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Overview of major molecular alterations during progression from Barrett's esophagus to esophageal adenocarcinoma

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Esophageal adenocarcinoma (EAC) develops in the sequential transformation of normal epithelium into metaplastic epithelium, called Barrett's esophagus (BE), then to dysplasia, and finally cancer. BE is a common condition in which normal stratified squamous epithelium of the esophagus is replaced with an intestine-like columnar epithelium, and it is the most prominent risk factor for EAC. This review aims to impartially systemize the knowledge from a large number of publications that describe the molecular and biochemical alterations occurring over this progression sequence. In order to provide an unbiased extraction of the knowledge from the literature, a text-mining methodology was used to select genes that are involved in the BE progression, with the top candidate genes found to be *TP53*, *CDKN2A*, *CTNNB1*, *CDH1*, *GPX3*, and *NOX5*. In addition, sample frequencies across analyzed patient cohorts at each stage of disease progression are summarized. All six genes are altered in the majority of EAC patients, and accumulation of alterations correlates well with the sequential progression of BE to cancer, indicating that the text-mining method is a valid approach for gene prioritization. This review discusses how, besides being cancer drivers, these genes are functionally interconnected and might collectively be considered a central hub of BE progression.

Keywords: Barrett's esophagus; esophageal adenocarcinoma; cancer progression; genomic alterations

Introduction

Since 1950, when Norman Barrett first described the gastroenterological condition known as Barrett's esophagus (BE),[1] a substantial number of research papers describing different aspects of BE, including surveillance, origin, treatment, and occurrence, have been published. Many of those have focused on the progression of BE to cancer. Historically, researchers followed one gene that, in their perspective, was worth investigating. In most cases, small-scale approaches, including polymerase chain reaction, western and Southern blotting, enzyme-linked immunosorbent assay, immunohistochemistry, and small interfering RNA transfection assays, were used. Usually, a target protein was carefully selected on the basis of the existing evidence of its importance in the progression and validated using BE/esophageal adenocarcinoma (EAC) cell lines, fresh frozen tumor samples, and/or biopsies and mouse models.

The aim of this review is to reanalyze the existing literature using a high-scale text-mining tool, prioritize genes that have the highest appearance in the relevant literature, and systematize and summarize the existing knowledge about the mechanism of BE progression. A better understanding of the molecular alterations occurring during progression from BE to EAC is greatly needed to improve prevention and tumor diagnosis. In addition, the most appropriate therapeutic targets are those alterations that occur early in the development of disease. The discovery of causative alterations not only occurring early in the pathogenesis but also covering the majority of the progressing samples is not a trivial task.

A text-mining tool was used to retrieve a list of the most prominent genes participating in BE progression to cancer. Thereafter, the literature

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Table 1. A list of the most significant genes retrieved by the text-mining approach that appeared in at least two abstracts

Gene name	Function	Alteration	Alias names	PMIDs
CDH1	Cell adhesion in epithelial tissue	Reduced expression	E-cadherin	12203119, 18286686
CDKN2A	A stabilizer of the tumor suppressor protein p53; cell cycle G_1 control	Mutation, LOH, inactivation by hypermethylation	p16, INK4A; p14, ARF	11910361, 17330261, 18410530, 23695891, 25280564, 9834265
GPX3	Detoxification of hydrogen peroxide	Downregulation, hypermethylation	Glutathione peroxidase 3	16229808, 18664505
CTNNB1	Creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells; transcriptional factor	Nuclear accumulation, reduced expression	β-Catenin	10907314, 12203119
NOX5	A superoxide-generating protein	Upregulation	NADPH oxidase 5	17403674, 17947454
TP53	Cell cycle regulation; apoptosis induction; DNA repair	LOH, mutations, deactivation, positive p53 staining	p53	10907314, 16537709, 17330261, 18410530, 19302208, 22629421, 25280564, 8692948

Note: A gene name is accompanied by the summary of its function, genomic/molecular alteration during progression of Barrett's esophagus to cancer, alias names used in the abstracts, and PMID of publications where this gene was identified by PubTator.

describing the role of the prioritized genes in cancer progression was intensively studied. The principal focus was given to the following aspects: (1) the type of molecular alteration(s) in the prioritized gene and the disease stage in which it was first detected, (2) the percentage of the patients at each disease stage that carried these alterations, (3) the mechanism responsible for the alterations (if known), and (4) how this alteration might be involved in tissue transformation and carcinogenesis.

Using PubTator,[2] a web-based text-mining tool, we retrieved 47 publications based on the search keywords: [Barrett esophagus] AND [progression] AND [mechanism] (as of February, 2016). PubTator automatically identified gene names, diseases, chemicals, and other entities in every abstract. Fourteen abstracts that did not contain any gene names were excluded. The manual validation of the retrieved list showed that seven out of 33 publications were not relevant to the subject of the study, as they described the progression of the normal esophageal epithelium to BE. As we were primarily interested in genes associated with BE progression, these publications were excluded. Seventy-seven gene names were retrieved from 26 publications. After manual curation, the number of genes dropped to 52, mostly due to the fact that the same gene was mentioned several times in its different alias forms (e.g., p53 and TP53). Six genes appeared in two or more publications (Table 1). Figure 1 shows the main steps of the text-mining pipeline. It should be noted that output from PubTator was used only as a guide for gene prioritization, while this review was written based on a significantly wider range of publications.

TP53 in EAC and its precursors

TP53, a gene located at chromosome 17p13, is the most important tumor suppressor in many different types of cancer. It encodes a nuclear protein of 393 amino acids that contains five domains: an unfolded N-terminal transactivation domain followed by a highly structured DNA-binding domain and tetramerization domains.[3]

TP53 protein regulates fundamental aspects of cellular stress responses, monitors the integrity of the genome, and halts cell cycle progression at G_1 if the genome is damaged, allowing time for DNA repair. TP53 promotes apoptosis if the genome cannot be repaired for any reason. As such, it is not surprising that *TP53* is the top gene on the list, appearing in eight publications (Table 1).

