Abstract. The F-box proteins (FBPs) in esophageal tumorigenesis are pivotal as they govern a broad array of basic physiological responses including cell growth, cell death and DNA damage repair. Esophageal cancer (EC) is a common and highly aggressive cancer worldwide. Aberrant stabilization of crucial proteins participates in esophageal tumorigenesis. Recently, growing evidence has shown that FBPs play a critical role in oncogenesis, invasion, metastasis and prognosis assessment of EC. In this review we summarized published data on the roles of known FBPs, their respective substrates and the key signaling pathways, in the development of EC, aiming to uncover new ways for the rational design of targeted therapies in EC.

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1. Brief introduction to EC

Esophageal cancer is the sixth leading cause of cancer-related mortality and the eighth most common cancer with more than 455,800 new esophageal cancer cases and 400,200 deaths according to global cancer statistics (1). The incidence rates of EC have been increasing (2). Tobacco use and alcohol consumption are the main risks for EC (3). The two main types of EC are squamous cell carcinoma (SCC) and adenocarcinoma. In the highest-risk area, which stretches from Northern Iran through the Central Asian republics to North-Central China, 90% of cases are squamous cell carcinomas (4). Despite many advances in EC treatment, over the last several decades, the prognosis for patients with advanced EC remains poor. The overall 5-year survival ranges from 15% to 25% (5). Poor outcomes in patients with EC are related to diagnosis at advanced (metastatic or disseminated) stages. New treatment methods and strategies are necessary. Genome instability with alterations in the expression of proteins is a hallmark of cancer (6). Extensive study in this field has led to a better understanding of the molecular mechanism by which FBPs regulate cellular processes and of how their deregulations contribute to carcinogenesis. In this review, we summarize the related FBPs involved in EC, focusing on the function and the substrates of each related F-box protein in EC.

2. The ubiquitin-proteasome system

Intracellular protein degradation plays an important role in the regulation of the cell cycle, signal transduction, and disposal of improperly folded proteins. The ubiquitin-proteasome pathway is the major system for protein degradation (7). Ubiquitin Proteasome System (UPS) is an evolutionary conserved protein degradation mechanism that is involved in various physiological responses such as cell cycle control, DNA replication, transcription, and cell signaling (8,9). Ubiquitin (Ub) is a 76-amino acid protein that is covalently conjugated to a lysine residue in proteins. Similarly to phosphorylation, ubiquitination is reversible and linked to deubiquitination.

Ubiquitin-proteasome pathway. Ubiquitin attachment to substrates is accomplished by the coordinated activity of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin-protein ligase (E3). The degradation of proteins by the UPS is mainly comprised of three steps. The first step is that Ub is activated by the E1 enzyme by creation of the thioester linkage between Ub and the cysteine residues of E1 in an ATP and Mg^{2+}-dependent manner. Then, E2 accepts the activated ubiquitin molecule from E1 and with a help from an E3 ubiquitin ligase transfers

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it to the lysine residue of a target protein. In the third step, the ubiquitin proteins are recognized and degraded by 26 proteasome to several small peptides (Fig. 1). Ubiquitin-mediated proteolysis is instigated by the attachment of K48 polyubiquitin chains to substrates, which provides a signal for recognition and degradation by the proteasome. E3 ubiquitin ligases are a large family of proteins that are engaged in the regulation of the turnover and activity of many target proteins. E3 determines the target protein specificity, and it is the reason why the deregulation of E3 ligase often leads to cancer development (10).

