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Review

Differential Splicing Generates New Transmembrane Receptor and Extracellular Matrix-related Targets for Antibody-based Therapy of Cancer

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Abstract. *Alternative splicing has been shown to be deregulated in cancer and a link to growth stimulation has been established. Here we describe transmembrane and extracellular matrix-related targets generated by alternative splicing with a restricted pattern of expression in normal tissues and a deregulated pattern of expression in cancer as possible targets for therapeutic intervention with antibody-related agents. We focus on isoforms of transmembrane and extracellular matrix proteins, such as CD44, Claudin 18, L1 cell adhesion molecule and epithelial cellular adhesion molecule, fibronectin, tenascin, osteopontin and versican as well as transmembrane tyrosine kinases, such as fibroblast growth factor receptors, epidermal growth factor receptor and receptor d´origin nantais.*

The identification of deregulated and highly specific targets for treatment of cancer can potentially translate into therapy with high efficacy and a favourable side-effect profile. Breakthroughs in this context are compounds inhibiting oncogenic driver mutations such as the fusion protein consisting of BCR and ABL (BCR-ABL) for treatment of chronic myeloid leukemia (1), activating mutations of the epidermal growth factor receptor (*EGFR*) for treatment of non-small cell lung carcinoma (NSCLC) patients (2), treatment of gastrointestinal stromal tumor patients with tyrosine kinase c-kit (CD117) inhibitors (3), and of melanoma patients with activating V600E B-rat fibrosarcoma (*BRAF*) mutations with BRAF inhibitors (4) and of NSCLC patients harboring the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4 ALK) fusion protein with ALK inhibitors (5). Several reports have indicated that splicing is deregulated in cancer (6-8). In the following, we focus on the identification of transmembrane and extracellular matrix-related targets arising in cancer due to alternative splicing and their potential for targeting with antibody-related agents. The differentially spliced proteins discussed in this review are summarized in Figure 1, the transmembrane receptors discussed are shown in Figure 2.

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Splicing

The splicesosome, a complex of five small nuclear ribonucleic acid (snRNA) and hundreds of auxiliary proteins, mediates intron excision and subsequent exon ligation (9). Differential splicing can be regulated by several mechanisms. One of the mechanisms is making use of exonic splicing enhancers and exonic splicing silencers which increase inclusion or exclusion of the corresponding exons into the mature RNA. These elements act by specific binding to regulatory proteins such as serine-arginine (SR)-rich proteins (5). In the case of CD44, it has been shown that differential splicing is regulated by the proliferation status of the cells: resting lymphocytes predominantly express CD44 isoforms lacking the variant exon-encoded sequences, but after stimulation of proliferation, variant encoded exons are included (10, 11). Stimulation of the rat sarcoma-map kinase kinase-extracellular signal regulated kinase (Ras-MEK-ERK) pathway results in the inclusion of variant exons into the mature mRNA (12). In this system,

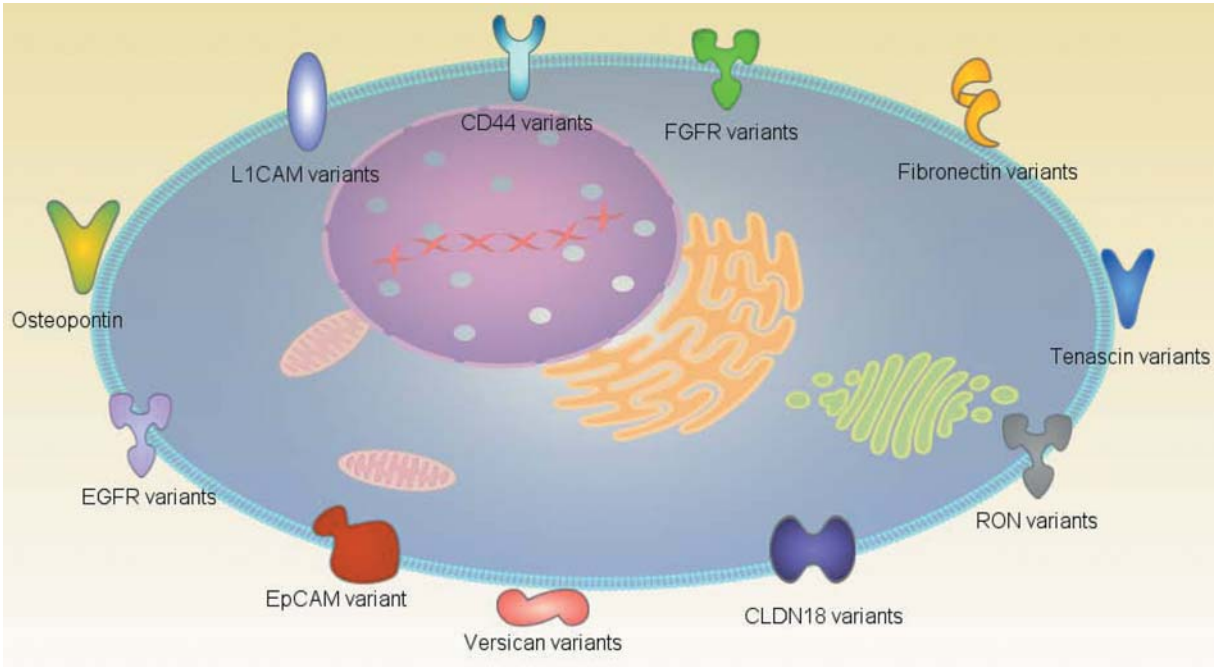

Figure 1. *Outline of differentially spliced gene products described in this review. For visual presentation Ingenuity Pathway Analysis (IPA) Path Designer tool was used (www.ingenuity.com). ECD: Extracellular domain; TM: transmembrane domain; CYT: cytoplasmic domain; variants of the following transmembrane proteins are shown: fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), receptor d´origin nantais (RON), epithelial cellular adhesion molecule (EpCAM), L1 cell adhesion molecule (L1CAM), Claudin18 and CD44. Versican (VN), fibronectin (FN) and tenascin (TN) and their splice variants represent extracellular matrix-related proteins.*

differential splicing is regulated by *trans*-acting factors and one of these factors, SAM68, is regulated by phosphorylation by ERK, a target of RAS-mediated signaling (12, 13). It has also been shown that the carboxy-terminal domain of RNA polymerase II plays an important role in differential splicing (14). The carboxy-terminal domain of human RNA polymerase II consists of 52 heptapeptides with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, with 326 potential phosphorylation sites which are important in writing and reading epigenetic information, such as transcript initiation, capping, elongation, splicing, mRNA 3´processing and small nuclear RNA processing. Regulation of alternative splicing by histone modification was noted for the fibroblast growth factor receptor 2 (*FGFR2*) gene (15). Histone marks can affect splicing outcome by recruitment of splicing regulators through chromatin binding proteins pointing to an adaptor system for the reading of histone markers by the pre-mRNA splicing machinery. The adapter system involved in differential splicing of the *FGFR2* gene consists of H3-K36me3, its binding protein morf-4 related gene on chromosome 15 (MRG15) and the splicing regulator polypyrimidine tract-binding protein (PTB) which interacts with the RNA. It is tempting to speculate that other combinations of adapter systems exist that act on other alternatively spliced exons.

