Bile reflux and hypopharyngeal cancer (Review)

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Abstract. Laryngopharyngeal reflux, a variant of gastro-esophageal reflux disease, has been considered a risk factor in the development of hypopharyngeal cancer. Bile acids are frequently present in the gastroesophageal refluxate and their effect has been associated with inflammatory and neoplastic changes in the upper aerodigestive tract. Recent *in vitro* and *in vivo* studies have provided direct evidence of the role of acidic bile refluxate in hypopharyngeal carcinogenesis and documented the crucial role of NF-κB as a key mediator of early oncogenic molecular events in this process and also suggested a contribution of STAT3. Acidic bile can cause premalignant changes and invasive squamous cell cancer in the affected hypopharynx accompanied by DNA damage, elevated p53 expression and oncogenic mRNA and microRNA alterations, previously linked to head and neck cancer. Weakly acidic bile can also increase the risk for hypopharyngeal carcinogenesis by inducing DNA damage, exerting anti-apoptotic effects and causing precancerous lesions. The most important findings that strongly support bile reflux as an independent risk factor for hypopharyngeal cancer are presented in the current review and the underlying mechanisms are provided.

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Abbreviations: HM, hypopharyngeal mucosa; HHCs, human normal hypopharyngeal cells

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1. Introduction

Tobacco smoke, chronic alcohol use and infection with human papillomavirus type 16 (HPV-16) are known risk factors for laryngopharyngeal cancer (1-4); however, there is a growing interest in identifying other risk factors.

Laryngopharyngeal reflux (LPR), a variant of gastro-esophageal reflux disease (GERD), has also been considered a potential risk factor in the last decade that may exert a carcinogenic effect on the upper aerodigestive tract (5-11). Recent findings have clarified the role of bile reflux as an independent risk factor in hypopharyngeal carcinogenesis through preclinical and clinical models (12-18), and a model of the molecular mechanism of the bile-induced tumorigenic effect has been proposed (19-25). Bile is produced in the liver and stored in the gallbladder (26) and its primary role is to assist in lipid digestion and absorption. Regurgitation of duodenal bile contents into the stomach and esophagus is known as bile reflux. The present review provides the latest knowledge regarding the association of bile reflux with hypopharyngeal cancer.

2. Bile as a potential carcinogen

The caustic nature of bile has long been recognized (27). In ancient times, the medical theory that excess, deficiency or ectopic bile in the body may affect human health was first stated by the father of medicine, Hippocrates (27). This medical theory remained popular for centuries through the writings of Galen (129-201 AD) (28) but was decisively displaced newly published theories of cellular pathology by Virchow and Rather in 1858 (29). In the past century, in 1938-40, Cook (30) proposed the possible role of bile acids in cancer. Years later (1974-1993) several studies supported the role of bile acids as carcinogens causing gastrointestinal cancer (31-34). In parallel, clinical studies provided the first evidence of mixed gastric and bile (duodenal) fluids in refluxate of patients with GERD (35-37).

During this period, Gotley *et al* (38) determined an increased amount of conjugated bile acids in 87% of aspirates using high-performance liquid chromatography, suggesting an association between bile and acid concentration and esophageal mucosal injury. Nehra *et al* (35), Kauer *et al* (39,40) and Domellof *et al* (41) also characterized the concentration and composition of bile fluid in aspirates of patients with GERD. Of note, Fein *et al* (42) were the first who provided solid evidence of bile fluid as an independent carcinogen in the gastrointestinal tract by using a rat model and demonstrating that bile acids are able to induce esophageal adenocarcinoma.

Until the present day, the clinical prevalence and magnitude of bile reflux have remained to be fully determined; however, there is growing evidence of bile contents in GERD refluxate (31,43-45). According to Covington *et al* (46), bile-containing enterogastric reflux is much more common than previously assumed. The increased information linking LPR and inflammatory/neoplastic disease of the upper aerodigestive tract as well as the lack of evidence for the carcinogenic effect of bile-containing refluxate into laryngopharynx led to further investigation.

Considerable research efforts to explore the carcinogenic effect of bile in the upper aerodigestive tract were made, including clinical and experimental studies. Galli *et al* (5) and Sasaki *et al* (11) suggested that during LPR, bile fluid reaches the epithelium of the upper aerodigestive tract, which may contribute to the development of inflammatory and neoplastic events. Furthermore, Lewin *et al* (47) reported a close association between LPR and patients with premalignant lesions or early carcinomas of the larynx. It is worth mentioning that although there have been efforts to link the effect of other gastroesophageal refluxate contents, such as pepsin, with pre-neoplastic events in the larynx and pharynx, the conclusions have been divergent (48-54). A recent study indicated a possible contributory effect of slightly acidic or neutral pepsin to the inflammatory and neoplastic effects of LPR (54), but there is still no direct evidence of carcinogenesis induced by pepsin.

At the beginning of 2016, a series of *in vitro* and *in vivo* experiments questioned the role of bile reflux in hypopharyngeal carcinogenesis (12-14). A study by our group from 2016 (13) established a murine model of wild-type mice, *Mus musculus* (C57BL/6J) and provided the first evidence that bile acids may cause preneoplastic lesions in the hypopharyngeal mucosa (HM). Using this model and long-term exposure to bile acids, the progressive mutagenic effect of biliary refluxate causing invasive cancer was subsequently observed (15,16). These and other studies provided direct evidence that bile fluid is a carcinogen, capable of inducing hypopharyngeal squamous cell carcinoma (HSCC) (15,16). The significance of these findings was in line with clinical findings derived from bile reflux-related HSCC (18). Specifically, a clinical pilot study demonstrated a characteristic bile-related molecular phenotype that was similarly identified in bile-exposed murine HM, which clearly differed from adjacent non-pathologic tissue (18).

