

Role of fibroblast growth factor receptor-2 splicing in normal and cancer cells

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1. ABSTRACT

Types 1-4 of fibroblast growth factor receptors (FGFR) are all expressed in various cancers. Because of its prominent role in carcinogenesis and cancer progression, FGFR-2, is being considered as a novel target in cancer treatment. Owing to the alternative splicing of its extracellular domain, FGFR-2 exists in two variants: IIIb and IIIc. FGFR-2 IIIb is mainly expressed in normal epithelial cells, as well as in oral mucosal, esophageal, gastric, colorectal, pancreatic, pulmonary, breast, endometrial, cervical, and prostate cancers. The IIIc variant of FGFR is expressed in mesenchymal cells, and during epithelial-mesenchymal transition (EMT), is expressed in colorectal, pancreatic, bladder, cervical, and prostate cancers. The FGFR IIIb and IIIc variants bind different forms of FGFs and exert autocrine and/or paracrine effects in cancers. Recent reports indicate that switching from IIIb to IIIc variants correlates with the aggressiveness of the cancers via EMT. Here, we discuss the expression, role, and regulatory mechanisms of IIIb and IIIc variants of FGFR in cancers.

2. INTRODUCTION

Fibroblast growth factors (FGFs) are heparin-binding growth factors including FGF-1, FGF-2, FGF-3

(proto-oncogene Int-2), FGF-4 (HST/K-FGF), FGF-5, FGF-6, FGF-7, FGF-8 [androgen-induced growth factor (AIGF)], glia activating factor (GAF; FGF-9), FGF-10, FGF-11 [FGF homologous factor 3 (FHF-3)], FGF-12 (FHF-1), FGF-13 (FHF-2), FGF-14 (FHF-4), and FGF-16 through FGF-23 (1). Human FGFs, which are comprised of ~150–300 amino acids, have a conserved ~120 amino acid residue core, and show ~30–60% amino acid identity (1–3).

FGFs exert their biological activities by binding to high-affinity tyrosine kinase FGF receptors (FGFRs) on the surface of cells and low-affinity heparan sulfate proteoglycans that enhance ligand presentation (1, 4). Expression of FGFRs and their corresponding ligands (FGFs) contributes to tumor progression in human malignancies by enhancing angiogenesis and proliferation via both autocrine and paracrine effects (5–7). FGFRs are single transmembrane receptors, containing extracellular, transmembrane, and intracellular domains. The extracellular domains of FGFRs are usually composed of 3 immunoglobulin-like domains (Ig I-III), whereas their intracellular tyrosine kinase region is interrupted by a non-kinase intervening sequence (8). FGFRs consist of four members, named FGFR-1, 2, 3, and 4,

that are encoded by distinct genes (1). FGFR-1, 2, 3, and 4 genes are localized in chromosomes 8q12, 10q26, 4p16.3, and 5q35.1, respectively (1). Alternative splicing of the C-terminal half of the third Ig-like domain generates the IIIb and IIIc variants of FGFRs1-3, but FGFR4 does not possess such alternative exons (9, 10) (Fig.1). Appropriate tissue-specific expression of FGFR-2 IIIb or FGFR-2 IIIc, in conjunction with the presence of appropriate ligands, is crucial for fetal development, maintenance of cellular homeostasis, and other functions in postnatal status.

Mice null for the FGFR-2 gene die early during embryogenesis with no formation of limb buds (11). Those null for the FGFR-2 IIIb variant while retaining FGFR-2 IIIc, survive to birth. FGFR-2 IIIb null mice show dysgenesis of the kidneys, salivary glands, adrenal glands, thymus, pancreas, skin, otic vesicles, glandular stomach, and hair follicles; minor defects in the skull; and agenesis of the lungs, anterior pituitary gland, thyroid, teeth, and limbs (12–14). FGFR-2 IIIb is considered to be required for the growth and maintenance of limb bud formation, but not for limb bud initiation (12). Conditional FGFR-2 IIIb knockout in the rodent epidermis leads to increased macrophage infiltration in the dermis and adipose tissue, epidermal thickening accompanied by basal-layer dysplasia and parakeratosis, and the promotion of chemically induced squamous-cell carcinoma. To create a loss-of-function phenotype of FGFR-2 IIIc, Eswarakumar *et al.* (15) introduced a point mutation into the IIIc exon, which introduced a frame-shift and created a translation stop codon, without influencing the expression of FGFR-2 IIIb. Loss of FGFR-2 IIIc results in a viable recessive phenotype with craniosynostosis and retarded development of the axial and appendicular skeleton, causing dwarfism and misshapen skull. The knockout mice remain alive for over a year, but remain significantly smaller than normal mice.