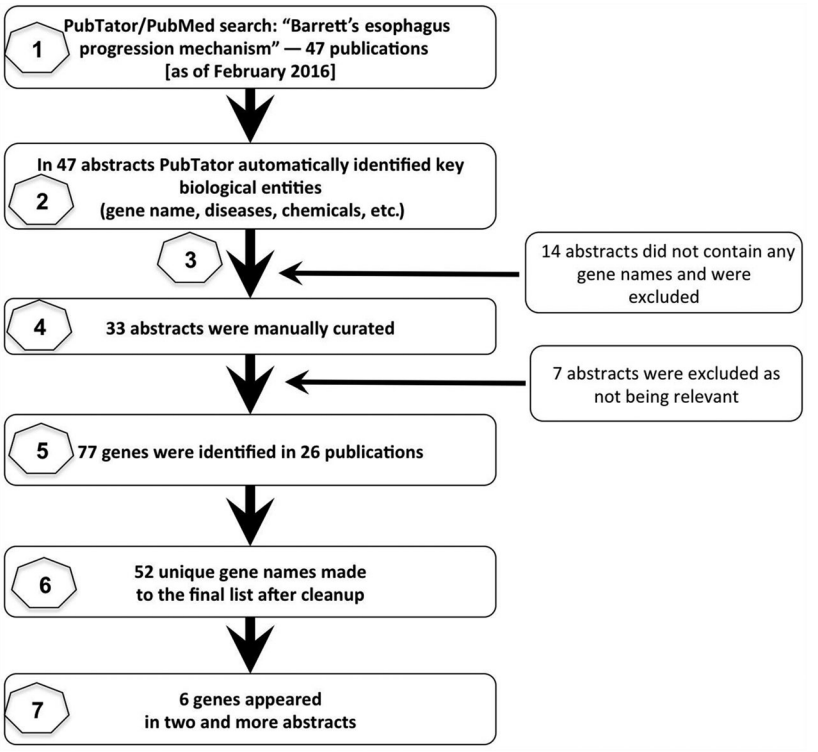

Figure 1. Text-mining pipeline using PubTator consists of the following steps: (1) retrieval search results using a keyword query; (2) annotation of the biological entities in the listed abstracts; (3) excluding publications that do not have a desired biological entity; (4) manual curation of the abstracts, ensuring that they all relevant to the subject of interest; (5) gene list generation based on white-listed articles; (6) excluding ambiguity—ensuring that every gene appears on the list only once; and (7) (optional) prioritizing genes based on their frequency; for example, parsing genes that appear in two or more publications.

In cancer, the function of TP53 is usually altered through *TP53* somatic mutations, loss of heterozygosity (LOH), and, more rarely, by DNA methylation. Impaired TP53 activity promotes accumulation of DNA injuries in cells, which may lead to carcinogenesis. The tumor suppressor gene *TP53* was one of the first to be investigated in Barrett's-associated neoplasms, and it is still the most enigmatic element of BE progression.[4] Some controversial results have been published on this topic in the last two decades. For example, patients with LOH of *TP53* are 16 times more likely to progress to EAC, according to the Seattle Barrett's Esophagus Study (United States),[5] but a group from the Netherlands stated that LOH in *TP53* was not a significant predictor of progression.[6]

Although LOH is a disease-specific event found rarely, if ever, in normal squamous epithelium, allelic loss of 17p is a relatively early event that occurs in BE tissue; it was reported in numerous publications at frequencies ranging from 0% to 25%.[5–9] Across five studies, LOH of the 17p region was detected in 17.8% of all analyzed BE samples (Table 2). It was stated that not only the presence of LOH at *TP53* but also the size of the clones with BE lesions can predict the progression to esophageal cancer.[9] Allelic loss of 17p can be considered a common event in EAC patients; it was detected in 31% of EAC samples in Refs. 10–11, 75% in Ref. 8, 78.9% in Ref. 12, and 91.6% in Ref. 7. In summary, 61.1% of EAC samples were affected (Table 2). In addition, allelic loss of 17p was detected in 59.3–77.7% of high-grade dysplasia (HGD) samples.[5,8]

In addition to undergoing LOH, *TP53* is often inactivated with a second hit—a mutation of another allele. *TP53* is the most significantly mutated gene in EAC.[13] In general, 50% of all missense mutations in the DNA-binding domain of TP53 fall within six "hotspots" (residues R175, G245, R248, R249, R273, and R282) and are

Table 2. Summary of the molecular alterations in EAC samples and its precursors

Gene	Alteration	Percentage of patients with indicated alteration [number of studies] (mean % across all analyzed samples)		
		Barrett's esophagus	Dysplasia	Esophageal adenocarcinoma
TP53	LOH	0.0–25.0% [5] (17.8%)	59.3–77.7% [2] (61.7%)	31.5–91.6% [5] (61.1%)
	Positive staining	0.0–66.6% [4] (30.3%)	55.5–66.0% [2] (60.0%)	65.0–87.5% [4] (72.0%)
	Mutations	1.5% [1]	72.0% [1]	68.0–72.0% [3]
CDKN2A	9p21 LOH	0.0–74.2% [9] (59.1%)	62.0–77.8% [3] (67.6%)	33.0–90.9% [8] (57.3 %)
	Methylation of *p16INK4A*	3.0–65.8% [4] (25.1%)	30.0–80.9% [4] (63.6%)	36.3–81.8% [4] (58.1%)
	Methylation of *p14ARF*	0.0% [1]	0.0% [1]	20.0% [1]
	Mutations	9.0% [1]	13.9% [1]	8–13.8% [2]
CDH1	Abnormal expression (reduced, absent, cytosolic, disorganized)	0.0–16.6% [3]*	0–100.0% [3]*	74.5–100.0% [4]*
CTNNB1	Nuclear localization	0.0% [1]	–	61.0–63.3% [2]
APC	LOH	0.0% [2]	27.2% [1]	29.4–54.5% [3] (42.1%)
	Methylation	39.5% [1]	–	78.0–95.2% [3] (86.9%)
GPX3	Expression reduction	–	–	52.0–90.5% [2]
	Methylation	61.9% [1]	81.8% [1]	88.2 % [1]

frequent in almost all cancer types. All missense mutations in *TP53* accumulated exclusively in the DNA-binding domain,[13,14] which is consistent with previous observations.[15] These mutations disrupt the proper conformation of TP53 so that mutant forms lack the ability to activate transcription in a sequence-specific manner.[16] A recent comprehensive mutational profiling study identified that the inactivation of TP53 occurred in a stage-specific manner: only 1.5% of the nondysplastic BE cases in Ref. 17, but up to 72% of EAC samples in Ref. 18, were affected by missense mutations.

Under normal conditions, *TP53* generates a short-lived product that can barely be detected using immunohistochemical (IHC) methods. The mutant *TP53* produces a more stable protein product, and the extended half-life makes it possible to detect nuclear accumulation of TP53 by IHC. No evidence of TP53 nuclear accumulation in squamous epithelium samples was found in Ref. 19, and only 7% of normal tissue samples had positive staining for TP53 in Ref. 20. BE samples contained positive staining for TP53 in 0% of cases in Refs. 4 and 19, 62.5% of cases in Ref. 20, and 60.9% of cases in Ref. 22. We were not able to identify any reasons for such a huge discrepancy on the basis of the description of the patient cohorts provided by the authors. Staining in EAC samples was more consistent, at 65% in Ref. 12, 71% in Ref. 20, 75% in Ref. 19, and reaching 87.5% in Ref. 4. Summarizing the four studies, 72% of EAC samples were positive for TP53 staining.

However, positive staining is not a secure proof of the existence of a mutation.[21] In a case–control study with a large cohort of BE patients, the odds of diffuse or intense TP53 staining were substantially elevated in biopsies from patients who developed EAC compared with controls (odds ratio 11.7; 95% CI 1.93–71.4).[23] Taking these data into account, the British Society of Gastroenterology suggested adding TP53 immunostaining to the histological assessment of dysplasia in the guidelines on the diagnosis and management of BE.[24]

Furthermore, other *TP53* alterations include frameshift mutations (7%) and nonsense mutations (13.7%).[13] *TP53* splicing sites were affected in 2.7% of EAC samples.[13] Altogether, 68.8–72.7%

of EAC samples were affected by somatic mutations in *TP53*,[13,14,17] which is significantly higher than nondysplastic samples and comparable to HGD samples (Table 2). In fact, *TP53* was one of two mutated genes that clearly defined the boundaries between the BE and HGD stages.[17]

In summary, the majority of EAC samples has either deleterious mutations in *TP53* or inactivation of *TP53* via LOH. It is the only gene in EAC that is not only altered in up to 87% of EAC samples (Table 2) but for which the alterations accumulate in a stage-specific manner. Therefore, reactivation of TP53 might be a particularly effective strategy for cancer therapy. For example, PRIMA-1 is a direct TP53 reactivator and one of the most promising drugs.[25]

CDKN2A in EAC and its precursors

CDKN2A was the second most frequent gene on the list generated by the text-mining method using PubTator (Table 1). The *CDKN2A* genomic locus has one of the richest histories of cancer research, with more than 9000 citations in PubMed queried for "CDKN2A and cancer." This rate is comparable to *TP53*.