3. Bile refluxate composition and acidity as critical factors of neoplastic events

Bile refluxate composition. According to Nehra *et al* (35) and Kauer *et al* (39,40), the majority of bile acids in esophageal aspirates of patients with GERD are conjugated with taurine or glycine and may be sulfated. Specifically, glycine conjugates of cholic, deoxycholic and chenodeoxycholic acids are the predominant bile acids aspirated from the esophagus of patients with GERD (the ratio of glycine to taurine conjugates in normal human bile is 3:1). It has also been indicated that unconjugated secondary bile acids, such as deoxycholic acid (DCA), may be present in the esophageal refluxate, particularly in patients with erosive esophagitis and Barrett's esophagus (35,41,55). It is known that pH affects the solubility of each bile component in various manners. At acidic pH (≤4), the conjugated bile acids are more un-ionized and therefore capable of penetrating or interacting with the cell membrane (taurine conjugates: pKa=1.8-1.9; glycine conjugates: pKa=4.3-5.2) (56). At pH <3.0, bile salts tend to precipitate, whereas between pH 5.5 and 7.0, most conjugated primary bile acids are found to be ionized and therefore relatively inactive. However, unconjugated secondary bile acid DCA remains unionized (pKa 5.5-6.2) and may therefore exert its harmful effects, causing mucosal injury even at a less acidic pH (12,16,17,55). According to Stamp (57), duodenal fluid, at a less acidic pH, may also contribute to gastrointestinal tract tumorigenesis. Specifically, glycine conjugates may remain un-charged and therefore be harmful to the epithelial cells at a less acidic pH. Ireland *et al* (58) indicated that duodenal fluid significantly contributes to the carcinogenic potential of methyl-N-amyl nitrosamine, particularly at less acidic pH. In addition, as bile acids are natural detergents when in high concentration, they may interact with the cell membrane even at a neutral pH.

Bile refluxate concentration. Although the bile composition is a crucial factor of bile reflux-related tumorigenesis, the concentration of bile may also potentiate its oncogenic effect. This view may be supported by previous *in vivo* studies indicating that the application of bile reflux components, chenodeoxycholic acid or DCA, at pharmacologic concentrations, both at a neutral pH of 7.0, to murine laryngopharyngeal mucosa was able to cause early premalignant changes, such as hyperplasia and dysplasia, as well as marked activation of NF-κB and its related oncogenic molecular phenotype (13,14).

Bile refluxate acidity. It appears that acidity of LPR refluxate is a critical factor for bile to induce a harmful effect on laryngopharyngeal mucosa (39,59,60). In the clinic, intraesophageal pH monitoring has been used extensively to identify reflux episodes. Although pH varies during gastroesophageal reflux episodes, according to Ulualp *et al* (61), 24-h ambulatory pH monitoring in the pharynx of patients with reflux laryngitis confirmed that a drop below pH 4.0 is common and is considered diagnostic of a reflux event, suggesting that acid may contribute to duodenogastric-induced inflammatory and neoplastic events. Lillemoe *et al* (62) demonstrated the injurious effect of the various duodenal components on rabbit esophageal mucosa at strongly acidic pH, supporting a synergism between bile and HCl. A study by our group from 2019 (17) documented that the tumorigenic effect of bile on hypopharyngeal cells is pH-dependent. Other studies from our group (13-15) also demonstrated that a strongly acidic pH (≤4.0) serves a critical role in the bile-induced tumorigenic

effect in murine laryngopharyngeal mucosa. It was indicated that chronic intermittent exposure of murine HM to a mixture of bile salts at a strongly acidic pH of 3.0 was able to progressively induce premalignant changes, microinvasion and invasive squamous cell carcinoma, causing increased DNA damage and oncogenic molecular alterations (15). Specifically, it was demonstrated that histopathologic changes caused by acidic bile were accompanied by underlying molecular alterations, such as increased levels of i) oxidative DNA/RNA damage and double-strand break (DSB) markers, ii) p53 and cell proliferation markers (Ki67, cytokeratin 14, and p63), as well as iii) alterations of the expression of cell adhesion molecules, like E-Cadherin and β-catenin, and iv) activation of NF-κB and other cancer-related transcription factors, such as signal transducer and activator of transcription 3 (STAT3) (13-15). However, hypopharyngeal cells or mucosa exposed to the same mixture of conjugated primary bile acids at neutral pH (7.0) produced hyperplastic or mild dysplastic changes and significantly less intense underlying molecular changes compared to acidic bile salts (12,13,17). In parallel, it was indicated that chronic exposure to acid alone or concentrated glucose was not able to produce any histological changes (13,15). The negative or reduced effect of acid alone and/or bile salts at neutral pH, compared to acidic bile salts, indicates that the latter is particularly injurious.

Primary and secondary bile acids in refluxate. It is clear that the presence of conjugated primary bile acids in a highly acidic refluxate exerts a tumorigenic potency on the long-term exposed upper aerodigestive tract. This theory may explain findings from our group (13,15) indicating that chronic local exposure of murine HM to a mixture of conjugated primary bile acids, at concentrations previously measured in patients with GERD (35,40,43,45,57) at a strongly acidic pH (≤4.0), is able to progressively cause precancerous lesions and invasive cancer. Specifically, as taurine conjugates are active at low pH (≤4.0), it appears that taurine-conjugated bile acids may be responsible for the tumorigenic effect of bile at lower pH (15,17). There is also recent *in vitro* evidence supporting that acidity (pH ≤4.0) and bile composition may have a role in the progression of HSCC (63).

The above observations strongly support that controlling the pH during reflux episodes may have a protective effect by reducing the risk of bile-induced hypopharyngeal cancer. However, there is epidemiologic evidence that numerous patients with refractory GERD may also experience symptoms at a weakly acidic pH of 5.5-6.0 (60,64). Since unconjugated DCA and glycine-conjugated bile acids may be partially active at a weakly acidic pH, it appears that as the pH grows less acidic, approaching pH 5.5, the partially activated primary bile acids and the activated DCA may exert their influence (17). A recent study by our group (16) supported that DCA and glycine-conjugated bile acids are potent activators of DNA damage and oncogenic pathways in HM in a weakly acidic environment. Previous findings have demonstrated a similar association between DCA and its tumorigenic activity in the esophagus and colon (31,65,66). Regarding the hypopharynx, it has been documented that bile at a weakly acidic pH (5.0-5.5) with or without DCA, similarly to a strongly acidic pH 3.0, is able to increase the risk of bile-related hypopharyngeal neoplasia by promoting premalignant lesions, DNA damage and oncogenic molecular alterations, compared to controls (16). Of note, it was indicated that long-term exposure to a weakly acidic control (pH 5.5) was not able to induce any histological changes (16). This observation strongly supports that the oncogenic properties of biliary esophageal reflux on laryngopharyngeal mucosa may not be fully modified when antacid therapy is applied.

Although further exploration with clinical evidence is expected to strengthen these previous preclinical observations, investigation of the mechanism by which bile refluxate exerts its oncogenic properties is expected to contribute not only to a better understanding of the pathophysiology of hypopharyngeal cancer but also to alternative therapeutic strategies for patients with refractory GERD, using specific inhibitors of relevant molecular pathways or bile receptors.