Ubiquitin enzymes. Human genome encodes 2 E1s, approximately 30 E2s and more than 600 E3s (11). E3 ligases determine the substrate specificity for ubiquitylation and subsequent degradation. The largest family of E3 ubiquitin ligases is the RING type SCF E3 ligases (RING-E3s). RING-E3s can further be subdivided into RING-containing single peptide E3s, Cullin-RING ligases (CRLs) and their derivatives, U-box E3s (11). CRLs complex is the largest family of RING-E3s in the family, which contains eight members: namely CRL1, CRL2, CRL3, CRL4A, CRL4B, CRL5, CRL7 and CRL9. Within the eight CRLs, CRL1 is so far the best-characterized member, which is also designated as the SKP1-cullin 1 F-box protein (SCF). The best characterized of CRLs family E3s is Skpl-Cullin1 F-box (SCF). The best characterized of CRLs family E3s is Skpl-Cullin1 F-box (SCF) complex (12,13). All SCF E3 ligases share a similar structure in which CUL binds to SKP1 and an F-box protein at the N-terminus and a RING protein RBX1 or RBX2 at the C-terminus. SCF complex consists of four structural and functional components: Skp1 (adaptor protein), Cul1, ROC1/Rbx1 (RING protein), and F-box protein, the latter of which serves as a receptor for target proteins.

3. The F-box protein families

F-box is a widespread protein motif of ~40-50 amino acids and it functions as a site of protein-protein interaction. F-box proteins are categorized within three families based on the recognizable domains beyond the F-box domain, which comprise the Leu-rich repeat (L) family, WD40 domain (W) family and the F-box only (O) family (Fig. 2). So far, 69 FBPs were identified in the human genome (14) and newer members of the F-box protein family are being discovered. Extensive studies have been heavily focused on only four FBPs SKP2, FBXW7, β-TrCP1, β-TrCP2, and the other FBPs largely remain functionally mysterious.

The FBXW family. The FBXW family is composed of 10 proteins, all the members contain WD40 repeat domains. Proteins involved in protein-protein interaction and containing WD40 repeats comprise the first class of FBPs (FBXWs). There are 10 family members of FBXWs. This class mainly targets proteins involved in cell cycle regulation and tumorigenesis and thereby modulating their outcome. The typical representatives of FBXW family are βTrCP1 (FBXW1), βTrCP2 (FBXW11), hCdc4 (FBXW7). The roles of β-TrCP in cancer differ according to its substrates. Loss of FBXW7 has been found in many cancers and it is associated with poor prognosis. FBXW4 is mutated, lost and underexpressed in a variety of human cancer cell lines and clinical patient samples (15). FBXW8 is the only known F-box protein to form an E3 complex with Skp1, Rbx1 and Cul7, which does not bind SKP1 alone but selectively interacts with Skp1-FBXW8 heterodimer (16).
The FBXL family. Some F-box proteins harbor an F-box and leucine-rich repeats (LRRs). For this reason, the 22 FBPs with LRRs are designated as FBXLs. These motifs are 20-29 residue sequences that are frequent in many proteins providing a structural framework for protein-protein recognition mechanism. SKP2 (also known as FBXL1) can serve as an exemplified family member that has been comprehensively studied and well characterized with respect to its substrates. SKP2 is overexpressed in various types of human cancers, which supports its role as a proto-oncoprotein. FBXL10 over expression was observed in human pancreatic cancer (17). FBXL5 inhibited the metastasis of gastric cancer (18).

The FBXO family. FBXO proteins do not possess either WD40 or LRR domains, but usually have other protein-protein interaction domains such as zinc-finger, carbohydrate interacting (CASH), proline-rich domains, Sec7, cyclin-box, Traf-domain-like and calponin homology (CH) (19). FBXO family is the most diverse subfamily of FBPs. Those proteins have other different or unknown domain in the C-terminal region. There are 37 members in the FBXO family. Currently, emerging experimental and clinical data have begun to reveal some interesting biological functions on FBXO proteins. FBXO5, an anaphase-promoting complex/cyclosome inhibitor, can control tumor cell proliferation. FBXO5 overexpression causes mitotic catastrophe and genomic instability and potentially contributes to tumorigenesis (20). FBXO11 targets BCL6 for degradation and functions as a tumor suppressor in diffuse large B-cell lymphomas (21).

