Prognostic role of TLR4 and TLR2 in hepatocellular carcinoma

Running head: Prognostic role of TLR4 and TLR2 in hepatocellular carcinoma

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18 **Introduction**

19 Hepatocellular carcinoma (HCC) is the second most common cause of cancer mortality [1]. The overall 5-
20 year survival of HCC is only 8%; with curative surgery, the survival rate increases up to 40% [2]. However,
21 only 15-30% of newly diagnosed HCC cases can be treated with curative intent surgery [2] and new
22 prognostic markers are needed for clinical decision-making.

23 Toll-like receptors (TLR) are innate immunity receptors which recognize pathogen-associated molecular
24 patterns and enable rapid responses against invading pathogens [3]. The expression of TLR4 has been
25 associated with an enhanced ability of invasion and metastasis and poor prognosis in HCC [4,5]. The
26 promoting role of TLR2 in cell proliferation, lymph node metastasis, tumour invasion and correlation to
27 TNM stages in HCC has been observed [6,7].

28 The aim of this study was to investigate the prognostic role of TLR4 and TLR2 in HCC patients in the
29 population of Northern Finland.

Materials and methods

Study design and data collection

The study was a retrospective cohort study in a single tertiary care institution in Northern Finland. Design and data collection has been reported earlier [8]. A total of 273 patients with histologically confirmed HCC were treated in Oulu University Hospital between January 1983 and March 12, 2018. Of these, representative paraffin-embedded tumour samples were available from 203 patients for analysis and were included in the cohort. The 8th edition of TNM classification was used in staging. Patient survival data was acquired from 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).

Detailed description of used immunohistochemistry and tissue microarray has been presented in Supplementary material.

Immunohistochemical staining of TLR4 and TLR2 was evaluated by two independent investigators (V.K. and N.K.), blinded from the clinical data. Cytoplasm intensity, cytoplasm percentage (e.g. percentage of cells with detectable cytoplasmic expression), nuclei percentage and membrane percentage were evaluated. Median values were used as cut-offs for further analysis. For more accurate description, see Supplementary material. Examples of TLR4 and TLR2 cytoplasm intensity and nuclear staining are presented in Supplementary Figure 1.

Outcomes

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

Statistical analysis

For categorical data analysis $\chi 2$-test was used. The threshold for significance was set at $P < 0.05$. In all continuous variables, median and interquartile range are presented. Mann-Whitney U was used when comparing continuous variables. Cohen's kappa was calculated to analyze interobserver agreement where values between 0.01–0.20 indicate none to slight, 0.21–0.40 fair, 0.41– 0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect agreement [9]. If interobserver difference was less than one point in intensity or less than 30% in the proportion of positive cells, agreement was reached. Groups were defined according to median values. Kaplan-Meier method was used to compare survival between groups and log-rank test was used to analyze statistical differences between groups. Cox regression analysis 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 index (A, B or C), year of operation/diagnosis (1983-2005, 2006-2018), tumour grade (1-2, 3) and stage (1, 2 or higher). In adjusted model 2, treatment (surgery, local ablation, transarterial chemoembolization, palliative treatment) was added. Statistical analysis was performed with IBM SPSS statistics 24.0 (IBM Corp., Armonk, NY).

Results

Patients

Of 203 HCC patients, 37 (18.2%) were treated with surgery, 21 (10.3%) with local ablation (radiofrequency ablation or percutaneous ethanol injection), 34 with transarterial chemoembolization (TACE) and 111 (54.7%) with best supportive care or palliative treatment. Median age was 71 years (IQR 64-79) with male dominance (71.9%). Median follow-up time was 0.8 years (IQR 0.2-2.0). Most patients (60.6%) had Child Pugh score A. Median tumour size was 65.5mm (IQR 40.0-100.0) and 11 (55.7%) patients had tumour stage II or higher. The overall 5-year survival of the patients in the study cohort was 14.0% and disease-specific survival 22.8%.

TLR4 and TLR2 stainings

Cytoplasmic, nuclear and membrane staining of TLR4 and TLR2, including good interobserver agreement in hepatocellular carcinoma, has been described in Supplementary material.

