PERSONALIZED MEDICINE AND NEUROSURGERY

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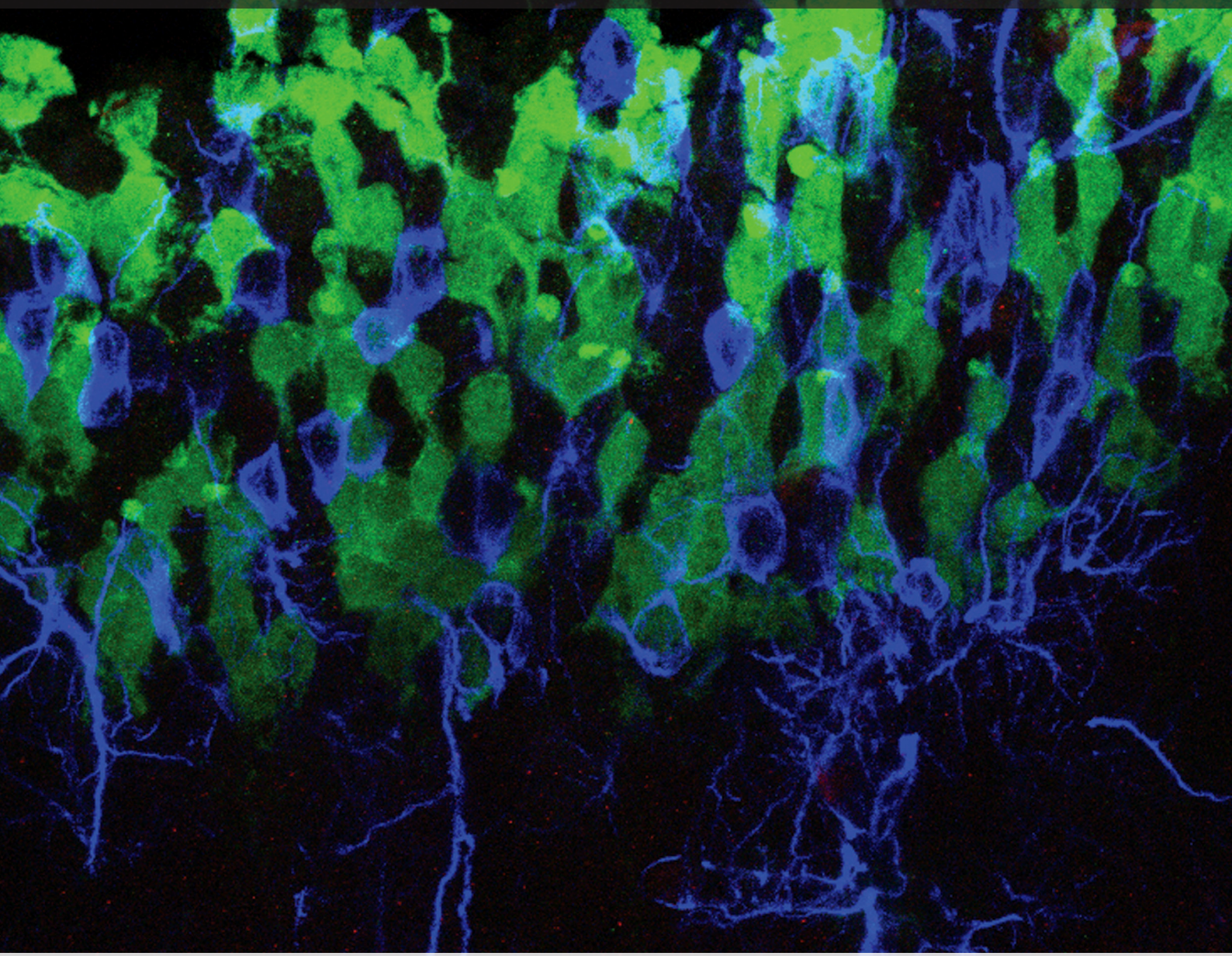

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PERSONALIZED MEDICINE AND NEUROSURGERY

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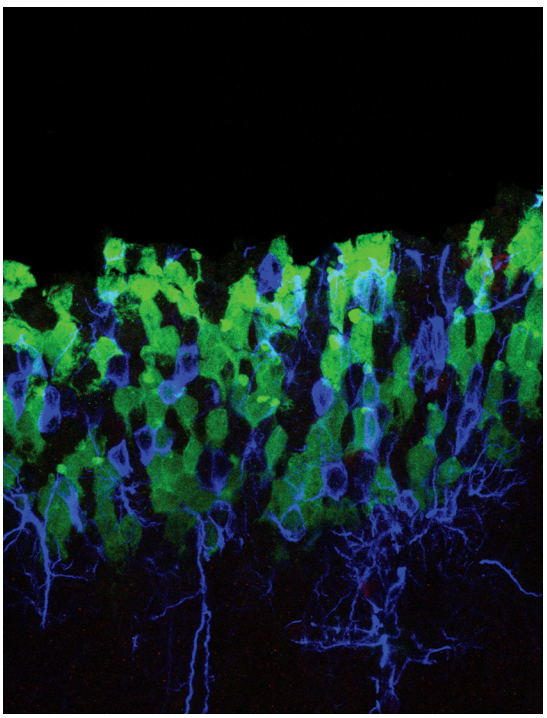

Neural stem cells of the 3rd ventricle reveal their glial nature in a P4 GFAP-CreERT2-YFP transgenic mouse. Fluorescent staining shows the induced expression of YFP (Green) in the cell body, while their basal processes express GFAP (Blue). A minor population expresses the quiescence marker ID1 (Red). Corresponding cell populations are found in the human postnatal brain, and defects in their genomic regulation of quiescence are critical drivers in the formation of pediatric brain tumors.

Image taken with Leica SPE laser scanning microscope.

Used with permission from Dr. Roberto Fiorelli, PhD.

The Precision Medicine Initiative, which was instituted by President Barack Obama on January 20, 2015, highlighted the importance that advances in genomics and related "-omic" approaches have made to science and medicine, and it set the stage for their federally funded and mandated integration into the delivery of health care. Whether these advances comprise large-scale approaches, such as The Cancer Genome Atlas, which provides a modern classification of cancers based on molecular profiles, or genealogy initiatives, which seek to trace the movement of our early ancestors out of Africa, genomic technology has taken us closer to developing targeted therapies and a refined understanding of our evolutionary journey. It is against this backdrop that we summarized some of the recent advances in the field of precision medicine, or personalized medicine, as they pertain to neurosurgery. In this e-Book collection provided by Frontiers in Surgery: Neurosurgery, we present a collection of articles by leaders in the field of neurosurgery that highlight domains using a personalized approach for the treatment of patients or avenues when personalization is possible and when it will likely alter the care of patients with neurological diseases.

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EDITORIAL
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Editorial: Personalized Medicine and Neurosurgery

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Keywords: personalized medicine, editorial, neurosurgery, precision medicine initiative, contributions

Editorial on the Research Topic

Personalized Medicine and Neurosurgery

The Precision Medicine Initiative, which was instituted by President Barack Obama on January 20, 2015, highlighted the importance that advances in genomics and related "-omic" approaches have made to science and medicine, and it set the stage for their federally funded and mandated integration into the delivery of health care. Whether these advances comprise large-scale approaches, such as The Cancer Genome Atlas, which provides a modern classification of cancers based on molecular profiles, or genealogy initiatives, which seek to trace the movement of our early ancestors out of Africa, genomic technology has taken us closer to developing targeted therapies and a refined understanding of our evolutionary journey. It is against this backdrop that we summarized some of the recent advances in the field of precision, or personalized, medicine as they pertain to neurosurgery. In this e-Book collection provided by *Frontiers in Surgery: Neurosurgery*, we present a collection of 13 articles by leaders in the field of neurosurgery that highlight domains using a personalized approach for the treatment of patients or avenues when personalization is possible and when it will likely alter the care of patients with neurological diseases.

The contributions in this collection are broadly divided into three sections pertaining to vascular neurosurgery, oncology, and spinal disorders. In the realm of vascular neurosurgery, contributions from Fennell et al. summarize the biology of saccular aneurysms and avenues for development of new therapies for this disease process. Achrol and Steinberg continue the discourse by highlighting emerging therapies for subarachnoid hemorrhage and sequelae of aneurysm rupture, both areas where great advances are possible. Baranoski et al. review the emerging literature on cerebral cavernous malformations, a relatively common etiology of hemorrhagic stroke and one with treatment likely to be altered by the introduction of small molecule inhibitors targeting cavernous malformation formation.

The oncology collection is rich with both surgical and molecular advances that have made treatment of primary and metastatic tumors of the central nervous system safer and more effective. Ghinda and Duffau present their experience with intraoperative mapping for personalization of treatment of low-grade gliomas. Belykh et al. summarize the advances in intraoperative fluorescence imaging that have allowed for more aggressive and extensive resection of tumors in the brain. Razavi et al. provide a summary of mechanisms of immune evasion characteristic of glioblastoma multiforme. Bi et al. provide an authoritative review on the genomics of meningiomas, focusing on the diagnostic and prognostic implications of new information. Schunemann et al. summarize the state of the art in the management of von Hippel–Lindau disease. Mooney et al. and Hardesty and Nakaji complete the section with a review of pituitary adenoma biology and a review of the mechanisms of brain metastasis, respectively.

The section on spinal disorders includes a collection of papers by spinal neurosurgeons at Barrow Neurological Institute. Walker et al. review advances in biomarker development for spinal cord injury, and Martirosyan et al. and Martirosyan et al. describe novel findings on the biology of degenerative disc disease and the role of microRNA markers in predicting spinal cord injury.