Recent studies have shown that gene amplification, abnormal activation, or single nucleotide polymorphisms (SNPs) of FGFR-2 play important roles in cancer progression (16–19). Gene amplification or missense mutations of FGFR-2 occur in gastric, lung, breast, ovarian, and endometrial cancers and melanomas (20–26). SNPs of intron 2 in FGFR-2 are associated with an increased risk of breast (27, 28) and endometrial cancers, and activating mutations of FGFR-2 have been identified in endometrial cancers (24, 29). Amplification and overexpression of FGFR-2 is strongly associated with the poorly differentiated, diffuse type of gastric cancer, which has an especially unfavorable prognosis (30).

Concerning FGFR-2 IIIb or IIIc variants and cancer, there is a lot of evidence correlating epithelial-mesenchymal transition (EMT) with migration and metastasis of cancer cells (31).

The role of FGFR-2 IIIb expression is different in each type of cancer. Increased expression of FGFR-2 IIIb seems to be related to cell transformation and tumor progression in some cancers. On the other hand, decrease of FGFR-2 IIIb expression was reported to be associated with increased FGFR-2 IIIc expression. Recent studies, including our own, have shown that FGFR-2 IIIc expression was closely correlated with carcinogenesis and tumor progression in several types of cancers (32, 33).

This review summarizes the correlation of alternative splicing of FGFR-2 variants to EMT in cancer, and clarifies whether they are potential therapeutic targets for cancer.

3. STRUCTURE OF FGFR-2

FGF-FGFR binding activates intracellular signaling cascades. Mitogenic signaling is mediated through tyrosine phosphorylation of key substrates, including activation of the mitogen-activated protein kinases such as ERK-1 and ERK-2 via the ras pathway (34, 35). Binding of the specific ligands to FGFR-2 results in receptor dimerization, with subsequent autophosphorylation of tyrosine residues within the intracellular domain and recruitment and phosphorylation of substrate proteins such as phospholipase C-gamma (PLC- gamma) and FGFR receptor substrate (FRS2) (36, 37).

More than 20 alternative splicing variants of FGFR-2 have been identified in the extracellular and intracellular regions (38). Alternative splicing of the extracellular domain of FGFR-2 results in the generation of variants containing all three Ig-like domains (referred to as the alpha isoforms), or only Ig II and Ig III (referred to as the beta isoform (39, 40). Alternative splicing of intracellular domains generates C1, C2, and C3 variants (41). The major splicing event of FGFR-2 occurs in the carboxyl-terminal half of the third Ig-like domain in the extracellular domain (D3). The two types of FGFR-2 variants termed FGFR-2 IIIb (NM_022970.3/Variant 2/4657 bp) and FGFR-2 IIIc (NM_000141.4/Variant 1/ 4654 bp) are generated by alternative splicing of exons 9 and 10 (9, 42, 43). When the C-terminal half of D3 is encoded by exon 8, the FGFR2 IIIb variant is generated, while the FGFR-2 IIIc variant is generated when the C-terminal half of D3 is encoded by exon 9 (Fig.1). The homology of IIIb and IIIc regions of FGFR-2 is 62% and 51% at the mRNA and protein levels, respectively. The Intronic Splicing Enhancer/Intronic Splicing Silencer-3 (ISE/ISS-3), which is located in intron 8 downstream of a UGCAUG motif of FGFR-2, regulates the FGFR-2 splicing via binding of FOX-2 or Epithelial Splicing Regulatory Protein (ESRP) 1 and 2 (44–46). The ISE/ISS-3 functions specifically in epithelial cell types to enhance splicing of the upstream exon 8 and silence