TLR4 correlation with clinicopathological variables

TLR4 cytoplasm intensity was not associated with any clinicopathological variables. In TLR4 nuclei percentage, differences between groups were observed in BMI ($p=0.004$), treatment method ($p<0.001$), tumour recurrence ($p=0.009$), local tumour recidivism ($p<0.001$), alcohol consumption ($p=0.018$) and cirrhosis ($p=0.034$). The baseline characteristics of TLR4 groups are presented in Supplementary Table 1.

TLR2 correlation with clinicopathological variables

TLR2 cytoplasm intensity was associated with given treatment ($p=0.028$), year of treatment ($p=0.002$) and tumour unifocality ($p=0.033$), TLR2 nuclei percentage was associated with given treatment ($p=0.002$) and local recidivism ($p=0.020$).

Outcomes

TLR4 and TLR2, 5-year survival

Overall and disease-specific 5-year survival, TLR4 cytoplasm intensity

Overall 5-year survival was 11.9% in strong TLR4 cytoplasm intensity group and 18.3% in weak TLR4 cytoplasm intensity group, p=0.050 (Figure 1A**)**. Disease-specific 5-year survival was 14.3% in strong TLR4 cytoplasm intensity group and in weak TLR4 cytoplasm intensity group 33.7%, p=0.004 (Figure 1B**).**

Overall and disease-specific 5-year survival, TLR4 percentage of positive nuclei

Overall 5-year survival was 7.5% in high TLR4 nuclei percentage group and 22.7% in low TLR4 nuclei percentage group, p=0.019 (Figure 1C). Disease-specific 5-year survival was 12.1% in high TLR4 nuclei percentage group and 35.8% in low TLR4 nuclei percentage group, p=0.009 (Figure 1D).

Overall and disease-specific 5-year survival, TLR2 cytoplasm intensity staining

Overall 5-year survival was 14.4% in strong TLR2 cytoplasm intensity group and 15.2% in weak TLR2 cytoplasm intensity group, p=0.986. Disease-specific 5-year survival was 23.2% in strong TLR2 cytoplasm intensity group and 24.3% in weak TLR2 cytoplasm intensity group, p=0.873**.**

Overall and disease-specific 5-year survival, TLR2 percentage of positive nuclei

Overall 5-year survival was 27.9% in high TLR2 nuclei percentage group and 14.0%, in low TLR2 nuclei percentage group, p=0.238. Disease-specific 5-year survival was 49.6% in high TLR2 nuclei percentage group and 22.5% in low TLR2 nuclei percentage group was, p=0.130.

TLR4 cytoplasm intensity, percentage of positive nuclei, cox regression analysis

For results of multivariable analysis see Table 1. In both adjusted models, TLR4 cytoplasm intensity was associated with 5-year disease-specific mortality (model 1 HR 1.95%, 95% CI 1.30-2.92, model 2 HR 1.53, 95% CI 1.02-2.28), Table 1. TLR4 percentage of positive nuclei associated with risk for 5-year overall (HR 1.52, 95% CI 1.06-2.16) and disease-specific (HR 1.78, 95% CI 1.19-2.68) mortality in model 1, Table 1.

Multivariable analysis was not performed in TLR2 due to non-significant differences in crude survival between groups.

Discussion

The results of this study suggest that TLR4 expression is a prognostic factor in HCC. Strong cytoplasmic TLR4 intensity and high TLR4 nuclei percentage were associated with poor 5-year overall and disease-specific survival. In multivariable analysis, strong cytoplasmic TLR4 intensity remained prognostic in both models.

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, so we were not restricted to register data, which is often the case in large scale studies. We were able to perform regression analysis with previously recognized confounding factors in adjusted model 1 where both TLR4 cytoplasm intensity and nuclear percentage were independently prognostic. To

include the effect of the given treatment, adjusted model 2 was performed. However, treatment strongly overlaps with other covariates such as stage, cirrhosis and Child-Pugh index, but to avoid false positive results, adjusted model 2 was used as the primary analysis despite the possibility of over-adjustment. However, despite strong adjustment, TLR4 cytoplasm expression remained prognostic. The long time period of 35 years (1983-2018) may cause confounding due to the improvements in HCC treatment and staging over the years. Nevertheless, limitations were taken into account by adjusting with relevant confounding factors. To our knowledge, the present study is the largest to examine TLR4 and TLR2 in HCC. We have previously validated the used TLR antibodies with Western blot from human liver [10] suggesting that immunostaining in the current study should work as intended. However, validation studies with other patient cohorts and methods are still needed.