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With further advances in next-generation genomics technology and a decrease in its cost, physicians are likely to find that genomic information constitutes a pillar in their diagnostic and prognostic assessment of patients. Certainly, a much larger body of work focusing on other disease states could have been included in a collection such as this, but we sought to begin with a concise and select group of surgical neurological diseases. We hope to see our colleagues applying and taking the lead in the development of precision approaches to neurosurgical patients.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Cerebral Cavernous Malformations: Review of the Genetic and Protein–Protein Interactions Resulting in Disease Pathogenesis

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Mutations in the genes *KRIT1*, *CCM2*, and *PDCD10* are known to result in the formation of cerebral cavernous malformations (CCMs). CCMs are intracranial lesions composed of aberrantly enlarged "cavernous" endothelial channels that can result in cerebral hemorrhage, seizures, and neurologic deficits. Although these genes have been known to be associated with CCMs since the 1990s, numerous discoveries have been made that better elucidate how they and their subsequent protein products are involved in CCM pathogenesis. Since our last review of the molecular genetics of CCM pathogenesis in 2012, breakthroughs include a more thorough understanding of the protein structures of the gene products, involvement with integrin proteins, and MEKK3 signaling pathways, and the importance of CCM2–PDCD10 interactions. In this review, we highlight the advances that further our understanding of the "gene to protein to disease" relationships of CCMs.

Keywords: cavernous malformation, CCM, *CCM1*, *CCM2*, *CCM3*, *KRIT1*, *PDCD10*

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INTRODUCTION

Cerebral cavernous malformations (CCMs) are intracranial lesions comprised of low flow and abnormally dilated capillary endothelial channels with increased permeability that predispose these vessels to episodes of thrombosis and focal hemorrhage, resulting in seizures and neurologic deficits.

Loss-of-function mutations in the genes Krev interaction trapped 1 (*KRIT1* or *CCM1*), cerebral cavernous malformation 2 (*CCM2*), and programed cell death protein 10 (*PDCD10* or *CCM3*) result in the formation of CCMs. Although a role for these three genes in the formation of these intracranial vascular lesions has been established since the 1990s, additional works have further elucidated the molecular mechanisms by which mutations in these genes and the resultant aberrant proteins interact, leading to the formation of CCMs.

The three CCM proteins coded by *KRIT1*, *CCM2*, and *PDCD10* form a trimeric protein complex. Germline loss-of-function mutations in any of these genes may lead to the formation

Abbreviations: CCM, cerebral cavernous malformation; FAT, focal adhesion targeting; HP1, hydrophobic patch 1; ICAP1, integrin cytoplasmic-associated protein-1; MLC, myosin light chain; ROCK, Rho-associated coiled-coil-forming kinase; SMURF1, Smad ubiquitin regulatory factor 1.

of CCMs. Therefore, it is reasonable to assume that a molecular pathway exists that requires all three proteins to function together correctly for proper cellular function. Moreover, research is demonstrating how each component protein is capable of interacting with numerous other signaling and cytoskeletal molecules allowing for a diverse range of functions in molecular signaling pathways *via* unique protein–protein interactions.

In this review, we highlight some of these recent advances that further our understanding of the "gene to protein to disease" relationships of CCMs. This work is meant to expand upon and to serve as an update to the previous review from this institution published in 2012 (1).

KRIT1 (CCM1)

In 1994, Kurth et al. were the first to begin successful mapping of a causative gene for CCMs (2). Utilizing linkage analysis, these authors identified the q11–q12 region of chromosome 7, specifically a 33-centimorgan (cM) region from D7S502 to D7S479, as potentially being responsible for CCM formation in a large Hispanic family. Concurrently, Marchuk et al. identified linkage between CCM and a sequence on the proximal long arm of chromosome 7 between D7S502 and D7S515 (3), and Gunel et al. successfully identified the D7S699 locus as linked to CCM (4). The potential region for the precise location of the *CCM1* gene was further refined to a 4-cM segment of the human 7q21-q22, bounded by D7S2410 and D7S689 (5, 6).

Krev interaction trapped 1 was subsequently identified as the *CCM1* gene in an analysis of multiple affected Hispanic families (7, 8). This finding was confirmed in a 1999 study involving French families with hereditary CCMs (9).

Krev interaction trapped 1 encodes the 736-amino acid peptide. It is the largest of the three CCM proteins and is comprised of an N-terminal Nudix domain with three NPxY/F motifs, an ankyrin repeat region, and a C-terminal FERM domain (band 4.1 protein, ezrin, radixin, and moesin) (10). Although KRIT1 has no known catalytic activity, it binds and interacts with scaffolding and signaling molecules.

KRIT1 and Integrin Activation

Krev interaction trapped 1 interacts with integrin cytoplasmic-associated protein-1 (ICAP1) (10). Integrins are transmembrane receptors whose functions include cellular attachment to the extracellular matrix as well as established roles in cell-to-cell signal transduction, embryogenesis, and tissue formation and repair. ICAP1 is one of the few established suppressors of integrin activation (10, 11). The N-terminal of KRIT1 binds to ICAP1 *via* the first of its three NPxY/F motifs and an unpredicted binding motif encompassing residues H172 to R185 (10). Liu et al. demonstrated that ICAP1 utilizes the same binding domain to interact with KRIT1 as it does with β-integrin peptides (10). Therefore, ICAP1 cannot bind to integrin and suppress its activation when it is bound to KRIT1, resulting in increased integrin activation while ICAP1 is bound to KRIT1.

Interestingly, it also appears that KRIT1 stabilizes ICAP1 and that the loss of KRIT1 results in decreased ICAP1 levels and, therefore, increased integrin activation (12). Faurobert et al. found that the loss of KRIT1 or CCM2 resulted in ICAP1 destabilization and a subsequent increase in β1 integrin activity (12). Furthermore, they found that endothelial cells that are lacking KRIT1 or CCM2 do not properly interact with the extracellular matrix, and that this anomalous interaction with the extracellular matrix may impair endothelial barrier function and result in increased RhoA-dependent contractility.

It is possible that mutations leading to aberrant KRIT1–ICAP1 interactions could produce abnormal integrin activation and consequently disrupt normal tissue development. Although the precise role of the KRIT1–ICAP1 interaction on integrin activity in endothelial cells has not been fully elucidated, it is clearly an area that warrants further investigation.

Work by Renz et al. has demonstrated that CCM proteins are involved in the modulation of the β1-integrin signaling cascade that regulates angiogenesis (13). These authors showed that loss of CCM proteins in endothelial cells results in the β1-integrin-dependent overexpression of the transcription factor, Krüppel-like factor-2 (KLF2). This overexpression of KLF2 subsequently results in increased activation of epidermal growth factor-like domain-containing protein 7 (EGFL7) and angiogenesis. This work suggests that CCM proteins are critical regulators of endothelial quiescence, and that loss of proper CCM signaling can result in aberrant angiogenesis. It is possible that this CCM-mediated regulation of KLF2 is further regulated by a CCM2–MEKK3 [mitogen-activated protein kinase kinase kinase-3 (MAP3K3)] interaction (14–16).

Krev interaction trapped 1 localizes at endothelial cell–cell junctions. The loss of KRIT1 results in impaired endothelial cell–cell junctions and loss of integrity associated with increased Ras homolog gene family (member A), RhoA, and protein activity (17–20).

KRIT1 and the Heart of Glass Receptor

The heart of glass receptor (HEG) is a transmembrane protein that plays a role in cardiovascular development. The loss of HEG results in aberrant cardiovascular morphogenesis (19, 21). Work by Gingras et al. demonstrated that the C-terminal FERM domain of KRIT1 binds to HEG and that this interaction is critical for the proper localization of KRIT1 at endothelial cell–cell junctions (19). Inhibition of this interaction results in failure of KRIT1 to localize at the endothelial cell–cell junctions, which results in aberrant cardiovascular development.

Interestingly, recent work by Zheng et al. demonstrated that postnatal mice with conditional knockout of HEG in endothelial cells do not form cavernous malformations (21). However, postnatal mice with conditional knockout of CCM2 in endothelial cells rapidly develop CCMs in the central nervous system. These authors were also able to demonstrate the absence of HEG mutations in a cohort of human patients with sporadic CCMs (sporadic CCMs are single isolated lesions that occur in the absence of germline mutations in *KRIT1*, *CCM2*, or *PDCD10*). Together, these findings suggest that HEG–CCM interactions are critical for embryonic cardiovascular development and growth and that CCMs arise due to postnatal HEG-independent CCM signaling aberrations in the endothelium of the central nervous system.

KRIT1 and Notch Signaling

Krev interaction trapped 1 is also linked to Notch signaling. Wustehube et al. found that KRIT1 inhibition results in decreased Notch pathway activity while KRIT1 overexpression leads to upregulation of the Notch pathway as demonstrated by increased DLL4 expression (22). This reduction in Notch signaling in KRIT1-deficient endothelial cells results in irregular vascular sprouting and abnormal angiogenesis. Schulz et al. demonstrated that silencing of KRIT1 in endothelial cells resulted in decreased Notch3 activity in cocultured brain pericytes (23). Additionally, these authors found that DLL4 proteins stimulated Notch3 receptors on human brain pericytes and that activated Notch3 induced the expression of *PDGFRB2*, *N-Cadherin*, *HBEGF*, *TGFB1*, *NG2*, and *S1P* genes. Upregulated Notch3 signaling in pericytes promoted proper pericyte–endothelial cell interactions, stimulating proper angiogenesis (23). Pericytes devoid of functional Notch3 signaling failed to suppress aberrant angiogenesis adequately. Therefore, proper Notch–KRIT1 interactions and subsequent endothelial cell–pericyte interactions are important to maintain proper vascular development, and dysregulation of Notch signaling and the Notch3–KRIT1 interaction may contribute to the pathogenesis of CCMs.

KRIT1 and Regulation of Reactive Oxygen Species

Krev interaction trapped 1 also interacts with pathways that regulate the degradation of reactive oxygen species. A lack of KRIT1 results in the decreased expression of superoxide dismutase-2, a reactive oxygen species scavenging molecule, which leads to increased levels of reactive oxygen species, AKT (protein kinase B) phosphorylation, and AKT-dependent forkhead box protein O1 (FOXO1) phosphorylation (24).

Choquet et al. have demonstrated that increased levels of reactive oxygen species and oxidative stress, as marked by deregulation of cytochrome P450, may contribute to increased severity of CCM disease (25). These authors found that patients with concomitant mutations in the cytochrome P450 family of proteins and CCMs tended to have more lesions, larger lesions, and higher rates of intracranial hemorrhage.