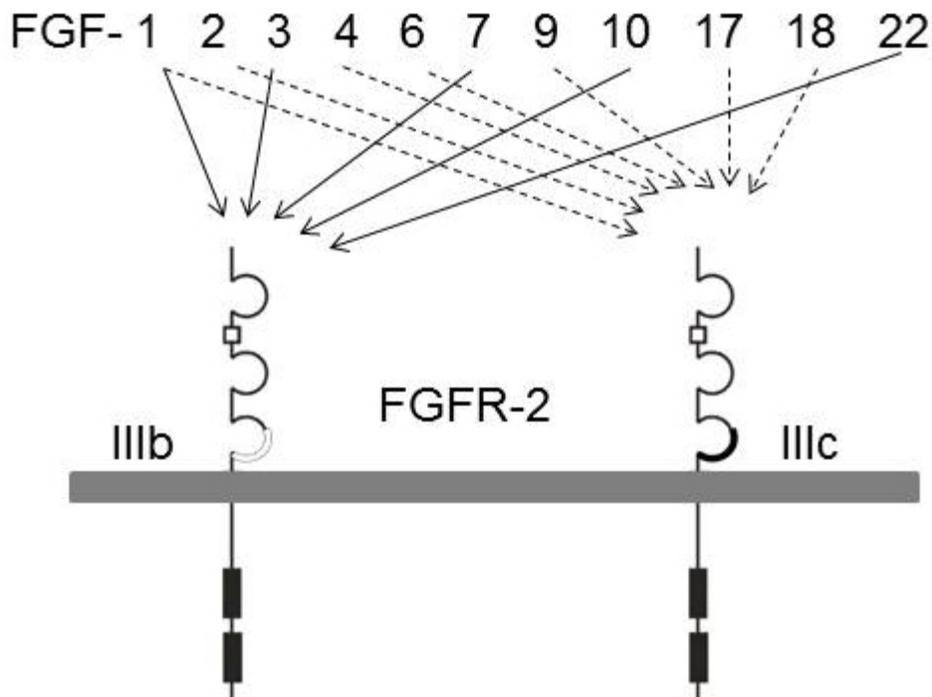


Figure 2. Binding of FGF to IIIb and IIIc isoforms of FGFR-2. FGFs 1, 3, 7, 10, and 22 bind to IIIb of FGFR-2 with high affinity, whereas FGFs 1, 2, 4, 6, 9, 17, and 18 bind to IIIc with high affinity.

enhances growth of normal intestinal epithelial cells and hepatocytes (49, 50). In noncancerous esophageal tissues, FGFR-2 IIIb was localized in epithelial cells from the basal region of the epithelium to the lower one-third of the epithelium and its localization was broader than that of Ki-67, one of the markers for proliferating cells (51). Levels of FGF-7 mRNA synthesized by stromal cells and FGFR-2 IIIb mRNA in epithelial cells markedly increase, and a single injection of recombinant human FGF-7 enhances cell proliferation and accelerates ulcer healing (52). FGFR-2 IIIb immunoreactivity was weakly detected in the luminal surface of normal gastric epithelial cells (53). In addition, FGFR-2 IIIb immunoreactivity was strongly detected in the nucleus and cytoplasm of many parietal cells in the stomach. FGFR-2 IIIb immunoreactivity was also localized in the luminal surface of normal colorectal epithelial tissues (54). These findings suggest that FGFR-2 IIIb plays important roles not only in epithelial cell proliferation in the GI tract, but also in cell differentiation. In fetal pancreatic tissues, FGF-7 induces beta-cell expansion through the activation of ductal cell proliferation and their subsequent differentiation into beta-cells (55). In normal human pancreatic tissues, we found that FGFR-2 IIIb immunoreactivity was detected in the cytoplasm and/or membrane of islet cells and, pancreatic ductal cells (56). FGFR-2 IIIb was localized in islet cells in rat tissues and the intensity was stronger in glucagon-secreting alpha cells among the islet cells (57). FGFR-2 IIIb was weakly localized in the surface

of squamous epithelial cells, but strongly expressed in vascular smooth muscle cells in uterine cervical tissues (58). FGFR-2 IIIb was not detected in normal lung tissues (59).

4.2. Cancer cells and tissues

FGFR-2 IIIb over-expression has been detected in a variety of cancers. FGFR-2 IIIb mRNA is expressed in carcinoma cell lines derived from multiple tissues, including breast, colon, stomach/esophagus, pancreas, prostate, oral mucosa, and uterus. Overexpression of the FGFR-2 IIIb variant have been reported in various cancers, including breast, endometrial, cervical, lung, esophageal, gastric, pancreatic, and colorectal cancer (CRC) (51, 54, 56, 58–64). The role of the FGFR-2 IIIb variant between types of cancer has been controversial. FGFR-2 IIIb was expressed in esophageal cancer cells in 41% of patients, and FGFR-2 IIIb expression correlated with the well-differentiated cell type of esophageal cancer (51). FGF-7 induced proliferation in FGFR-2 IIIb positive esophageal cancer cells. The different roles of FGFR-2 IIIb in various cancers have not been well characterized; however, the differences may be due to the affinity of different ligands for FGFR-2 IIIb or their effects on other FGFRs. In gastric cancer tissue, FGFR-2 IIIb was expressed in the cell membrane and cytoplasm of cancer cells in 36.5% of the cases (53). FGFR-2 IIIb expression