In previous studies, TLR4 expression has been linked to early recurrence, poor disease-specific survival and associated with higher microvascular invasion [4]. Also negative results have been reported [6,11]. TLR4 in nuclei has been previously observed for example in esophageal cancer, for example, also associating with survival [12].

TLR2 expression in cytoplasm and cell nuclei have been previously reported [13]. TLR2 has been associated with grade of differentiation, stage and disease-free survival [6]. Suppression of TLR2 has resulted in lower tumour cell proliferation, invasion and migration [7]. In most cited studies, TLR2 expression levels were higher in HCC and correlated with poor prognosis, but we were unable to repeat this finding which can be due to different etiology in Western and Eastern populations (alcohol vs. hepatitis infection).

The relationship between inflammatory response and cancer progression has been known for years [14]. In several animal models inflammatory cells and cytokines, such as NF-κB, TNF-α and various interleukins, have shown the ability to promote carcinogenesis with their anti-apoptotic effects, induction of oxidative damage to DNA, and the induction of tissue repair response [14–17]. Toll-like receptors are known for their role in host defense, but increasing evidence suggests also a role in cancer progression [15]. Infection, or

injury can induce inflammation, which can promote tumorigenesis through chronic tissue damage and the subsequent induction of tissue repair [15]. The unregulated TLR-regulated tissue repair response can drive tumour growth and progression in a positive feedback of unregulated tissue injury and repair, which can trigger TLR-dependent inflammatory responses [15]. Multiple mechanisms for TLRs role in cancer promotion have been suggested [5,15,18–20]. Also, the potential role of TLRs in cancer immunotherapy has gained a lot of interest [21]. The underlying mechanism behind nuclear translocation of TLR4 and the correlation with prognosis is unclear. TLR4 contains several sequences indicating nuclear localization [22]. Alternatively, nuclear carrier proteins might be related to nuclear translocation, but no such proteins have been identified. Translocation of membrane-bound TLRs to nucleus might be due to an increased amount of these proteins and related to signaling activity [12].

Alcohol consumption leads to the activation of innate immunity via TLRs signaling, and TLR4 signaling seems to contribute with the development of alcohol liver disease [23]. Alcohol ingestion or high fat intake disrupts the protective mucosal barrier due to the overgrowth of intestinal bacteria or disruption of intestinal barrier functions, resulting in higher intake of endotoxins, which leads to activation of TLR signaling cascades that regulate inflammatory response and the release of various cytokines [23,24]. In our study, TLR4 expression associated with cirrhosis and alcohol consumption. Previously, hepatic TLR4 and TLR2 expression has been observed to be higher in hepatitis and cirrhosis tissue samples than in HCC [25]. Also, high intake of alcohol leads to increased levels of LPS, which is the key factor for alcohol-induced liver injury and it is known that alcohol is a major risk factor in population of Northern Europe. This might be the explanation why in this study the expression of TLR4, but not TLR2, was found to be a risk factor for poor prognosis [26].

The results of the present study have clinical and research-related implications. Our study showed that strong cytoplasmic TLR4 expression is an independent factor for poor prognosis in HCC. High TLR4 nuclei expression percentage seems to have prognostic impact in HCC, but in this cohort poor prognosis was seen

only in unadjusted analysis, and multivariable analysis when given treatment was not included as a confounder. Replication studies are needed in the future to examine the prognostic role of TLR4 in HCC, especially in different subgroups based on received treatment. Furthermore, optimal cut-offs need to be determined. Based on this study, TLR4 is a useful biomarker for poor prognosis both in surgically resected tissue samples as well as from core biopsies with good interobserver agreement. TLR2 nuclear percentage was detected in 90.6% patients, but only 9.1% had high TLR2 nuclei percentage. The absolute survival difference between high and low TLR2 nuclei percentage was nearly 30%, but without statistical significance. The role of TLR2 nuclear percentage in HCC needs further studies.

