


# The Role of CCNI in Esophageal Adenocarcinoma: What We Have Learned From the Lab

Cancer Control  
 Volume 29: 1–9  
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 DOI: 10.1177/10732748221074734  
[journals.sagepub.com/home/ccx](https://journals.sagepub.com/home/ccx)  


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## Abstract

**Background:** Esophageal cancer is one of the most common and deadliest cancers in the world, particularly esophageal adenocarcinoma. There has never been a special drug to treat it.

**Purpose:** This article summarizes the work that we have done in our laboratory about the role of CCNI in esophageal cancer and gives a new perspective of CCNI biology.

**Research Design:** This is a review article. **Study Sample:** The work was done using validated cell lines and fixed human tissue slides.

**Data Collection and Analysis:** This is a review article, therefore, no data collection or analysis was involved.

**Results:** CCNI is a matricellular protein supporting adhesion, migration, and survival in normal cells, but in the esophageal cancer cells, it induces TRAIL-mediated apoptosis. CCNI promotes TRAIL and its death receptor expression but down-regulates the decoy receptors and survivin in a p53-dependant manner. It was thought that CCNI relies on TNF to induce apoptosis, but our study found that these two molecules antagonize each other. CCNI promotes TNFR1 cleavage and uses the soluble product to block TNF signaling, while TNF upregulates PGLYRP1 to overcome this obstacle because PGLYRP1 is a secreted protein that competes with TNF for TNFR1 binding. As a result, when CCNI and TNF are present together in the vicinity of esophageal tumors, they cancel each other out.

**Conclusions:** Based on our laboratory study, CCNI has much potential to be a candidate for the treatment of esophageal cancer.

## Keywords

CCNI, trail, TNF, esophageal cancer, apoptosis

Esophageal cancer is one of the most common and deadliest malignancies in the world. It is ranked as the eighth in global incidence and the sixth in mortality.<sup>1</sup> The odds for men to have esophageal cancer in their lifetime is about 1 in 78 and for women is 1 in 212. Most of the cases of esophageal cancer are either squamous cell carcinoma (ESCC) or adenocarcinoma (EAC). While the former has been the dominant one historically, accounting for nearly 90% of the incidence, the EAC diagnosis has gone up by 6-fold in the last 30 years and now becomes the fastest growing cancer in the world. Anatomically, ESCC affects the epithelial lining of the mid-esophagus, while EAC is mostly found in the lower portion, near the stomach. Despite the closeness in their locations, these two have little in common except both favoring men over women. There are about 2.5-fold more male ESCC patients than

females and 4.4-fold more for EAC. Genetically, however, ESCC is more like the squamous cell carcinoma of the head

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and neck, while EAC shows more characteristics of gastric adenocarcinoma.

Smoking has been found as the main risk factor for ESCC development, especially when it is associated with alcohol consumption.<sup>2</sup> Approximately 90% of the ESCC patients had a history of smoking and drinking.<sup>3</sup> Take China as an example, which has 18% of the world population and the largest tobacco market in the world.<sup>4</sup> Even though more and more people are trying to quit smoking nowadays, 45.3% of Chinese men still smoke regularly.<sup>5</sup> Moreover, a majority of smokers also have a great passion for hard liquor, as they often say, “tobacco and alcohol are inseparable” and they must be consumed together to be fun. This is especially true in the Northern provinces of China where ESCC affects nearly 0.8% of the residents.<sup>6</sup> The famous “esophageal cancer belt,” which starts from northern Iran, through the central Asian countries, all the way up to northern China. For this reason, more than 50% of the world’s ESCC patients are Chinese.

While tobacco and alcohol addiction has been the main contributor to the steady growth of ESCC, obesity or overweight creates more chances for the occurrence of EAC,<sup>7</sup> as the excessive body weight puts constant pressure on the stomach, squeezing the stomach fluid (often containing bile salts from the duodenum) to break the lower esophageal sphincter barrier, rushing into the esophagus, and resulting in gastroesophageal reflux disease (GERD). A recent study showed that GERD increases the risk of EAC by 8.6-fold.<sup>8</sup> Currently, GERD affects nearly 2.5% of the Chinese population and 25.8% of Americans,<sup>9</sup> making it the most common gastrointestinal diagnosis given in hospital visits.