Table 1. FGFR-2 IIIb in normal tissues

Organ	Positive	Negative
Colorectum	Luminal surface of the epithelium	
Esophagus	Basal to lower one-third of the epithelium	
Lung		Bronchial cells, alveolar cells
Pancreas	Islet cells, alpha cells (rat)	Ductal cells
Stomach	Luminal surface of the epithelium, parietal cells	
Uterus	Surface of squamous epithelium, vascular smooth muscle cells	

in gastric cancer cells was significantly associated with early-type macroscopic findings, shallow invasion of the gastric wall, and expansive growth type. In contrast, decreased expression of FGFR-2 IIIb in gastric cancer cells was associated with the proliferation and invasion of gastric cancer cells and a poor prognosis for the patient. FGFR-2 IIIb-specific antibody and probes for *in situ* hybridization analysis showed that expression of FGFR-2 IIIb increased in CRC. FGFR-2 IIIb was expressed in 62.5% of CRC patients and localized at the center of the cancer nests (54). A high level of FGFR-2 IIIb expression was associated with a well-differentiated histological type and shallow wall invasion. As determined by *in situ* hybridization, both FGFR-2 IIIb and FGF-7 mRNA are expressed in CRC cells, and FGF-7 mRNA was recognized in neuroendocrine cells lying close to CRC cells (63). FGF-7 induced an increase in VEGF-A expression; thus, FGF-7 and its receptor FGFR-2 IIIb may be involved in tumor angiogenesis in CRC (65). Furthermore, FGF-7 enhances adhesion to type-IV collagen, one of the main components of the vascular basement membrane, via downregulation of Integrin alpha 2 and activation of ERK1/2 and focal adhesion kinase (FAK) signaling pathways (66). Co-expression of FGFR-2 IIIb and FGF-10 has been reported in CRC cells, and FGF-10 increased the growth rate of FGFR-2 IIIb-positive CRC cell lines (64). These findings suggest that interactions of FGFR-2 IIIb, FGF-7, and FGF-10 may play important roles in CRC proliferation and tumor angiogenesis via autocrine and/or paracrine manners. We reported that FGFR-2 IIIb was detected in 42 % of the pancreatic cancer cases, whereas FGF-7 immunoreactivity was detected in 34% of the cancer patients (62). The expression of FGFR-2 IIIb and FGF-7 correlated with venous invasion, vascular endothelial growth factor (VEGF)-A expression, and a poor prognosis, and may promote venous invasion and tumor angiogenesis in pancreatic cancers. Another ligand for FGFR-2 IIIb, FGF-10, induced pancreatic cancer cell migration and invasion via FGFR-2 IIIb (67). Moderate FGFR-2 IIIb-*in situ* hybridization signals were observed in pancreatic cancer cells. FGFR-2 IIIb was expressed in 86% of uterine cervical cancer cases, and histologically, 94% of patients with the keratinizing type and 79% of patients

with the non-keratinizing type of cervical cancer (58). Furthermore, FGFR-2 IIIb was prominently localized in proliferating reserve cells and squamous metaplastic reserve cells adjacent to cervical cancer cells. In contrast, FGFR-2 IIIb was not detected in cervical ductal cells. Strong immunoreactivity to FGFR-2 IIIb was observed in lung tumor cells in 31 of 61 specimens (59). In lung adenocarcinoma, cells staining with the FGFR-2 IIIb antibody were scattered throughout the tumor region, whereas in squamous cell carcinoma (SCC), FGFR-2 IIIb-positive cells were often clustered in tumor cell nests (59). A stable transfectant of the FGFR-2 IIIb gene in salivary adenocarcinoma cells induced cancer cell differentiation and apoptosis (68).