CCM2 and PDCD10

The identification of families with hereditary CCMs, but no *KRIT1* mutations, highlighted the possibility of the involvement of genetic loci other than *KRIT1* in the pathogenesis of CCMs (26, 27). Indeed, evidence began to emerge that linked two additional chromosomal regions in families with CCMs – one segment on 7p and one on 3q (26). The *CCM2* gene was successfully mapped to 7p15-p13, spanning an 11-cM region between D7S2846 and D7S1818. Within in this region, Liquori et al. identified eight genes that were the most likely to be involved in CCM pathogenesis, including one gene that contains a phosphotyrosine-binding domain and was predicted to interact with *KRIT1* (28). In 2004, the *CCM2* gene was confirmed by Denier et al. and was identified as being located on 7p13, containing 10 coding exons (27). *CCM2* codes for the 444-amino acid protein CCM2/malcavernin, which contains a predicted N-terminus phosphotyrosine-binding domain and a C-terminal helical domain (28, 29). Endothelial cells require CCM2 for proper cytoskeletal structure, cell–cell interactions, and vessel lumen formation *via* the interaction of many critical signaling pathways.

The third CCM locus, PDCD10/CCM3, is located on 3q25.2-27. This locus was identified within a 22-cM interval flanked by D3S1763 and D3S1262 (26). The role of *PDCD10* in CCM pathogenesis was first proposed in 2005 by Bergametti et al. (30). They screened 20 unrelated families with CCMs, but found no mutations in *KRIT1* or *CCM2*.

Programed cell death protein 10, located on 3q26.1, is a highly conserved gene containing seven coding and three non-coding exons, which result in a 212-amino acid protein (PDCD10). This protein is ubiquitously expressed and has an N-terminal dimerization domain and a C-terminal focal adhesion targeting (FAT)-homology domain (31). PDCD10 is the third member of the CCM protein complex and binds directly to CCM2 as well as several other signaling molecules (31, 32).

CCM2, CCM2L, and Interactions with the MEKK3 Pathway

One of the roles of CCM2 in the CCM signaling pathway is to solidify endothelial cell–cell junctions and stabilize vascular structures. A significant development in the study of CCM2 and its functions was the discovery of its paralog. Termed CCM2-like (CCM2L), this peptide has a high sequence homology to CCM2 and is selectively expressed in endothelial cells during periods of angiogenesis (33). Although there appears to be some similarity in their functions, the loss of CCM2L in *Xenopus* (frog) results in a phenotype similar to that of CCM2 knockouts, and the CCM2L-null phenotype can be rescued by overexpression of CCM2 (34); the two molecules are not entirely homologous. Because the three CCM proteins bind to each other to form a trimeric complex, CCM2L directly competes with CCM2 for binding to KRIT1, and therefore, subsequently inhibits CCM2-mediated endothelial cell–cell adhesion stability by uncoupling these upstream components of the CCM pathway from CCM3 (33). Interestingly, CCM2L does not compete with CCM2 for binding to PDCD10. These results suggest that CCM2L and subsequent modulation of the CCM pathway are molecular mechanisms by which endothelial cells maintain vessel stability and induce postnatal vessel growth.

Work by Cullere et al. in 2015 further elucidated the relationship between CCM2 and CCM2L and their mechanisms of action (15). These authors demonstrated that both CCM2 and CCM2L could bind to and inhibit MEKK3 in a complex with KRIT1. MEKK3 and its downstream targets and effectors function in key signaling pathways, including those involved in endothelial–mesenchymal transition, cell proliferation, and cellular migration. Moreover, the MEKK3 pathway plays a critical role in early cardiovascular development (35). Binding of both CCM2 and CCM2L to MEKK3 inhibits its activation and prevents its ability to phosphorylate MEK5 (dual specificity mitogen-activated protein kinase kinase 5), a downstream target. Lack of CCM2 also results in increased activation of extracellular regulated kinase 5 (ERK5), a mitogen-activated protein kinase 5, in endothelial cells. ERK5 is ubiquitously expressed in endothelial cells, where it is thought to play a role in cell survival and maturation. These findings

suggest that both CCM2 and CCM2L are capable of regulating the activity of MEKK3, and therefore augmenting multiple major signaling pathways that have essential cellular functions.

The mechanism of CCM2 interaction with MEKK3 was discovered in 2015 by Wang et al. (16). They described the crystal structure of the C-terminus of the CCM2 peptide and found that it contains a five-helix domain followed by a C-terminal tail that forms a separate, isolated helix capable of interacting with the other five helical domains. Furthermore, they discovered that the MEKK3 N-terminal helix binds the C-terminus of the CCM2 peptide, successfully revealing the mechanism for CCM2-MEKK3 interaction and signaling.

Zhou et al. determined the role of CCM proteins in the MEKK3 pathway (14). They demonstrated that CCM deficiency results in increased endothelial cell expression of transcription factors KLF2 and KLF4 *via* lack of inhibition of the MEKK3 signaling cascade. They also showed that exogenous inhibition of MEKK3 in CCM-deficient organisms is capable of rescuing the CCM-deficient phenotype.

CCM2–PDCD10 Binding and Mutual Complex Stabilization

Perhaps the most intriguing recent discovery involving the CCM proteins includes the interaction between CCM2 and PDCD10. The three CCM proteins bind together to form a trimeric molecule, and while the binding relationships between KRIT1 and CCM2 – and its paralog CCM2L – have been well described (33, 34), the molecular mechanism and functional importance of the CCM2-PDCD10 binding were only recently identified. Draheim et al. successfully demonstrated both the mechanism of interaction between the CCM2 and PDCD10 molecules and that this interaction is required for their activity (36).

The crystalline structure of PDCD10 has an N-terminal dimerization domain and a C-terminal FAT-homology domain. Within this FAT domain is a highly conserved surface termed "hydrophobic patch 1" (HP1) that is critical for PDCD10 binding with numerous molecules including CCM2 (31, 37). However, the mechanism by which this domain interacted with CCM2 was unknown. Draheim et al. showed that the structure of CCM2 contains short helical sequences called LD motifs, and it is at these LD motifs that CCM2 binds PDCD10 *via* its highly conserved HP1 region of the PDCD10 FAT domain (36). Furthermore, the authors demonstrated that this binding stabilizes the CCM2–PDCD10 complex and prevents proteasomal degradation of the protein complex. The loss of proper binding of CCM2 to PDCD10 due to aberrations in the binding domains results in abnormal endothelial cell function, demonstrating that these domains are both essential for CCM2–PDCD10 interaction, CCM protein signaling, and endothelial cell function (36).

ADDITIONAL DEVELOPMENTS

KRIT1, CCM2, PDCD10, and RhoA–ROCK Signaling

Loss of KRIT1 function results in impairment of endothelial cell–cell junctions, with a loss of integrity and an associated increase in RhoA activity (17–20). Activated RhoA levels are also increased in endothelial cells lacking the normal function of CCM2 or PDCD10 (17, 38–40).

Activated RhoA results in actin polymerization and stress fiber formation, in part *via* the RhoA effector molecule Rho-associated coiled-coil-forming kinase (ROCK). ROCK, a serine-threonine kinase, polymerizes actin and increases actomyosin contractility *via* inhibition of myosin light chain (MLC) phosphatase. Inhibition of any of the CCM proteins results in increased levels of phosphorylated MLC, increased stress fiber formation, and the inability of endothelial cells to properly migrate, form three-dimensional tubal structures, and create a stable impermeable monolayer (20, 39–41). All of these anomalies in CCM-knockout mice were successfully rescued by inhibition of ROCK, further supporting the role of RhoA–ROCK signaling in the CCM phenotype (20, 39–41).

The precise mechanism by which CCM proteins interact with the RhoA pathway remains to be fully elucidated. Some possibilities include the interaction of KRIT1 with β1 integrin signaling (10, 12).

Cerebral cavernous malformation 2 may selectively promote E3 ubiquitin ligase-mediated degradation of RhoA *via* interaction with Smad ubiquitin regulatory factor 1 (SMURF1) (42). Crose et al. found that cells lacking CCM2 possessed increased levels of RhoA, but not increased levels of other known SMURF1 substrates, indicating that disruption of CCM2 does not inhibit SMURF1 itself, but rather the interaction of SMURF1 with RhoA (42).

Zheng et al. showed that RhoA activity increases when STK25 (a GCKIII serine-threonine kinase and known binding partner of PDCD10) is knocked down, which could be a potential mechanism for increased RhoA activation in PDCD10-deficient endothelial cells (38).

PDCD10 and Neuronal Migration

Additional functions of CCM proteins are being identified. Louvi et al. discovered that PDCD10 has a pivotal role in neuronal migration *via* suppression of RhoA signaling. They demonstrated that PDCD10 activity is required for proper radial glia and pyramidal neuron migration through the subventricular zone (43). Loss of PDCD10 resulted in dysregulation of the actin and microtubule cytoskeleton and adversely affected cellular morphology and migration. This dysregulation may be a result of CCM-mediated regulation of RhoA signaling.

PDCD10 Mutations Associated with Increased CCM Severity

Although loss-of-function mutations in any of the three CCM genes may result in CCM formation, different mutations result in varying degrees of disease severity. Patients with CCMs harboring PDCD10 mutations have a significantly greater disease burden and severity compared to those with KRIT1 or CCM2 mutations. Cigoli et al. found that patients with PDCD10 mutations had an earlier onset of disease symptomology compared to those with KRIT1 or CCM2 mutations. Shenkar et al. demonstrated that patients with familial PDCD10 mutations had a significantly more aggressive clinical CCM disease phenotype than patients with KRIT1

or CCM2 familial disease or sporadic lesions (44). Patients with PDCD10 mutations had an increased number of lesions and also presented with lesion hemorrhages earlier in life. Moreover, these authors found additional PDCD10 aberrations in addition to the CCMs, including scoliosis, cognitive disability, and skin lesions, further suggesting that PDCD10 plays other roles in tissue development aside from endothelial cell formation (43, 44).