CDKN2A is located on chromosome 9p21. The earliest report on 9p21 alteration in EAC was made by the Meltzer group in 1994.[26] A high prevalence of LOH on chromosome 9p was previously shown for other cancers, such as glioma and mesothelioma.[27,28] Meltzer's paper confirmed that EAC is not an exception, and LOH at chromosome 9p occurred in 47% of EAC samples.

Initial findings brought a lot of attention to this area from different research groups and institutions, with research publications predominantly confirming previously published results. However, the frequency of 9p21 LOH in an EAC population was very inconsistent, shown to be 33% in Ref. 29, 44% in Ref. 30, 47% in Ref. 26, 59% in Ref. 31, 63.6% in Ref. 32, 68% in Ref. 33, 89% in Ref. 7, and up to 91% in Ref. 34. In summary, 57.3% of all analyzed EAC patients carried this alteration (Table 2).

Surprisingly, 9p21 LOH is a very early event in cancer progression. Approximately half of the analyzed BE tissue samples contained LOH at 9p21: 34% in Ref. 30, 49% in Ref. 35, 50% in Refs. 33 and 36, 58.8% in Ref. 9, and reaching 74.2% in Ref. 6. However, at least two papers reported no or almost no 9p21 LOH events in BE tissue.[7,37] It would be beneficial to identify a reason for such a huge discrepancy: cohorts of BE patients might have been exposed to different factors (absence/presence of gastroesophageal reflux disease (GERD), length of GERD, smoking status, etc.); intestinal mucosa samples might have been derived from either progressed or nonprogressed patients or affected by differences in the methodology used (publications reporting a higher rate of alterations tend to be published later). Overall, 59.1% of all analyzed BE samples contained 9p21 LOH (Table 2).

HGD was also assessed, with the following outcome: 62% of patients with HGD showed 9p21 LOH in Ref. 35, 75% in Ref. 37, and 78% in Ref. 33. Altogether, almost 68% of samples analyzed in three different studies were considered positive for 9p21 LOH alteration.

No differences in accumulation of 9p21 LOH in BE, HGD, and EAC cohorts were detected (Table 2), although it was shown that this genomic alteration was significantly associated with progression.[6] In another study, the 9p region was the most mutated locus in the nonprogressing patients, affecting 17% of this cohort, but at the same time it remains one of the most predictive factors of BE progression, affecting >50% of progressing patients.[38]

In summary, 9p21 LOH seems to be a very early event in the sequence of the BE $\rightarrow$ dysplasia $\rightarrow$ cancer progression pipeline, and thereby it cannot play the role of an ideal predictor of progression.[39] A significant fraction of BE patients carried this genomic alteration, and the ratio of the affected patients grows insignificantly in the HGD cohort but not in EAC patients.

In the mid-1990s, an extremely important discovery was made: the 9p21 locus has a highly unusual genomic organization.[40] *CDKN2A* contains two upstream exons, 1α and 1β, which are driven by two distinct promoters. Transcription generates alternative transcripts that share common downstream exons, 2 and 3 (Fig. 2, upper panel). Although two transcripts share two exons, the open-reading frames remain distinct in the shared exon 2, and this results in two absolutely distinct protein products. The transcript initiated from the proximal promoter (1α) encodes CDKN2A–p16INK4A, a member of the INK4 protein family. The second transcript, initiated from the upstream exon 1β, encodes CDKN2A–p14ARF (ARF stands for alternative reading frame).

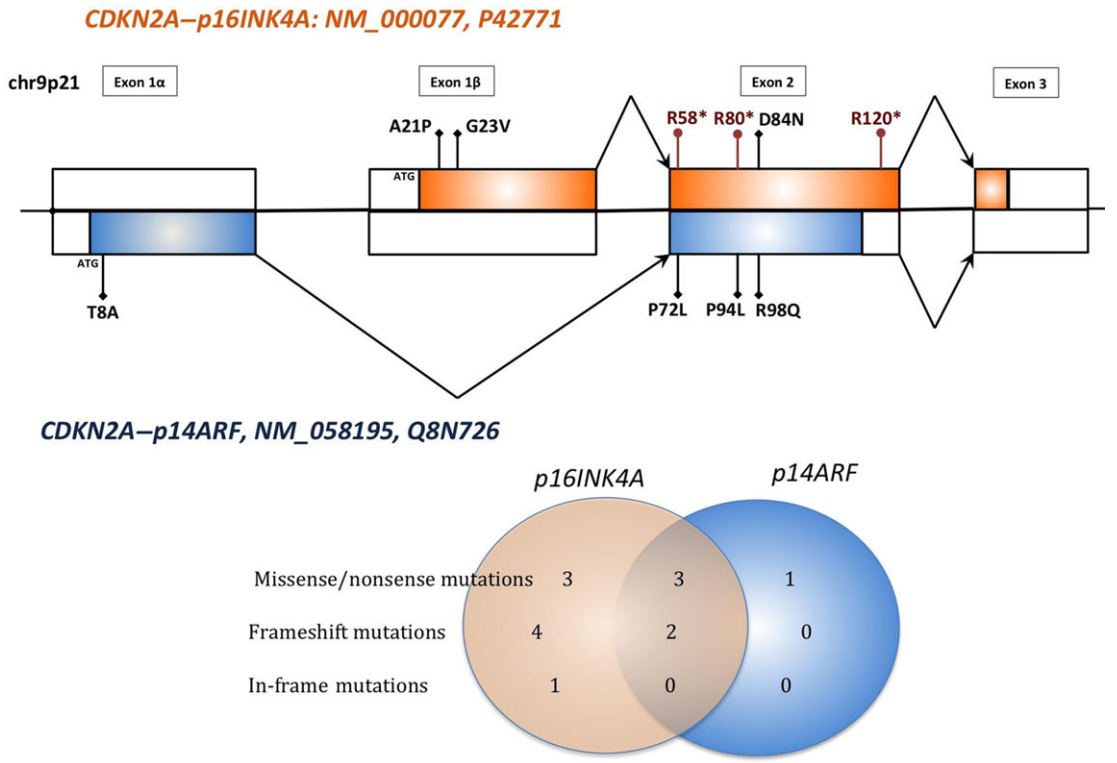

Figure 2. (Upper panel) Schematic view of the exon structure of *CDKN2A* demonstrating the types of alterations identified in Ref. 13. The locations of missense mutations and nonsense mutations are denoted by brown circles and black diamonds, respectively. (Lower panel) Venn diagram shows the overlap between coding mutations in *p16INK4A* and *p14ARF* transcripts.

