Editorial

# Bad neighborhoods: apoptotic and necroptotic microenvironments determine liver cancer subtypes

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Cancer and cell death are inextricably intertwined. Impaired cell death is a prerequisite for cancer development, and artificial induction of cell death is a key strategy in cancer therapy. At present, clinical approaches to kill tumor cells mostly rely on the induction of apoptosis. For many years, apoptosis has been regarded as the only physiologic form of cell suicide. However, over the last two decades, it has become clear that alternative forms of regulated cell death exist which operate via distinct molecular mechanisms. Therefore, these novel forms of non-apoptotic cell death represent putative therapeutic options to overcome apoptosis resistance of cancer cells, potentially paving the way to more effective tumor therapies (1-3). Representing the currently best studied form of non-apoptotic cell death, necroptosis is elicited by triggers such as death receptors, toll like receptors (TLR) or sensors for intracellular nucleic acids (1,4,5). Their signals converge on the phosphorylation/activation of receptor-interacting serine/threonine protein kinase 3 (RIPK3). Phosphorylation of mixed-lineage kinase domain-like (MLKL) by RIPK3 results in oligomerization and membrane translocation of MLKL and subsequent rupture of the plasma membrane. The release of damage-associated molecular patterns (DAMPs) from the bursting necroptotic cells then promotes inflammation and activation of the innate and adaptive immune response (4-6).

At present, it is unclear whether artificial induction of necroptosis will have beneficial or detrimental effects in cancer patients. On one hand, necroptosis-induced inflammation can stimulate antitumoral immune responses, ideally leading to complete cancer eradication. On the other hand, a necroptotic inflammatory microenvironment may actually promote cancer growth and metastasis (1,7).

Adding a new facet to this picture, Seehawer and colleagues (8) now show that the apoptotic or necroptotic death of hepatocytes in the nearby liver microenvironment can determine the subtype of liver cancers [hepatocellular carcinomas (HCC) or intrahepatic cholangiocarcinomas (ICC)]. HCC are the most prevalent tumor type in the liver, being responsible for approximately 80% of cases whereas ICC account for approximately 15% (9). Although both cancer subtypes can arise from the same cell type, i.e., the hepatocyte, they show substantial differences with regard to their histology, metastatic potential and prognosis. So far, the mechanisms that determine the decision for one or the other cancer subtype have remained largely unknown.

To study liver tumorigenesis *in vivo*, Seehawer and colleagues generated transposon constructs co-expressing oncogenic drivers that model the induction of the MEK-ERK or the PI3K-mTOR signaling pathways combined with the frequent upregulation of the myelocytomatosis (*Myc*) gene seen in human HCC. They transfected these oncogenic drivers together with a transposase-encoding plasmid into the livers of *p19Arf*$^{-/-}$ mice (a model showing increased incidence of tumors) and found that delivery via hydrodynamic tail-vein (HDTV) injection led to tumors showing all characteristics of multifocal HCC. Surprisingly, the same oncogenic drivers caused liver tumors exhibiting properties of unilocular ICC or mixed HCC/ICC when delivered via *in vivo* electroporation.

By *in vivo* lineage tracing, the authors demonstrated that both HCC and ICC developed from the same cellular

origin, i.e., from differentiated hepatocytes. To address why transposon delivery via HDTV leads to HCC but transfection of the same constructs via electroporation lead to ICC, they performed delivery via HDTV and mock-electroporated a defined area of the HDTV-transfected liver 2 hours later. Irrespective of the original HDTV delivery, tumors arising from the electroporated liver area proved to be ICC or combined HCC/ICC. In contrast, tumors from other areas of the liver were HCC. Next, the authors addressed whether the switch from the HCC to the ICC tumor subtype in the electroporated liver area was caused by genetic mutations introduced by the electroporation procedure. However, exome sequencing from laser-dissected purified HCC and ICC tissues extracted from mixed HCC/ICC of electroporated livers did not reveal any striking differences in the mutational profiles of HCC *vs.* ICC tumors.

Based on these findings, the authors hypothesized that the two delivery methods might differentially shape the liver microenvironment to determine lineage commitment towards HCC or ICC. Yet, both HDTV and electroporation equally induced inflammatory reactions in the liver tissue with no significant differences in hepatic stellate cells, Kupffer cells and infiltrating inflammatory and immune cells such as T cells, B cells, monocytes and neutrophils. This suggested that immune composition did not contribute to lineage commitment in liver cancer.