PDCD10 and Meningiomas

Programed cell death protein 10 mutations are becoming increasingly identified in other disorders of tissue development. A particularly exciting discovery is the predisposition of patients with PDCD10 mutations to develop meningiomas in addition to CCMs. Several reports in the literature demonstrate that patients with familial PDCD10 mutations have developed late–onset meningiomas in addition to multiple CCMs (45–47). Such reports highlight the potential functional diversity of CCM proteins in tissue development.

Endothelial-to-Mesenchymal Transition in CCMs and Potential Role of Anti-inflammatory Agents

Another recent intriguing development in the study of CCMs is the discovery that PDCD10-deficient endothelial cells in CCMs undergo endothelial-to-mesenchymal transformation (48). This transformation is the result of the loss of PDCD10-mediated regulation and subsequent upregulation of β-catenin signaling. Bravi et al. also found that once this change occurred in the endothelial cells of CCMs, TGF-β/BMP signaling was subsequently required for the progression of the disease (48). The authors also found that this endothelial-to-mesenchymal cell transformation occurred in sporadic CCM lesions in addition to the familial and animal model lesions (49). While these findings are interesting from a pathogenic standpoint, they are even more intriguing because they suggest potential therapeutic options for the treatment and prevention of CCMs. Indeed, Bravi et al. found that the anti-inflammatory drugs sulindac sulfide and sulindac sulfone, which attenuate β-catenin transcription activity, reduced aberrant vascular malformations in a murine PDCD10-deficient model of CCMs (48).

CONCLUSION

Significant research findings from 2000 to 2015 have further enhanced our understanding of the pathogenesis of CCM formation. The use of advanced sequencing technologies to characterize genomic mutations and the identification of new signaling pathways and protein–protein interactions have led to great strides in understanding the molecular genetics involved in the development of CCMs. However, many unanswered questions remain, and future studies are clearly needed to improve our understanding of CCM pathogenesis. "Gene to protein to disease" mechanisms involved in the pathogenesis of CCMs should shed further light on potential therapeutic targets.

AUTHOR CONTRIBUTIONS

All the authors made substantial contributions to the conception or design of the work.

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CORRECTION
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Corrigendum: Cerebral Cavernous Malformations: Review of the Genetic and Protein–Protein Interactions Resulting in Disease Pathogenesis

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Keywords: cavernous malformation, CCM, *CCM1*, *CCM2*, *CCM3*, *KRIT1*, *PDCD10*

A corrigendum on

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by Baranoski JF, Kalani MYS, Przybylowski CJ, Zabramski JM. Front Surg (2016) 3:60. doi: 10.3389/fsurg.2016.00060

In the original article, the reference *Cigoli et al.* is missing from the section "Additional Developments", sub-section "*PDCD10 Mutations Associated with Increased CCM Severity*".

The text of the subsection should read:

Although loss-of-function mutations in any of the three CCM genes may result in CCM formation, different mutations result in varying degrees of disease severity. Patients with CCMs harboring PDCD10 mutations have a significantly greater disease burden and severity compared to those with KRIT1 or CCM2 mutations. Cigoli et al. found that patients with PDCD10 mutations had an earlier onset of disease symptomology compared to those with KRIT1 or CCM2 mutations (50). Shenkar et al. demonstrated that patients with familial PDCD10 mutations had a significantly more aggressive clinical CCM disease phenotype than patients with KRIT1 or CCM2 familial disease or sporadic lesions (44). Patients with PDCD10 mutations had an increased number of lesions and also presented with lesion hemorrhages earlier in life. Moreover, in addition to the CCMs, these authors found PDCD10 aberrations, including scoliosis, cognitive disability, and skin lesions, further suggesting that PDCD10 plays other roles in tissue development aside from endothelial cell formation (43, 44).

The authors apologize for this error and state that this oversight does not change the scientific conclusions of the article in any way.

REFERENCE

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Biology of Saccular Cerebral Aneurysms: A Review of Current Understanding and Future Directions

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Understanding the biology of intracranial aneurysms is a clinical quandary. How these aneurysms form, progress, and rupture is poorly understood. Evidence indicates that well-established risk factors play a critical role, along with immunologic factors, in their development and clinical outcomes. Much of the expanding knowledge of the inception, progression, and rupture of intracranial aneurysms implicates inflammation as a critical mediator of aneurysm pathogenesis. Thus, therapeutic targets exploiting this arm of aneurysm pathogenesis have been implemented, often with promising outcomes.

Keywords: biology, inflammation, intracranial aneurysm

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INTRODUCTION

Intracranial aneurysms are common abnormalities of the brain (1–30). The reported prevalence was 3.2% in a homogeneous Finnish population and up to 5% in others (31, 32). The overall risk of rupture is about 1% (33, 34). At 40–65%, the overall lethality of subarachnoid hemorrhage (SAH) resulting from cerebral aneurysm rupture is significant (31, 35, 36). Thus, SAH remains a challenging clinical issue (31, 32, 37–43). Of patients who survive the initial ictus, ≤50% face significant morbidity (31, 38, 40, 44, 45).

The true natural history of cerebral aneurysms is incompletely understood. Types of cerebral aneurysms include giant, fusiform, and saccular. In this review, we focus on saccular aneurysms. Although much of the aneurysm biology remains unknown, a growing body of literature addresses their formation, progression, and rupture.

CLINICAL RISK FACTORS

Risk factors for intracranial aneurysms include the epidemiological risk factors of female sex, smoking, hypertension, and family history, which is the strongest indicator of rupture among non-modifiable risk factors. Compared to the general population, first-degree relatives of persons with intracranial aneurysms or previous SAH have a risk 3–7 times higher and tend to have ruptured aneurysms at younger ages than those with sporadic aneurysms (37, 38, 40, 42, 46–48). In a cohort of 142 patients with 181 unruptured aneurysms followed from the 1950s until 1997–1998 for death or SAH, the annual incidence of hemorrhage was 1.3% (36). Cumulative rates of bleeding were 11% at 10 years, 23% at 20 years, and 30% at 30 years. Associated risk factors were aneurysm diameter and age. Smoking was an independent covariate related to rupture risk.

Abbreviations: IL, interleukin; NF-κB, nuclear factor-κB; SAH, subarachnoid hemorrhage; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cell.

ANATOMICAL AND CIRCULATORY FACTORS

Aneurysms develop at branch points of high intravascular turbulence and abnormal vessel wall shear stress. They arise in areas with complex arterial vascular geometry, particularly bifurcations and curvatures that contribute to increases in wall shear stress. Although formation is linked to diffuse genetic/familial, environmental, and immunologic risk factors, saccular aneurysms seldom occur in random locations (31, 43). They tend to arise in sites similar to where giant and fusiform aneurysms form, with comparable and predictable geometric and anatomical properties. Vascular flow is turbulent or laminar. Turbulent flow has random variations in temporal and spatial components, with inconsistent predictability (43). Laminar flow typically occurs in large, straight vessels and is synonymous with normal physiological conditions but can be more complex or "disturbed," occurring in areas of arterial bifurcations or poststenotic areas (49–52). These perturbations in flow often result in endothelial dysfunction, aiding aneurysm formation (31, 43). The endothelial response to wall shear stress appears to cause a cascade of gene signaling, morphological, and phenotypic changes that result in the initiation, progression, and rupture of intracranial aneurysms.

The locations of aneurysms are relatively consistent, with most cerebral aneurysms in the circle of Willis (43). However, considerable anatomical variability results from population-level differences in the individual geometry of the circle of Willis. Only 40% of people have a characteristic "complete" circle of Willis (43, 53). Unlike most large extracranial arteries, the bifurcation apex in cerebral vessels does not have consistent histologic media. Furthermore, the cerebral bifurcation apex has significantly less structural support from perivascular tissue (43, 54). Hemodynamic data suggest that deviations from optimal geometric constructs predispose specific vessels to aneurysm formation.

Approximately 90% of cerebral aneurysms occur in the anterior circulation, commonly (30–35%) the anterior communicating artery complex, followed by the internal carotid artery (30%) and associated branches (posterior communicating, ophthalmic arteries). Lastly, 22% occur in the middle cerebral artery and 10% in the posterior circulation (basilar apex, superior cerebellar artery, posterior inferior cerebellar artery) (40). These locations correlate with the distribution of intracranial atherosclerosis and areas of suboptimal hemodynamic patterns (40, 43). Known anatomical differences in familial aneurysms also account for approximately 10% of SAHs (38). Familial aneurysms typically are multiple and occur in the middle cerebral artery.

ANEURYSM FORMATION AND THE ROLE OF INFLAMMATION

Numerous immunologic factors may influence the formation of intracranial aneurysms and their progression and rupture.

Pathology

The pathophysiological underpinnings of a saccular cerebral aneurysm may lie in an atherosclerotic pathway. Animal modeling points to damage of the internal elastic lamina that may define early aneurysm formation and change (55–60). Further atherosclerotic changes within the aneurysm wall are also described (61, 62). Structural differences occur in both small and large saccular aneurysms. Small aneurysms have diffuse intimal thickening, with proliferating vascular smooth muscle cells (VSMCs) and a preponderance of macrophages and lymphocytes. Larger aneurysms have more advanced atherosclerotic changes, particularly with phenotypic changes in VSMCs, lipid-laden macrophages, and lymphocytic infiltration.

Our current understanding of atherosclerosis as a contributor to cerebral aneurysm formation and progression is rooted in efforts to define abdominal aortic aneurysms (63–66). Individuals with both cerebral and abdominal aneurysms share comorbid risk factors, such as smoking and arterial hypertension. Immunologic response and chronic inflammation are key pathogenic features of atherosclerosis (67–73). These immunologic responses suggest that inflammatory mediators could be linked to the formation, progression, and rupture of cerebral aneurysms (31).

Vessel Wall Changes

Histologic changes in aneurysm formation include vessel wall damage as a precursor. Normal vessel walls are organized into distinct layers, while aneurysmal vessel walls have fewer distinct layers characterized by disintegration of the internal elastic lamina, progressive disorganization of the muscular media, intimal hyperplasia, and progressive irregularity of the luminal surface (74–79). Healthy cerebral vessels have a mix of collagen and connective tissue (type I, III, and IV), fibronectin, and laminin. Type I collagen exists mostly in adventitia and fibronectin in the media of normal vessels (80). However, vascular remodeling changes the vessel wall. Type I collagen increases and fibronectin is dispersed in the wall, while the levels of type III and IV collagen and laminin decrease (54).