Obesity used to be more common in the Western world, however, as the economy in the developing countries is improving year after year, more and more Asians and Latin Americans have joined this community. Take China as an example. A recent study reported that at least 46 million Chinese adults are obese, and another 300 million are overweight.<sup>9</sup> In addition, 15% of the children in China have a body mass index above 25. As a result, the global obese population has doubled since 1980,<sup>10</sup> reaching 650 million worldwide, plus 2 billion in the overweight category. This warrants the rise of the GERD incidence.

Chronic GERD can lead to a metaplastic transformation in the esophagus, from squamous epithelium to intestinal columnar phenotype, known as Barrett’s Esophagus (BE), because the columnar epithelium is more tolerant to the acid/bile attacks than the squamous mucosa. When we eat, the ingested food first mixes with the stomach acid, then with the duodenal bile. When they reach the intestine, the ingested materials contain both acid and bile. As a result, the intestinal cells have already developed adaptation to such conditions. The esophagus in a normal individual, on the other hand, does not have such an opportunity to meet with the stomach or duodenal contents because they are blocked by the lower esophageal sphincter. Based on this interpretation, BE can be viewed as an adaptation of the esophageal cells to acid/bile

exposure. Nevertheless, this metaplastic transformation is not a simple phenotypic switch. It plants the seeds for cancer. To obtain and maintain their new identities, the esophageal epithelial cells have to make many genetic and epigenetic adjustments. They keep doing so every time the acid/bile refluxate gets into the esophagus until one day they reach a point-no-return, then EAC grows. According to multiple studies,<sup>1,11</sup> BE increases the risk for EAC by 400-fold, compared to regular individuals.

For a long time, acid reflux had been thought to be the cause of EAC. However, later studies,<sup>12,13</sup> including both *in vitro* and *in vivo*, revealed that acid alone only triggers inflammation, not BE or EAC at all; on the contrary, it might even protect the esophageal epithelium against malignancy. It is the bile brought up from the duodenum during acid reflux that causes the cellular transformation from squamous to columnar then to adenocarcinoma. According to a clinical assessment,<sup>14</sup> the bile concentration in the esophagus of some GERD patients can be ~10 times higher than it in healthy individuals. More convincing evidence was found in animal studies, which demonstrated that 87% of the rats with surgically created duodenal reflux developed EAC in less than a year post-operation, while only 30% of the rats with gastric-duodenal reflux had such an outcome. For those rats with only gastric reflux, no sign of cancer was ever found.<sup>15</sup> Consistent with the *in vivo* study, our *in vitro* experiments showed that culturing normal esophageal epithelial cells in a bile-containing medium resulted in the expression of the intestinal markers, for example, MUC2, KRT20, and VIL1, an indication of intestinal metaplasia, while lowering the pH of the culture medium only triggered an inflammatory response,<sup>13</sup> such as upregulation of cytokines (e.g., TNF, TRAIL), and production and the accumulation of matrix proteins (e.g., MMP, CCN1).

Based on a recent analysis,<sup>16</sup> the global incidence of esophageal cancer was increased by 16.8% from 2007 to 2017 and is likely to have another 77.4% increase by the year 2035, mostly in Asia, Africa, and South America. The possible reason for this rise can be several, but the leading factor for ESCC is still the steady consumer market for tobacco and alcohol, and for EAC is the fast-growing obese population. We think this is going to be one of the top global concerns in the near future.

## CCN1 Biology

CCN1 (Cyr61) has been the main focus of our laboratory investigation for the past two decades. It is the prototype of the CCN (cell communication network) family, which contains six matricellular proteins with unique features in between the soluble growth factors and the structural extracellular matrix proteins. These molecules mainly support cell adhesion, migration, and survival. They are often highly expressed in the wounded tissue where inflammatory cytokines are abundant.<sup>17,18</sup> CCN1 functions through a variety of integrin receptors or heparan sulfate proteoglycans. Up to date, there