Likewise, the amount of cell death in HDTV- and electroporation-transfected livers was equal, and the authors demonstrated that the dying cells were hepatocytes. However, Seehawer and colleagues noticed that the type of cell death induced by the two delivery methods was different. In HDTV-transduced livers, apoptosis was predominant, whereas in livers transduced by electroporation, high levels of phosphorylated MLKL as well as positive signals for total and phosphorylated RIPK3 in the electroporation area argued for a necroptotic death of hepatocytes.

Given that necroptosis is a strongly proinflammatory type of regulated cell death, Seehawer *et al.* argued that DAMP release from necroptotic hepatocytes might stimulate neighboring immune cells to release cytokines which then might drive the decision for ICC over HCC in electroporated livers. Indeed, cytokine expression profiling revealed that multiple cytokines displayed an increase in their expression in ICC relative to the levels observed in HCC. To further validate their hypothesis, the authors pre-treated mice with necrostatin-1, an inhibitor of necroptosis before applying electroporation. Consistent with an inhibition of necroptosis, the authors found significantly reduced MLKL phosphorylation and overall cell death in sections from electroporated livers, accompanied by a switch towards apoptosis. More importantly, necrostatin-1 pretreatment lowered the levels of most cytokines that had previously shown an increase after electroporation and shifted the tumor subtype back to HCC.

However, results obtained with necrostatin-1 have to be interpreted with some caution. RIPK1, the target of necrostatin-1, has pleiotropic functions that extend beyond necroptosis (10). In addition, necrostatin-1 is no longer considered a specific inhibitor of necroptosis (11). To further strengthen their cause, Seehawer and colleagues therefore generated and analyzed mice with a hepatocyte-specific knockout of the *Mlkl* gene which causes necroptosis deficiency. Consistent with the necrostatin-1 experiments, the authors found a reduction of electroporation-associated cytokines and development of HCC instead of ICC after electroporation of oncogenic drivers into the livers of these mice. Conversely, tumors with an increased proportion of ICC developed in a mouse model of bile duct ligation-mediated liver damage where cell death is primarily necroptotic. Implicating TLR, the authors showed that development of ICC was switched to HCC in TLR-deficient mice, with TLR2 and TLR4 on immune cells being the most crucial receptors. Validating the relevance of their findings in the human system, the authors investigated the mRNA expression of necroptosis- and apoptosis-related genes and found a necroptosis signature (with elevated *RIPK3* expression) in ICC *vs.* an apoptosis signature in HCC patients. Overall, these data strongly argue that necroptosis favors the formation of ICC over HCC in oncogenically transformed hepatocytes.

Investigating whether the underlying mechanism might depend on necroptosis-induced epigenetic changes, Seehawer and coworkers identified the carcinogenesis-associated transcription factors TBX3 and PRDM5 as potential commitment factors. The authors found reciprocal expression patterns of both factors in HCC *vs.* ICC. In functional genetic experiments, enforced expression of TBX3 together with RNA interference-mediated suppression of PRDM5 shifted tumors arising after electroporation from ICC to HCC whereas suppression of TBX3 combined with enforced expression of PRDM5 switched tumors developing in HDTV-transfected livers from HCC to ICC. Remarkably, the expression patterns of the *Tbx3* and *Prdm5* genes in human HCC and ICC reflected those observed in the mouse models. These

 

data clearly show that TBX3 and PRDM5 synergize in determining lineage commitment in primary liver cancer, and Seehawer *et al.* were able to comprehensively define associated downstream regulatory networks as well as chromatin remodeling enzymes involved in the epigenetic regulation of the *Tbx3* and *Prdm5* genes.

In summary, the work of Seehawer and colleagues provides evidence that an apoptotic *vs.* a necroptotic cytokine microenvironment directs the lineage commitment of oncogenically transformed hepatocytes into ICC *vs.* HCC. In their model, DAMPs released by necroptotic hepatocytes stimulate immune cells to secrete cytokines which then epigenetically control the expression of *Tbx3* and *Prdm5* as two key regulatory genes. Given that the underlying mechanisms are conserved also in humans, their study also provides insight why common risk factors such as a western lifestyle and fatty liver disease can result in two distinct tumor subtypes with distinct appearances and distinct prognoses.

As with any seminal study, in parallel to many new insights, many new questions arise: what are the triggers of necroptosis? How do TLR participate in this model? Which specific cytokines regulate *Tbx3* and *Prdm5* expression? Which molecular pathways are involved in this regulation? What is the role of the identified target genes of TBX3 and PRDM5? And, probably most important: how can we translate these insights into better therapies? Clearly, we can expect exciting new answers and insights in the future.

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## Footnote

*Conflicts of Interest*: The author has no conflicts of interest to declare.

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