4. Key role of NF-κB in bile reflux-related hypopharyngeal carcinogenesis

Several epidemiologic studies have supported the role of LPR in the neoplasia of the upper aerodigestive tract (5-11,67,68). However, the exact mechanism of LPR-related laryngopharyngeal carcinogenesis has remained elusive and unexplored until the last decade (69-71). Studies including that by Huo *et al* (66) indicated that exposure of esophageal cells to DCA produced elevated levels of NF-κB *in vitro*, suggesting the role of NF-κB as a key molecule in esophageal cancer (72-75). Thus, these observations supported the hypothesis of a possible mechanistic role of NF-κB in cancer of extraesophageal sites, such as hypopharynx.

NF-κB is a transcriptional factor complex consisting of homo- and heterodimers of five members of the Rel family [RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100)] (76). The canonical pathway of NF-κB activation includes phosphorylation of IκB-α, which leads to nuclear translocation of the heterodimers p50/Rela or p50/cRel and consequent binding to the promoters of target genes and regulation of their expression. Constitutive activation of NF-κB has been observed in various cancer types, linking inflammation to the neoplastic transformation of the epithelium (77-79). In the initiation and progression of head and neck squamous cell carcinoma (HNSCC), several oncogenic pathways have been identified. These commonly include epidermal growth factor receptor (EGFR)/Ras/RAF/MAPK, PI3K/Akt1/mTOR, IKK/NF-κB, STAT3 and Wnt/β-catenin (80-89). It has been indicated that HNSCC exhibits abundant NF-κB activation and several studies indicate that NF-κB is upregulated in premalignant lesions and invasive cancer (80,90-94).

The role of NF-κB in acidic bile reflux-related laryngopharyngeal carcinogenesis was first demonstrated in the last decade through *in vitro* and *in vivo* experimental models (12-25). A study by our group from 2017 (21) documented the key role of NF-κB in mediating acidic bile-induced oncogenic molecular events in human hypopharyngeal cells (HHCs). Subsequently in 2018, a study by our group (22) also demonstrated that NF-κB is a crucial factor in controlling the levels of small regulatory molecules, such as microRNA (miRNA/miR) markers, in HHCs. A series of *in vitro* and *in vivo* studies also suggested that NF-κB inhibition may prevent inflammatory and neoplastic events in HHCs and HM, including STAT3 activation and significant deregulations of

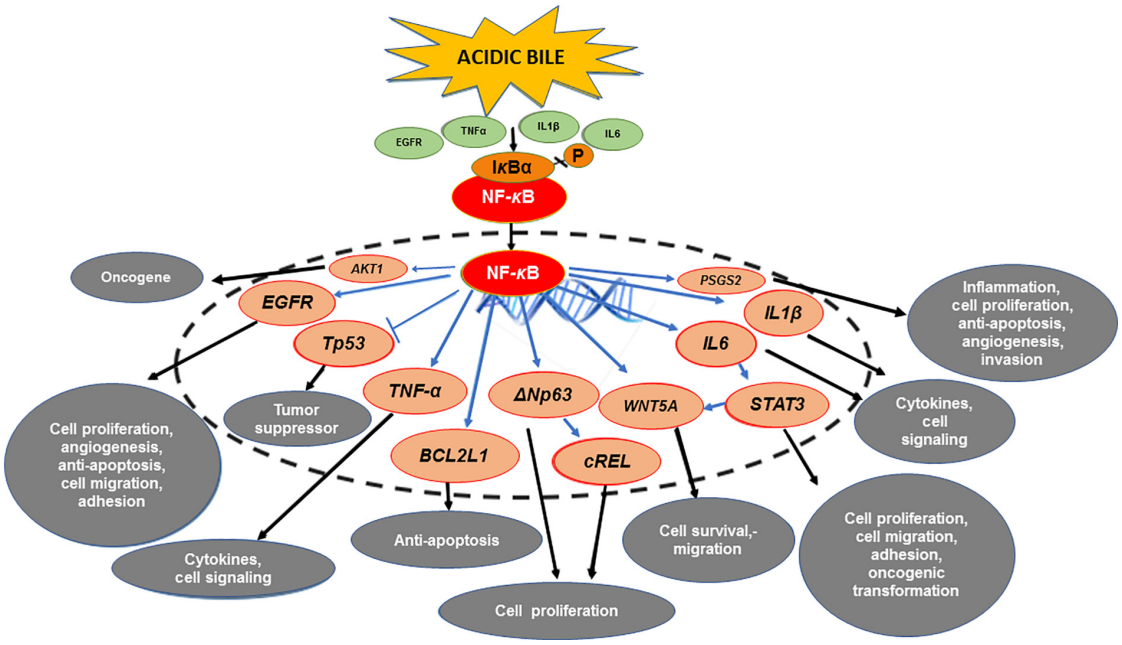

Figure 1. The mechanistic role of NF-κB in the mRNA oncogenic phenotype induced by bile reflux in hypopharyngeal carcinogenesis. Acidic bile induces constitutive activation of NF-κB via TNF-α, EGFR or TLR, which promotes the transcriptional activation of genes with inflammatory, anti-apoptotic or oncogenic function, such as *IL6*, *IL1β*, *TNF-α*, *BCL2L1*, *EGFR*, *cREL*, *STAT3*, *ΔNp63* and *WNT5A*. Activation of NF-κB under acidic bile exposure also induces overexpression of *AKT1*, suggesting acidic bile may contribute to the PI3K/AKT1 downstream pathway, which is frequently activated in HNSCC. In addition, NF-κB is able to upregulate the expression of *PTGS2* (COX-2), supporting its regulatory role in inflammatory and cancer-related downstream signaling pathways. Finally, acidic bile-induced NF-κB activation may prevent the upregulation of wild-type *TP53* expression.

several cancer-related genes and miRNA markers (19,23-25). Other non-specific stress factors, such as highly concentrated glucose or acidic pepsin, were not capable of inducing activation of genes with oncogenic function previously linked to HNSCC *in vitro* (12,13,53).