are 18  $\alpha$  and 8  $\beta$  integrins known in humans, forming 24 pairs of heterodimers, among which, at least 8 pairs have been reported to mediate CCN1 actions, including  $\alpha_6\beta_1$ ,  $\alpha_2\beta_1$ ,  $\alpha_{11}\beta_1$ ,  $\alpha_D\beta_2$ ,  $\alpha_M\beta_2$ ,  $\alpha_V\beta_3$ ,  $\alpha_V\beta_5$ , and  $\alpha_{IIb}\beta_3$ .<sup>19-25</sup> Depending on which pair of integrins CCN1 chooses, the result can be the opposite. For instance, when CCN1 binds integrin  $\alpha_V\beta_3$  in fibroblast, it promotes cell proliferation, but if switching the receptor to integrin  $\alpha_V\beta_5$ , CCN1 induces apoptotic cell death.<sup>24</sup> On the other hand, for the same action but in different cells, CCN1 also selects different integrin receptors to finish the same job. For instance, to support cell adhesion, CCN1 uses integrin  $\alpha_V\beta_3$  in endothelial cells,<sup>23</sup>  $\alpha_{IIb}\beta_3$  in platelets,<sup>25</sup>  $\alpha_6\beta_1$  in fibroblasts,<sup>24</sup> and  $\alpha_M\beta_2$  in monocytes.<sup>22</sup> The high specificity of integrin signaling gives CCN1 the edge for interventions. Recent studies have generated a number of integrin targets for cancer therapy, and some of them are already in the process of drug development.<sup>26</sup>

When normal cells attach to CCN1, it usually activates cyto-protective pathways against apoptosis. However, in the presence of abundant TNF cytokines (e.g., TNF, FASL, TRAIL), CCN1 was found to induce apoptotic cell death. It is believed that CCN1 promotes the generation of reactive oxygen species (ROS), which inactivates MAPK phosphatases (e.g., DUSP1), allowing TNF to activate JNK or p38 signaling. Then JNK eliminates c-FLIP through ITCH-mediated ubiquitin-proteasome degradation, while p38 promotes BAX-mediated cytochrome c release from the mitochondria, both leading to apoptosis.<sup>27</sup> In another word, CCN1 induces apoptosis by unmasking the toxicity of TNF cytokines. In cancer cells, on the other hand, CCN1 acts like a double-edged sword. It supports tumor growth in breast cancer<sup>28</sup> and pancreatic cancer,<sup>29</sup> while in endometrial cancer,<sup>30</sup> non-small cell lung cancer,<sup>31</sup> and EAC,<sup>32</sup> CCN1 induces tumor cell apoptosis. In prostate cancer cells, CCN1 was found to be both protective and destructive, depending on the availability of TRAIL.<sup>33</sup>

Over the years, we have done quite a bit of study on CCN1 in the context of gastrointestinal abnormalities and gained a much better understanding of its role in esophageal cancer. We like to share with you some progress that we have made in this field, including published and unpublished data.

## CCN1 Sensitizes EAC Cells to TRAIL-Mediated Apoptosis

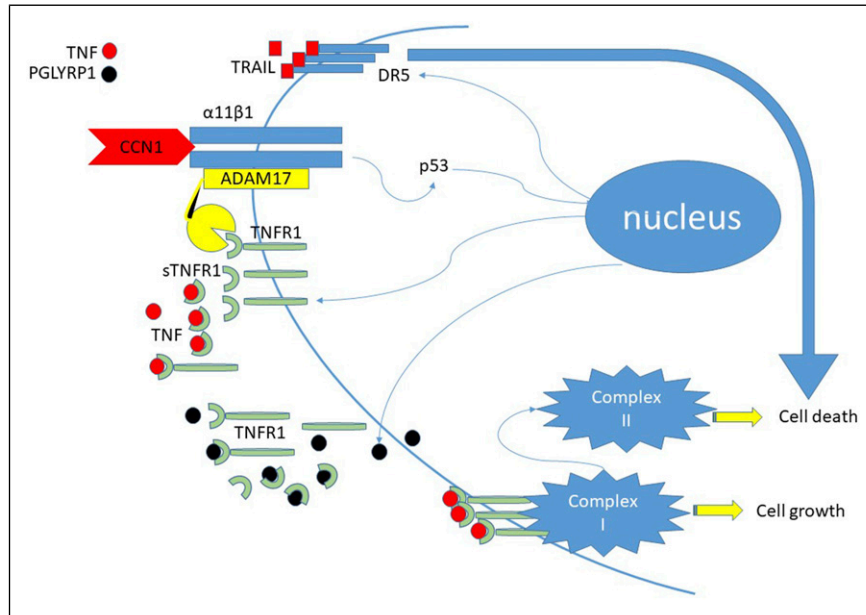
There has never been a special drug to treat esophageal cancer. Since GERD is the primary cause of EAC, people have been focusing on GERD treatment to control EAC development. Currently, the main strategy to treat GERD is using medications (e.g., Proton Pump Inhibitor, or PPI) to suppress acid secretion in the stomach or using a surgical procedure to tighten the lower esophageal sphincter, but neither is a clear winner so far. On the contrary, more and more studies show severe side-effects in association with PPI use, including the reduction in vitamin absorption,<sup>34</sup> susceptibility to infections,<sup>35</sup> bone fracture,<sup>36</sup> and even increased risk for EAC.<sup>37</sup>