5. ROLES OF FGFR-2 IIIc

5.1. Normal cells and tissues

FGFR-2 IIIc expression in human normal tissues has not been extensively reported. In our immunohistochemical analysis using FGFR-2 IIIc-specific antibody, FGFR-2 IIIc was localized in some endothelial and smooth muscle cells in small-sized blood vessels (Table 1). In gastrointestinal tracts, FGFR-2 IIIc was localized in fundic glands in the stomach, epithelial cells in the duodenum and stromal fibroblasts in the colorectum. FGFR-2 IIIc was not localized in gastric foveolar epithelial cells, Brunner's glands in duodenum or epithelial cells in colorectum. In normal colorectal tissues, weak FGFR-2 IIIc expression was detected in superficial colorectal epithelial cells, but no FGFR-2 IIIc expression was detected in the proliferative zone of the colorectal epithelium (69). FGFR-2 IIIc was localized in hepatocytes, but not localized in bile ducts. In pancreas, FGFR-2 IIIc was weakly localized in islet cells, but not in ductal cells or acinar cells (70). In other major tissues, FGFR-2 IIIc was localized in renal tubules, but not in the glomeruli of kidneys. FGFR-2 IIIc was localized in cardiomyocytes and pericardial cells. In lungs, FGFR-2 IIIc was localized in bronchial and alveolar cells. FGFR-2 IIIc protein was not or very faintly localized in squamous epithelial cells and its mRNA was expressed in basal cells of the squamous epithelium of uterine cervix (71).

Table 2. FGFR-2 IIIc in normal tissues

Organ	Positive	Negative
Heart		
	Cardiomyocytes	
	Pericardial cells	
Lung		
	Bronchial epithelial cells	
	Alveolar epithelial cells	
Liver	Hepatocytes	Biliary ductal cells
Pancreas		
	Islet cells (weak)	Ductal cells
		Acinar cells
Blood vessel		
	Endothelial cells	
	Smooth muscle cells	
Colorectum	Stromal fibroblasts	Epithelium
Duodenum	Duodenal epithelium	Brunner's glands
Kidney	Renal tubules	Glomeruli
Stomach	Fundic gland	Foveolar epithelium

5.2. Cancer cells and tissues

FGFR-2 IIIc expression has been reported in prostate cancer, ovarian cancer, oral squamous cell carcinoma, breast cancer, bladder cancer, non-small-cell lung cancer, colorectal cancer, and pancreatic cancer (32, 33, 69, 70, 72–75). Loss of FGFR-2 IIIb expression was associated with activation of FGFR-2 IIIc expression, and/or a shift to more virulent behavior. A class switch from FGFR-2 IIIb to IIIc is related to the progression of prostate cancers (76). Furthermore, FGFR-2 IIIc expression in prostate and bladder cancer cells induced epithelial-mesenchymal transition (EMT) and a switch in splicing, which may play crucial roles in cancer metastasis (74, 77, 78). FGFR-2 IIIc-positive cells were detected by immunohistochemistry in the following lesions, listed in order of increasing percentage: hyperplastic polyps < low-grade adenomas < high-grade adenomas < carcinomas (69). In CRC cases, FGFR-2 IIIc immunoreactivity was highly expressed in 26 of 95 patients with CRC (27%), and its expression was correlated with distant metastasis of the cancer. FGFR-2 IIIc-transfected CRC cells showed increased growth, soft agar colony formation, migration, and invasion. FGFR-2 IIIc was expressed in 4 of 4 CRC cell lines, and FGFR-2 IIIc-transfected CRC cells formed larger tumors in subcutaneous tissues and the cecum of nude mice. FGFR-2 IIIc was expressed in 83 of 117 pancreatic ductal adenocarcinoma cases, which correlated with decreased duration until development of liver metastasis after surgery (70). FGFR-2 IIIc was expressed in 6 of 6 pancreatic ductal adenocarcinoma

cell lines and FGFR-2 IIIc stably transfected cells exhibited increased proliferation *in vitro* and formed larger subcutaneous and orthotopic tumors, the latter producing more liver metastases. We have previously reported that the expression levels of FGFR-2 IIIc positively correlated with the presence of pre-cancerous lesions in the uterine cervix, termed cervical intraepithelial neoplasia (CIN) (71). Furthermore, stable transfection of FGFR-2 IIIc in cervical cancer cell lines induced cancer cell growth. Therefore, FGFR-2 IIIc correlates with the carcinogenesis and aggressive growth of cervical cancer.