Structural and pathological changes occur in the endothelium and VSMCs. Functioning vascular endothelium promotes vasodilation and is antiatherogenic; it also inhibits platelet adhesion and accumulation, VSMC proliferation and leukocyte adherence, and pro-inflammatory cascades. Recent evidence points to damage of the vascular endothelium as the inciting event, leading to the creation, inflammatory cascade, progression, and rupture of intracranial aneurysms (81–83). The key inciting event in endothelial injury may be hemodynamic stress (76).

Perturbations in the vascular endothelium appear constant in both experimental and human intracranial aneurysms (75, 77, 81, 84–89). Damage to the vascular endothelium incites morphologic and pathologic changes likely occurring in stages. The earliest changes (e.g., partial loss of endothelium) occur upon aneurysm formation and the latest (e.g., intimal swelling) upon progression. Initial morphologic and functional changes in the endothelium could be a response to shear stress. Endothelial cells become elongated and realign with directional blood flow. Changes also occur in the development of actin stress fibers that may alter endothelial cell density or migration (90, 91). Hemodynamic stress may alter acute and chronic inflammatory signaling pathways. Shear stress appears to activate mediating pathways of inflammation within endothelial cells [prostaglandin E(2)–E-prostanoid(2)

(PGE(2)–EP(2))]. It also may amplify the chronic inflammatory pathway *via* nuclear factor-κB (92).

Changes in vessel walls are punctuated by changes in the vascular endothelium that occur in concert with phenotypic and morphologic changes in VSMCs supporting the media layer of the intracranial vasculature and providing structural support to vessel walls. Dynamic changes and eventual loss of the media layer contribute to aneurysm formation and rupture (80). Histologic evidence suggests that normally contractile VSMCs respond to environmental cues by undergoing phenotypic changes causing them to resemble a pro-inflammatory, pro-remodeling, and dedifferentiated phenotype (93–95). Normal differentiation of cerebral VSMCs includes high levels of largely contractile proteins comprising smooth muscle-myosin heavy chains, smooth muscle alpha-actin, and semicarbazide amine oxidase, which regulate VSMC differentiation (54, 96–105). An early morphologic finding was related to phenotypic changes in these proteins. The spindle-like VSMCs change into spider-like cells that migrated to and proliferated in the media, resulting in myointimal hyperplasia (99). These changes may be punctuated by the previously mentioned hemodynamic factors, macrophage and endothelial cell-derived mediators [tumor necrosis factor (TNF)-α, interleukin (IL)-β, nitric oxide, and growth factors], environmental factors, and genetic changes (54, 100, 102, 104). This punctuated VSMC transition results in proliferation of a pro-inflammatory phenotype of VSMCs. The pro-inflammatory phenotype is characterized by reduced levels of the contractile elements of VSMCs: smooth muscle-myosin heavy chains, smooth muscle alpha-actin, and semicarbazide-sensitive amine oxidase (54, 102). Further changes in the increase in transcription factors (Ets-1, p47phox, IL-6, monocyte chemoattractant protein-1, reactive oxygen species, matrix metalloproteinases, cathepsins), promoting inflammation, recruiting reactive oxygen species, and matrix remodeling, are identified as potentially promoting aneurysm progression (96, 98, 103, 106). Ultimately, these changes result in decreased expression of collagen biosynthesis and further loss of VSMCs, weakening the aneurysm wall and predisposing to aneurysm rupture (31).

Specific Inflammatory Pathways

The specific immunologic pathways and mediators involved in aneurysm formation remain partially understood. However, the immunologic effect can be divided into three areas linked to endothelial cells, VSMCs, and leukocytes. A common pathway for aneurysm formation is linked to certain leukocytes with distinct pathways of influence and known associated inflammatory mediators catalyzed by endothelial injury (31). The immunologic function is mediated by endothelial dysfunction, and the primary inflammatory mediators are NF-κB, Ets-1, MCP1, IL-1β, nitric oxide, angiotensin II, phosphodiesterase-4, and PGE(2)–EP(2) (**Figure 1**) (31). Dysfunctional major pathways of VSMCs include pro-inflammatory and pro-matrix remodeling, along with phenotypic modulation and associated apoptotic cell death. The major inflammatory mediators involved in VSMCs are IL-1β, p47phox, Ets-1, MCP1, angiotensin II, reactive oxygen species, matrix metalloproteinase, and cathepsins (31, 84, 107). Leukocytes, particularly mast cells and T-cells, influence aneurysm formation *via* a chronic inflammatory pathway associated with vessel wall

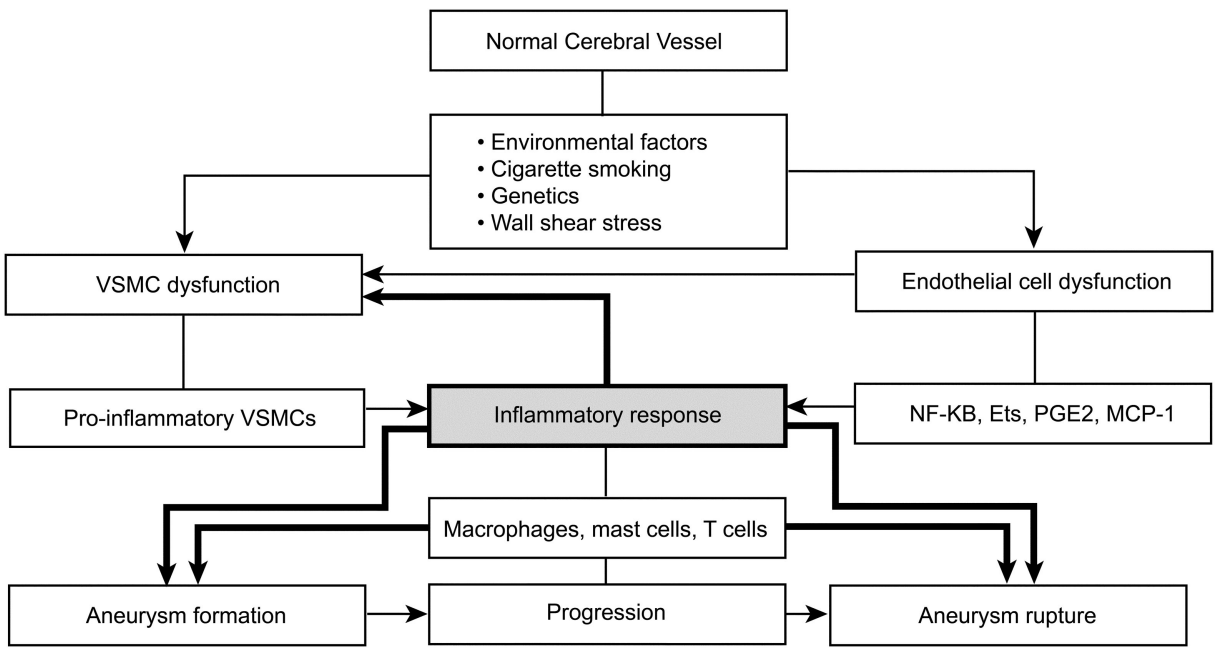

FIGURE 1 | **Environmental factors and immunologic pathways and mediators involved in aneurysm formation**. Shading emphasizes the contribution of inflammation to the process of aneurysm formation. VSMC, vascular smooth muscle cell; NF-κB, nuclear factor-κB; Ets, E-twenty-six family transcription factors; PGE2, prostaglandin E2; MCP1, monocyte chemoattractant protein 1. *Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA*.

remodeling and damage, with subsequent apoptotic cell death. Several inflammatory mediators are associated with leukocytes: TNF-α, IL-1β, IL-6, TLR4, Fas, nitric oxide, complement, IgG, IgM, basic fibroblast growth factor, TGF-α + β, vascular endothelial growth factor, reactive oxygen species, matrix metalloproteinases, and cathepsins (31, 108, 109). Understanding how these specific inflammatory mediators function opens the door to treatments targeting these major inflammatory pathways (31).

GENETIC FACTORS

Genetic factors contribute to the formation, progression, and rupture of intracranial aneurysms. Several studies have used microarray polymerase chain reaction to characterize the nature of these lesions. Despite further elucidating the gene expression profiles of these lesions, these studies have been limited by the significant variability of lesion types, stage of progression, location, and rupture status of lesions (31). The variability and small sample sizes in published gene expression studies impede generalizations to the intracranial aneurysm population.

Microarray data have yielded more than 500 differentially expressed genes in intracranial aneurysm tissue (31, 110). The two most significantly associated gene ontology terms identified were in antigen processing and immune response. Additional processing of aneurysm tissue revealed significant involvement, confirmed by real-time polymerase chain reaction, in integrin signaling, chemokine signaling, complement and coagulation cascades, nitric oxide signaling, and IL-10 signaling. These studies showed a convincing correlation of major histocompatibility complex II gene overexpression in aneurysm tissue that associated antigen-presenting cells, particularly macrophages and monocytes, with intracranial aneurysm formation (31). Gene analysis of a rodent aneurysm model has shown associations in pathways involved with proteinases, reactive oxygen species, chemokines, complement, adhesion molecules, and apoptotic pathways in both the intima and media of aneurysm walls (31, 111). These data also showed differential expression of endothelial cells and VSMCs, suggesting a different role in the process of aneurysm formation (31, 112).

Gene expression patterns were more recently studied in groups of ruptured and unruptured aneurysms (31, 113), with 686 upregulated and 740 downregulated genes identified in the ruptured cohort. Upregulated pathways were numerous, most notably in response to turbulent blood flow, chemotaxis, leukocyte migration, oxidative stress, extracellular matrix degradation, and vascular remodeling. Additionally, enriched genes encoding TLR, NF-κB, hypoxia-induced factor 1A, and Ets transcription factor-binding sites were identified. These findings suggest that, although both aneurysm groups have an immunologic pedigree, ruptured and unruptured aneurysms likely have different immunologic biology.