The impact of alternative splicing on cancer cell metabolism was revealed recently (16). Under oxygen abundance, quiescent cells derive energy from glucose by oxidative phosphorylation, whereas tumor cells convert glucose primarily into lactate, a phenomenon known as aerobic signaling with impact on tumor growth (17-19). This is partly achieved by isoform expression of pyruvate kinase based on differential splicing. The adult isoform PKM1 promotes oxidative phosphorylation, whereas the embryonic isoform PKM2 which is expressed in cancer promotes aerobic glycolysis. The two isoforms result from mutually exclusive alternative splicing of PKM pre-mRNA, resulting in inclusion of either exon 9 (PKM1) or exon 10 (PKM2).

FGFR Splice Variants

FGFRs (FGFR1, 2, 3, 4) respond to 18 fibroblast growth factors (FGFs) (20, 21). The extracellular domains contain three immunoglobulin-like domains (D1, D2 and D3). Domains D2 and D3 are involved in ligand binding and specificity, whereas domain 1 is absent from several isoforms and has an auto-inhibitory function. In addition, the receptor family shares a transmembrane domain and a split intracellular kinase domain. Phylogenetically, they are related

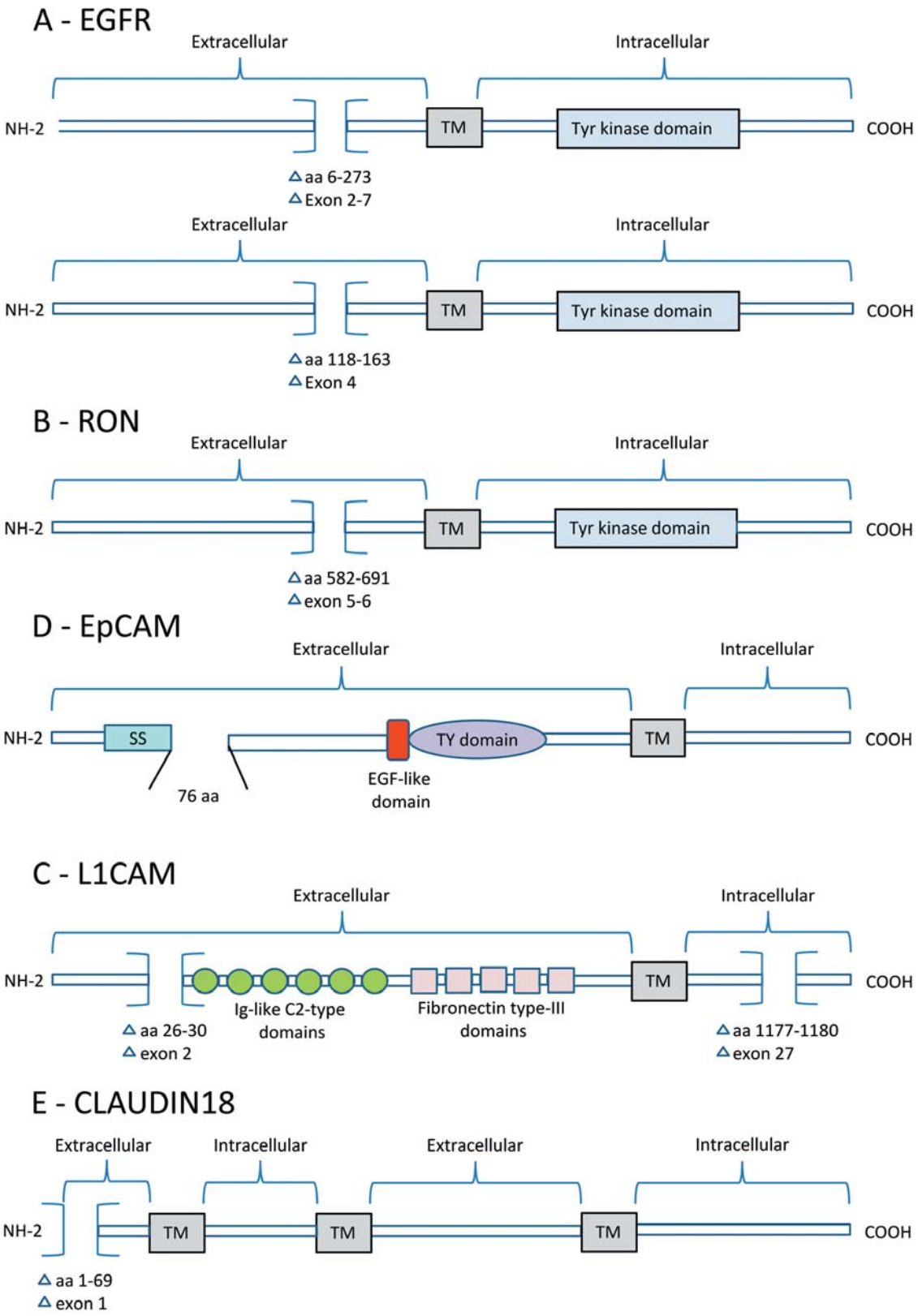

Figure 2. *Summary of transmembrane molecules with tumor-related alterations due to differential splicing. Location and size of the deletion or insertion is specified. TY: Thyroglobulin type-1 domain; SS: signal sequence; aa: amino acids.*