Bile reflux-induced NF-κB-related mRNA oncogenic phenotype. There is evidence that numerous types of cancer arise from sites of chronic inflammation (95). Specifically, a wide array of chronic inflammatory conditions predisposes susceptible cells of epithelial origin to neoplastic transformation (carcinomas) as a multistep process (focal proliferation of dysplastic cells with potential progression to malignant carcinoma). An example includes reflux esophagitis that may lead to DNA damage, development of Barrett's esophagus and esophageal carcinoma (96). In certain cases, the progenitors of the inflammation are known, such as bacterial infections or gastric acids that have been associated with increased risk of adenocarcinoma of the stomach and esophagus, respectively (97,98). It may be assumed that bile acids interact directly with HM, as described in a paragraph below, but may also be the progenitors of an LPR-induced chronic inflammatory microenvironment associated with an increased risk of hypopharyngeal cancer. It has been indicated that a chronic inflammatory microenvironment or harmful stimuli are able to induce a constitutive activation of NF-κB, which may lead to a cascade of molecular alterations. Specifically, constitutive activation of NF-κB may lead to subsequent transcriptional activation of genes that are implicated in a variety of signaling pathways via aberrant overexpression of cytokines, transcription factors and growth factor receptors, such as TNF-α, TLR and EGFR (76,77,79-89,93,94,99-103).

Preclinical studies from our group (12-25) documented that acidic bile is able to induce activation of NF-κB and significant overexpression of several cancer-related genes. Specifically, acidic bile was reported to induce significant transcriptional activation of anti-apoptotic *BCL2* and other genes previously linked to HNSCC, such as *STAT3*, *EGFR*, *WNT5A*, *TNF-α*, *ΔNp63*, *cREL*, *IL6*, *IL1β*, *AKT1* and *PTGS2* (82-94,102-111) (Fig. 1). Furthermore, a clinical pilot study revealed that bile-related HSCC had significantly higher levels of NF-κB and differential expression of the above genes compared to the adjacent non-pathologic tissue or bile-negative HSCC, providing further evidence of the central role of NF-κB (18).

Targeting NF-κB negatively affects the NF-κB signaling pathway and has been indicated to be an encouraging strategy to improve anticancer therapies (112). Several pharmacologic and dietary inhibitors of NF-κB are considered promising therapeutic options, demonstrating chemo-preventive or chemosensitizing properties in head and neck cancer (99,112). BAY 11-7082 [(E)-3-(4-methylphenylsulphonyl)-1-propenenitrile] is a reliable inhibitor of the NF-κB pathway that has been widely used in numerous studies exploring the effect of NF-κB (112,113). It has been suggested that BAY 11-7082 offers the most rapid and potent anti-tumor effect among other NF-κB inhibitors (112) and it may be used as a sensitizer for anti-cancer treatment (114,115). Furthermore, curcumin is a turmeric natural supplement with known antioxidant, anti-inflammatory and anti-cancer properties, previously demonstrated to have potential chemo-preventive effects in head and neck malignancies (116), by blocking NF-κB activation and halting the proliferation of cancer cells (117) due to its pleiotropic properties (118).

Both BAY 11-7082 and dietary curcumin have been used in *in vitro* and *in vivo* experimental studies to investigate the underlying mechanism of bile reflux-induced carcinogenic effect into the hypopharynx. Studies by our group (19,25) suggested that application of BAY 11-7082 effectively suppressed cell proliferation rates, the activation of NF-κB and related oncogenic mRNA profiles induced by acidic bile exposure. This oncogenic phenotype included the significant overexpression of anti-apoptotic *BCL2* and other genes implicated in the initiation and progression of HNSCC, including *TNF-α*, *EGFR*, *STAT3*, *ΔNp63*, *cREL*, *IL6*, *IL1β*, *AKT1*, *PTGS2* and *WNT5A* (82-85,87,89-94,102-111) (Fig. 1). Parallel evidence that acidic bile stimulus is able to activate NF-κB and its related pathways in HHCs arose from the *in vitro* treatment with curcumin, which successfully blocked the transcriptional activity of NF-κB (20), similar to BAY 11-7082 (21,23). A study by our group from 2020 (24) documented the preventive and therapeutic properties of curcumin on murine HM against the acidic bile effect, thus shaping the future translational development of effective targeted therapies using topical non-pharmacologic inhibitors of NF-κB.

Strong evidence that NF-κB activation is able to influence the acidic bile-induced oncogenic mRNA profile inspired a further study on whether synchronizing its inhibition with acidic bile exposure is significant. Thus, a study by our group from 2019 (23) reported the temporal characteristics of NF-κB inhibition in blocking the acidic bile-induced oncogenic molecular events in HHCs. A series of studies also documented that topical application of BAY 11-7082 or curcumin to HM, either before, after or simultaneous to acidic bile exposure, successfully prevented or suppressed cell proliferation and NF-κB-related molecular events (19,24,25).

These results revealed that the upregulation of *RELA*, *BCL2*, *STAT3*, *EGFR*, *WNT5A*, *TNF-α*, *IL6* and *PTGS2* is directly promoted by acidic bile through NF-κB, shortly after its exposure (19,24,25) (Fig. 1), and strongly suggested that it may be clinically feasible to topically apply NF-κB inhibitors, without any precise synchronization with acidic bile exposure, to prevent acidic bile-induced oncogenic molecular changes.

Interactions between NF-κB activation and other factors. The application of NF-κB inhibitors also revealed important information about possible interactions between acidic bile-induced NF-κB activation and other central molecules in head and neck cancer (Fig. 1).

The NF-κB/STAT3 crosstalk has been indicated to be fundamental in inflammation-associated carcinogenesis in head and neck cancer (108,119,120). Application of NF-κB inhibitors successfully prevented the acidic bile-induced activation and nuclear translocation of STAT3, which is an important regulator of cell proliferation, and reduced the transcriptional levels of *IL6* and *STAT3*, in treated hypopharyngeal cells (19-25) (Fig. 1). These data strongly support the theory that the acidic bile-induced activation of IL-6/STAT3 is NF-κB-dependent (108) (in a paragraph below, the role of STAT3 in bile-induced carcinogenesis is discussed). In addition, prior findings implied strong interactions between NF-κB and STAT3 in acidic bile-exposed premalignant HM (13,14), further supporting the theory that the inflammatory response induced by acidic bile may increase the risk of laryngopharyngeal cancer.

EGFR is frequently overexpressed in HNSCC (121,122). Crosstalk between NF-κB and downstream pathways of EGFR has been observed (123,124). Although the exact role of EGFR in bile-related hypopharyngeal carcinogenesis has remained to be elucidated, the application of NF-κB inhibitors resulted in the successful suppression of acidic bile-induced overexpression of *EGFR*, supporting the interactions between NF-κB and EGFR pathways during this process (Fig. 1). In addition, as both STAT3 and EGFR are important contributors to HNSCC pathogenesis (80,121), the above observations further emphasized the requirement to develop a therapeutic strategy for targeting NF-κB in head and neck malignancies and particularly in bile reflux-related HSCC.