Known genetic conditions and familial relationships are also associated with higher rates of intracranial aneurysms. Autosomal polycystic kidney disease, Ehlers–Danlos syndrome, neurofibromatosis 1, and alpha1-antitrypsin deficiency are linked with aneurysm formation (31, 40). Thus, if there are definable immunologic pathways and common identifiable genomic markers, then multiple avenues may be available for preictal intervention.

FUTURE DIRECTIONS AND TREATMENTS

Given our expanding understanding of the contribution of inflammatory factors to aneurysm formation, great efforts have been made in investigating non-interventional treatments. Much of the non-interventional therapeutic research to date has been conducted in animals, with the most promising data from studies on inhibiting the NF-κB pathway.

Multiple animal trials have sought to exploit the anti-inflammatory effect of statins. Statins can block different stages of the inflammatory reaction, decrease degeneration in the vessel, and slow intracranial aneurysm progression (3, 31, 85, 114). Unfortunately, other data indicate variable results with different doses of pravastatin (88). At lower doses (5 mg/kg/day), pravastatin reduced overall endothelial damage and inhibited aneurysm formation in rats (88). The reverse was noted at higher doses of pravastatin (25 and 50 mg/kg/day) and at lower doses of simvastatin (5 mg/kg/day), where there was enhancement of aneurysm growth, and with high-dose pravastatin, even induction of aneurysm rupture (31, 88). The adverse effects of statins were accompanied by increased apoptotic caspase-3 levels and TUNEL-positive cells. Positive but disparate results have also been found with a phosphodiesterase-4 inhibitor and several angiotensin II receptor blockers (3, 31, 81, 85, 115).

The most impressive animal data involve NF-κB inhibition in rats. A drastic decrease in inflammatory response and a 60% decrease in aneurysm incidence were found with NF-κB inhibition (31, 116). Whether the litany of animal data will have a translational impact remains to be seen. Retrospective data from the International Study of Unruptured Intracranial Aneurysms showed that patients who used aspirin three times weekly had a lower risk of aneurysm rupture versus those who did not use aspirin (117), perhaps because of the known anti-inflammatory effects of aspirin.

There are multiple, largely rat, studies of cathepsin inhibitors, MCP1 inhibitors, matrix metalloproteinase inhibitors, mast cell degranulation inhibitors, and free radical scavengers. These agents have diversely positive effects on factors, such as aneurysm incidence, size, media thickness, and internal elastic lamina score (2, 31, 97, 114, 118). The positive animal data continue to mount, prompting great hope it will translate into positive clinical therapies.

CONCLUSION

There is still much to learn about aneurysm biology. Experimental animal data support inflammatory pathways as a key factor in aneurysm formation, progression, and rupture, but concrete non-surgical therapeutic targets remain elusive. Continued research and understanding of the biology and immunology of aneurysms have been pivotal in broadening our current understanding and will play an important role as we continue to improve the treatment of this pathology.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

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Personalized Medicine in Cerebrovascular Neurosurgery: Precision Neurosurgical Management of Cerebral Aneurysms and Subarachnoid Hemorrhage

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Cerebral aneurysms are common vascular lesions. Little is known about the pathogenesis of these lesions and the process by which they destabilize and progress to rupture. Treatment decisions are motivated by a desire to prevent rupture and the devastating morbidity and mortality associated with resulting subarachnoid hemorrhage (SAH). For patients presenting with SAH, urgent intervention is required to stabilize the lesion and prevent re-rupture. Those patients fortunate enough to survive a presenting SAH and subsequent securing of their aneurysm must still face a spectrum of secondary sequelae, which can include cerebral vasospasm, delayed ischemia, seizures, cerebral edema, hydrocephalus, and endocrinologic and catecholamine-induced systemic dysfunction in cardiac, pulmonary, and renal systems. Increased focus on understanding the pathophysiology and molecular characteristics of these secondary processes will enable the development of targeted therapeutics and novel diagnostics for improved patient selection in personalized medicine trials for SAH. In unruptured cerebral aneurysms, treatment decisions are less clear and currently based solely on treating larger lesions, using rigid aneurysm size cutoffs generalized from recent studies that are the subject of ongoing controversy. Further compounding this controversy is the fact that the vast majority of aneurysms that come to clinical attention at the time of a hemorrhagic presentation are of smaller size, suggesting that small aneurysms are indeed not benign lesions. As such, patient-specific biomarkers that better predict which aneurysms represent high-risk lesions that warrant clinical intervention are of vital importance. Recent advancements in genomic and proteomic technologies have enabled the identification of molecular characteristics that may prove useful in tracking aneurysm growth and progression and identifying targets for prophylactic therapeutic interventions. Novel quantitative neuroimaging technologies have also recently emerged, capable of non-invasive characterization of hemodynamic factors, inflammation, and structural changes in aneurysmal walls. The combined use of these quantitative neuroimaging and molecular-based approaches, called *Radiogenomics*, is a technique that holds great promise in better characterizing individual aneurysms. In the near future, these radiogenomic techniques may help improve quality of life and patient outcomes *via* patient-specific approaches to the treatment of unruptured cerebral aneurysms and personalized medical management of secondary processes following aneurysmal SAH.

Keywords: biomarkers, cerebral aneurysm, neuroimaging, radiogenomics, subarachnoid hemorrhage

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INTRODUCTION

Cerebral aneurysms are common vascular lesions with prevalence in autopsy studies as high as 5% (1). The most common clinical presentation of cerebral aneurysms is rupture leading to subarachnoid hemorrhage (SAH) (2). The estimated incidence of SAH from ruptured intracranial aneurysms in the United States is one case per 10,000 persons (2, 3). An estimated 10% of these patients die before reaching medical attention with the 30-day mortality rate reaching as high as 45%. The 30% of patients who do survive suffer significant disability (3–5).

Aneurysms that present with SAH represent unstable lesions with significant risk of re-rupture, with recurrent hemorrhage within the first 24 h in as many as 4%, and in as many as 20% within the first 2 weeks of the initial event, if left unsecure (2). Symptomatic unruptured aneurysms presenting with new cranial nerve palsies or brainstem dysfunction are at increased risk of rupture, as high as 6% per year, and should be treated (6).

Recent advances in genomic and proteomic technologies have enabled the identification of molecular characteristics that may prove useful in tracking aneurysm growth and progression to guide treatment of unruptured aneurysms. Novel quantitative neuroimaging technologies have also recently emerged, capable of non-invasive characterization of hemodynamic factors, inflammation, and structural changes in aneurysmal walls. The combined use of these quantitative neuroimaging and molecular-based approaches, called *Radiogenomics*, is a technique that holds great promise in better characterizing individual aneurysms.

Beyond securing the aneurysm from risk of rupture, the treatment of patients with aneurysmal SAH includes managing a significant spectrum of secondary sequelae, which can include cerebral vasospasm (CV), delayed ischemia, seizures, cerebral edema, hydrocephalus, and endocrinologic and catecholamine-induced systemic dysfunction in cardiac, pulmonary, and renal systems. Optimizing management of these complex multisystem factors is critical for improving the 30-day mortality rate (as high as 45%) and the proportion of significantly disabled survivors (as high as 30%). An increased focus on understanding the pathophysiology and molecular characteristics of these secondary processes will enable the development of targeted therapeutics and novel diagnostics for improved patient selection in personalized medicine trials for SAH.

CURRENT CONTROVERSIES IN THE MANAGEMENT OF CEREBRAL ANEURYSMS

The management of asymptomatic unruptured aneurysms is the subject of ongoing controversy. A recent prospective observational cohort study, The International Study of Unruptured Intracranial Aneurysms (ISUIA), in which 1,692 patients were preselected to be conservatively followed, reported that the subgroup with the smallest aneurysms (defined in this study as <7 mm) had an observed 5-year rupture rate of 0% during the interval they were followed (1). Controversy surrounding the methodology of this study exists because, unlike a true natural history study, patients may have been preselected for inclusion on the basis of their surgeons' opinions that these aneurysms were less likely to rupture. Consistent with this, the rupture rates of this observational cohort were significantly lower than in other studies of unruptured cerebral aneurysms (2, 7–10). Another controversy was the ISUIA-reported risk of morbidity associated with microsurgical clipping of unruptured aneurysms as 15.7% after 1 year, which raised significant concerns when compared to the literature reporting surgical morbidity in the range of 3–7% (2). The result of inappropriately generalizing the ISUIA data of a preselected subset of aneurysms has nonetheless had the important effect of at least temporarily discouraging the treatment of many unruptured cerebral aneurysms. The result this will have on actual patient outcomes in real-world populations remains to be seen. In the interim, it is vitally important to generate improved biomarkers that move past arbitrary size cutoffs so that clinicians can better characterize rupture risk in individual lesions and thus improve decision-making for each unique patient.

Moving beyond the question of when to intervene, the issue of how to intervene is also the subject of much controversy, with options including microsurgical clipping and endovascular coiling. The International Subarachnoid Aneurysm Trial (ISAT) reported prospectively randomizing 2,143 patients, who presented with ruptured aneurysms, to either clipping or coiling (11). an important caveat of this analysis is that these 2,143 patients represented only a fraction of the total 9,559 patients the study initially assessed with aneurysmal SAH. The vast majority of real-world aneurysmal SAH patients (77.6%) were excluded upfront from this analysis, based on inclusion criteria that resulted in an analysis of a minority of aneurysmal SAH patients. The clinical characteristics of the resulting study demonstrated the profound effects of this selection bias, including 90% having favorable clinical grade, 95% having aneurysms in the anterior circulation, and 90% of aneurysms being <10 mm. Generalizing these findings may be inappropriate, and in fact many contributors to the ISAT trial have themselves pointed out significant issues with data transparency and need for secondary sources of data on this critical topic (12). As a result of these significant limitations of ISUIA and ISAT, and despite the impact they have already had on current treatments, whether to observe, surgically treat, or endovascularly manage intracranial aneurysms remains controversial.

Whether the increased durability of clipping outweighs its slightly higher risks compared to coiling is unknown. In fact, even ISAT investigators reported that the rehemorrhage rates and recoiling rates in subsequent analyses of their data indicate significant problems with the study's original conclusions (12). Nevertheless, endovascular technology is likely to continue to advance with indications and outcomes likely to be constantly changing.