4. The emerging roles of SCF<sup>skp2</sup> E3 ligases in EC

SKP2, located on 5p13 chromosome, was discovered in 1995 by Beach and his collaborators (22). The SCF<sup>skp2</sup> E3 ligase contains at the N-terminus an F-box domain which facilitates its binding to SKP1 and CUL1 and at the C-terminus a leucine-rich repeat domain for substrate recognition. SKP2, S-phase kinase associated protein 2, plays a crucial role in cell cycle progression by promoting the degradation of many key regulatory proteins, including p27, p16, p21, p57, E2F-1, TOB1, RBL2, cyclin D/E, BRCA2, FOXO1, RASSF1A. As the majority of the substrates are tumor suppressor proteins, it can be asserted that SKP2 functions as an oncogene (23-26). P27 is a negative regulator of the cell cycle that plays an important role in tumor suppression. Loss of p27 results in uncontrolled proliferation and promotes tumor progression (20,27). Taken together, these findings indicate that the SKP2-mediated reduction, as a result of enhanced degradation of tumor suppressor p27, contribute to the development of cancers including esophageal cancer (28). Therefore, the p27 degradation inhibitors present an attractive target for drugs. The involvement of SKP2 overexpression in metastasis has been reported in many tumors including melanoma (29), nasopharyngeal carcinoma (30), pancreatic cancer (31), breast (32) and colorectal cancer (33). Collectively, these data suggest SKP2 to be a proto-oncoprotein.

The involvement of SKP2 as a common driving factor in carcinogenesis has been proven. Fukuchi et al (24) first elucidated the role of SKP2 in tumor progression, in which they suggested that SKP2 might be a prognostic factor in early stage ESCC. They analyzed SKP2 and p27 expression in surgical specimens obtained from 32 patients, and they found that SKP2 expression showed an inverse location and correlation to p27 expression in early compared to advanced ESCC. There was an inverse relationship between SKP2 and p27 in 6 ESCC cell lines, but not cyclin E, cyclin D1 and E2F-1. DNA amplification is one of the mechanisms activating SKP2 gene in ESCC. Amplification and elevated expression of SKP2 was
correlated significantly with tumor stage and positive lymph node metastasis in ESCC (34). Downregulation of SKP2 by antisense treatment induced apoptosis and inhibited invasion and migration in lung cancer cells (35). Wang et al. (36) found that SKP2 expression levels was increased in ESCC tissues. Elevated expression of SKP2 correlated significantly with tumor stage and positive lymph node metastasis, and high expression of SKP2 promoted the radioresistance of ESCC cancer cells in part through Rad51 pathway. These alterations in the various ways of the carcinogenesis appeared in different stages of EC. The expression of SKP2 protein increased during esophageal squamous cell cancer progression from normal esophageal tissues to esophageal intraepithelial dysplasia (EID) and ESCC, which indicated SKP2 as a potential diagnostic mark in clinical settings (37). Liang et al. (38) showed that SKP2 expression was not correlated with lymph node metastasis, but correlated with local tumor invasion, which was not consistent with the observation of Wang et al. (36). SKP2 expression might be a new prognostic biomarker for tumor recurrence in ESCC patients.

Molecular mechanisms by which SKP2 induces tumor growth have not been fully elucidated. Some studies on the relationship between PI3K/Akt pathway and SKP2 have been reported. Akt is a downstream molecule of PI3K in response to growth factor stimulation, and activated Akt promotes cell survival by suppressing apoptosis (39). PI3-K/Akt pathway leads to elevated SKP2 expression and subsequent enhanced p27 destruction in human cancers. Inhibition of Akt1 activity in breast cancer cells could downregulate SKP2 expression (40). SKP2 is the main determinant in the PI3K/Akt-dependent regulation of p27(kip1) in the prostate cancer cell lines PC3 and DU145 (41). Reichert et al. (42) showed that in pancreatic ductal adenocarcinoma cells, PI3K/Akt signaling was linked to SKP2 gene transcription via control of a cis-acting element, E2F1, binding to the proximal human SKP2 gene promoter. Whereas, the degradation of E2F1 in S-G2 phase was mediated by SCF<sup>kpc</sup> complex (43). Others have also reported that PI3K/Akt signaling controls SKP2 transcription in different cellular systems (44). However, whether SKP2 also can affect the PI3K-Akt pathway still remains unclear. Wang et al. (34) showed that decreased SKP2 reduced pAkt expression, and that the PI3K/Akt pathway is the downstream target of SKP2. These results suggest a possible negative feedback loop between PI3K/Akt and SKP2 that may help to maintain the balance between cell survival and apoptosis. Further research exploring the molecular mechanisms by which SKP2 affects the PI3K/Akt pathway awaits further investigation.