Conclusion

TLR4 cytoplasmic expression is independently prognostic in HCC. Role of TLR4 cytoplasmic and nuclear expression in different treatment subgroups need further studies.

Additional information

Authors' contributions

Valtteri Kairaluoma: investigation, drafted the manuscript, formal analysis, conceptualization, methodology, software, writing - original draft, writing - review & editing, visualization, validation, project administration.

Olli Helminen: investigation, formal analysis, conceptualization, methodology, software, investigation, writing - review & editing, visualization, resources, funding acquisition, supervision, project administration.

Niko Kemi: investigation, formal analysis, conceptualization, methodology, software, writing - review & editing, visualization, validation.

Heikki Huhta: acquired the data, performed the experiments, formal analysis, conceptualization, methodology, software, writing - review & editing, visualization.

Vesa-Matti Pohjanen: acquired the data, investigation, formal analysis, conceptualization, methodology, software, writing - review & editing, visualization, supervision.

Ethical 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.

Data availability

Anonymized data is available from the corresponding author upon request. Sharing the data will require additional ethical approval.

Declaration of Interest statement

Authors declare that they have no conflicts of interest.

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303 Table 1. Overall and disease-specific 5-year mortality, TLR4 cytoplasm intensity and TLR4 percentage of
304 positive nuclei. Hazard ratios (HR) with 95% confidence intervals (CI) of mortality comparing patients with
305 HCC treated in Oulu University Hospital 1983-2018. Follow-up ended December 31, 2017. In patients
306 treated in 2018, follow-up ended 30 days after surgery.

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	Weak TLR4 cytoplasm intensity (n=176) HR (95% CI)	**Strong TLR4 cytoplasm intensity (n=176) HR (95% CI)**	**Low TLR4 nuclei percentage (n=179) HR (95% CI)**	**High TLR4 nuclei percentage (n=179) HR (95% CI)**
5-year overall mortality				
Crude	1 (reference)	1.40 (0.99-1.96)	1 (reference)	1.49 (1.07-2.08)
Adjusted model 1[a]	1 (reference)	1.56 (1.01-2.23)	1 (reference)	1.52 (1.06-2.16)
Adjusted model 2[b]	1 (reference)	1.24 (0.87-1.77)	1 (reference)	0.75 (0.50-1.12)
5-year disease specific mortality				
Crude	1 (reference)	1.74 (1.19-2.54)	1 (reference)	1.65 (1.13-2.41)
Adjusted model 1[a]	1 (reference)	1.95 (1.30-2.92)	1 (reference)	1.78 (1.19-2.68)
Adjusted model 2[b]	1 (reference)	1.53 (1.02-2.28)	1 (reference)	0.82 (0.52-1.30)