For these reasons, the Food and Drug Administration of the United States has repeatedly issued warnings on this line of drugs. For people who still have GERD symptoms after medications, tightening the lower esophageal sphincter by surgery is an option. However, only 5% of GERD patients choose to do so and the follow-up study shows that two-thirds of them come back on medication again later.<sup>38</sup>

One of the common problems with cancer treatment is targeting. A good anti-cancer drug should not only be able to kill the transformed cells, but also be harmless to the normal ones. TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand) has such characteristics. In the laboratory study, TRAIL has been found to selectively kill a variety of cancer cells that are resistant to conventional chemotherapy while leaving normal cells unaffected, because it triggers extrinsic apoptosis through death receptors DR4 or DR5, which are almost exclusively expressed on cancer cells.<sup>39</sup> However, more and more clinical studies found that some cancer cells are resistant to TRAIL treatment, despite the abundance of DR4 or DR5 expressed on them. Several possible mechanisms have been postulated to explain this phenomenon. Expression of decoy receptors, namely, DCR1, DCR2, and OPG, is on the top of the list. Overexpression of any one of the decoy receptors can effectively silence the ligand because compared to DR4 and DR5 these decoy receptors have a higher affinity to TRAIL. They can paralyze the death receptors completely by forming either homo-oligomers or hetero-oligomers.<sup>40,41</sup> This has been found true in breast cancer,<sup>42</sup> lung cancer,<sup>43</sup> leukemia,<sup>44</sup> and ovarian cancer.<sup>45</sup> Secondly, while TRAIL triggers apoptotic pathways, it can also activate NF- $\kappa$ B, which promotes cell survival by transcribing several anti-apoptotic proteins including c-FLIP, Bcl-x<sub>L</sub>, IAP1/2, and survivin. Among them, c-FLIP (cellular FLICE-inhibitory protein) antagonizes procaspase-8/-10 and thereby aborting the apoptotic process<sup>46</sup>; the members of the IAP (inhibitor of apoptosis) family can inactivate caspases<sup>47</sup>; Bcl-x<sub>L</sub> protects the mitochondrial integrity so that CASP8/10-mediated Bid cleavage is unable to induce cytochrome c release.<sup>48</sup>

Preconditioning the tumor cells with a second agent has been found very helpful in overcoming TRAIL resistance.<sup>40,41</sup> A good sensitizer has to meet the same criteria as for the anti-cancer drug, that is, not being harmful to the normal tissue. Based on our laboratory investigation,<sup>32</sup> CCN1 seems to fit the description, at least for EAC treatment and prevention.

In response to GERD episodes, CCN1 expression is highly upregulated in the esophageal epithelium of the patients, then gets weaker and weaker as the condition moves towards malignancy, and eventually disappears in EAC tumors.<sup>32</sup> Forced overexpression of CCN1 in EAC cells leads to TRAIL-mediated apoptosis. We found that CCN1 upregulates TRAIL and DR5 expression but downregulates the decoy receptors in EAC cells (Figure 1), which clears the path for TRAIL signaling. In the normal esophageal epithelial cells, on the other hand, CCN1 does the opposite, promoting DCR2 and



**Figure 1.** Role of CCN1 in esophageal adenocarcinoma. CCN1 activate p53, which transcribes TRAIL and DR5, mediating EAC cell apoptosis. Meantime, CCN1 upregulates TNFR1 expression and ADAM17 activity through integrin  $\alpha_{11}\beta_1$ , which cleaves TNFR1 into sTNFR1, interfering with TNF signaling. TNF fights back by upregulating PGLYRP1, which absorbs the extracellular TNFR1 species, allowing TNF to bind the membrane-bound TNFR1 receptor and supporting cell survival.