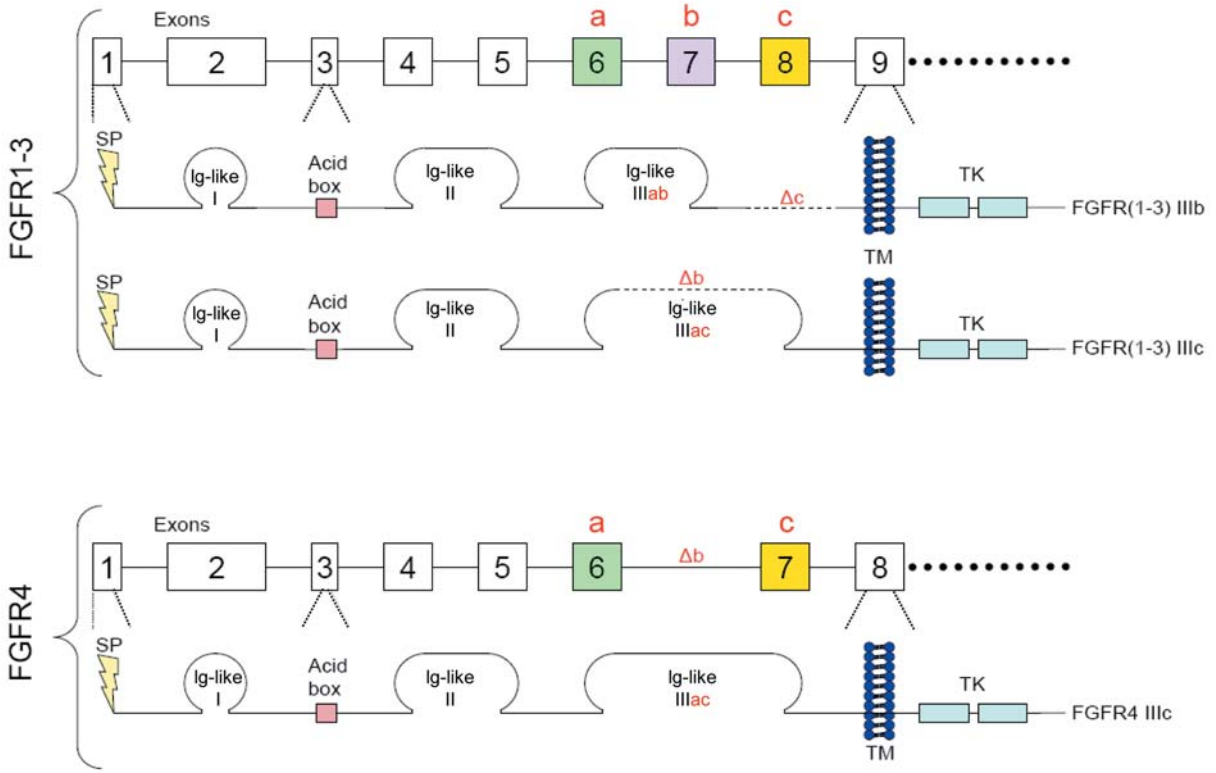

Figure 3. *Splice variants of fibroblast growth factors 1-4 (FGFR1-4). The upper panel shows FGFR1-3 variants and in the lower panel the only known FGFR4 splice variant is shown. Exons involved in differential splicing are shown in green, pink and yellow. SP: Signal peptide; Ig-like: immunoglobulin-like domain; TM: transmembrane domain; TK: tyrosine kinase domain. An equivalent to exon7 (pink) of FGFR1-3 is missing from the FGFR4 gene as shown by analysis of the human genome assembly GRCh37/hg19 (February 2009).*

to the vascular endothelial growth factor receptors (VEGFRs) and to the platelet-derived growth factor receptors (PDGFs). FGFs bind with high affinity to heparan sulfate proteoglycan on the cell surface and to specific FGFRs. Differential splicing of the FGFR family is illustrated in Figure 3. Ligand binding specificity is generated by alternative splicing of domain D3. The first half of domain 3 is generated by the invariant exon IIIa which is spliced to either exon IIIb or IIIc and both of them are spliced to the transmembrane domain. FGFR2 occurs as isoform IIIb which binds FGF1, 3, 7 and 10 and as isoform IIIc which binds FGF1, 2, 4, 6 and 9 (22). Epithelial tissues predominantly express the IIIb isoform, whereas mesenchymal tissues express isoform IIIc. *FGFR2* amplification was found in 3-10% of gastric carcinomas and was associated with a poor prognosis (23). Gastric cancer patients with FGFR2IIIb and FGF7 double-positive tumors had a much worse prognosis than patients with single-positive tumors, pointing to the functional role of this loop in a subset of gastric cancer patients (24). *FGFR2* amplification and overexpression has also been observed in a subgroup (6 out of 145=4%) of triple-negative breast tumors. Evaluation of breast and gastric cancer cell lines with FGFR2 amplification revealed preferential expression of FGFR2IIIb isoform (25). GP369, a FGFR2IIIb-specific antibody was shown to specifically inhibit ligand-induced phosphorylation of FGFR2IIIb, downstream signaling and FGF2 driven proliferation *in vitro* (25). GP369 also inhibits growth of cancer xenografts with activated FGFR2 signaling. In bladder carcinoma cells, an isotype switch from the epithelial isoform of FGFRIIIb to the mesenchymal isoform IIIc has been observed (26). Since FGFRIIIc binds to a variety of ligands (FGF1, 2, 4, 6, and 9) in contrast to FGFRIIIb, which binds FGF1,3,7 and 10, these tumor cells might become sensitive to FGFs they were not previously responsive to. Proof-of-concept experiments with a monoclonal antibody specific for FGFRIIIc are not yet available. For FGFR4, only the IIIc isoform has been identified (Figure 3).

Epidermal Growth Factor Receptor Variants III (EGFRvIII) and de4 EGFR

The important role of EGFR in cancer biology is well documented (27). EGFRvIII harbors an in-frame deletion of exons 2 to 7 (268 amino acids) of the extracellular domain

producing a 150 kDa protein that is constitutively activated in a ligand-independent manner (28). Deletion of amino acids 6-273 of the extracellular domain results in insertion of a novel glycine at the fusion junction (Figure 2A upper panel). Constitutive kinase activity and prolonged signaling of EGFRvIII are mediated by impaired endocytosis and degradation by inefficient ubiquitinylation (29). EGFRvIII is expressed in 20-30% of glioblastomas and is a negative prognostic indicator for this disease (30). EGFRvIII was found to be expressed in 42% (n=33) of head and neck squamous cell carcinomas (HNSCC), where it contributes to enhanced growth and resistance to targeting of wild-type EGFR and correlates with reduced survival (31). In addition, expression of EGFRvIII has been reported in breast, non-small cell lung, ovarian and prostate carcinomas, but not in normal tissues, which underlines its importance as a tumor-selective agent (32). Several groups have developed antibodies targeting EGFRvIII. Anti-EGFRvIII antibody Y10 enhanced survival of mice in an intracranial *in vivo* model with melanoma cells transfected with a murine homolog of EGFRvIII (33). Moreover, enhanced survival of rats bearing intracranial gliomas was observed when a boronated EGFRvIII-specific antibody (L8A4) was administered intracranially (34). MAB 806 is a monoclonal antibody which interacts with EGFRvIII and with EGFR overexpressed due to amplification (35, 36). MAB 806 was shown to inhibit glioma xenografts expressing EGFRvIII or overexpressing wild-type EGFR in subcutaneous and intracranial models (37, 38). MAB 806 does not bind to normal tissues and a phase I trial with this chimerized antibody has been completed (39). The antibody showed excellent targeting of tumor sites in all patients, no evidence of normal tissue uptake and no significant toxicity.

In splice variant de4 EGFR, exon 4 is removed (45 aa of the extracellular domain) and a novel glycine is generated at the splice junction (Figure 2A, lower panel) (40). Expression of this variant was shown in glioma (4/40), prostate cancer (3/11) and ovarian cancer (3/9). Ligand-independent autophosphorylation and self-dimerization was observed. This variant likely enhances proliferation and transformation through constitutive activation of extracellular signal-regulated protein kinase/protein kinase B (ERK/PKB) and up-regulation of the expression level of transcription factor of the jun family (JUN), as well as β-catenin phosphorylation induced by overexpressed SRC resulting in the destruction of E-cadherin/catenin adhesive complexes facilitating migration.