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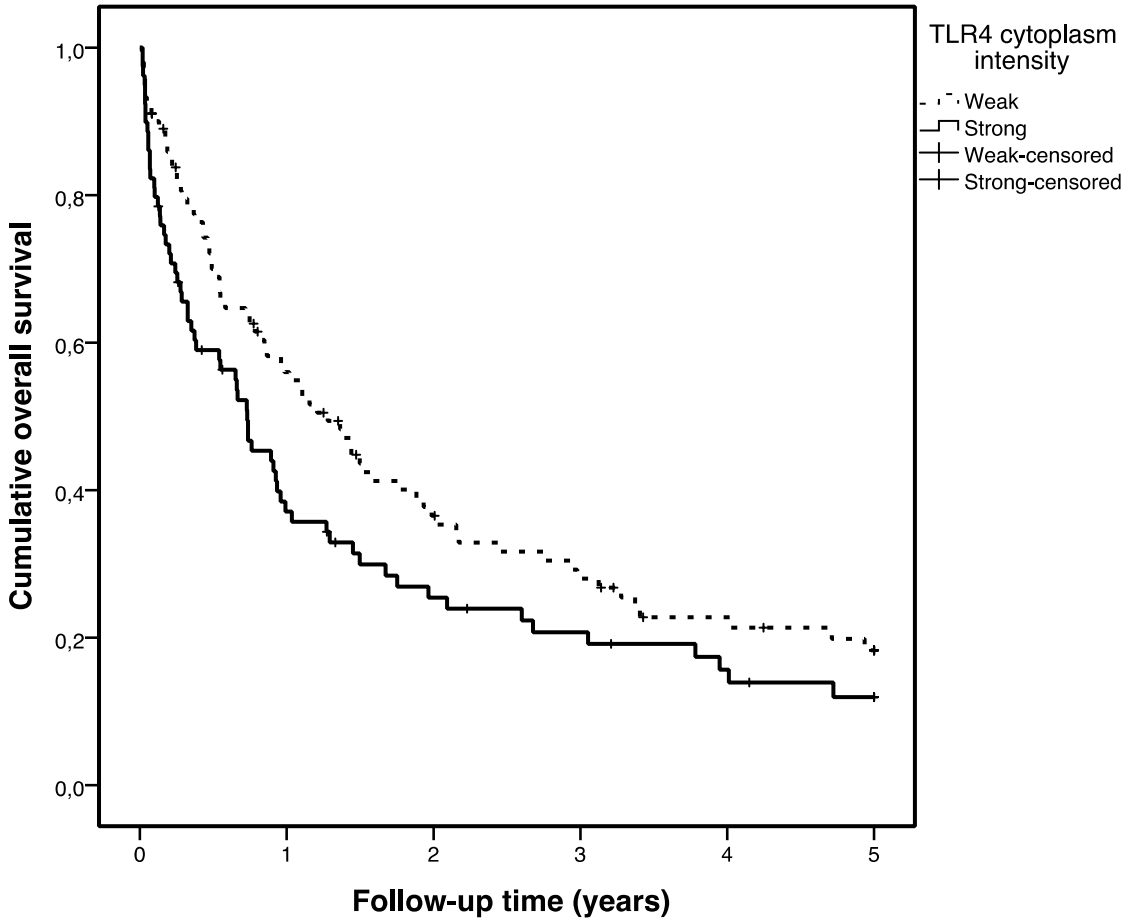
Figure Legends

Figure 1A. Overall 5-year survival, TLR4 cytoplasm intensity



316 Figure 1B. Disease-specific 5-year survival, TLR4 cytoplasm intensity

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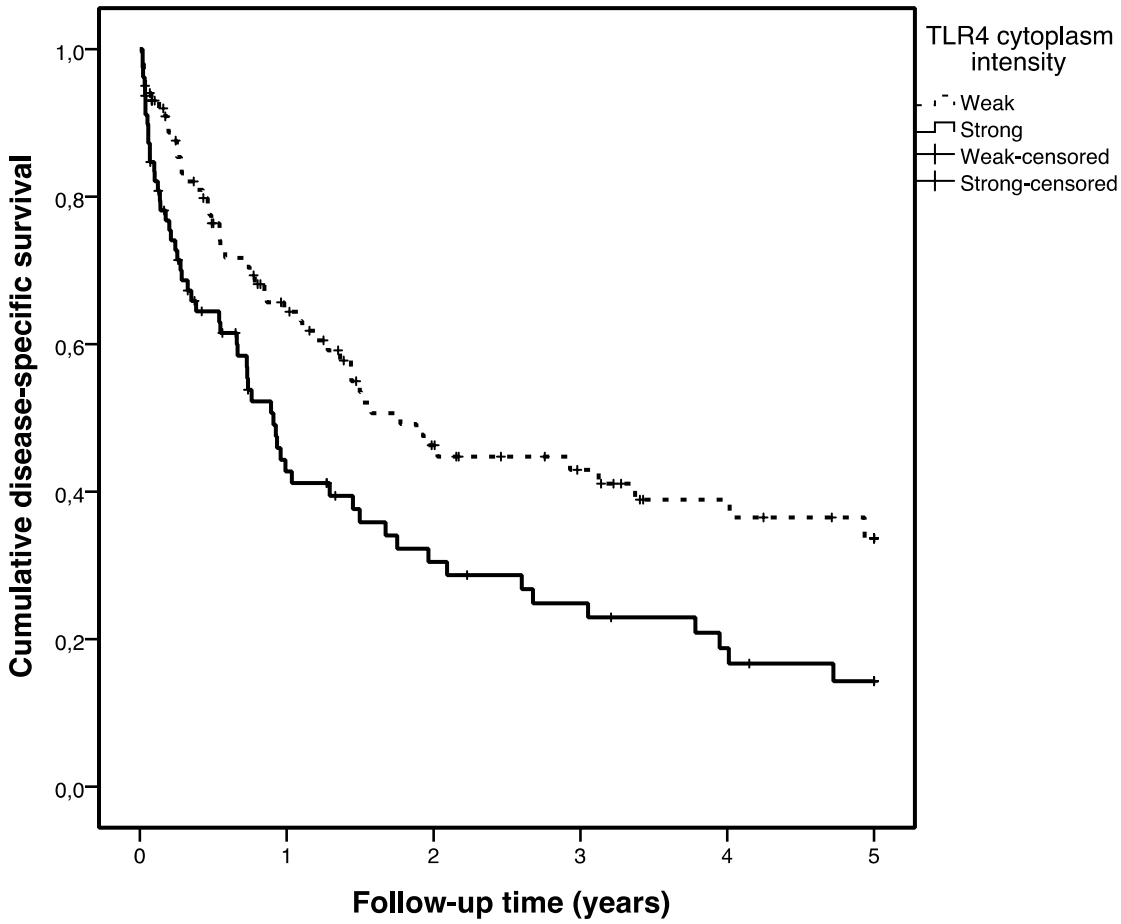