As such, patient-specific biomarkers that better predict which aneurysms represent high-risk lesions and which lesions are likely to respond best to a particular therapy are of vital importance.

EMERGING BIOMARKERS IN THE MANAGEMENT OF UNRUPTURED CEREBRAL ANEURYSMS

Although the pathogenesis of cerebral aneurysms is unknown, their development at stereotyped locations associated with specific hemodynamic factors suggests that regional blood flow patterns play a fundamental role in the pathophysiology of the disease (13–16), as recently reviewed by Can and Du (17). Using non-invasive quantitative imaging to characterize aneurysm morphology and computational fluid dynamics analyses of resulting hemodynamics, these studies have provided new insight into the key factors that play in a role in aneurysm progression and risk of rupture. Interestingly, bifurcation aneurysms were associated with high wall shear stress (WSS), suggesting that wall remodeling and degeneration *via* endothelial injury is of greatest relevance in these aneurysms. In contrast, sidewall aneurysms were associated with low WSS, suggesting that stasis of blood flow, and resulting endothelial dysfunction with pro-inflammatory-mediated degeneration of the aneurysm wall, may be more clinically relevant in these aneurysms (17). In paired analysis of unruptured aneurysms that went on to rupture, the hemodynamic factors associated with rupture risk included low shear index area (LSA), defined as the area of the aneurysm wall exposed to a WSS <10% of the mean parent vessel, which was observed to be higher in aneurysms that went on to rupture (i.e., a greater percentage of the aneurysm wall was exposed to low shear stresses). However, patients with ruptured aneurysms experienced a higher maximum WSS (17). Taken together, these data suggest that a significant area of low shear stress results in endothelial dysfunction and degeneration of the aneurysmal wall to the point of susceptibility, and that focally high WSS exerted against this background results in the subsequent rupture event. These hemodynamic parameters of LSA and WSS provide a more dynamic measure of the aneurysm than arbitrary size measurement cutoffs proposed by the ISUIA and ISAT studies, and these next generation parameters will likely play an increasing role in the patient-specific characterization of aneurysms and associated clinical decision-making in the future.

Recently, ferumoxytol-enhanced magnetic resonance imaging has shown promise in non-invasively characterizing aneurysm inflammation. Increased ferumoxytol uptake in aneurysm walls is a measure of myeloid cell inflammation, and has predicted aneurysm instability and an increased 6-month rupture risk in pilot studies. Thus, increased ferumoxytol uptake may serve as a biomarker for lesions warranting urgent intervention (18–20). As hemodynamic factors, such as high LSA, may result in a pro-inflammatory milieu, with subsequent endothelial apoptosis and aneurysmal wall degeneration (17), a combination of hemodynamic and inflammatory characterization by newer non-invasive neuroimaging modalities may become increasingly important in the patient-specific management of aneurysms in the near future.

EMERGING BIOMARKERS IN THE MANAGEMENT OF SECONDARY SEQUELAE OF SAH

Secondary sequelae of SAH include CV, delayed ischemia, seizures, cerebral edema, hydrocephalus, and endocrinologic and catecholamine-induced systemic dysfunction in cardiac, pulmonary, and renal systems. Currently there are no established biomarkers for preclinical diagnosis or monitoring of progression of these secondary sequelae.

Hydrocephalus can develop in up to 20% of patients who have aneurysmal SAH (2), requiring ventriculostomy for drainage of cerebrospinal fluid (CSF). There are currently no accurate predictors of shunt dependency after ventriculostomy placement in SAH, but emerging CSF-based biomarkers that reflect the rate of CSF clearance, as well as neuroimaging that quantifies CSF dynamics, hold promise in selecting patients for rapid removal of the external ventricular drain to minimize risks of ventriculitis.

Cerebral vasospasm is a major cause of morbidity and mortality in SAH and refers to intracranial vasoconstriction that may occur between 3 and 14 days after SAH. The pathogenesis of vasospasm is unknown and even with maximal therapy vasospasm can cause strokes and death (21). Approximately two-thirds of all patients with SAH who undergo cerebral angiography will demonstrate radiographic evidence of vasospasm, known as angiographic CV. Symptomatic (clinical) CV, defined as the development of new focal neurologic deficits in patients with SAH in association with angiographic CV and not attributable to other causes, occurs in approximately one-third of all patients with SAH. Approximately one-third of these patients with CV die from the CV-related infarcts and another one-third are left significantly disabled. Medical treatment of CV consists of orally administered nimodipine (60 mg every 4 h for 21 days), which has been shown to improve outcome after SAH (22). Patients are monitored with daily transcranial Doppler (TCD) velocities, and in patients who develop elevated TCDs and new neurologic deficits, triple-H therapy is initiated (hypertension, hypervolemia, and hemodilution) (3). Patients with persisting neurologic deficit undergo urgent catheter angiography to confirm the presence of vasospasm and if confirmed are treated with intra-arterial administration of smooth muscle relaxants, such as papaverine or nicardipine or with balloon angioplasty. These antispasmodic therapies can result in angiographically confirmed arterial dilatation in >90% of patients (23–25).

Multiple CSF biomarkers have been identified for the early diagnosis of symptomatic CV, as recently reviewed by Lad et al. (26), which may help guide patient-specific selection for personalized medicine trials aimed at preventing delayed ischemic neurologic deficits, such as protocols using earlier angiography for early intra-arterial smooth muscle relaxant therapy. Endothelin-1 has been shown to significantly increase days 4–7 after SAH in patients who develop symptomatic CV versus those who do not (27), and this increase predicts the occurrence of symptomatic CV (28). CSF interleukin (IL)-6 levels also significantly increase in the first 4–5 days after disease onset in patients with CV

compared to those with uncomplicated SAH (29). These data suggest that endothelin-1 and IL-6 could be useful diagnostic and predictive markers for CV and potentially useful tools for personalized medicine protocols in the treatment and prevention of symptomatic CV.

Subarachnoid hemorrhage can result in overactivity of the sympathetic nervous system and catecholamine surge with resulting multisystem dysfunction. Cardiac abnormalities after SAH are common, including electrocardiographic changes, elevations in cardiac enzymes, and left ventricular dysfunction in up to one-third of cases (30–32). These abnormalities appear to directly result from the excessive catecholamine release in response to the intracranial hemorrhage (33). In some patients, other adverse events from this catecholamine surge include pulmonary edema, hypotension requiring vasopressors, delayed strokes, and death (34). The combination of decreased cardiac contractility, increased pulmonary vascular permeability, increased pulmonary vascular pressure, and increased volume from resuscitation results in the development of this pulmonary edema, and increased preload results in stretching of the cardiac atrium and atrial natriuretic peptide release (peaks on day 2) (35). This natriuretic peptide acts on renal tubules, triggering sodium and volume loss, and without appropriate resuscitation, plasma sodium levels fall significantly by post-rupture days 4–6, which can be preempted by judicious volume and salt replacement. This has been shown to reduce the incidence of severe CV (36). The relationship between natriuretic and diuretic states after aneurysmal SAH and the subsequent development of CV, particularly with regards to activation of the renin–angiotensin–aldosterone system between days 4 and 6, warrant further study and may provide further biomarkers to guide patient-specific treatments that optimize sodium and fluid balance, address natriuretic and renin–angiotensin–aldosterone signaling dysfunction, and provide appropriate inotropic and vasopressor support during myocardial dysfunction and ventilator support during neurogenic pulmonary edema.

CONCLUSION

Patient-specific biomarkers that better predict which cerebral aneurysms represent high-risk lesions worthy of intervention are of vital importance. Personalized treatment strategies are also increasingly important in the management of secondary sequelae from SAH, including CV, delayed ischemia, seizures, cerebral edema, hydrocephalus, and endocrinologic and catecholamine-induced systemic dysfunction in cardiac, pulmonary, and renal systems. The combined use of these quantitative neuroimaging and molecular-based approaches, called *Radiogenomics*, is a technique that holds great promise in better characterizing individual aneurysms. In the near future, these radiogenomic techniques may help improve quality of life and patient outcomes *via* patient-specific approaches to the treatment of unruptured cerebral aneurysms and personalized medical management of secondary processes following aneurysmal SAH.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Genetics Underlying an Individualized Approach to Adult Spinal Disorders

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Adult spinal disorders are a significant cause of morbidity across the world and carry significant health and economic burdens. Genetic predispositions are increasingly considered for these conditions and are becoming understood. Advances in molecular technologies since the mid-1990s have made possible genetic characterizations of these diseases in many populations, and recent findings have provided insight into the underlying pathophysiologic mechanisms. These studies have made clear the genetic heterogeneity producing clinical phenotypes and suggest that individualized treatments are possible in the future. We review the genetics and heritability of cervical spondylotic myelopathy and ossification of the posterior longitudinal ligament and perform a systematic review of the genetics of adult lumbar degenerative scoliotic deformity, highlighting recent discoveries and the potential for personalized future therapeutics for these patients.

Keywords: cervical spondylotic myelopathy, genetics, genome-wide association study, heritability, intervertebral disc degeneration, ossification of posterior longitudinal ligament, proteomics

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INTRODUCTION

Degenerative diseases of the spine affect the majority of individuals over a lifetime, causing pain and neurologic dysfunction, and presenting a significant challenge to physicians. These clinical entities develop from a complex interplay of genetic and environmental factors that are incompletely understood. Patients with a given disease may appear similar radiographically, yet outcomes may be disparate regarding disease progression and responses to medical, rehabilitative, or surgical interventions. Although there is a role for surgery in certain conditions, patient selection must be carefully considered, given the risks associated with surgery. Better stratification of patients is needed to guide treatments.

Advances in molecular technologies over the last 15 years have provided much insight into the genetics of numerous diseases, including those of the spine. Candidate gene approaches and genome-wide association studies have shed light on underlying pathophysiologic mechanisms at work in the development of such diseases. The potential application of these studies to patient care includes providing a clearer picture of who will benefit from surgical intervention. Furthermore, they offer an exciting opportunity for future therapies targeting specific genetic aberrations that predispose to disease.