Furthermore, NF-κB inhibition had a strong effect in suppressing the acidic bile-induced overexpression of *WNT5A* (Fig. 1), a factor related to cancer-associated inflammation and epithelial-to-mesenchymal transition (111), indicating that NF-κB is able to mediate acidic bile-induced changes in hypopharyngeal cell-cell interactions.

In addition, COX-2 is regularly highly overexpressed during inflammatory and neoplastic processes (125) and is significantly overexpressed in acidic bile-exposed HM (24,25). *In vivo* application of NF-κB inhibitor significantly abrogated the acidic bile-induced overexpression of *PTGS2* (24,25) (Fig. 1), further supporting the regulatory role of NF-κB in early inflammatory and cancer-related pathways, such as COX-2 (126).

In addition to the above, the PI3K/Akt pathway (127) is one of the most frequently activated pathways in head and neck cancer (128). The successful suppression of acidic bile-induced *AKT1* overexpression using topical application of NF-κB inhibitor on murine HM suggested that NF-κB may mediate acidic bile-induced deregulations of PI3K/Akt downstream pathways (127) (Fig. 1).

In summary, the NF-κB pathway is a core central pathway that interacts with multiple upstream and downstream signaling pathways linked to the carcinogenic process. Using both a specific NF-κB inhibitor, such as BAY 11-7082, and a more pleiotropic NF-κB inhibitor, such as curcumin, it was documented that the acidic bile-induced deregulations of cancer-related genes or inflammatory factors are mediated by the NF-κB (Fig. 1). Furthermore, as curcumin is able to prevent the bile-related anti-apoptotic effect independently of the pH status, it may have an advantage over other NF-κB inhibitors. Of note, curcumin specifically reduced a lower percentage of analyzed NF-κB signaling genes compared to BAY 11-7082 (25 vs. 85%) (20,21). Thus, curcumin may confer a clinical advantage by preventing generalized suppression of NF-κB signaling, which is essential to the basic metabolic function of healthy mucosa and thereby reducing global toxicity (24,129).

Bile reflux-induced NF-κB-related miRNA oncogenic phenotype. miRNA molecules have also been important in both inflammation and cancer (130), modulating the expression of genes by causing target mRNA degradation or inhibiting their translation (131). The expression levels of certain miRNAs, such as 'oncomiRs' and 'tumor suppressor' miRNAs, have been indicated to be altered in tumor cells compared to normal cells (upregulated or downregulated), and capable of contributing to carcinogenesis, thereby demonstrating a significant

regulatory role in the multistep process of cancer initiation and progression (132).

There is further evidence that miRNA markers, such as 'oncomiR' miR-21 and 'tumor suppressor' miR-375, have a crucial role in the initiation and progression of HNSCC (14,22,133,134). Arantes *et al* (135) reported the fundamental role of miR-21 as a biomarker in head and neck carcinogenesis, while miR-375 has been proposed as a predictive biomarker for early diagnosis in laryngeal cancer (136). In addition, interactions between NF-κB and miRNA markers, such as miR-21, miR-34a and miR-451a, have been importantly described by others in human cancer cells, including HNSCC (104,137,138). Specifically, a cluster of miRNA markers was reported to be associated with NF-κB that may be associated with the aggressive biological behavior of HNSCC (104).

Explorations by our group (14,15,19,22,23,25) revealed that acidic bile caused deregulations of the expression of oncogenic miRNA markers, previously associated with laryngopharyngeal cancer (133-141). Specifically, the 'oncomiRs' miR-21, miR-192 and miR-155, and the 'tumor suppressors' miR-34a, miR-375, miR-451a, miR-99a and miR-504 were indicated to be significantly altered in exposed HHCs and murine laryngopharyngeal mucosa (14,15,19,22,23,25) (Fig. 2). Of note, results from our group (14,15) highlighted the role of miR-21 and miR-375 deregulations in acidic bile-related neoplasia.

In addition, findings from HSCC tumor specimens from patients with documented bile reflux supported its strong association with upregulation of 'oncomiR' miR-21 and downregulation of 'tumor suppressors' miR-34a and particularly of miR-375, along with strong positivity for NF-κB (18) (Fig. 2). Bile reflux-associated HSCC was also associated with a marked reduction of 'tumor suppressors' miR-489, miR-504 and miR-99a compared with their adjacent non-pathologic tissue (18), suggesting their involvement in the onset and progression of HSCC (142,143). Finally, bile exposure-associated HSCC exhibited differential expression of miR-489 and miR-504, and particularly of miR-375, compared to bile-negative HSCC, which had significantly lower NF-κB levels. A previous view by our group (18) suggested that these miRNA markers may have a distinct role in biliary reflux-associated hypopharyngeal cancer.

Application of BAY 11-7082 in HHCs or murine HM was proven to prevent miRNA deregulations caused by acidic bile, providing insight into the interactions of transcriptionally active NF-κB with cancer-related miRNA markers (19,22,23,25). Specifically, it was demonstrated that BAY 11-7082 is able to effectively reverse the acidic bile-induced downregulation of 'tumor suppressor' miR-451a and miR-99a, and upregulation of 'oncomiRs' miR-21, miR-155 and miR-192 (19,22,23,25) (Fig. 2), which are considered important markers for poor prognosis in head and neck cancer or linked to gastroesophageal reflux (22,135,144-150). Other studies have also indicated that NF-κB has a direct regulatory effect on the expression of miR-21 and miR-155 through their binding promoters (151,152). Through *in vitro* and *in vivo* applications of BAY 11-7082 on hypopharyngeal cells and mucosa, respectively, a direct effect of acidic bile on the above miRNAs was demonstrated shortly after exposure (22,23,25). This observation proposed the use of these miRNAs as biomarkers of early neoplastic events in acidic bile-exposed HM, strongly supporting the role of NF-κB as a mediator in this process. In addition, topical *in vivo* application of BAY 11-7082 either before, after or simultaneous to acidic bile significantly inhibited the acidic bile-induced upregulation of 'oncomiR' miR-192 (19,25), previously associated with GERD (148), and downregulation of 'tumor suppressor' miR-504, a promising target for HSCC (142) (Fig. 2). This observation also suggested the utility of these miRNAs as biomarkers of early neoplastic events in acidic bile-exposed HM.