Receptor d'origin Nantais (RON) Splice Variants

The RON tyrosine kinase is a member of the c-met proto-oncogene product (c-MET) proto-oncogene family (41). The ligand of RON was identified as macrophage-stimulating protein (42). RON is a 180 kDa heterodimeric protein composed of a 40 kDa extracellular α chain and a 145 kDa transmembrane β chain (43). RON is overexpressed in many types of tumors and involvement in tumor cell migration and proliferation, angiogenesis and interaction with the tumor microenvironment have been documented (44). Recently, cross-talk between RON and c-MET causing c-MET-mediated resistance has been demonstrated (45). Since RON is expressed in different cell types, such as macrophages and osteoblasts, tumor-specific versions of RON would increase specificity as a therapeutic target. A monoclonal antibody inhibiting ligand receptor interaction has demonstrated *in vivo* activity in several xenograft models and is presently being evaluated in clinical studies (46). Three RON splice variants exhibiting deletions in the extracellular domain of the β chain (RONΔ165, RONΔ160 and RONΔ155) (47, 48) have been described. RONΔ165 has an in-frame deletion of 49 amino acids corresponding to exon 11, preventing proteolysis and keeping the protein in the cytoplasm. RONΔ160 has a 109 amino acids deletion in the extracellular domain corresponding to exons 5 and 6; the mature protein is located on the cell surface. RONΔ155 is generated by combined deletion of exons 5, 6 and 11; the cellular location of RONΔ155 is presently unknown. All three RON variants are constitutively active and can induce scattering of tumor cells in a ligand-independent manner. The constitutive activity is probably based on dimerization due to unbalanced cysteine residues in the extracellular domain. RONΔ160 is expressed in colorectal cancer and might represent a specific target for treatment of this disease. Further study revealed that RONΔ160 is expressed in a panel of colorectal cancer cell lines (49).

Epithelial Cellular Adhesion Molecule (EpCAM) Splice Variant

EpCAM is a 40 kDa transmembrane glycoprotein expressed on the basolateral surface of normal epithelia. EpCAM is significantly up-regulated in several carcinomas and is evenly distributed on the tumor cell surface and therefore is a target for both active and passive immunotherapy (50). A novel in-frame EpCAM splice variant designated as DD-0232v3 has been identified (51). DD-0232v3 has 76 additional amino acids inserted after the signal sequence, with a potential Arg Gly Asp (RGD) domain and arginine/proline rich domains (Figure 2C). The functional contribution with respect to cell-to-cell and cell matrix interaction is under investigation. Cell surface biotinylation and immunofluorescence experiments confirm localization of this splice variant to the plasma membrane. Unlike EpCAM, this splice variant is practically undetectable in normal epithelia. The cancer-specific overexpression, cell surface localization and the potential to be internalized indicate that DD-0232v3 might be a promising target for antibody-related agents.

L1 Cell Adhesion Molecule (L1CAM) Splice Variant

L1CAM is a neuronal adhesion molecule which is overexpressed in many tumor entities (52) and was shown to be involved in proliferation, invasion and metastasis. L1CAM is composed of 28 exons and 27 introns and the molecular weight of its gene products ranges between 300 and 220 kDa (53). The extracellular domain consists of six Ig-like domains and five fibronectin-like domains (53). L1CAM occurs in two isoforms, full-length L1CAM and a variant in which exons 2 and 27 have been deleted (Figure 2D). It was found that ovarian carcinoma cell lines BW and GG predominantly express L1CAMΔ(2,27) (54). Exon 2 encodes a peptide of five amino acids that affects homophilic and heterophilic binding to neuronal ligands, which are important for growth promotion of neuronal cells (55), whereas exon 27 encodes a cytoplasmic sequence YRSLE which mediates clathrin-dependent endocytosis and regulates L1CAM density at the cell surface (56). The deletion of exon 2 generates a new junction in the extracellular domain of L1CAM with the potential to generate monoclonal antibodies specific for this variant. In addition to cancer cells, the variant lacking exons 2 and 27 was also described in B-cells (57, 58). Comparative expression analysis between B-cells and tumor cells for L1CAMΔ(2, 27) are not available. Recently it was shown that full-length L1CAM and not the L1CAMΔ(2, 27) splice variant promotes metastasis through gelatinase expression (59).

Claudin18 Splice Variants

Claudin18 is a member of the claudin family which are four membrane-spanning domain transmembrane receptors involved in the formation of tight junctions (60). The human Claudin18 gene makes use of two alternative first exons, resulting in isoforms CLDN18.1 and CLDN18.2, differing in the *N*-terminal 69 amino acids including the first extracellular loop (Figure 2E) (61). The isoforms exhibit different lineage commitment: CLDN18.1 is preferentially expressed in lung tissue, CLDN18.2 displays a pronounced tropism of expression for stomach (61). The two variants differ in only 8 out of the 51 amino acids of the first extracellular domain, nevertheless a monoclonal antibody specific for CLDN18.2 was derived (62). CLDN18.2 was found to be expressed in gastric, pancreatic, esophageal, ovarian and lung tumors. CLDN18.2 is repressed in the vast majority of normal tissues, but is re-expressed in the context of malignant transformation. A recombinant monoclonal antibody directed against CLDN18.2 is currently in phase II clinical studies. Active immunization with a virus-like particle vaccine, such as hepatitis B virus core antigen virus-like particles which display a surface epitope of CDN18.2, resulted in formation of auto-antibodies with specificity for the CLDN18.2 isoform (63). It was shown that the induced antibodies were able to kill CLDN18.2-expressing cells *in vitro* by complement-dependent and antibody-mediated cytotoxicity. Moreover, partial protective immunity against the challenge of mice with syngeneic tumor cells expressing CLDN18.2 was demonstrated.

CD44 Splice Variants

CD44 is encoded by a single gene consisting of 20 exons. A large variety of splice variants can be generated since 10 of the exons can be spliced differentially (Figure 4) (64). CD44 and its isoforms are class I transmembrane glycoproteins. The amino-terminal domain of the standard isoform (sCD44) is separated from the plasma membrane by a 46 amino acids stem structure and the stem structure can be enlarged by amino acids derived from the differentially spliced exons (v-exons). CD44 acts both as an oncogene and as a tumor suppressor. Its function depends on the ligand involved and probably on tumor-specific regulation and tumor-inherent mutations (65). Tumor suppressor function of CD44 is mediated by binding of hyaluronic acid by CD44 and subsequent formation of a complex with merlin, which inhibits signaling to the nucleus and the cell cycle, and the formation of variant splicing isoforms. In contrast, growth promotion by CD44 and its isoforms is mediated by binding to growth factors resulting in oligomerization and formation of a complex with ezrin, promoting signaling to the nucleus and cytoskeletal organization. All isoforms containing exons 6 and 7 tend to cluster. Involvement of CD44 and its splice variants in a number of oncology-related signaling phenomena has been described (66). Among these are involvement in HER2/HER4 activation by recruitment of matrix metalloproteinase 7 (MMP7) and its substrate heparin-binding epidermal growth factor, probably through the heparan sulfate side chain of exon 3 of CD44, co-receptor function by catalyzing the dimerization of HER2 and HER3, the presentation of FGF members to FGFRs through the heparan sulfate side chain of the v3 exon-encoded amino acids and in the activation of HGF/c-met signaling. Research into CD44 has been spurred after demonstration that two of its splice variants (CD44v4-7 and CD44v6-7) decisively mediate metastasis of a pancreatic carcinoma cell line (67, 68). Since the v6 variant is also expressed in activated lymphocytes, it was proposed that activated lymphocytes and metastasizing tumor cells bind to a specific ligand in the lymph nodes (69). It was argued that tumor cells are retained in the lymph nodes by this mimicry, can multiply in a specific niche and thus increase