5. The emerging roles of SCF<sup>FBXW7</sup> E3 ligases in EC

FBXW7, F-box and WD40 repeat-domain-containing 7, is an F-box protein that is responsible for substrate recognition by an SCF (Skp1-Cul1-F-box protein)-type ubiquitin ligase complex. FBXW7 was first identified in budding yeast in 1973 (45). FBXW7 is localized to chromosome region 4q32 and has three isoforms (FBXW7α, FBXW7β, FBXW7γ) (46). All three isoforms contain conserved interaction domains in the C-terminal and various isoform-specific domains in the N-terminal region. FBXW7 has pivotal roles in cell division, growth, and differentiation. Burgeoning amounts of literature strongly supported FBXW7 was a tumor suppressor in human cancers based on the following evidence: i) FBXW7 low expression was frequently found in various human cancers (47). ii) FBXW7 mediates the degradation of several major oncoproteins such as cyclin E (48), c-Myc (49), Notch (50) and c-Jun (50), which function in proliferation, differentiation, apoptosis, and metabolism. iii) Decreased FBXW7 protein is associated with poor prognosis as well as tumor metastasis (51-55). iv) FBXW7β is a p53 target gene and p53 mutations may reduce FBXW7 expression (56).

Furthermore, loss of FBXW7 in cancer cells might promote resistance to taxol and ABT-737 (57,58). These observations suggest that by upregulating FBXW7, drug resistance could be reversed. It is worth mentioning that most studies focus on discovering the ubiquitin targets of FBXW7 ubiquitin ligase pathway.

Increased number of substrates have deepened our understanding of the diverse oncogenic pathways that FBXW7 regulates. It is widely accepted that the FBXW7 gene mutations and allelic loss are the main mechanisms which downregulate FBXW7 protein expression and its tumor suppressor functions in cancer. Proteasome-mediated FBXW7 protein degradation is another mechanism (59,60). Over the past 5 years, our understanding of the FBXW7 has increased enormously. The first to explore FBXW7 in EC was Sterian et al. (61), they found that 54% of the esophageal adenocarcinomas showed allelic deletion in the chromosome 4q region. This was confirmed by exome sequencing on 113 ESCC tumor-normal pairs. The link between the expression of FBXW7 protein and prognosis has been verified in many cancers. In ESCC, decreased FBXW7 protein level may contribute to tumor progression and local invasiveness (62). In support of this notion, FBXW7 mRNA was significantly lower in ESCC cancer tissues than in non-cancer tissues, which is correlated with poor prognosis in ESCC (63). RNAi-mediated knockdown of FBXW7 in ESCC cells promotes proliferation in ESCC cell line KYSE70 (63). FBXW7 expression is under the control of several oncogenic micro-RNAs such as miR-27, miR-92, and miR-223 in numerous cancers. Overexpression of miR-223 increases genomic instability by modulating expression of FBXW7 (64). There was a significant inverse relationship between the expression levels of miR-223 and FBXW7 protein in ESCC, which indicated FBXW7 as a functional downstream target of miR-223 (54).

6. Other FBPs (β-TrCP, FBXL19, FBXO4, FBXO31 and FBXO32) involved in EC

FBPs as tumor suppressors in EC

FBXO32, FBXO32, known as atrogin-1, was firstly discovered by Gomes et al. (65) involved in muscle atrophy (66). FBXO32 may be a functional tumor suppressor in cancers. FBXO32 in tumors was significantly lower than that in corresponding normal tissues and it was associated with TNM stage, depth of invasion, distant metastasis or recurrence. While, the methylation frequency of FBXO32 in tumor tissues was significantly greater than that in corresponding normal tissues (67). Overexpressed FBXO32 induces apoptosis of the ESCC cell line TE13 (67). The frequency of FBXO32 methylation in gastric cardia adenocarcinoma (GCA) tumor tissues was significantly higher than that in corresponding normal
tissues and was associated with TNM stage, pathological differentiation, distant metastasis (68). The methylation status of FBXO32 could predict survival in cancers (67,69). Taken together, aberrant hypermethylation of FBXO32 may be one of the mechanisms that lead to loss, or down regulation of the gene in cancer.