6. REGULATION OF SPLICING

6.1. Normal cells

Alternative splicing vastly expands transcriptomic diversity, as evidenced by recent studies demonstrating that nearly all multi-exon human genes undergo alternative splicing (79, 80). The most well-known cis-element which regulates the splicing of FGFR-2 is an auxiliary cis-element, ISE/ISS-3 (Intronic Splicing Enhancer/Intronic Splicing Silencer-3) that functions specifically in epithelial cell types to enhance splicing of the upstream exon IIIb and silence the downstream exon IIIc (81). Binding of Fox-2 to ISE/ISS-3 element has been shown to play an important role in FGFR-2 splicing regulation (46). Recently epithelial splicing regulatory protein 1 (ESRP1) and ESRP2 were reported as RNA-binding proteins that participate in the enhancement of splicing of the

upstream exon IIIb while silencing the downstream exon IIIc by binding ISE/ISS-3 (47). For IIIc regulation, Tra2beta was reported to repress the selection of exon 9 (IIIc) (82). hnRNP was reported to repress inclusion of exon 9 (83), and hnRNP H and F interact with Fox2 to repress exon 9 inclusion (45).

6.2. Dysregulation in cancers

ESRP1 is a master cell type-specific splicing regulator critical for maintaining the epithelial cell identity and has been implicated in a variety of developmental and disease processes. Moreover, ESRP1 orchestrates an epithelial type of splicing regulatory program in EMT via regulating of splicing of FGFR-2, CD44, ENAH, and p120-catenin in breast, lung and colon cancer (84, 85). In human non-small cell lung cancer, ZEB1 and ZEB2 are key regulators of the EMT process (85), and ZEB1 induction dramatically led to a substantial reduction in the expression of ESRP1. In colon cancer, ESRP1 functions as a tumor suppressor involved in posttranscriptional regulation of a number of genes by exerting a differential effect on protein translation via 5'UTRs of mRNAs. In breast cancer, the function of ESRP1 is controversial. ESRP1 has been implicated in promoting CD44 alternative splicing, switching the expression from CD44s to CD44v. CD44s expression was up regulated in high-grade human breast tumors and was correlated with the level of the mesenchymal marker N-cadherin in these tumors (86). The regulation of CD44 alternative splicing causally contributes to EMT and breast cancer progression. Meanwhile, CD44v mRNA expression promoted by ESRP1 was also reported to enhance lung colonization by metastatic breast cancer cells. In relating ESRP1 expression and prognosis using gene expression profiles of breast cancer patients, a high level of ESRP1 expression was significantly associated with a lower overall rate of survival. ESRP1 is a regulator of the EMT process in pituitary adenomas causing acromegaly (87). In pancreatic cancer, ESRP1 regulates the expression patterns of FGFR-2 isoforms, attenuates cell growth, migration, invasion, and metastasis and is a favorable prognostic factor (88). The roles of ESRP1 in cancers, carcinogenesis, and EMT will be further examined in the future.

7. THERAPEUTIC TARGET FOR CANCER

FGFR-2 IIIb or IIIc variant specific drugs have not been applied in clinical treatment. Recently, AZD4547, a selective inhibitor of FGFR-1, 2, and 3 was developed and is under clinical investigation for the treatment of FGFR-dependent tumors (89). In the experimental studies, a small molecule, Ki23057, which inhibits autophosphorylation of FGFR-2 IIIb, decreased the growth of biliary tract cancer cells, gastric scirrhous carcinoma cells, and CRC cells *in vitro* and *in vivo* (90, 91). Combination treatment

with 5-fluorouracil (5-FU) and Ki23057 produced synergistic anti-tumor effects in an animal model of gastric cancer (92), and Ki23057 was more effective when used in combination with irinotecan, paclitaxel, and etoposide for drug-resistant gastric cancer cells (93). A recent study demonstrated that monoclonal antibodies to FGFR-2 IIIb or IIIc variants successfully inhibited the growth of gastric tumor xenografts (94). A mutation in the soluble ectodomain of FGFR-2 IIIc, S252W, suppressed cell growth, angiogenesis, and metastasis of human breast cancer and prostate cancer cell lines *in vitro* and *in vivo* (95). In pancreatic ductal adenocarcinoma, suppression of FGFR-2 IIIc using siRNA targeting FGFR-2 IIIc mRNA expression inhibited cell proliferation *in vitro* (70). In addition, an anti-FGFR-2 IIIc antibody inhibited the proliferation and migration of pancreatic cancer cells. Fully human anti-FGFR-2 IIIc monoclonal antibody inhibited the growth and migration of colorectal cancer cells *in vitro* (69).

8. CONCLUSION

FGFR-2 IIIb and IIIc variants play important and different roles on carcinogenesis and tumor progression in various cancers. Novel therapies against FGFR-2 IIIc or inhibition of alternative splicing from IIIb to IIIc isoforms may be an effective target for cancer therapy.

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