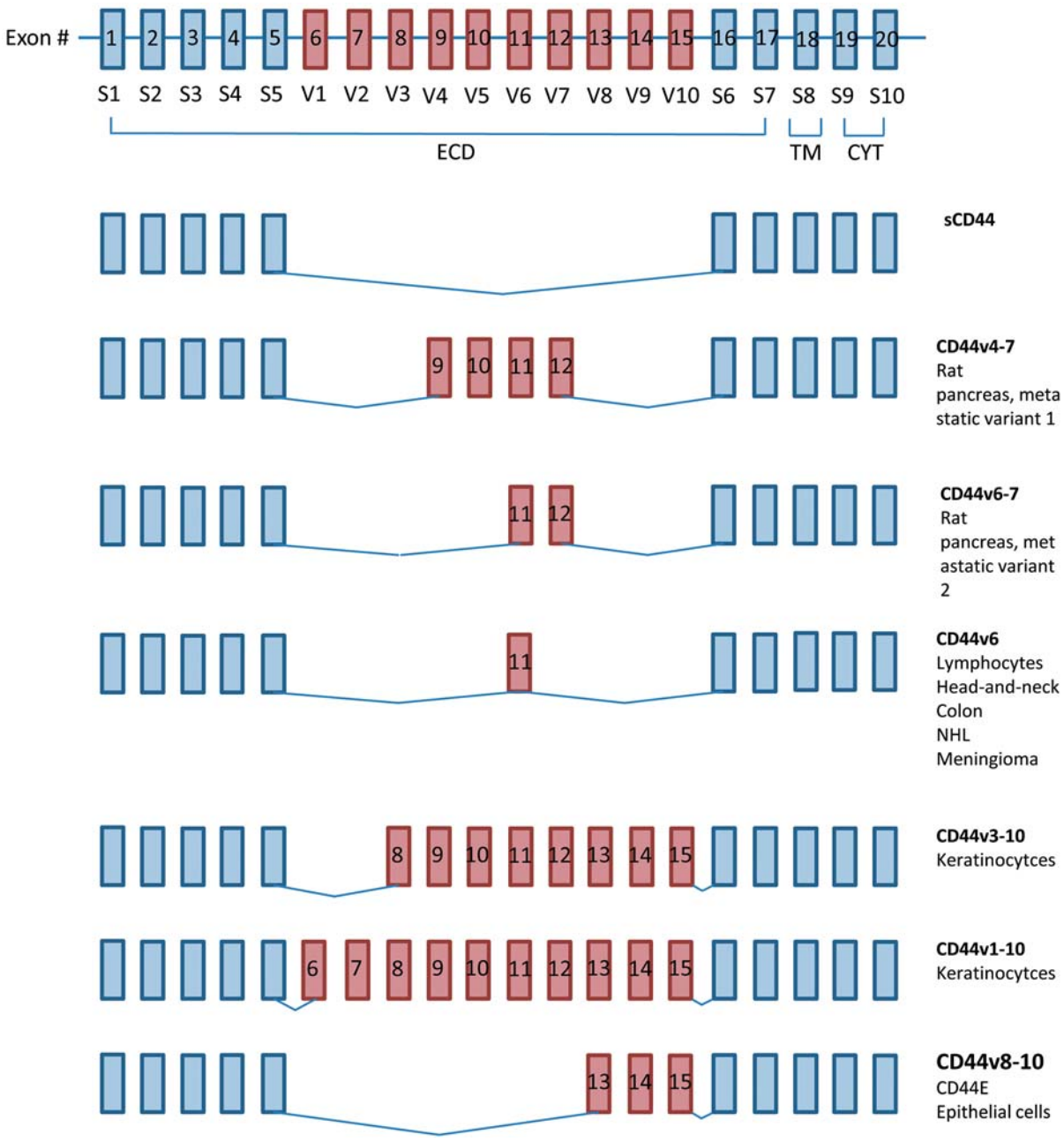

Figure 4. *Splice variants of CD44. At the top the exon-intron organization of the CD44 gene is shown. S1 to S10 designate exons of the standard isoform, V1 to V10 refer to exons identified in variant isoforms. Exons of the standard isoform of CD44 are shown in blue, exons of the variant isoforms are displayed in brown. Below, CD44 isoforms detected in various normal and tumor cells, as well as in human tumors, are shown. ECD: Extracellular domain; TY: thyroglobulin type-1 domain; CYT: cytoplasmic domain.*

the probability of successful metastasis. The expression of CD44 splice variants was identified as an early event in colorectal carcinogenesis and as a predictor of poor prognosis (70, 71). Stimulation of the RAS-MEK-ERK pathway induces the expression of v6-containing variants (72). Translational research has focused on splice variants containing the v6 exon. CD44v6 expression has been observed in cancer of the head-and-neck, lung, skin, cervix and esophagus and in normal tissues, expression of CD44v6 has been found in keratinocytes and normal mucosa and in myoepithelium of the prostate and the lung (72, 73). A high-affinity monoclonal antibody for