OPG while inhibiting TRAIL and its death receptors.<sup>32</sup> Based on these results, CCN1 seems to be an ideal candidate in assisting TRAIL to get its anti-cancer power back.

### Acid/Bile Exposure Triggers TRAIL-Mediated Apoptosis in Some EAC Cells, But Some Can Convert the Death Signal from TRAIL into a Stimulus for Survival

Upon the engagement of TRAIL with DR4 or DR5, procaspase-8 is recruited to the death receptor through Fas-associated protein with death domain (FADD), together forming a death-inducing-signaling complex (DISC) to initiate caspase cascade.<sup>51,52</sup> Within the DISC, procaspase-8 gets fully activated by forming homodimers. However, c-FLIP can compete with procaspase-8 to be a part of the DISC and inhibit CASP8 activation. As a homolog to CASP8, c-FLIP is expressed in three isoforms: c-FLIP<sub>L</sub>, c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub>. Among them, c-FLIP<sub>L</sub> closely resembles procaspase-8 only without catalytic activity due to the substitution of several amino acids, while c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> are equivalent to the prodomain of procaspase-8. All 3 isoforms, especially the two short ones, can bind to FADD to prevent the recruitment of procaspase-8.<sup>53</sup> When c-FLIP is low in the cells, CASP8 gets activated through homo-dimerization and initiates the apoptotic process.<sup>52</sup> However, if c-FLIP levels are high, the procaspase-8 in the DISC can be completely replaced by c-FLIP and consequently, procaspase-8 remains inactive in the cytosol.<sup>54,55</sup> On the other hand, when c-FLIP is moderately

expressed, both procaspase-8 and c-FLIP join the DISC by hetero-dimerization. In this case, CASP8 gets a partial cleavage, resulting in a product that is incapable to initiate the caspase cascade but can activate NF $\kappa$ B and promote cell survival.<sup>56</sup> TRAIL resistance due to c-FLIP interference has been documented in several cancers, including gastric adenocarcinoma,<sup>57</sup> colorectal carcinoma,<sup>58</sup> pancreatic cancer,<sup>59</sup> and several others.

Since CCN1 relies on TRAIL and DR5 to induce EAC cell apoptosis and EAC derives from chronic GERD, we decided to investigate how EAC cells respond to acid, bile, or acid/bile combination. Diversity was found among EAC cells in response to acid/bile exposure. Some EAC cells (e.g., OE19 and FLO-1) can live through chronic acid attacks (pH 4.5) but die of TRAIL-mediated apoptosis when bile is present.<sup>49</sup> Under acidic conditions, these cells overexpress the decoy receptors to interfere with TRAIL signaling. In addition, c-FLIP<sub>R</sub> is upregulated to block CASP8 activation. In the presence of bile, especially acidic bile, however, both the decoy receptors and c-FLIP<sub>R</sub> fail to rise because the acid/bile combination lowers the activity of protein kinase C, which stabilizes c-FLIP<sub>R</sub> through phosphorylation. Forced expression of c-FLIP<sub>R</sub> in these cells shuts down acid/bile-induced CASP8 activation and apoptosis. Our results also showed that it is c-FLIP<sub>R</sub> (not any other c-FLIP species) that replaces caspase-8 in the DISC, even though c-FLIP<sub>L</sub> is present in the cell as well. After c-FLIP<sub>R</sub> was wiped out by acid/bile treatment, c-FLIP<sub>L</sub> still did not make a move to join the DISC in homodimers or heterodimers with procaspase-8. In another word, c-FLIP<sub>L</sub> makes no contribution to the fate of these EAC cells under acid/bile conditions.



In some other EAC cells (e.g., OE33 and SK-GT-4), the same acid/bile combination does not induce apoptosis, despite the robust elevation of TRAIL and DR5, because TNFR1-associated death domain (TRADD) is also upregulated to replace FADD in the DISC formation.<sup>50</sup> Unlike FADD, TRADD does not have the death effector domain to interact with procaspase-8 directly. Instead, TRADD recruits TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein kinase 1 (RIPK1) to activate the NFκB-mediated cell survival pathway. In this case, c-FLIP, as a transcriptional target of NFκB, gets accumulated. In the end, TRAIL signaling is interrupted. This study provides a new explanation for TRAIL resistance in EAC.