P16INK4A controls the RB1-regulated G_1–S transition. P16INK4 ensures that RB1 remains in complex with the E2F transcription factors E2F1, E2F2, and E2F3 by inhibiting CDK4/6–cyclin D–mediated hyperphosphorylation of RB1.[41] These RB–E2F1–3 complexes recruit histone deacetylases to repress transcription of E2F1–3 target genes, leading to G_1 arrest. In the absence of inhibition by CDKN2A–p16INK4A, CDK4/6–cyclin D phosphorylates RB1, which results in the release of E2F1–3.[42] E2F1–3 then activates genes that are necessary for progression into S phase.[43]

CDKN2A–p14ARF functions as a potent growth suppressor: it activates TP53 by inhibiting MDM2, a very well-known inhibitor of TP53 activation. MDM2 ubiquitinates TP53 and triggers its degradation. TP53 activation leads to cell cycle arrest or apoptosis in cells.[44,45] Transcription of *CDKN2A–p14ARF* is directly stimulated by E2F1–3, resulting in upregulation of CDKN2A–p14ARF in response to aberrant mitogenic stimuli.[46]

CDKN2A–p16INK4A and CDKN2A–p14ARF are both important for cell cycle arrest in cellular senescence induced by oncogenic signaling or oxidative stress and are highly expressed in senescent cells.[47]

There is no doubt that homozygous deletion or LOH would encompass and inactivate (partially or completely) both proteins encoded in the 9p21 region. However, unsurprisingly, this is not the only existing mechanism of *CDKN2A* silencing in EAC. Epigenetic regulation and somatic mutations play essential roles in the inactivation of this gene, not only in EAC samples, but also in BE tissues. The frequency of *CDKN2A* inactivation by hypermethylation in Barrett's mucosa without neoplasia has been cited at 3%,[37] 7%,[29] 33%,[33] and 66%[35] (Table 2), although no methylation was detected in the normal squamous epithelium.[29,33,34] *CDKN2A* promoter hypermethylation is a frequent event in EAC: methylation was found in 36%,[34] 53%,[29] and 54%[48] of EAC cases in different studies, reaching as

high as 82% in Ref. 33. Methylation of CDKN2A in HGD samples is not significantly different from EAC but is significantly stronger than in BE tissue (Table 2).

Ironically, these publications only reported the methylation status of the *CDKN2A–p16INK4A* promoter. Only Vieth *et al.*[29] showed that hypermethylation of the *p14ARF* promoter is not an infrequent event in EAC, and, in 20% of EAC samples, this transcript was silenced by an epigenetic mechanism. Moreover, it looks like methylation can be independently regulated at the two promoters in *CDKN2A*.[49] For example, it was reported that *CDKN2A–p14ARF* promoter hypermethylation in hepatocellular carcinoma occurs independent of hypermethylation of *CDKN2A–p16INK4A*.[50]

A significant decrease in p14ARF expression with disease progression from normal epithelium to nondysplastic BE to EAC has been demonstrated.[51] In addition, only 25% of EAC samples showed detectable levels of p14ARF expression, whereas 80% of BE specimens and 100% of normal epithelium express this protein. Most likely, LOH, in combination with hypermethylation of the *p14ARF* promoter and somatic alterations, is responsible for this protein reduction. Patients positive for p14ARF had significantly better survival, with a very high hazard ratio of 10.85 (95% CI 3.24–36.37).[51]

Despite these complex mechanisms of CDKN2A downregulation, somatic single-nucleotide mutations and small indels still contribute significantly to CDKN2A disruption. After *TP53*, *CDKN2A* is the second most mutated gene in EAC.[13] However, this high frequency is the result of mutation accumulation in both proteins p14ARF and p16INK4A (Fig. 2, upper panel). For example, out of four missense mutations identified in EAC samples, only one is shared by both proteins, although it is translated into different outcomes (Fig. 2, upper panel): D84N in p16INK4A and R98Q in p14ARF. Two other mutations (A21P and G23V) located in exon 1β do not affect the coding region of p14ARF. On the contrary, p14ARF contains a mutation (T8A) in exon 1α that does not have an impact on the p16INK4A coding region.

Deleterious stop gains are accountable for one-third of all alterations in *CDKN2A* (6 out of 17: R58*, R80* in four different samples, and R120*). The incorporation of stop gains usually leads to significant damage to protein structure and function, but these *CDKN2A* mutations are only translated into stop codons in the *p16INK4A* transcript, whereas the *p14ARF* transcript is affected only by missense mutations (P72L and P94L) and one noncoding mutation in the 3′ UTR region at the same genomic location. As introduction of a premature stop codon is rarely ambiguous, truncated *p16INK4A* transcripts are most likely degraded. According to Mutation assessor, these mutations in CDKN2A–p14ARF have a non-neutral effect on protein functionality and might influence the TP53 activation pathway.

Small insertions and deletions are other sources of CDKN2A function disruption. Out of six frameshift deletions or insertions, only two are located in exon 2, shared between *CDKN2A–p14ARF* and *p16INK4A*, and these most likely affect the function of both proteins. Another four disrupt the protein sequence of p16INK4A but land either in intron regions or in the 3′ UTR region of *p14ARF* (Fig. 2, lower panel).

Nine percent of nondysplastic BE samples and 13.9% of HGD samples carried mutations in *CDKN2A*, including nonsense mutations and frameshift alterations.[17] These rates are comparable to the reported mutation frequency in EAC.

In summary, inactivation of *CDKN2A* in EAC can occur by deletion, methylation, and/or mutation accumulation, and not infrequently two or even three mechanisms are required to block CDKN2A protein function. *CDKN2A–p16INK4A* was shown to be totally inactive in >80% of EAC samples, meaning that most EAC neoplasms contain genetic and/or epigenetic alterations in this gene.[33] Seventy-five percent of EAC samples displayed undetectable levels of CDKN2A–p14ARF.[51] Notably, 9p21 LOH was detected in a significant portion of BE samples, suggesting that it is probably a very early event but not sufficient for cancer progression. The level of hypermethylation of the promoter of *CDKN2A–p16INK4A*, but not *CDKN2A–p14ARF*, is also very high, although the ratio of BE patients affected is not consistent across different studies (Table 2). Approximately every tenth BE patient harbors mutations in *CDKN2A*, but this rate is not stage specific and stays about the same in cancer samples.

In conclusion, both CDKN2A–p16INK4A and CDKN2A–p14ARF play undisputed roles in EAC development. Hopefully, in the future the genomic alterations, including somatic mutations or hypermethylation patterns, will be reported equally well for both *CDKN2A* transcripts.