β-TrCP. Human β-Transducin repeat-containing protein (β-TrCP), first identified in 1998 as a binding partner of HIV-1 Vpu protein in a yeast two-hybrid screening. The role of β-TrCP in cancers is tissue-dependent. Overexpressed β-TrCP1 or β-TrCP2 has been detected in multiple cancers, including gastrointestinal cancers, hepatoma-blastoma, colorectal cancer, pancreatic cancer, breast cancer and melanoma, suggesting a carcinogenic function for these two proteins. β-TrCP also participates in cell adhesion and migration (70). One typical substrate of β-TrCP is the IκB protein, the inhibitor of the NFκB pathway (71). Another substrate is β-catenin in Wnt pathway (72). IκB functions as a tumor suppressor. β-catenin has been identified to be upregulated in various types of human cancer, and is always correlated with poor prognosis and short survival (73,74). IκB and β-catenin exerting antagonistic functions make it hard to be an ideal drug target. β-TrCP is a member of the SCF family with both oncogenic and tumor suppressor properties. In most cases, β-TrCPs function as oncoproteins, whereas in a few others, they have displayed tumor suppressive functions. Studies investigating the role of β-TrCP in esophageal tumorigenesis are limited. Li et al (75) first evaluated the significance of β-TrCP in ESCC. Reduced expression of β-TrCP was observed in 24.4% (29/119) ESCC specimens. There was no correlation among the expressions of β-catenin and β-TrCP. No correlation between immunoeexpression of β-TrCP and clinicopathological parameters was found in ESCC patients (75).

FBXL19. Human FBXL19 (CXXC11) gene was first identified through an in silico search in 2004 (76). FBXL19 is one of the genes related to psoriasis and psoriatic arthritis (77,79). Interleukin-33 (IL-33) is an endogenous proinflammatory danger signal released from injured or dying host cells (80). ST2L, a member of the family of IL-1 receptors, has been identified as the receptor for IL-33. FBXL19 regulates the IL-33-ST2L axis, selectively mediating ST2L ubiquitination and degradation, to limit IL-33-induced pulmonary inflammation (81). FBXL19 exhibits an anti-tumor property by downregulation of small GTPase. SCFFBXL19 may affect cell motility by targeting Rac1 for its ubiquitination and degradation (82). Rac1 (ras-related C3 botulinum toxin substrate 1), a member of the small G-protein Rho family, triggers intracellular signaling such as cell proliferation and cytoskeletal reorganization. Overexpression of FBXL19 reduced both active and inactive forms of Rac1 protein. Inactivation or knockdown of Rac1 significantly reduces the cell migration rate in various cell types (83,84). Rac3 is a small GTPase multifunctional protein that regulates cell adhesion, migration, and differentiation, which has been considered as an oncogene in cancer. TGFβ1 is associated with poor prognosis of esophageal cancer (85). TGFβ1-mediated downregulation of E-cadherin contributes to tumor invasion and proliferation. Overexpression of Rac3 attenuated TGFβ1-induced E-cadherin OE19 elongation phenotype. Inhibition of Rac3 by FBXL19 attenuated the TGFβ1-induced decrease of E-cadherin in EC cell lines OE19 and OE33 (86). RhoA is a new substrate for FBXL19. RhoA phosphorylation by Erk2 promotes FBXL19-mediated RhoA degradation, since inhibition of the Erk pathway attenuates RhoA ubiquitination, while overexpression of Erk2 reduces RhoA stability. RhoA plays a critical role in regulation of cell growth and cytoskeleton rearrangement (87). Erk2 is a serine/threonine kinase involved in numerous cellular pathways, including growth and development, and ERK phosphorylation is triggered by activation of receptor tyrosine kinases in response to growth factor signals (88).

