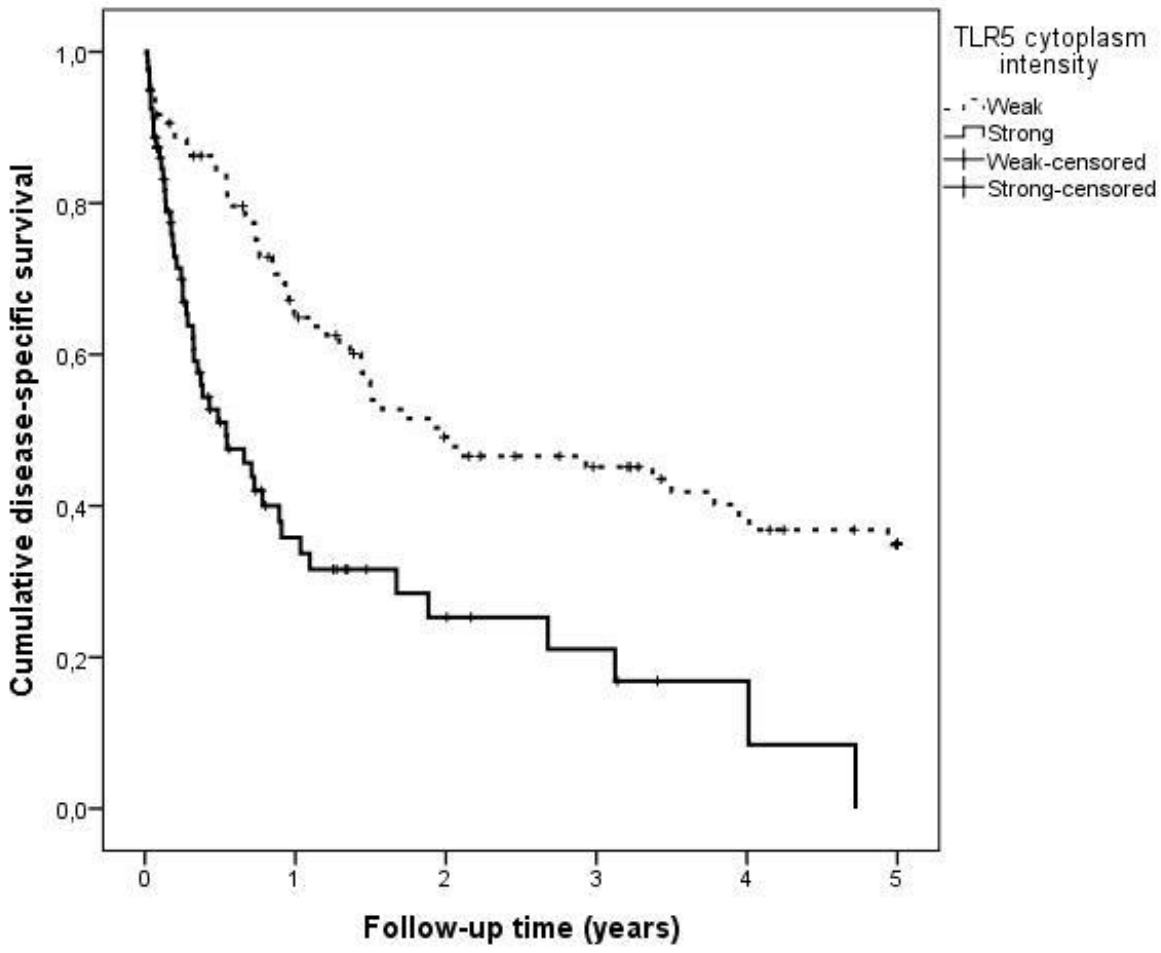
552 Figure 2B. Disease-specific 5-year survival, TLR5 cytoplasm intensity