In summary, the above preclinical data provided evidence of the role of NF-κB as a regulator of miR-192, miR-21, miR-155, miR-451a, miR-375, miR-99a and miR-504 (Fig. 2), and proposed these miRNAs as potential therapeutic targets of bile-related mutagenic evolution in the HM.

Interactions between NF-κB-related mRNA and miRNA phenotypes. Previous studies suggested that interactions between miRNAs and mRNA molecules may be NF-κB-dependent during carcinogenesis (141,153,154). Rokavec *et al* (153) proposed that miRNA molecules, such as miR-34a, interact with STAT3 in an NF-κB-dependent manner. According to Tili *et al* (154), permanent upregulation of miR-155 may mediate a prolonged inflammatory reaction leading to cancer.

It has been proposed that acidic bile-induced NF-κB activation, *BCL2* overexpression and significant alterations of oncogenic *EGFR*, *STAT3*, *TNF-α*, *IL6*, *IL1β* and *WNT5A* may directly or indirectly interact with cancer-related miRNA markers, such as 'oncomiRs' miR-21 and miR-155, as well as 'tumor suppressors' miR-34a, miR-375 and miR-451a (14-16,141) (Fig. 3). These observations suggest that inflammatory episodes caused by acidic bile may be associated with downstream oncogenic pathways and may be effectively prevented by NF-κB inhibition.

NF-κB as a challenging target for cancer therapy. Recent findings documenting the crucial role of NF-κB in bile reflux-related hypopharyngeal carcinogenesis, pose a challenge for researchers and clinicians on how to identify patients who are more likely to benefit from NF-κB inhibition treatment. An NF-κB-related gene expression signature associated with bile reflux in patients with HSCC (Figs. 1 and 2) may provide a better prediction for inhibition selection and also allow the development of diagnostic and prognostic biomarkers of NF-κB inhibition response.

Although NF-κB targeted therapy has been already applied in clinical practice with promising results in anti-cancer therapy (129,155,156), including HNSCC treatment (157,158), there is an increasing effort in the pharmaceutical industry to develop advanced NF-κB inhibitors (129,159,160). In particular, research focuses on the identification of IKK/NF-κB inhibitors for targeted therapy that would prevent NF-κB activation without affecting other signaling pathways and selectively affect malignant cells rather than normal cells. As one of the major adverse effects of using NF-κB inhibitors as anticancer drugs is their ability to impair innate immunity when applied in excessive and prolonged periods (129,159-161), research should also focus on both minimizing systemic toxicity and prevention

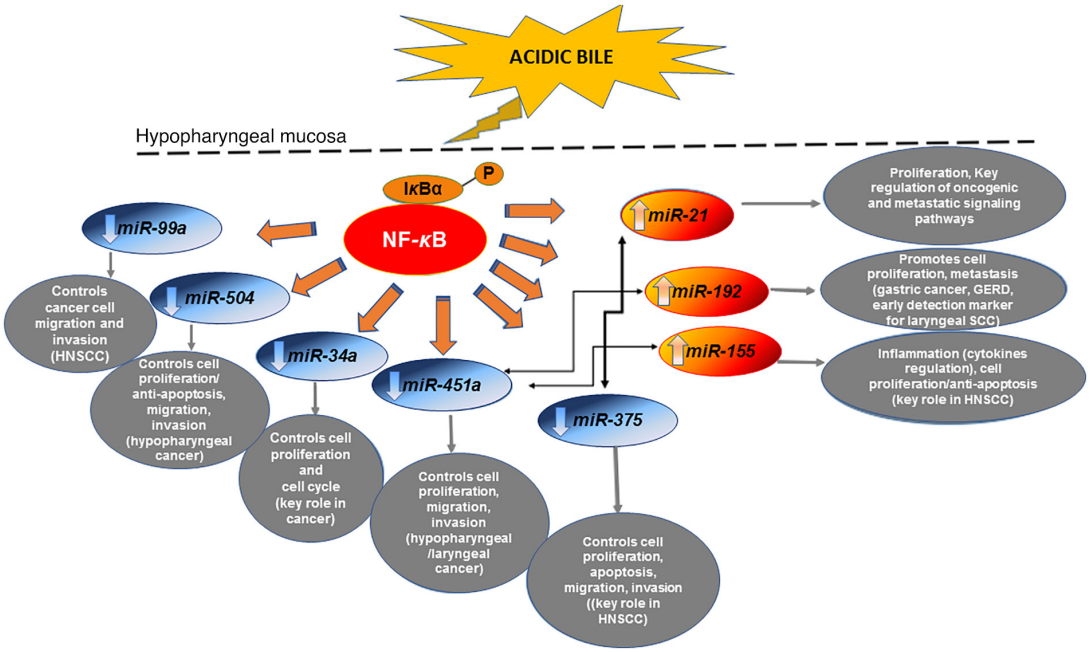

Figure 2. The mechanistic role of NF-κB in the miRNA oncogenic phenotype induced by bile reflux in hypopharyngeal carcinogenesis. Chronic stimulation of laryngopharyngeal mucosa by acidic bile induces constitutive activation of NF-κB, producing upregulation of 'oncomiRs' *miR-21*, *miR-155* and *miR-192*, previously associated with oncogenic signaling pathways in head and neck cancer and GERD. The acidic bile-induced activation of NF-κB in treated hypopharyngeal mucosa is capable of downregulating 'tumor suppressor' *miR-34a*, *miR-451a*, *miR-375*, *miR-99a* and *miR-504*, are known to control the cell cycle and are frequently affected in head and neck cancer. Acidic bile-induced expression levels of 'oncomiRs' exhibited an inverse correlation with 'tumor suppressor' miRNAs that appears to be regulated by NF-κB. miRNA/miR, microRNA; GERD, gastroesophageal reflux disease; HNSCC, head and neck squamous cell carcinoma.