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321 Figure 1C. Overall 5-year survival, TLR4 nuclei percentage

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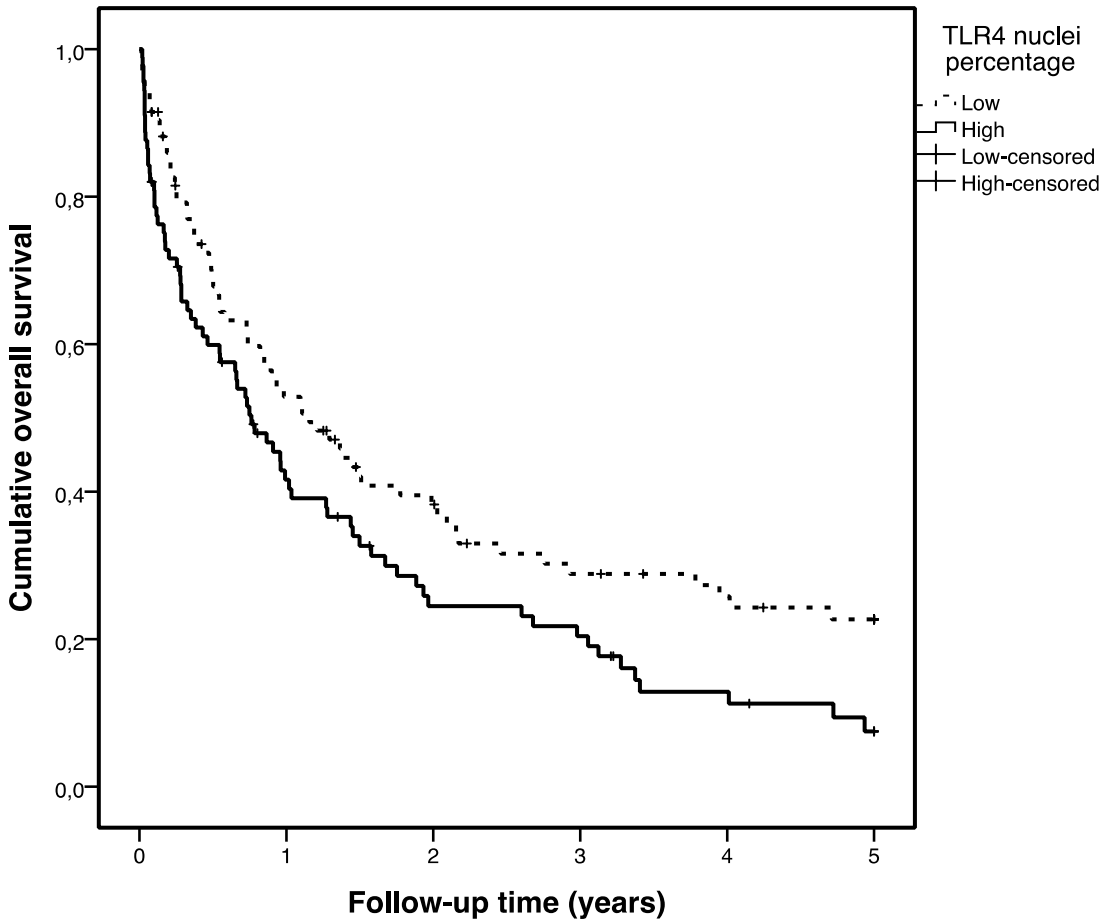