E-cadherin in BE progression

E-cadherin (named for "epithelial calcium–dependent adhesion"), or CDH1, is a calcium-dependent cell adhesion glycoprotein that plays an essential role in building the adhesion junctions in epithelial cells. It is encoded in the 16q22 genomic region and is composed of five extracellular cadherin repeats called EC1–EC5, a transmembrane region, and a cytoplasmic tail that binds β-catenin.[52] Only the first extracellular cadherin domains participate in the implementation of adhesive binding connection.[53]

The maintenance of adult tissue architecture is largely dependent on the function of cadherins: cadherin molecules expressed on neighboring cells associate at the cell surface in a Ca^{2+}-dependent manner through homophilic interaction, and this mediates cell–cell adhesions. These junctional structures share a common organization: they contain transmembrane adhesive components bound to cytosolic adaptor proteins that serve as a link to the cytoskeleton. Suppression of CDH1 was registered in many epithelial cancers, such as breast cancer, hepatocellular carcinoma, and esophageal squamous cell carcinoma.[54–56]

The first report on CDH1 expression in EAC samples was published more than 20 years ago in 1994 and stated that the expression of E-cadherin was significantly lower in patients with dysplastic tissue compared with normal esophageal epithelium; a further reduction of its expression was observed in EAC samples.[57] In addition, the authors noticed that, although CDH1 was still expressed on the surface of the intestinal metaplasia cells, its molecular weight was altered: from a single strong band at 120 kDa in normal tissue to several bands at 120, 108, 90, and 45 kDa. The highest band was hardly visible, and the most stain was observed at 108 and 45 kDa. The authors hypothesized that it might be a sign of an impaired glycosylation or a truncated protein. It is notable that the molecular weight of CDH1, estimated based on amino acid sequence, is 97 kDa. It seems that two upper bands (120 and 108 kDa) were fully and partially glycosylated protein forms, respectively, whereas the 45-kDa band is most likely a byproduct of protein degradation. Moderately differentiated adenocarcinoma samples had strong bands only at 108 and 45 kDa; no normal 120-kDa bands were visible. Poorly differentiated adenocarcinoma failed to express any E-cadherin.[57]

E-cadherin has membranous locations in normal epithelial tissue.[57,60–62] The most drastic changes in CDH1 expression level and localization were detected between metaplastic and EAC tissue types.[61] Expression of E-cadherin in columnar metaplasia without dysplasia was similar to esophageal squamous epithelium,[61,62] and in only 16.6% of BE cases immunostaining for CDH1 was partially found in the cytoplasm.[59] The CDH1 expression pattern in EAC tissue was very heterogeneous and significantly reduced or even absent in the majority of EAC samples.[58,61–63] Significant reduction in immunoreactivity in dysplastic tissues in comparison to normal epithelium was detected in the studies in Refs. 59 and 62 but not in Ref. 61 (Table 2).

As was previously mentioned, downregulation of E-cadherin leads to reduced cell adhesion. So, it is not a surprise that aberrant localization and/or reduced expression occur during the neoplastic progression of BE, and poorly differentiated tumors showed the greatest reduction in immunoreactivity. The primary cause of changes in CDH1 molecular weight is not clear, and it is not known whether impaired glycosylation of the protein prevents it from being inserted into the cell membrane or whether cytosolic mislocalization causes impaired glycosylation and partial degradation.

The major known mechanisms of E-cadherin silencing in the epithelial cancers are (1) LOH in chromosome region 16q22;[64] (2) inherited germline mutations in CDH, like in hereditary gastric cancer;[65] and (3) methylation of 5′ CpG dinucleotides within the promoter region of E-cadherin.[55] All of these mechanisms are only partially applicable to *CDH1* silencing during BE progression.

No inherited germline variations in relation to BE and/or EAC have been reported so far. In addition, *CDH1* is rarely mutated in EAC.[13,66]

The normal methylation status of *CDH1* in HGD and EAC patients was confirmed by two independent studies.[67,68] Nevertheless, we cannot completely ignore this silencing mechanism in EAC: hypermethylation of *CDH1* was detected in 65–84% of EAC cases, indicating that epigenetic silencing might be at least partially responsible for *CDH1* downregulation.[66,69]

It was established that reduced expression of *CDH1* in EAC samples is associated with the overexpression of *Slug* (*SNAI2*).[70] SNAI2 is a C2H2-type zinc finger transcriptional factor. In the normal tissue, this protein triggers epithelial-to-mesenchymal transitions, allowing epithelial cells to migrate from their place of origin and form tissues, such as the mesoderm.[71] It is known that SNAI2 regulates the expression of *CDH1* in the normal epithelial cell model.[72] SNAI2 also might be responsible for the acquisition of invasiveness during tumor progression and recurrence.[73]

It was shown that *SNAI2* is significantly overexpressed in EAC tissue in comparison to BE tissue samples, and its expression inversely correlated with *CDH1* expression.[70] The authors did not propose any mechanism of *SNAI2* upregulation during BE progression, although it was pointed out that the overexpression of fibroblast growth factor (FGF), transforming growth factor β (TGF-β), and Wnt might play roles in the induction of *SNAI2* expression. EAC is not the only cancer type where inverse correlation between CDH1 and SNAI2 was observed.[73] Breast cancer is probably the best example where this pair was intensively studied.[74]

In addition, loss of E-cadherin expression is not only associated with cancer progression but also with tumor stage invasiveness. CDH1 protein expression is significantly correlated with disease-free and overall survival of the EAC patients.[75]

CDH1 has to be localized on the plasma membrane to be able to carry out its function as a major gatekeeper of the epithelial cell. Not only reduction or absence of CDH1 expression but also redistribution of this protein in the cell might serve as hallmarks of BE tissue progression. Alteration in CDH1 expression during sequential progression of BE tissue to cancer is a relatively late event. CDH1 abnormal expression was detected in majority of EAC samples, whereas only a small number (if any) of BE samples had similar pattern. Data on CDH1 expression pattern in HGD are very inconsistent and require further investigation.

CTNNB1 in BE progression

β-Catenin (CTNNB1) has dual functions, regulating cell–cell adhesion and gene transcription.[76,77] This single molecule executes multiple functions through its precise subcellular compartmentalization and strict regulation. The major aspect of its regulation is the ability of β-catenin to form mutually exclusive protein complexes with three different partners: E-cadherin, adenomatous polyposis coli (APC), and lymphocyte-enhancer factor 1 (LEF1).[76] The competitive nature of these interactions ensures that transcriptionally active β-catenin is not subject to degradation and that the β-catenin that is a part of the cell–cell adhesion complex is not transcriptionally active.

In normal tissue, Wnt signaling is inactive, and β-catenin is a part of the protein complex that helps to form adherens junctions. As described above, cadherins form the cell–cell junctional structures. While in the adherens junction, cadherin molecules are able to recruit β-catenin molecules, which bind to the cadherin intracellular domain (Fig. 3). In turn, β-catenin associates with α-catenin that directly binds to actin filaments. This supporting structure is essential for maintaining epithelial cell shape and proper function. Owing to E-cadherin, β-catenin in the normal epithelial tissue (including esophageal) is located exclusively at the plasma membrane and protected from degradation.[78] The absence of E-cadherin in *CDH1*$^{-/-}$ embryonic stem cells leads to the accumulation of free β-catenin in the nucleus, that is, the absence of E-cadherin mimics Wnt signaling.[76] The concentration of free-form cytosolic β-catenin is strictly regulated by the so-called β-catenin destruction complex (Fig. 3). This complex includes two kinases, casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3A or GSK3B). Cytosolic β-catenin is subject to phosphorylation first by CK1 and then by GSK3. This targets β-catenin for ubiquitination and degradation by cellular proteasomes. GSK3 is able to function only in complex with its docking station AXIN1. AXIN acts as a scaffold protein bringing together GSK3 and its substrate β-catenin. The last member of the β-catenin destruction complex is APC. APC is a huge protein that has several docking sites for β-catenin–CK1–GSK3–AXIN complexes. Thus, with the loss of one or several elements of this destructive complex, the cell has an excess of free β-catenin and functions as if it is under continuous Wnt signaling.