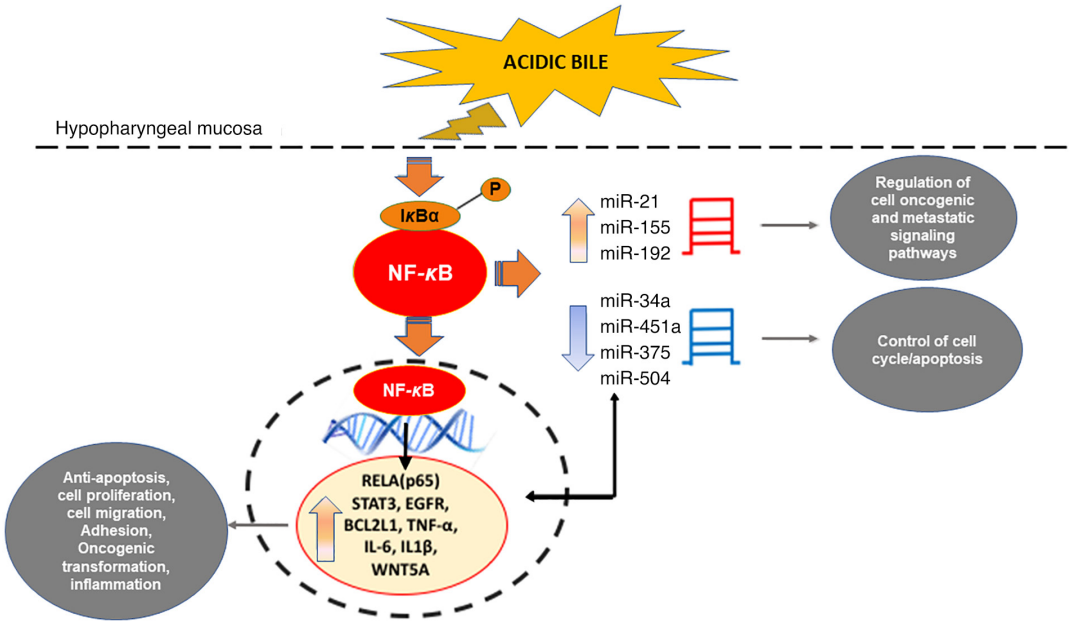

Figure 3. Schematic representation of proposed interactions between acidic bile-induced NF-κB activation and alterations of cancer-related mRNA and miRNA phenotypes in hypopharyngeal cancer. NF-κB inhibition provided evidence of strong interactions between acidic bile-induced and cancer-related oncogenic mRNA and miRNA phenotypes in treated hypopharyngeal cells. miRNA/miR, microRNA.

of long-term immunosuppression. Thus, an ideal inhibition of NF-κB should be transient and reversible, as well as effective when combined with other anti-cancer treatments. Prior *in vivo* explorations strongly supported the effectiveness of models using intermittent and topical treatment as opposed to prolonged and systemic treatment. Specifically, the marked efficacy of short-term topical treatment with NF-κB inhibitors, such as curcumin (2 mg/kg/day) and BAY 11-7082 (6.25 mg/kg/day), in suppressing bile reflux-induced early preneoplastic changes in the hypopharynx (19,24,25), strongly supports the view that non-systemic and transient NF-κB inhibition may be clinically feasible in preventing bile-reflux-related oncogenic effects. This area of research is progressing rapidly; however, another adverse effect is of

high importance prior to the targeting of IKK or NF-κB in the clinic. This refers to the enhanced production of IL-1β and related cytokines by inhibitors of NF-κB activation during bacterial infections (161), suggesting the short-term use of NF-κB inhibitors in combination with antibiotics.

5. Role of STAT3 in bile reflux-related hypopharyngeal carcinogenesis

The *STAT3* oncogene (162) is a transcription factor central to head and neck cancer (119,120,122). In addition to the significant role of NF-κB, recent preliminary data from our team indicate the important role of STAT3 in bile-related hypopharyngeal carcinogenesis by promoting early oncogenic molecular events, including cancer-related inflammatory molecules *IL6*, *TNF-α* and *RELA (p65)*.

Specifically, using three different inhibitors with each blocking a different step of STAT3 upstream signaling, such as nifuroxazide, SI3-201 and STA-21 (163-165), preliminary data from our group were obtained regarding the mechanism of the effects of bile. In detail, acidic bile is able to induce constitutive activation of STAT3 that may not be exclusively dependent on JAK/STAT3 upstream signaling (165), but it may also be stimulated by alternative signaling, such as EGFR. Although targeting STAT3, either by its knockdown or its pharmacological inhibition, had a minimal effect on nuclear or total phosphorylated NF-κB (p65 S536) protein levels, it was observed to contribute, among others, to the transcriptional activation of NF-κB. As mentioned above, previous findings of NF-κB inhibition, using BAY 11-7082, had determined a role of NF-κB in acidic bile-induced activation of STAT3 (19-25).

All of these observations suggest possible molecular crosstalk between the NF-κB and STAT3 transcription factor associated signaling pathways in bile reflux-related inflammation and tumorigenesis in the hypopharynx, as similarly proposed in HNSCC (108,119,120).

6. Bile-induced DNA damage

One of the principal questions regarding the effects of bile on cellular physiology was how bile refluxate induces DNA damage. Dvorak *et al* (59), suggested that bile at acidic pH may potentially induce DNA damage in esophageal cells, speculating that chronic exposure to bile acids at low pH may result in increased genomic instability, abnormal cell signaling and resistance to apoptosis. According to Goldman *et al* (166), bile in combination with acid, but not acid alone, immediately activates all three isoforms of nitric oxide (NO) synthase, a family of enzymes catalyzing the production of NO, which links chronic inflammatory diseases and reactive oxygen/nitrogen species (ROS/RNS) with cancer. They also indicated that bile in combination with acid increased intracellular acidification and DNA damage in esophageal cells, which may lead to mutations and cancer progression (166). Bernstein *et al* (31,167) proposed that de-conjugated secondary bile acids, such as DCA, are capable of inducing DNA damage, giving rise to cancer due to the accumulation of mutations. Specifically, DCA was determined to induce increased intracellular production of ROS/RNS, resulting in increased oxidative stress and DNA damage (64,168,169).