### CCN1 Induces p53-Dependent Downregulation of Survivin

Survivin is the smallest member of the IAP family. It essentially supports cell division through the regulation of chromosome and microtubule attachment<sup>61</sup> and thus plays a critical role during embryonic development. In adults, however, survivin is silenced in most of the terminally differentiated tissues. For this reason, survivin expression in adults is usually taken as a top indicator of cancer. Survivin is a downstream inhibitor of apoptosis. Overexpression of survivin disrupts the caspase cascade, stopping the apoptotic process before it finishes. Survivin can prevent caspase activation directly<sup>62</sup> or indirectly through binding to XIAP, another member of the IAP family.<sup>63,64</sup>

In our study, we found that EAC cells express survivin >500-fold higher than their normal counterparts, but it is silenced in the presence of CCN1.<sup>60</sup> Survivin is known as a direct target of p53, not for its expression but its repression.<sup>65</sup> This is one of the reasons why survivin is absent in human adults. As a powerful cancer suppressor, p53 keeps our inner environment in balance by regulating nearly 5000 genes involved in various cellular activities, including both pro- and anti-apoptotic genes. A majority of them, including the gene encoding for survivin, are negatively regulated by p53. However, TP53 is mutated in more than 70% of EAC tumors.<sup>66</sup> Even in the remaining 30% tumor cells, p53 levels are barely detectable. In the presence of CCN1, however, p53 gets stabilized and accumulated.<sup>60</sup> CCN1 induces ROS generation and accumulation, which causes DNA damage, triggering p53 activation.<sup>27</sup> This not only suppresses survivin expression but also promotes TRAIL and its death receptors because they are all the transcriptional targets of p53 (Figure 1).

### CCN1 Attenuates Bile-Induced Esophageal Metaplasia by Suppressing Non-Canonical NFκB Activation

As discussed above, chronic GERD induces esophageal metaplastic transformation from the squamous epithelium to the intestinal columnar epithelium, a premalignant condition of EAC. TNF-activated canonical NFκB signaling was

blamed for this because the intestinal phenotype is controlled by caudal type homeobox 2 (CDX2), which is a transcriptional target of NFκB.

The name of NFκB refers to five individual transcription factors including NFKB1 (p105), NFKB2 (p100), RELA, RELB, and REL. While p105 is constitutively processed into its active form p50 through proteasome degradation, the conversion of NFKB2 from p100 to p52 (the active form) requires a specific signal. Normally, these proteins are kept in silence in the cytoplasm in the form of homodimers or heterodimers by the inhibitor of kappa B (IκB). All of the TNF and TNFR family members can activate NFκB/p50-RELA, the predominant form for the canonical pathway, but only a few of them<sup>67,68</sup> are capable to trigger the process of NFKB2 conversion from p100 to p52. The best-characterized pair for activating the canonical pathway is TNF and TNFR1.<sup>69</sup> Upon TNF ligation, TNFR1 recruits TRADD, then TRAF2 and RIPK1 to form complex I (Figure 1), which further recruits 3 complexes, including the linear ubiquitin chain assembly complex (LUBAC), the TGFβ-activating kinase 1 (TAK1) complex, and IκB kinase complex (IKK). As a result, IKKβ (a component of the IKK complex) becomes activated to phosphorylate IκBα and causes its degradation, which allows p50-RELA to move into the nucleus to transcribe genes for cell survival. In the non-canonical pathway, on the other hand, the activated receptor binds TRAF2 directly and then itself is internalized to degrade TRAF3, which liberates NFκB inducing kinase (NIK), otherwise, NIK is constitutively degraded by TRAF3. Activated NIK phosphorylates IKKα (another component of the IKK complex), which in turn initiates NFKB2 processing and activation.

In response to chronic acid/bile exposure, we found that lymphotoxin α (LTA) rather than TNF is first upregulated by the bile to activate the canonical NFκB, and then LTA is gradually replaced by CD40 to activate the non-canonical NFκB pathway. As a result, CDX2 gets activated by the canonical NFκB and reinforced by the non-canonical NFκB to initiate the transcription of the intestinal genes in the esophageal epithelial cells. On the other hand, acid rather than bile promotes CCN1 expression, which downregulates both LTA and CD40 expression and thereby suppresses esophageal metaplasia. Based on this study,<sup>13</sup> BE development is likely due to CCN1 loss, at least partially.