Upon receiving Wnt signaling, β-catenin levels increase owing to stabilization against proteolysis. Cell stimulation with Wnt ligand (or downregulation of E-cadherin) leads to LEF1/β-catenin–based transcriptional activation. Unphosphorylated β-catenin can migrate into the nucleus and,

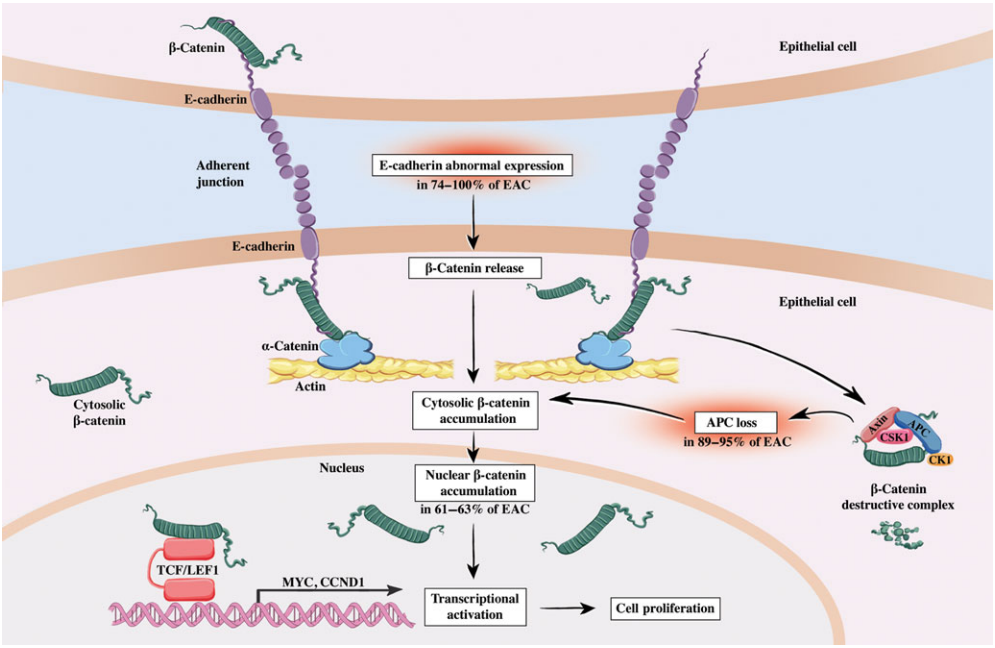

Figure 3. During the progression of Barrett's esophagus to cancer, CDH1/E-cadherin (purple protein molecule) is going through significant changes: its expression is either reduced until it is hardly detectable or it is disorganized. In this case, CTNNB1/β-catenin (green protein) loses its docking shelter and is released into the cytoplasm. In the normal/metaplastic cell, cytosolic β-catenin is quickly degradable by the so-called β-catenin destructive complex, but, if some elements of this complex are missing (like APC in the majority of EAC samples), β-catenin accumulates in the nucleus, which leads to the transcriptional activation and expression of its targets (like *MYC* and *CCND1*) and cell proliferation induction.

in interaction with members of the TCF/LEF1 transcription factor family, activate transcription of *MYC*, *CCND1*, and other genes and promote cellular proliferation.[79,80] Nuclear accumulation of β-catenin is a major indicator of β-catenin transcriptional activation.[81]

APC, whose function is to downregulate β-catenin, is an important component of the Wnt signaling pathway. In the absence of Wnt signaling, APC causes complex degradation of β-catenin, preventing its accumulation in the cytoplasm.

In normal esophageal squamous epithelium, β-catenin is located predominantly at the plasma membrane, with some cytoplasmic staining but no nuclear accumulation.[59,61,78,82] A predominantly membranous localization of β-catenin was also shown for BE.[82] In 3–13% of EAC samples, β-catenin was not detected at all,[78,83] leading to the hypothesis that loss of E-cadherin expression results in β-catenin release into the cytoplasm and subsequent phosphorylation and degradation through its regulation machinery (Fig. 3). Seven to 38% of EAC samples retain a normal β-catenin distribution and expression pattern;[78,83,84] however, in the remaining EAC samples (61–63%), strong nuclear accumulation of β-catenin was observed.[83,84] This might happen when cytosolic β-catenin is protected against degradation, either by (1) β-catenin mutation at the phosphorylation site and/or (2) deletion or downregulation of APC and/or AXIN (in this case, β-catenin migrates into the nucleus and triggers the expression of its targets). The S37 position in β-catenin was mutated in ∼2% of EAC samples.[13] This specific mutation eliminates a substrate for GSK-3 kinase and prevents β-catenin from degradation.[85] Methylation of the APC promoter and/or LOH of the 5q21 region where this gene is encoded are the major mechanisms of β-catenin-dependent transcriptional activation. LOH was detected in 0% of normal esophageal epithelium,[86] in 0% of BE samples distant from dysplasia,[7,87] in 27.2% of HGD samples,[88] and in 29–54.5% of EAC samples.[7,86,88] These data indicate that the accumulation of LOH at the APC locus occurs during the

whole sequential progression from normal squamous epithelium to EAC (Table 2). Hypermethylation of the APC promoter region affects almost all (78–95%) EAC samples,[48,68,89] whereas it was not detected in normal esophageal epithelium.[67,89] Methylation patterns of the APC promoters were detected in 39% of Barrett's metaplastic tissue samples, but this result was difficult to interpret, as a big portion of the normal gastric tissue samples carried the same pattern.[89] Somatic mutations in APC are rarely detected in EAC (~4–5% of samples).[13,90]

Thus, β-catenin nuclear accumulation is common (affecting more than 60% of EAC samples) and represents a relatively late event in BE progression (as no BE tissue was shown to be affected by β-catenin nuclear accumulation or deletion of APC, its major regulatory partner). No difference in β-catenin staining was observed in the nonprogressing BE samples versus those that progressed within the next several years (minimum 6 months of follow-up).[23] These data support the "late arising" hypothesis, that β-catenin nuclear accumulation is an indication of progressing tissue. The principal outcome of β-catenin accumulation in the nucleus is its transcriptional activity. CCND1 expression (one of the major transcriptional targets of β-catenin) was present in almost all tissues (including BE), but high expression (>50% cells positive) was displayed mostly in HGD and EAC (46.7% and 42.3%, respectively).[91] MYC is another major transcriptional target of β-catenin. Similar to CCND1, immunochemical staining showed weaker nuclear expression for MYC in BE and low-grade dysplasia samples compared to HGD and EAC.[92]

Surprisingly, the presence of abnormal β-catenin (including loss of membranous staining and the appearance of nuclear staining) in tissue samples of EAC patients correlates with better survival.[84] The reason behind this observation is not clear, but it is consistent with similar observations in colorectal cancer, where obese patients positive for nuclear β-catenin had significantly better overall survival,[93] and in medulloblastoma, where the WNT signature molecular subgroup has the best prognosis.[94]