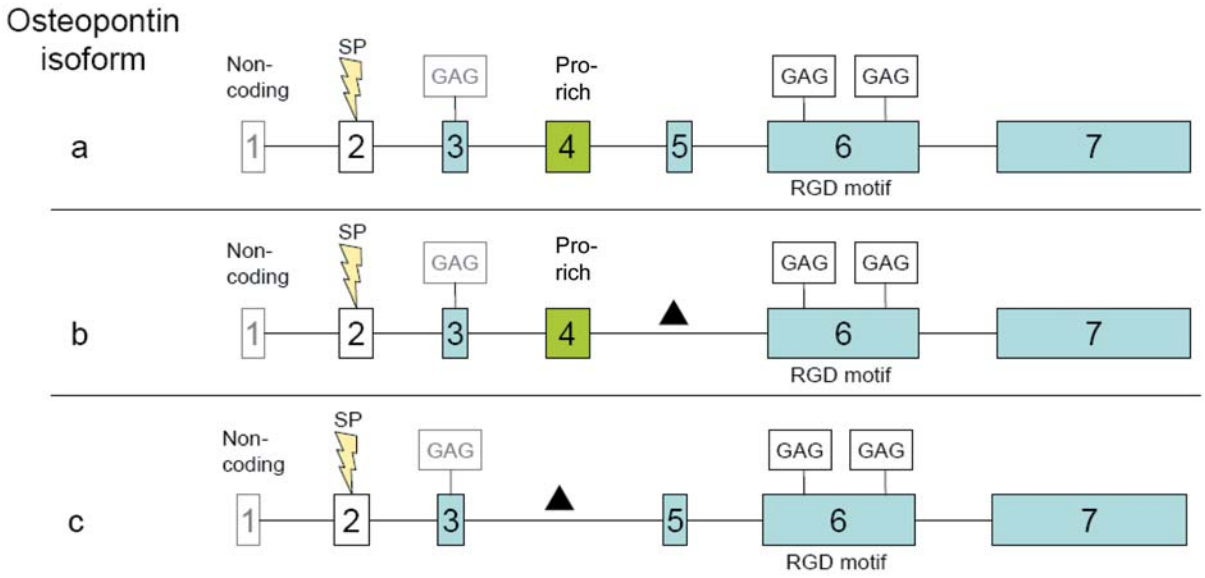

Figure 5. *Splice variants of osteopontin (OPN). The exons of the osteopontin gene are highlighted as boxes. a, b and c refer to OPN splice variants a, b and c. Exon 5 and exon 4 are missing in OPN variants b and c, respectively; a corresponds to the full-length protein. Exons marked in blue contain serine-phosphate-rich domains and differential splicing is indicated by a black triangle. SP: Signal peptide; GAG: glycosamino-glycan attachment site; Pro-rich: proline-rich domain; RGD motif: RGD-binding site.*

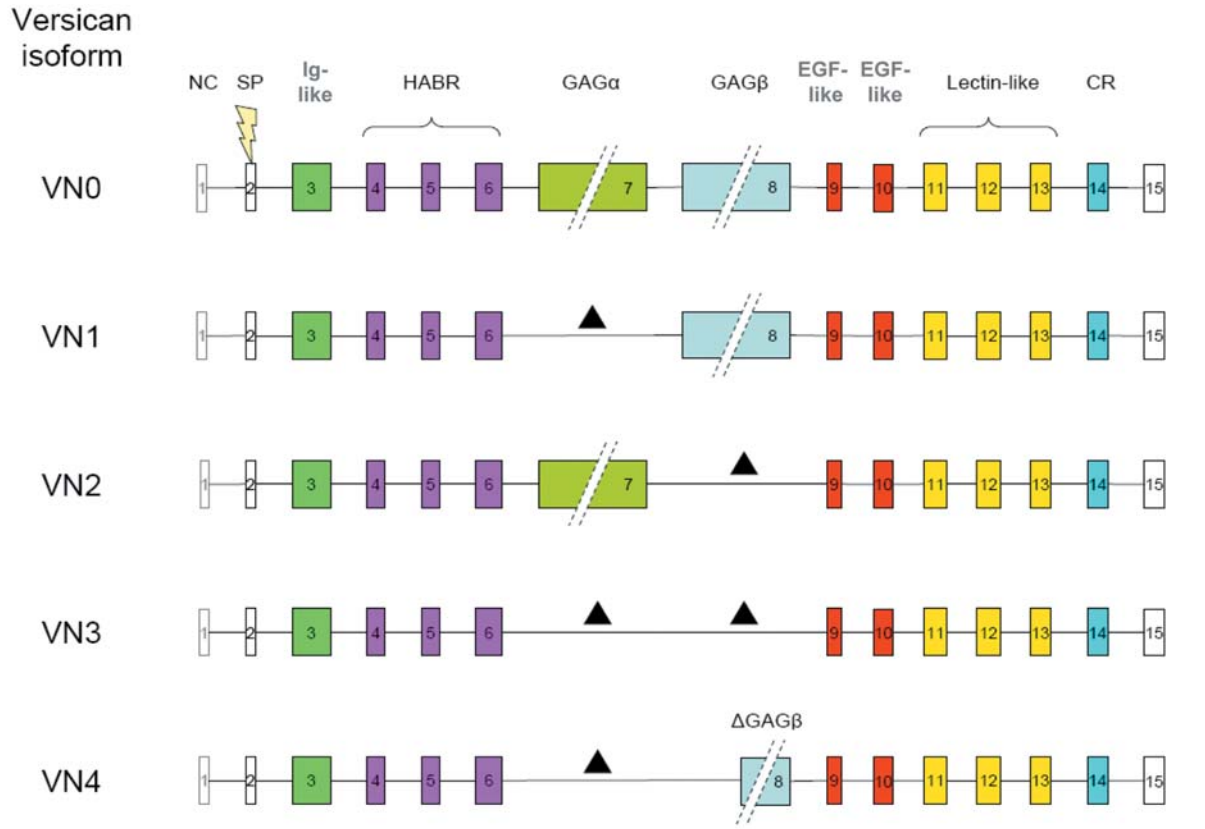

Figure 6. *Versican (VN) splice variants. The exons of the VN gene are shown as boxes. VN0 indicates the VN gene and its exons, VN1 to VN4 are variants of VN generated by differential splicing. Black triangles indicate differential splicing and stippled lines indicate that the sizes of the marked exons are not drawn to scale. CR: Complement-regulatory protein-like domain; EGF-like: epidermal growth factor-like domain; GAGα,β: glycosamino-glycan attachment sites containing exons; ΔGAGβ: truncated exon β containing glycosamino-glycan attachment sites; HABR: hyaluronic acid-binding region; NC: non-coding; SP: signal peptide.*

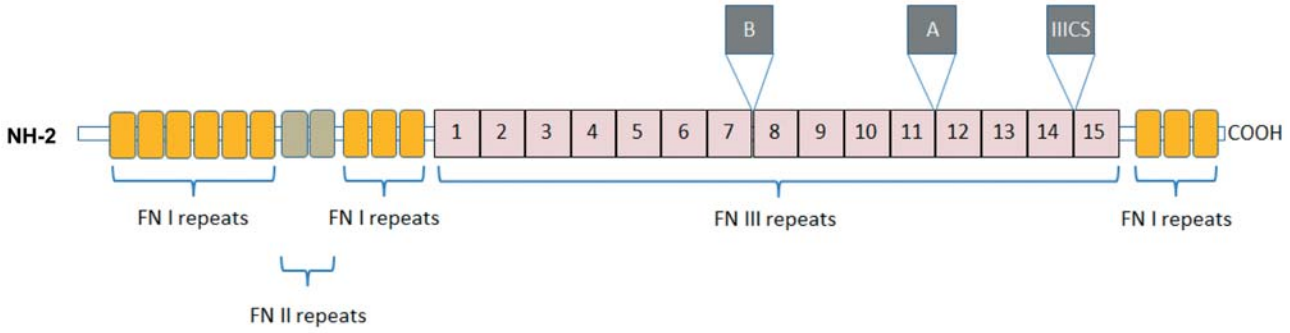

Figure 7. *Topology of fibronectin (FN) and its tumor-related splice variants. FN I, FN II and FN III repeats are highlighted by a different color code, differentially spliced exons (A, B and IIICS) are indicated in black.*