### CCN1 Promotes TNFR1 Cleavage to Neutralize TNF, While TNF Promotes PGLYRP1 to Absorb the Extracellular TNFR1 and Thereby Antagonizes CCN1-Induced Apoptosis [unpublished]

As mentioned above, CCN1 was reported to induce apoptosis in fibroblasts through unmasking TNF toxicity. We performed a similar study using EAC cells. The result is quite different. At first, CCN1 inhibits TNF expression but promotes TNFR1 expression in EAC cells. In return, TNF has a negative impact on CCN1 as well. When these two factors are present together

in the vicinity of the EAC cells, they cancel each other out.<sup>70</sup> CCN1 promotes TNFR1 expression but also promotes the activity of a disintegrin and metalloproteinase 17 (ADAM17) through integrin  $\alpha_{11}\beta_1$ , which cleaves TNFR1 into soluble TNFR1 fragments to undermine TNF activity. TNF, on the other hand, greatly upregulates peptidoglycan recognition protein 1 (PGLYRP1), a secreted protein with a high affinity for TNFR1. PGLYRP1 absorbs the extracellular TNFR1 species and thereby interrupting CCN1 function (Figure 1).

Both TNF and TNFR1 can function in either membrane-bound (mTNF, mTNFR1) or soluble forms (sTNF, sTNFR1). While sTNF and mTNF both can function through the membrane-bound TNFR1, sTNFR1 works as decoy receptors to interfere with TNF signaling. In addition to sTNFR1, a cell also constitutively releases a certain amount of full-length TNFR1 molecules to the extracellular space to interfere with TNF signaling by the same mechanism as sTNFR1.<sup>71,72</sup>

PGLYRP1 is a relatively new immune protein targeting Gram-negative bacteria.<sup>73</sup> Upon bacterial infection, PGLYRP1 is released by the immune cells to bind to the peptidoglycan in the bacterial wall and present the microorganisms to the phagocytes for elimination. Accidentally, this protein was found to compete with TNF for TNFR1 binding and also capable to form a 1:1 complex with HSP70, a major supporter of cell survival, and then inducing cell death through TNFR1.<sup>74</sup> This work was further supported by a later discovery of a 140 kD super complex in murine fibroblasts,<sup>75</sup> which is equivalent to the total size of 3 protein combinations, PGLYRP1 (22 kD), HSP70 (70 kD), and TNFR1 (55 kD), in 1:1:1 ratio. In EAC cells, however, we did not see such a complex containing PGLYRP1. Instead, we discovered a bigger complex with a molecular mass above 170 kD, which was recognizable by the antibodies against either one of these 3 proteins. We speculate that the PGLYRP1 in this complex is a dimer because a glycosylated PGLYRP1 dimer in these tumor cells is around 55 kD. Furthermore, we found this complex exists in the EAC cells regardless of the cell condition, although it is enhanced substantially by TNF treatment. This complex was not only seen in the cellular extracts but also more abundant in the conditioned cell culture media, especially under TNF treatment. We also identified another complex in the media with a molecular weight around 44 kD containing both PGLYRP1 and TNFR1, but not HSP70. We think this is formed by PGLYRP1 (22 kD) and a sTNFR1 fragment (22 kD) released by ADAM17 cleavage. Usually, these two types of extracellular TNFR1 are released to interfere with TNF signaling so that it would not be able to reach the cells. In EAC cells, we found that PGLYRP1 bound both forms of the extracellular TNFR1 and ironically, TNF highly promoted this action. This made us speculate that TNF might use this competitor just to clear the decoy TNFR1 receptors floating in the extracellular space so that TNF itself would be easier to get to the membrane-bound TNFR1 receptors. To verify this thought, we used shRNA to knock PGLYRP1

down in the EAC cells and then tested for its effect on TNF signaling. The results showed that loss of PGLYRP1 severely impaired TNF-induced I $\kappa$ B $\alpha$  phosphorylation and degradation, indicating that TNF upregulates PGLYRP1 just to clear its path to cell signaling (Figure 1).