GPX3 and NOX5 in BE progression

Surprisingly, the last two proteins identified by the text-mining approach belong to the same biological pathway (Table 1): they both regulate the levels of reactive oxygen species (ROS) in cells. ROS are byproducts of the metabolism of molecular oxygen. They include superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$). ROS are normally present in all aerobic cells, and their balance is tightly regulated. DNA damage by ROS is well known as one of the major causes of cancer.[99,100] ROS may damage DNA, RNA, lipids, and proteins, leading to increased mutation and altered functions of enzymes and other proteins (e.g., activation of oncogene products and/or inhibition of tumor suppressor proteins). Not surprisingly, elevated ROS levels have been reported in BE.[101] ROS are important factors mediating acid reflux–induced DNA damage and are key mediators linking inflammation and Barrett's carcinogenesis.[102,103]

The first gene, *GPX3*, encodes a selenoprotein, a part of the glutathione peroxidase family responsible for the detoxification of hydrogen peroxide (H_2O_2). GPX3 is secreted primarily by kidney proximal convoluted tubule cells and carries antioxidant activity in the extracellular space (primarily in the plasma). GPX3 is one of a few selenoproteins in human cells with a selenocysteine in the catalytic center. Expression of GPX3 was confirmed in human gastrointestinal tissue, including the esophageal squamous epithelium.[95] GPX3 utilizes glutathione, thioredoxin, and glutaredoxin as electron donors to reduce a broad range of hydroperoxides.[96]

Conversely, the second gene, *NOX5*, which encodes the superoxide-generating protein NOX5, is a member of the NADPH oxidase family. NOX5 is expressed in some tissues where ROS production plays a role in the stimulation of the immune system, signal transduction, and modification of the extracellular matrix, but NOX5 is not a regular housekeeping protein of the esophageal squamous epithelium.[97,98]

GPX3 mRNA expression level was reduced in 90.5% of EAC samples in comparison to normal squamous tissue.[104] In addition, the same paper showed that *GPX3* promoter hypermethylation was detected in 61.9% of BE samples, 81.8% of dysplastic samples, and 88.2% of EAC samples. *GPX3* hypermethylation patterns were detected in about 17% of the normal squamous epithelium samples. The authors argued that this result was overestimated and most likely due to cross-contamination

with BE tissue. Moreover, GPX3 protein expression was measured using a western blotting approach and found to be inversely correlated with *GPX3* promoter hypermethylation. In conclusion, intestinal metaplastic cells are gradually but consistently losing their natural mechanism of ROS detoxification. More than half of the tested EAC samples in another, more recent work demonstrated absent to weak staining for GPX3, whereas all normal squamous tissue showed medium to strong signal for this protein.[105] A significant inverse correlation between DNA methylation and mRNA expression for *GPX3* was confirmed as well.

GPX3 is a selenium-containing protein, and this microelement is required for normal functioning of GPX3. It was shown that patients with BE had significantly lower plasma concentrations of selenium,[106] although a population-based study failed to show that a diet containing selenium has a protective effect against cancer progression in BE patients.[107] Moreover, BE patients with high serum selenium level (upper three quarters) had significantly lower risk of LOH of *TP53*.[108] No association with LOH of *CDKN2A* was found.[108]

The above-mentioned findings indicate that (1) progression of the BE patients to dysplasia and cancer is associated with a loss of *GPX3* expression due to hypermethylation; (2) this loss most likely causes an elevated ROS accumulation and DNA breakage; and (3) high levels of serum selenium might serve as a protective factor against LOH in *TP53* and, as a consequence, cancer progression.

The exact mechanism whereby the *GPX3* promoter is hypermethylated during BE progression is not clear, but it is known that cigarette smoking (another risk factor for EAC development) can induce profound epigenetic changes in a number of tumor suppressor genes, including *GPX3*, in oral squamous cell carcinomas.[109]

The unusual behavior NOX5 in BE was predominantly discovered and described by Cao and colleagues.[110–113,117–119] During the last 10 years, Cao and his team have published over 10 papers describing NOX5 and its role in BE progression.

One of the first observations was that the level of *NOX5* mRNA was significantly higher in BE with HGD than in normal mucosa and BE without dysplasia, although the number of tested samples was relatively low.[110] In addition, this paper confirmed that an acid-induced upregulation of NOX5 was responsible for H_2O_2 overproduction. In addition to H_2O_2 overproduction, overexpression of NOX5 in Barrett's cells significantly increased thymidine incorporation, suggesting that cell proliferation was enhanced.[111] Platelet-activating factor, one of the inflammatory mediators, as well as activation of STAT5, was reported to be a connecting link between the acid treatment and NOX5-dependent H_2O_2 production.[112] The work published several years later described a stunning correlation between acid treatment, H_2O_2 overproduction (through NOX5 overexpression,) and *CDKN2A* promoter hypermethylation and downregulation of its expression.[113] Although the study was performed in cell lines and never confirmed in clinical samples, that was a first attempt to connect genomic alterations that are known to be vital for cancer progression with expression fluctuations of cell proteins occurring under the influence of acid treatment. The authors hypothesized that acid reflux may play a significant role in the progression from BE to EAC. This hypothesis is consistent with the observation that treatment with proton pump inhibitors (PPIs) reduced the risk of cancer progression in BE patients when compared with patients who have never received this kind of treatment.[114,116] However, contrasting these studies, a significant preventive effect of PPIs with respect to the risk of HGD or EAC was not observed in Ref. 115.

Finally, Cao's group published a mechanism explaining the relationships between NOX5 activation and CDKN2A inactivation.[117] They introduced another key element in EAC progression, DNMT1, a methyltransferase that regulates tissue-specific patterns of methylated cytosine residues and is predominantly responsible for methylation of CpG islands in DNA (Fig. 4). NOX5 overexpression in acid-stimulated cells is responsible for increased promoter activity and upregulation of DNMT1, which is in turn responsible for the hypermethylation of the *CDKN2A* promoter (Fig. 4).[117] This conclusion was formed on the basis of the following experimental results: (1) knockdown of DNMT1 prevents acid-induced *CDKN2A* promoter hypermethylation in a human Barrett's cell line, (2) *DNMT1* mRNA expression level is significantly higher in the EAC tissues in comparison to normal squamous esophageal and Barrett's mucosa tissue, and (3) overexpression of NOX5 significantly increased *DNMT1* promoter activity.