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325 Figure 1D. Disease-specific 5-year survival, TLR4 nuclei percentage

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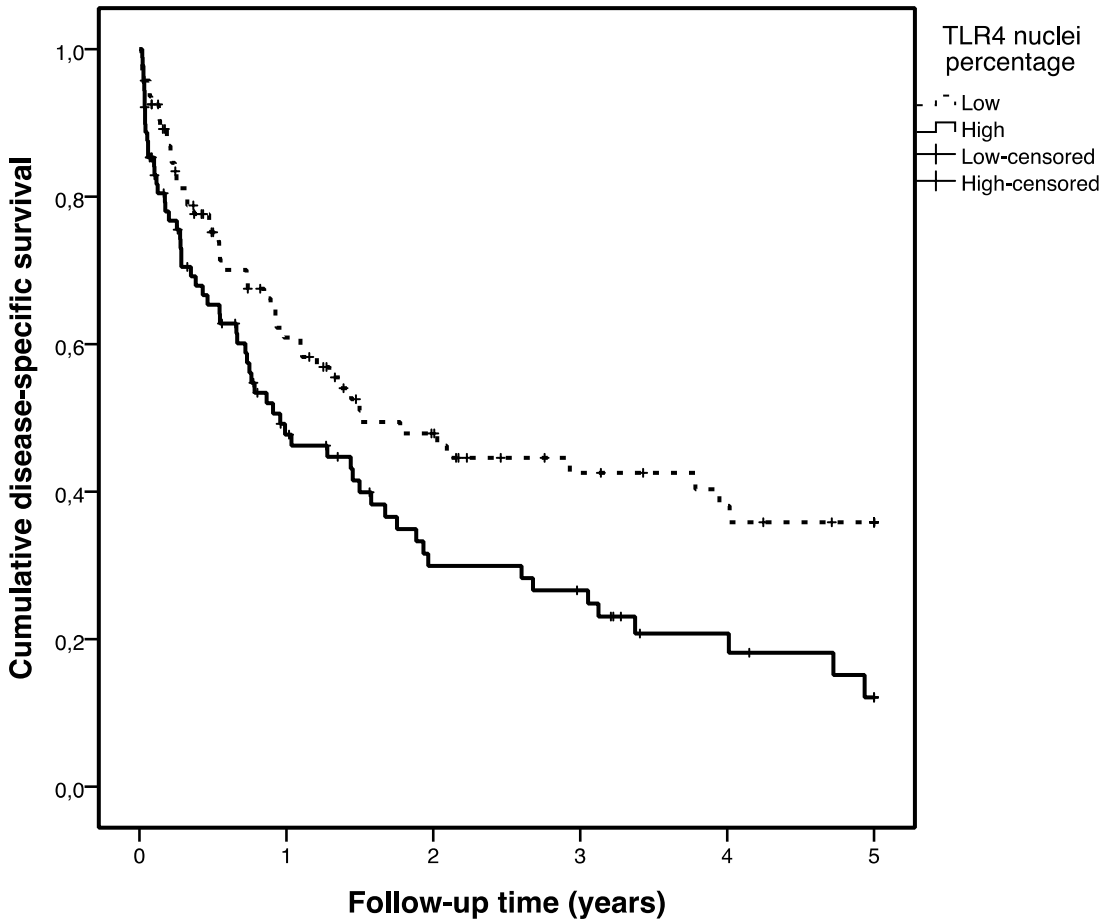

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