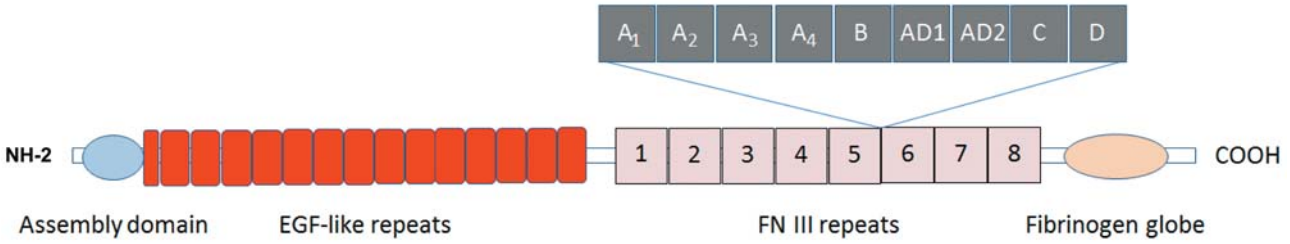

Figure 8. *Topology of tenascin (TN) and its tumor-related splice variants. Assembly domain, epidermal growth factor (EGF)-like repeats, fibronectin II (FN II) repeats and the fibrinogen globe domain are highlighted by a different color code. Differentially spliced exons (A1, A2, A3, A4, B, AD1, AD2, C and D) are indicated in black.*

immunotherapy was derived (74). A phase I study with (186)Re-labeled humanized antibody BIWA4 (bivatuzumab) in patients with head-and-neck squamous cell carcinoma demonstrated antitumor effects in patients with incurable, bulky disease (75). A phase I dose escalation study with the conjugate bivatuzumab mertansine in patients with incurable squamous cell carcinoma of the head-and-neck or esophagus revealed massive apoptosis of skin keratinocytes and one patient died after two infusions due to epidermal necrolysis. Therefore clinical development was discontinued (76). Binding to CD44v6 on skin keratinocytes was responsible for the severe skin toxicity. Subsequent studies have revealed expression of CD44 variants on human skin keratinocytes (77).

Osteopontin (OPN) Splice Variants

OPN is member of the small integrin-binding N-linked glycoproteins (78), a family of five integrin-binding glycophosphoproteins. Bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein are additional members of the family. The topology of human OPN and its splice variants is shown in Figure 5. OPN is expressed in bone and other tissues, is involved in bone remodelling and is overexpressed in a variety of types of cancer (78). OPN plays a role in almost all steps of tumor progression, including invasion, metastasis and angiogenesis (78). The RGD domain of human OPN promotes tumor growth through activation of nuclear factor kappa-light-chain enhancer of activated B-cells (NFκB) signaling and focal adhesion kinase (FAK) phosphorylation (79). OPN promotes angiogenesis by interaction with integrin αvβ3 which is significantly up-regulated on the surface of proliferating endothelial cells (80). Tumors overexpressing OPN often exhibit pathological microcalcifications and have a strong propensity for bone metastasis. Some of the observed phenomena might be mediated by specific isoforms of OPN. Three isoforms, OPNa, b and c, have been described (Figure 5). OPNa contains coding information from all exons, OPNb and c lack exon 5 and 4, respectively. OPNc isoform was shown to contribute to progression of ovarian carcinoma (81). This isoform was found to be overexpressed in tumor samples and to activate OvCAR-3 cell proliferation, migration, invasion, anchorage-independent growth and tumor formation *in vivo*, in contrast to isoforms a and b. Expression of prometastatic splice variant OPNc was detected in human pancreatic

ductal adenocarcinoma (82). In glioma cells, all splice variants of OPN protect glioma cells from apoptosis, isoforms a and c promote invasion through alteration of the levels of urokinase plasminogen activator and MMP2 and MMP9 (83). OPNc was identified as a marker highly specific for transformed breast cancer cells (84). In contrast, in hepatocellular carcinoma, OPNa and b isoforms were found in tumor tissue, whereas expression of the OPNc isoform was found only in normal tissue (85). Migration of hepatocellular carcinoma cells was promoted by isoforms OPNa and b, but not by isoform c. Taken together, these observations indicate that inhibition of OPN isoforms with antibodies or other agents would seem a promising approach for treatment of cancer, however, the functional role of OPN isoforms might be context-dependent.

Versican (VN) Splice Variants

VN is a chondroitin sulfate proteoglycan, a molecule in which polymeric side chains (glycosaminoglycans, GAG) are covalently attached to a core protein (86). VN and its four splice variants are located in the extracellular matrix (ECM) and are involved in ECM assembly, in proliferation, migration, survival and cell adhesion (87). VN binds to components of the ECM such as fibulin-1 and -2, hyaluronan, fibronectin, tenascin (TN)-R, type I collagen, P- and L-selectin, and several chemokines (88). Its expression is regulated by receptor tyrosine kinases (RTK), phosphoinositol 3-kinase (PI3K/PKB), sonic hedgehog and canonical wingless-int signaling (86). VN and its splice variants are shown in Figure 6. In VN0, an Ig-like domain is followed by hyaluronic acid-binding and GAG attachment sites encoded by exons 7 and 8 in the middle of the protein and EGF-like repeats, a lectin-like sequence and a complement-regulatory protein-like domain at the carboxy-terminal portion, resulting in a 2389 amino acid long core protein (89). Two chondroitin sulfate-carrying segments, GAGα and GAGβ, are present. The VN1 isoform lacks the GAGα segment, the GAGβ segment is not present in the VN2 isoform and the VN3 isoform lacks both GAG segments. The VN gene consists of 15 exons and isoforms VN1, VN2 and VN3 are generated by differential splicing of exons 7 and 8 (86). Isoform VN4 was found to be overexpressed in breast cancer (90). A total of 378 amino acids of its exon 8 are sandwiched between exon 6 and the beginning of exon 9, generated by differential splicing. Since VN expression can be assessed by immuno-histochemistry with antibodies which recognize all splice variants, the investigation of expression of VN isoforms in normal and tumor tissues remains to be addressed systematically. VN expression has been documented in fibroblasts, keratinocytes, arterial smooth muscle cells, connective tissue of various organs and in the peripheral and central nervous system (91). In a few cases, isoform distribution has been analyzed. VN0 and VN1 seem to be the main isoforms in VN-expressing tissues, whereas VN2 was found preferentially in the brain (92-94). VN is overexpressed in many types of tumors such as prostate, breast, brain and melanoma, when compared with corresponding normal tissues (90). In breast cancer, all VN isoforms including the newly discovered isoform VN4 were shown to be overexpressed at the RNA level (90). It remains to be investigated whether specific splice variants are expressed in tumor subtypes, with a restricted pattern of expression in normal tissues. If so, isoform-specific antibodies might be generated and evaluated as antibody-related cytotoxic agents. Recently, VN was identified as a tumor-derived macrophage activator (95) in the Lewis lung cancer model. Interaction of VN with toll-like receptor 2 (TLR2) and TLR6 on macrophages resulting in the production of inflammatory cytokines which enhance metastasis was demonstrated.