FBXO4. Cyclin-dependent kinases (CDK) drive cell-cycle progression, control transcriptional processes, and thus, regulate cell proliferation. Cyclin D1, the allosteric regulator of CDK4/6, is an integral mediator of growth factor-dependent G1 phase progression. Cyclin D1 overexpression occurs frequently in human cancer including esophageal carcinomas. Both CDK4 and CDK6, when complexed with cyclin D1, promote cell cycle progression (89). FBXO4 is an F-box constituent of SCF ubiquitin ligases that directs ubiquitylation of cyclin D1 (90), therefore, FBXO4 was considered as a tumor suppressor. FBXO4 promotes the ubiquitination and degradation of TRF1, which is important for maintaining telomere length. This role might make FBXO4 a factor in extending the lifespan of nascent transforming cells (91). A study from Barbash et al detected 14% hemizygous, missense mutations in the primary human esophageal carcinoma (92). FBXO4 loss predisposes mice to NMBA (an esophageal carcinogen) induced papilloma formation, which could be reversed by CDK4/6 inhibitors. This results confirmed the suppressor role of FBXO4 and FBXO4/cyclin D1 pathway in esophageal tumorigenesis (91).
and FBXO31 exerts anti-apoptotic effects on cancer cells in response to stress stimuli (94).

5. Conclusion and future direction

Extensive study of UPS has led to a better understanding of the molecular mechanism by which E3 ligases contribute to EC. FBPs are the core components of PBPs. Two F-box proteins SKP2 and FBXW7 have showed strong clinical relevance in the initiation and development of EC. Other FBPs such as β-TrCP, FBXL19, FBXO4, FBXO31 and FBXO32 have been proved to be involved in EC (Fig. 3) (101). Considering that F-box proteins can bind with a diversity of substrates and that each substrate may be regulated by many different E3 ligases depending on cell type. Therefore, identifying critical substrates of each F-box protein is paramount for understanding tumorigenesis and discovering therapeutic targets. However, it should be stressed that FBPs deregulate an entire network of proteins. Two main substrates of β-TrCP are IκB in the NF-κB pathway and β-catenin in Wnt pathway (71,72). IκB, inhibitor of NF-κB, functions as a tumor suppressor. β-catenin has been identified to be an oncogene in cancers. Intriguingly, some FBPs can be controlled by their substrates. More complicated substrates feed back to control FBPs. FBXW7α interacts with C/EBPβ and targets it for degradation (62), but C/EBPβ inhibits FBXW7 expression and promotes mammary tumour metastasis (102). Consistent with these findings is a recent study by Sancho and colleagues which show that, FBXW7 repression by hes5 (a Notch target gene) creates a feedback loop that modulates Notch-mediated intestinal and neural stem cell fate decisions (103).

In conclusion, this review provides only a glimpse into the mechanisms through which FBPs are involved in EC. However, all the current understanding is just the tip of the iceberg. The growing details in understanding of SCF-based protein targeting of a diverse array of fundamental substrates provide a great opportunity for cancer therapeutic development targeting FBPs. It has become clear that some FBPs, such as SKP2 and FBXW7, are promising targets for EC therapy. The proteasome inhibitor bortezomib, which blocks the entire protein degradation, highlighted the therapeutic potential of targeting this protein degradation system (104). However, bortezomib has been used in clinical trial for cancer treatment but with limited success. Selective inhibitors targeting a particular E3 ligase or a certain F-box protein may be more effective, but extensive research is to be continued (105). To this end, there are still many important remaining questions to be resolved. We need to identify the physiological substrates for many orphan F-box proteins. To assess whether there is crosstalk between individual F-box proteins calls for research in this emerging area involving functional delineation of FBPs and their cellular context-specific substrates in human EC. A better mechanistic understanding of FBP-regulated network of proteins in initiation and progression of cancer will be a research hotpot in the future.

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References


Figure 3. Main FBPs and their major downstream targets in EC. SKP2 and FBXO31 are potential oncogenes in EC (in the red background). FBXW7, β-TrCP, FBXL19, FBXO4 and FBXO32 are potential downstream suppressor genes in EC (in the green background).