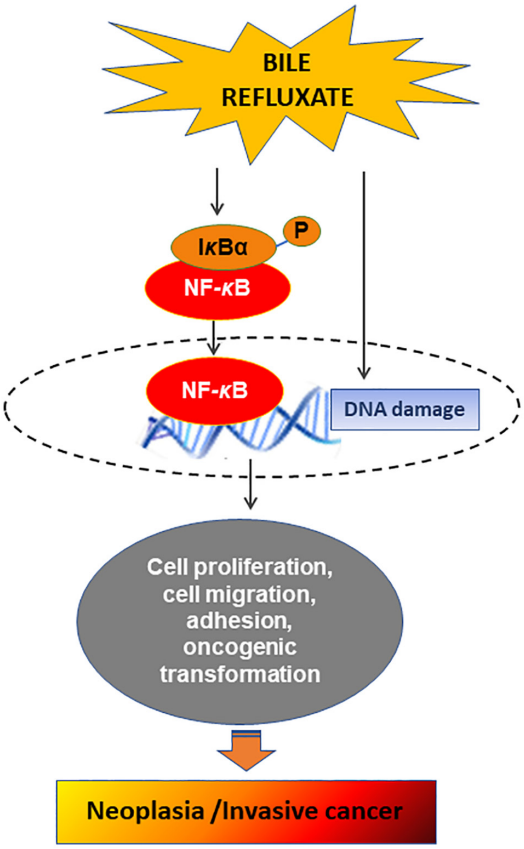

Figure 4. Schematic representation of the proposed mechanism of bile reflux-induced hypopharyngeal cancer. Bile refluxate is capable of inducing DNA damage, such as double-strand DNA breaks and oxidative damage and significant activation of NF-κB and its related anti-apoptotic pathways, leading to malignant transformation of laryngopharyngeal mucosa and invasive cancer.

Recent studies by our group (15,16,19) documented that conjugated bile acids led to the upregulation of γ-H2AX (pS139). γ-H2AX is a consistent marker of DNA DSBs (170,171), which was profoundly increased in bile-treated hypopharyngeal cells or HM at acidic pH, compared to neutral pH, acid alone or neutral control conditions (15,19). Subsequently, it was documented that bile-treated HM at weakly acidic pH, with or without DCA, also induced DNA damage in exposed HM (16). Specifically, premalignant and malignant lesions caused by acidic bile demonstrated increased levels of nuclear γ-H2AX, as well as DNA/RNA oxidative damage (Fig. 4) (15,16).

According to previous findings, increased oxidative damage may result in high levels of ROS or DSBs incurring direct DNA damage (168,172,173), which may potentially lead to tumor-initiating mutations in head and neck cancer (174). All of these results advocate the theory that acidic bile-induced DNA damage may contribute to its mutated phenotype (Fig. 4). In addition, ROS is able to activate several cancer-associated signaling pathways, including NF-κB (164), concluding that acidic bile may contribute to evasion of apoptosis and/or proliferation of mutated hypopharyngeal epithelial cells, resulting in malignant lesions of HM.

In parallel, chronic exposure of murine hypopharyngeal epithelium to acidic bile was observed to induce a systematic release of inflammatory molecules, such as IL-6 and TNF-α, which are considered central to head and neck

carcinogenesis (91). This systematic release of inflammatory molecules is able to maintain the constitutive release of other cancer-related cytokines in the microenvironment of the exposed epithelium. It is known that the chronic inflammatory microenvironment may lead to the production of activated inflammatory cells that may also serve as sources of ROS and reactive nitrogen intermediates (RNI), which are capable of inducing DNA damage and genomic instability and so promote mutations in neighboring epithelial cells (175,176). In addition, inflammatory cytokines contribute to increased intracellular ROS and RNI production in pre-malignant cells. In conclusion, the chronic inflammatory microenvironment caused by bile reflux may be one of the main factors in hypopharyngeal carcinogenesis.

7. Possible interactions of bile refluxate with hypopharyngeal mucosa

How acidic bile interacts with HM to exert its harmful effect, causing DNA damage and promoting NF-κB-related anti-apoptotic processes, leading to its malignant transformation, has remained to be fully elucidated. According to Li and Cao (177), bile acids may interact with membrane receptors, such as Takeda G-protein coupled receptor (TGR5). Their study also indicated that TGR5 is able to mediate bile reflux-induced DNA damage in esophageal cells (177). Another cell membrane receptor that is able to interact with bile acids is the sphingosine-1-phosphate receptor 1, known as S1PR1 or S1P1 (178,179). Both above-mentioned receptors were previously associated with lower esophageal cancer related to bile reflux (180,181). In particular, TGR5 was reported to be expressed in both adenocarcinoma and squamous cell carcinoma of the lower esophagus (181), while S1PR1 has been associated with squamous cell carcinoma of the head and neck (179).

Other studies provided evidence that bile acids are able to activate nuclear farnesoid X receptors (FXRs) (182,183), suggesting their contribution to pre-neoplastic changes (183) of the lower esophagus. Although several nuclear receptors (NRs) have been identified in the head and neck (184), an association between NRs and bile acids has not yet been described in HNSCC. Prior studies suggested a mutually antagonistic relationship between FXR and NF-κB activation (185,186) and proposed FXR receptors as useful targets for esophageal adenocarcinoma (182). However, the exact mechanism by which FXR affects the expression of proinflammatory molecules, such as NF-κB in either the lower or upper esophagus remains elusive and the role of FXR in bile reflux-related carcinogenesis deserves further exploration. Further investigation in the hypopharynx may identify specific receptors activated by acidic bile and clarify the role of NRs, such as FXR, and cell membrane receptors, such as TGR5 and S1PR1, in this process.

8. Conclusion

Recent *in vitro* and *in vivo* data provide evidence on bile reflux-associated hypopharyngeal carcinogenesis. The composition of biliary refluxate, such as conjugated bile acids, and acidity are pivotal factors in promoting DNA damage, as well as histologic and molecular changes in the HM, most likely through the constitutive activation of NF-κB. Chronic acidic bile exposure can cause increased oxidative damage, DSBs, overproduction of cytokines and cell-cell interaction changes, which are critical elements of tumor initiation and progression (187), possibly through derangements in both pre-neoplastic/neoplastic cells and their microenvironment. In parallel, acidic bile-induced constitutive activation of NF-κB can promote oncogenic mRNA and miRNA phenotypes, contributing to the proliferation of mutated cells and thus giving rise to the malignant transformation of the exposed HM. Primary data also support the contributing role of STAT3 in this process. Further investigation of the proposed mechanisms mediating bile-induced DNA damage, the tumor microenvironment and downstream oncogenic signaling pathways in HM, as well as the identification of specific receptors that may interact with bile, will contribute to innovative approaches to the diagnosis and prevention of laryngopharyngeal malignancies, as well as to the improvement of current therapeutic approaches to LPR-related carcinogenesis.

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Authors' contributions

DPV and SGD were involved in the conceptualization of this review article. DPV, SGD, PGD and BLJ were involved in searching the literature. DPV, PGD and SGD were involved in the writing of the original draft. DPV, SGD, PGD and BLJ reviewed and edited the article. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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