Fibronectin (FN) Splice Variants

FN is a dimeric protein linked by a pair of disulfide bonds. Soluble plasma FN is produced by hepatocytes and insoluble FN is a component of the ECM (96). As outlined in Figure 7, FN is composed of type I, II and III repeats. Two intracellular disulfide bonds form within each type I and type II module, type III modules are seven-stranded β-barrel structures that lack disulfides (97). Modules can be classified as binding sites for collagen, integrins, heparin and FN. An FN dimer can bind to integrins, such as α5β1, in order to organize the actin cytoskeleton to promote cell contractability (98). Several splice variants of FN have been identified. In transformed cells, the splicing pattern of FN becomes altered, leading to FN variants containing IIICS, extra domain (ED)-A and ED-B sequences (99). FN ED-B has attracted much attention because it accumulates selectively around new blood vessels in tumors (99). It is inserted between type III repeats 7 and 8, comprises 91 amino acids and is derived from a single exon. The sequence of this domain is identical in human, rat, mouse, rabbit and dog, however, a specific role in angiogenesis has not yet been defined. Expression of the ED-B domain in tumors of the head-and-neck, colon, glioblastoma, breast and skin has been demonstrated (99, 100). No staining was detected in normal tissues with the exception of some blood vessels in the ovaries and in proliferative phase endometrium. Splice variant III-A is generated by inclusion of a type III repeat and the third isoform, oncofetal glycosylated FN, is characterized by distinct post-translational glycosylation of the differentially spliced IIICS domain (99).

Translational research has focused on monoclonal antibodies directed against ED-B of FN, but auto-antibodies directed against all of the described isoforms are available (101). Synthetic antibody repertoires have allowed the identification of antibodies specifically binding to ED-B. This includes the single-chain Fv fragment (scFv) L19, which is an affinity-matured version of scFv E1 based on combinatorial mutagenesis of amino acids in the hypervariable loops (102). L19 was shown to localize specifically to tumor blood vessels in animals and humans (103, 104). A large number of therapeutic derivatives of L19 have been established and evaluated, such as conjugates of fluorochromes and radiosensitizers, procoagulant agents, enzymes, radionuclides and cytokines (97). Fusion of L19 to cytokines can improve the therapeutic index of cytokines. Different formats of L19, among them a complete human IgG1 and a human small immunoprotein (SIP) comprising the variable regions of L19 and a CH4 domain of human IgE, were constructed, expressed and their biodistribution in animals was monitored (105).

An L19-tumor necrosis factor α (TNFα) fusion protein was shown to exhibit antitumor activity markedly superior to untargeted TNFα in syngeneic tumor models (106). An immuno-cytokine between scFv (F8) specific to the extra A domain of FN and different combinations of the subunits (p35 and p40) of IL12 was constructed and based on the preclinical data, clinical evaluation is under consideration (107). Previously it was shown that an L19-IL12 fusion protein led to infiltration of the tumor microenvironment with macrophages, natural killer (NK) cells and lymphokine-activated killer (LAK) cells (108). A fusion protein between L19 and interleukin 2 (IL2) gave rise to an improved therapeutic index for IL2, pronounced antitumor activity and infiltration of experimental tumors with macrophages, LAK, NK cells and T lymphocytes (109). A phase I study was conducted with immunocytokine L19 IL2 (110). Five cohorts of patients with progressive solid tumors (n=21) received an intravenous infusion of L19 IL2 ($5\text{-}30\times10^6$ IU IL2 equivalent dose) on days 1, 3 and 5 every 3 weeks. Stable disease in 17/33 patients (51%) and 15/18 with metastatic renal carcinoma (83%) was observed after two cycles. Preliminary evaluation suggests clinical activity in patients with metastatic renal cell carcinoma, and selective expansion of NK cells in treated patients was demonstrated.

TN Splice Variants

TNs are glycoproteins of the ECM. Four members of the TN gene family have been identified: TN-C, TN-R, TN-X and TN-W (111). The topology of TN and its splice variants are summarized in Figure 8. The large isoform of TN-C is strongly overexpressed in tissues undergoing remodeling, such as wound healing and cancer, and is poorly expressed in normal tissues (112). TN-C is composed of an assembly domain, 14 1/2 EGF-like repeats, 8 constant fibronectin III homology repeats, 8 alternatively spliced FN III homology repeats and a fibrinogen globular domain (113). Through alternative splicing, nine type III homology repeats can be included or omitted in the mature mRNA generating structurally and functionally different TN-C isoforms (114). For the analysis of distribution of TN-C isoforms specific antibodies are available (115). There is growing evidence of deposition of TN-C in different types of cancer such as breast, colon, lung, renal, head-and-neck, chondrosarcoma and urothelial carcinoma (116). Antibody phage display technology was used to identify antibodies P16 and P12 specific for the alternatively spliced domains A1 and D of the large isoform of TN-C (117). Making use of the small SIP format, immunodistribution studies in xenografted animals were performed and accumulation of SIP (F16) in U87 glioblastoma was demonstrated, whereas it was rapidly cleared from other tissues. A monoclonal antibody to domain C of TN-C was shown to selectively target subcutaneously grafted U87 glioblastomas in mice (118). The G11 antibody was expressed as scFv and SIP format, as well as scFv IL2 fusion protein and revealed selective tumor uptake with a tumor/blood ratio of 11.8/1 at 24 h. These results are the first step for development of the G11 antibody for imaging and selective delivery in patients with glioma and lung tumors.

Conclusion

Already in 2000, six hallmark capabilities of cancer, namely sustaining proliferative signaling, evading growth suppressors, activation of invasion and metastasis, enabling of replicative immortality, induction of angiogenesis and resistance to cell death, had been noted (119). New emerging hallmarks and enabling characteristics, such as deregulation of cellular energetics, genomic instability and mutation, tumor-promoting inflammation and avoidance of immune destruction, have been added to the scenario (120). We have described that differential splicing has an impact on many of these processes, focusing here on transmembrane receptors and components of the ECM. The magnitude and specificity of expression for some of the described targets remains a critical issue and deserves further investigation. As outlined, clinical studies making use of the target characteristics generated by differential splicing will probably demonstrate proof-of-concept of this approach. Further targets due to differential splicing are emerging. Four novel splicing events were found in the extracellular domain of the rearranged during transfection (RET) transmembrane tyrosine kinase in papillary thyroid carcinoma (121). Several splice variants of the human urokinase plasminogen activator receptor (uPAR) have been described including one lacking exons 4 and 5 (uPAR4/5)

which encodes a uPAR in which domain 2 of the extracellular part is missing (122). mRNA steady-state levels of this splice variant were correlated with prognosis of breast cancer (123, 124). Further validation studies need to be performed to elucidate their relevance for antibody-based therapy of cancer.

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