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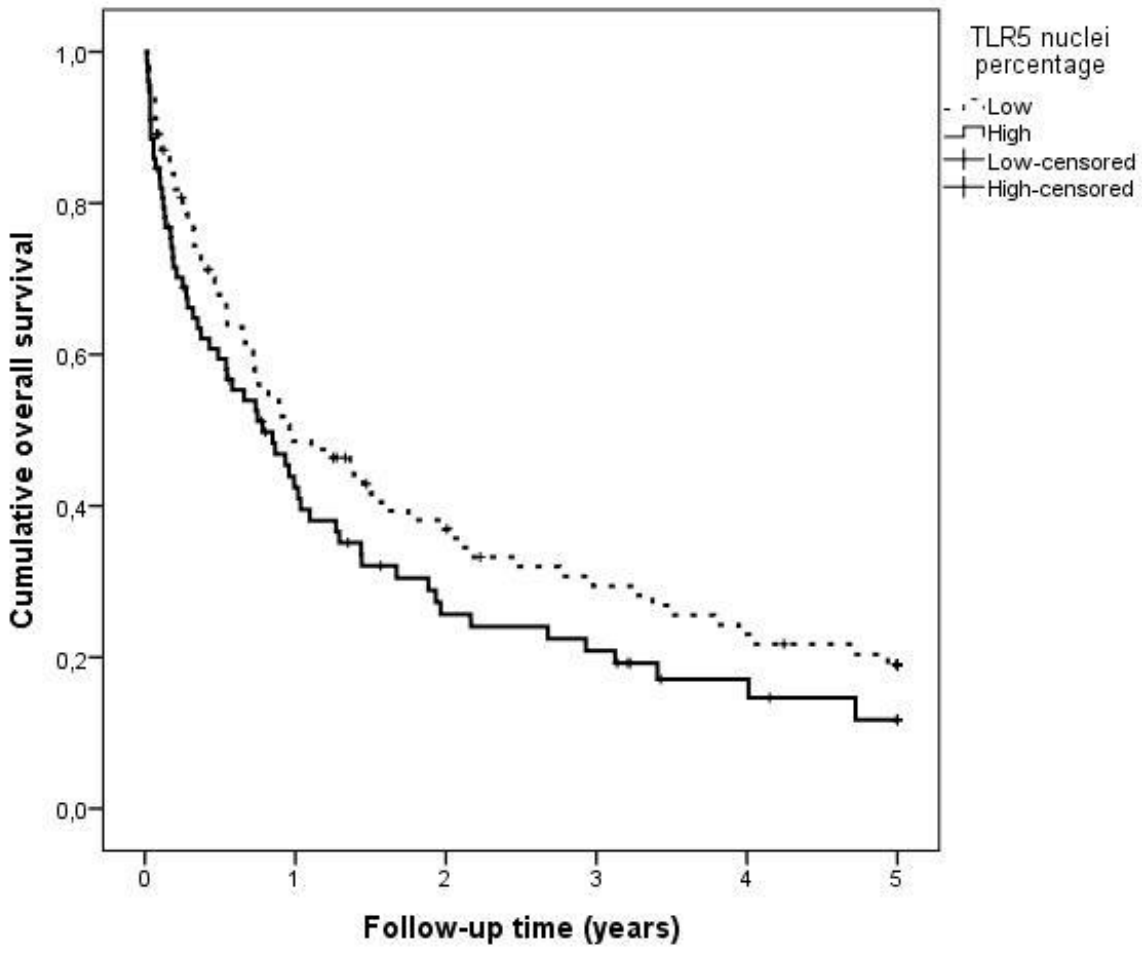
554 Figure 2C. Overall 5-year survival, TLR5 nuclei percentage



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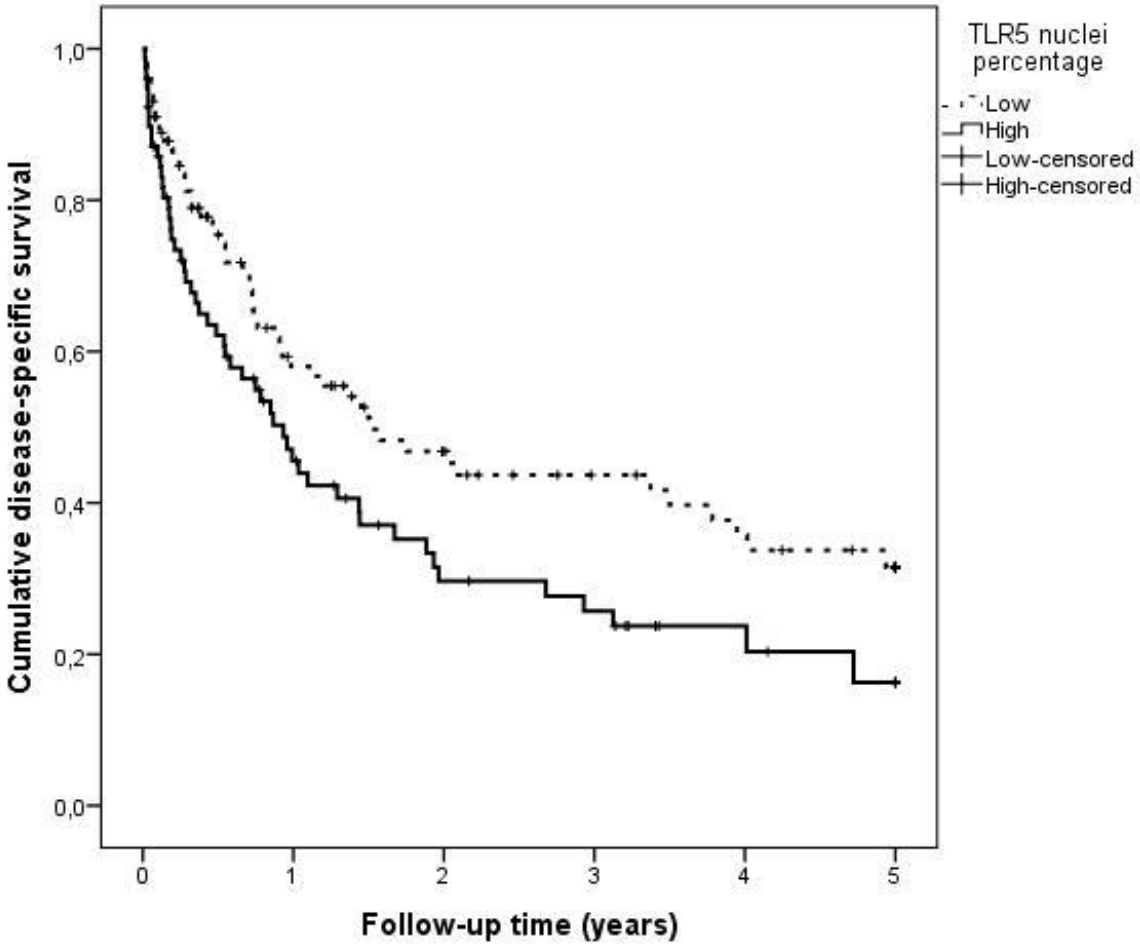
556 Figure 2D. Disease-specific 5-year survival, TLR5 nuclei percentage



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