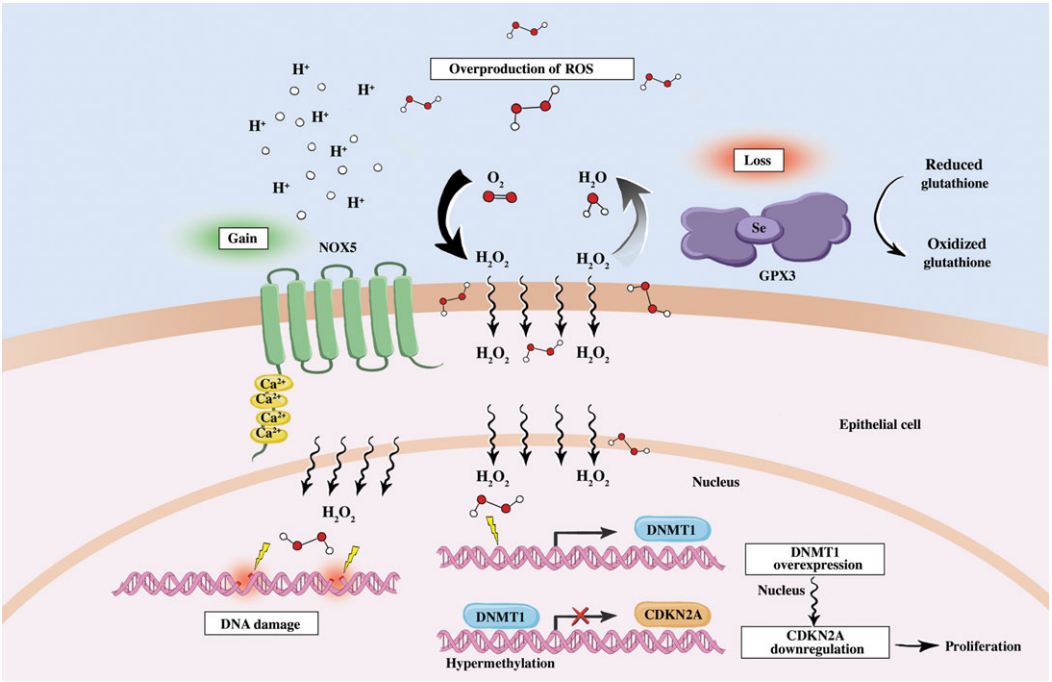

Figure 4. Exposure of the esophageal mucosa to gastric and bile acids leads to the accumulation of reactive oxygen species (ROS). Most likely, this is achieved by gradual downregulation of GPX3, a protein that is responsible for hydrogen peroxide detoxification, and upregulation of NOX5, a superoxide-generating protein. This leads to DNA breakage and *CDKN2A* hypermethylation, thereby contributing to the progression from BE to EAC.

These results hint that there is probably at least an indirect link between CDKN2A and DNMT1. In their conclusions, the authors hypothesized that an NFKB1 element might be missing in this chain.

In his latest paper on NOX5 mechanisms, Cao proved that acid-induced activation of the Rho kinase ROCK2 is a link connecting NOX5 upregulation and H_2O_2 overproduction.[118]

In addition, it was shown that acid-dependent NOX5 stimulation is not only a cause of *CDKN2A* promoter hypermethylation but also significantly contributes to acid-induced DNA damage.[119] Pretreatment with NOX5 inhibitor significantly reduced this effect.

In conclusion, we can hypothesize that downregulation of GPX3 with simultaneous upregulation of NOX5 might lead to a synergetic effect in elevation of ROS in the BE tissues and mediate acid-induced DNA damage, which, in turn, might lead to the increased accumulation of genomic alterations. This model is consistent with the observation that the somatic mutation load in DNA from benign intestinal metaplasia samples is comparable to that of breast and prostate cancer samples.[18] Most of these DNA breakages are harmless, but a chance to introduce a deleterious mutation into an oncodriver (like *SMAD4*, *TP53*, or *CDKN2A*) and start a progression process is higher than in the tissue with normal levels of ROS. At the same time, we have to keep in mind that the majority of the experiments with NOX5 were done in cell line models, which are usually simplified models, have their own limitations, and cannot always be correctly extrapolated into a mechanism of the human disorder.

Conclusions

BE is a relatively common metaplasia that confers a significant risk for the development of EAC. The prevalence of BE in Sweden (as a good representative of Western countries) was estimated at the level of 1.6% (95% CI 0.8–2.4) in the adult population aged 20–80 years.[120] It means that, in the Western world, 16 adults out of every 1000 people are at risk of one of the deadliest cancers. Although the rate of BE patient progression to cancer is very low (~0.22% of BE cases will progress to EAC per year),[121] we

cannot ignore it, as the survival rate of the EAC patients is devastating (22% over a 5-year period) and overall median survival is poor (10 months).[122]

This review was not intended to provide an overview of the potential biomarkers of BE progression, but rather to give an unbiased analysis of the published data on the genes that are deeply involved in BE progression, as well as to find possible functional connections between prioritized genes.

It is widely accepted that carcinogenesis in BE occurs in a stepwise manner from metaplasia to low-grade dysplasia, HGD, early adenocarcinoma, and, finally, metastatic carcinoma.[123] This makes it an excellent model system for investigation of the multistep nature of tumorigenesis. A specific combination of genomic, transcriptomic, and epigenetic changes is responsible for this multistep transformation of normal squamous esophageal epithelium to BE, dysplasia, and adenocarcinoma. We reasoned that text mining is a comprehensive integrated approach to prioritizing the genes responsible for BE progression to enable the elucidation of the mechanism of cancer progression. It is remarkable that a text-mining approach prioritized genes implicated in a wide range of biological pathways: DNA repair (*TP53*), cell cycle (*TP53*, *CDKN2A*), Wnt signaling (*CTNNB1*), cell adhesion (*CDH1*), and detoxification (*GPX3*, *NOX5*). Progression of BE to EAC is thought to be an unpredictable process, and the time of progression is variable, most likely because a number of (at first sight) unrelated mechanisms are involved in tumorigenesis.

Owing to unknown reasons, in the metaplastic cells exposed to an acidic environment, the natural mechanism of protection against reactive molecules is getting blocked by downregulation of GPX3 and upregulation of NOX5. Acid-stimulated NOX5 is a source of overproduction of ROS in BE that leads to increased accumulation of mutations in general and, in particular, in *TP53*. It prevents cell apoptosis due to DNA damage, and, as a consequence, clones with altered *TP53* get an evolutionary priority in survival. Another side effect of NOX5 upregulation is hypermethylation of the *CDKN2A–p16INK4A* promoter and, as a consequence, enhanced proliferation. An incompletely understood mechanism of CDH1 downregulation leads to the remarkable cell shape change and release of β-catenin into the cytoplasm and eventually to its nuclear accumulation. This (together with CDKN2A elimination/ablation) leads to the abnormal proliferation through expression of MYC and CCND1. The presence of one or several of these mechanisms is sufficient for cell progression and dysplastic changes.

All six genes prioritized by the text-mining approach accumulate genomic, transcriptomic, and/or proteomic alterations in the large subpopulation of EAC patients, and this subpopulation correlates well with progression stage. It denotes that each of the six genes plays a certain role in BE progression. In addition, all six genes are functionally interrelated, which might indicate that they can serve as an essential core for BE progression.

In spite of the fact that ~70% of EAC samples have a damaged copy of *TP53*, some progressed tissue still can bear wild-type copies of *TP53*. In contrast, most BE patients will never have EAC in their lifetime,[124] but, for example, hypermethylation of *GPX3* and 9p21 LOH was observed in a large subpopulation of BE patients. It gives us a hint that (1) one genomic alteration is not sufficient to initiate cancer progression and (2) the progression mechanism of BE to cancer is more complex. There are a number of additional factors that might influence the time and/or steps of progression, including inflammation via activation of NF-κB,[125] deregulation of telomerase expression through TERT upregulation,[126] enhanced angiogenesis via VEGFA upregulation,[127] apoptosis prevention via BCL2 overexpression,[128] and cell signaling disruption through, for example, genomic alterations in SMAD4,[17] among others. This could be accompanied by aneuploidy, clonal expansion, and genomic instability. Only incorporation of all these (and maybe other unknown) factors will produce a full image of BE progression.

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Conflicts of interest

The author declares no conflicts of interest.

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