CCN1, TNF, and PGLYRP1 are all involved in the inflammatory response. CCN1 supports wound healing by promoting cell proliferation and migration, while PGLYRP1 protects the wound from bacterial infection, as evidenced by our discovery that PGLYRP1 expression was up by 1359-fold in the esophageal epithelial cells in response to GERD episodes. Therefore, these three extracellular molecules have many chances to interact with each other physiologically. During wound healing, they may have the same goal—to restore tissue integrity. In cancer, however, each of these 3 seems to have its own “mind.” TNF generally supports tumor growth by activating NF $\kappa$ B. Only when it is highly accumulated in the tumor microenvironment, its toxicity may be triggered to kill the tumor cells. CCN1 tries to keep TNF down by inducing the sTNFR1 release, allowing the cancer cells to die of apoptosis. TNF fights back by promoting PGLYRP1 expression to neutralize sTNFR1. It becomes a power play when these two are together and the outcome depends on who wins the battle.

## Closing Remarks

CCN1 is a highly contextual protein. It is quite normal if some cancer cells are found to grow better in the presence of CCN1, but for EAC, CCN1 seems to be an ideal anti-cancer agent. First of all, CCN1 is harmless to normal cells. It supports wound healing during esophagitis and prevents esophageal metaplastic transformation in response to GERD episodes. It only selectively facilitates apoptosis in cancer cells by altering TRAIL receptor and survivin expression. Secondly, the story of CCN1 and TNF helping each other in apoptosis induction may be true in normal cells, but in cancer cells, especially in EAC cells, that is not the case. They antagonize each other in multiple ways. In addition to the inhibitory effect on expression that they have for each other, CCN1 also blocks TNF signaling by increasing the extracellular pool of TNFR1. TNF, on the other hand, fights back by upregulating PGLYRP1 to neutralize the soluble TNFR1 species. TNF likes to support EAC cell growth, while CCN1 tends to kill them.

Thus far, most of our studies are done *in vitro* using cell lines and tissue specimens. *In vivo* models are absolutely necessary for bringing a therapeutic idea from bench to bed. For this goal, we have been conducting some *in vivo* investigations on the role of CCN1 in EAC using surgically created EAC rats. Histological examination revealed typical features of esophagitis at 4 weeks after surgery, including markedly thickened epithelium, elongation of the lamina propria papillae into the epithelium, and basal cell hyperplasia. At week 10, numerous goblet cells were identified in the esophageal

mucosa, an indication of intestinal metaplasia. At week 40, some mucinous EAC appeared. During the course of EAC development, CCN1 was found heavily increased in the esophageal epithelium in the first few weeks when esophagitis was developing and continued its overexpression in a less degree along with BE progress. When EAC appeared, however, CCN1 expression became barely detectable. These results are consistent with what we found in human EAC specimens. The main beauty of this animal model is that we can see the entire process from normal to cancer in less than a year, giving us the edge for therapeutic interventions. We hope to publish this part of the work in the next paper.

## Appendix

### Abbreviations

ADAM17	a disintegrin and metalloproteinase 17
BE	Barrett's Esophagus
CDX2	caudal type homeobox 2
c-FLIP	cellular FLICE-inhibitory protein
CTF	c-terminal fragment
DISC	death-inducing signaling complex
EAC	esophageal adenocarcinoma
ESCC	esophageal squamous cell carcinoma
FADD	Fas-associated protein with death domain
GERD	gastroesophageal reflux disease
IAP	inhibitor of apoptosis protein
I $\kappa$ B $\alpha$	NFKB inhibitor alpha
IKK	I $\kappa$ B kinase
LTA	lymphotoxin alpha
mTNF	membrane-bound TNF
mTNFR1	membrane-bound TNFR1
NF $\kappa$ B	nuclear factor kappa B
NIK	NF $\kappa$ B inducing kinase
PGLYRP1	peptidoglycan recognition protein 1
PPI	proton pump inhibitor
RIPK1	receptor-interacting protein kinase 1
ROS	reactive oxygen species
sTNF	soluble TNF
sTNFR1	soluble TNFR1
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRADD	TNFR1-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand.

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### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Inner Mongolia Research Foundation for Natural Sciences (2021MS08007), Wu Jieping Medical Science Foundation (320.6750.19089-91), and the National Natural Science Foundation of China (81960445).

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