Another article in this issue of *Frontiers* highlights the genetics of intervertebral disc (IVD) disease, which may play a partial role in almost all degenerative spine disorders and, therefore,

Abbreviations: AIS, adolescent idiopathic scoliosis; CSM, cervical spondylotic myelopathy; DS, degenerative scoliosis; IVD, intervertebral disc; OPLL, ossification of posterior longitudinal ligament; SNP, single nucleotide polymorphism.

is not discussed here. Alternatively, we review the genetics of several of the most common spinal disorders, including cervical spondylotic myelopathy (CSM), ossification of the posterior longitudinal ligament (OPLL), and adult scoliosis. Rather than enumerate specific single nucleotide polymorphisms (SNPs) and other genetic anomalies, we highlight the overarching mechanisms uncovered by recent studies and discuss the potential for development of personalized approaches to treating these diseases in the future.

METHODS

Literature Review

We performed a systematic review of the literature to evaluate the contributions of current evidence on the genetics of adult degenerative scoliosis (DS). A recent systematic review that evaluated the genetic contributions for CSM and OPLL by Wilson et al. (1) was found and therefore was not performed in this study. The inclusion criteria included the studies comparing genetic variables in humans with this disease. Only studies in the English language were included. MEDLINE was queried with the terms "genetics" and "adult degenerative scoliosis" for articles published from 1966 to September 26, 2016. Studies focusing on other forms of scoliosis were excluded, as were those focusing solely on IVD degeneration. These queries returned 24 studies. The citation information for each result was examined by two of the authors for relevant studies. Six potentially relevant studies were identified, and the abstracts (and, if necessary, full manuscripts) of these studies were reviewed. The references of all reviewed manuscripts were also reviewed to identify other potential studies. Six studies met the inclusion criteria and were the focus of the present study. The studies were published between 2011 and 2015. All of the studies represented Level III evidence (small, non-randomized case–control studies). Results of the included studies were extracted and interpreted by the two reviewing authors.

CERVICAL SPONDYLOTIC MYELOPATHY

Cervical spondylosis is a nearly ubiquitous finding that occurs with aging as IVD degeneration, ligamentous laxity, facet hypertrophy, and osteophyte formation contribute to narrowing of the spinal canal (2). CSM occurs when neural elements of the spinal cord are compressed. Nevertheless, many patients incidentally show radiographic evidence of spinal cord compression but remain clinically asymptomatic (3). Although the exact reasons for this remain unknown, a potential explanation relates to the dynamic nature of the cervical spine and cord, and that static compression does not correlate exactly with the micropathological changes that occur in this disease (4).

Multiple human and animal studies have implicated various mechanisms in the acute and chronic pathophysiology of CSM (5). Direct mechanical forces result in static and dynamic injuries to neuronal and glial cells (6, 7). This injury is likely paralleled by ischemic changes seen in the disease caused by obstructed spinal cord perfusion and consequent microvascular changes (7–10). Several studies have also suggested a perpetuating cycle of ischemia related to blood–spinal cord barrier breakdown and dysregulation of the neurovascular unit (11, 12). Vascular permeability promotes edema through the release of inflammatory molecules and other potentially cytotoxic proteins into the cord parenchyma (13). This edema may potentiate neuronal damage and play an active role in the chronic degenerative component of the disease (14, 15). Additionally, glutamatergic toxicity (16), free radical-mediated cell injury (17, 18), and apoptosis (19) are also suggested as aggravating secondary injury pathways in the disease.

Heritability of CSM

An appreciation for the genetics of a disease is important for understanding how it is passed from one generation to the next. Although environmental factors undoubtedly play a role in the multifactorial pathogenesis, they allow providers and patients to assess the probability that the patient could develop the disease. Meaningful genealogy, however, is difficult to evaluate, and studies are limited (20). A study by Wilson et al. systematically reviewed the literature documenting the heritability of CSM and OPLL (1). Several authors have suggested genetic susceptibility of cervical spondylosis in twin–twin comparison studies (21–23). However, only one study has successfully used a population-based methodology to show inheritance patterns among non-twins (24). Patel et al. examined a database of 2 million Utah residents and found 486 patients with CSM and compared them with 1000 case controls (24). They used an index measuring genetic distance between pairs of patients to quantify familial clustering and found a statistically significant relationship related to the disease. Moreover, they identified a greater than five times relative risk of developing the disease among first-degree relatives. Studies corroborating these findings in other populations need to be done, but the data suggest heritability among the studied individuals.

Genetics of CSM

As methods for evaluating genomics have evolved, SNPs and proteomics have become easier to evaluate, and the literature regarding their contributions to CSM has grown. Nevertheless, identifying individual components of CSM is difficult as different genes spur degenerative changes that lead to spondylosis but not necessarily myelopathy. For example, Wang et al. have associated two different genetic polymorphisms with CSM (25). First, they identified two polymorphisms of the vitamin D receptor gene (*VDR*), *Apa*I and *Taq*I, which are related to the presence of CSM and the magnetic resonance imaging–based severity of disease in Chinese patients (25). They also found a strong link between CSM and the tryptophan allele (Trp2) of the collagen 9A2 gene, as well as smoking exposure (26). These polymorphisms promote IVD degeneration independently (27–32), a process that can cause central canal stenosis. Neither of these studies delineated how these genetic changes compared in patients with cervical stenosis with and without myelopathy. This also underlines the putative effects of environmental stressors on pathogenesis.

Another genetic relationship has been drawn between apolipoprotein E, a protein that plays a critical role in the repair and regeneration processes of multiple central nervous system

diseases. Specifically, the ε4 allele of the apolipoprotein E gene is implicated in impairment of these repair mechanisms. In a study by Setzer and colleagues, 106 patients with radiographic cervical stenosis were collected prospectively, and the ε4 allele was strongly associated with the development of CSM, independent of imaging findings, and other confounders (the allele was not related to the degree of stenosis) (33). The group showed that the allele also had negative effects on treatment outcomes in 60 of the patients who underwent surgical decompression (34). These results suggest that this genetic link portends a worse prognosis both for developing the disease and recovering from it. Large-scale studies evaluating the clinical usefulness of this association are necessary before its application can become widespread. Still, this knowledge may provide clues to the pathogenesis of the disease and potential therapeutic targets.

OSSIFICATION OF THE POSTERIOR LONGITUDINAL LIGAMENT

Ossification of the posterior longitudinal ligament is a condition of ectopic bone formation within the posterior longitudinal ligament, typically occurring at the cervical spine levels. It was first described as a disease of aging in Asian populations, with a prevalence of approximately 1–4%, though the prevalence is reported to be as high as 1.7% in Caucasian populations (35). About 17% of individuals with OPLL present with cervical myelopathy, while 29% of asymptomatic OPLL patients go on to develop myelopathy over the next three decades (36). Additionally, OPLL adds complexity to the treatment of cervical spondylosis and, ultimately, affects the surgical approach to treating symptomatic patients (37). Studies of the natural history of OPLL are clouded by the common presence of other coexisting degenerative spinal pathologies.

Little is known about the exact pathophysiologic mechanisms underlying OPLL. Multiple factors are suspected to play roles in the ectopic bone formation, including numerous biomechanically and metabolically mediated growth factors and cytokines (38). *In vivo* findings from human OPLL samples demonstrate degenerative elastic and cartilaginous fibers with metaplastic, hypertrophic cartilage cells (38, 39). Neovascularization, vascular endothelial growth factor-positive metaplastic chondrocytes, and abnormal collagen expression are thought to play a role in the spreading endochondral ossification front (38, 39). Additional studies are required to expand our understanding of OPLL and likely will be influenced by the wealth of genomic and proteomic results.

Heritability of OPLL

Several genetic studies have been performed to establish the heritability of OPLL. A study of 347 families of patients with OPLL found a 26% prevalence in parents and a 28% prevalence in siblings (40). In this study, the relative risk of first-degree relatives developing the disease was statistically significant and greater than five times that of the expected incidence in the general population. Another study looking at approximately 100 patients and relatives with OPLL found a prevalence of 27% with a relative risk of seven times that of the general population (41). Although a high segregation rate among siblings and a high prevalence of disease in parents suggest an autosomal dominant pattern of inheritance, neither study showed autosomal dominant (or recessive) inheritance on further analysis. Likewise, a polygene inheritance hypothesis was also rejected in these studies (40, 41). Altogether, these data suggest a high rate of heritability but not in a predictable fashion that would allow for practical genetic counseling.

Genetics of OPLL

Multiple genes have been targeted as possible contributors to the pathogenesis of OPLL. One of the first to be investigated was the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP1*) gene, a transmembrane metalloenzyme that regulates soft-tissue calcification and bone mineralization *via* the production of inorganic pyrophosphate, a known inhibitor of calcification (42). *ENPP1* was first implicated after studies in *ttw* mice showed altered gene expression causing tiptoe walking (43). The mice harbor a naturally recessive mutation that results in ectopic spinal ligament ossification and myelopathy that mirrors the disease traits in human OPLL (43). Several case–control studies in humans have examined SNPs in the *ENPP1* gene, the main enzyme that controls inorganic pyrophosphate in osteoblasts and chondrocytes. The results have linked various polymorphisms to disease susceptibility, severity, and location, but the results have been inconsistent regarding which SNPs are involved (44–46). Nonetheless, these findings implicate *ENPP1* as a possible therapeutic target. Further work needs to be done to elucidate the exact mechanisms by which it is modified in OPLL.

Collagen molecules have also received significant attention in the genetic research for OPLL. Mutations in type XI collagen within the *COL11A2* gene are thought to affect the formation of fibril networks in the extracellular matrix and change the conformation of Type II collagen, which is responsible for bone and cartilage formation (38). Two large genome linkage studies found five different SNPs in *COL11A2* that correlated with disease presence, and one was present in both reports (47, 48). Type VI collagen is also associated with multiple SNPs in chromosome 21, localizing to the *COL6A1* gene (49). Other studies have shown similar findings and linked this SNP to ossification of the ligamentum flavum and diffuse idiopathic skeletal hyperostosis (50, 51). However, a study of these *COL6A1* SNPs in a Korean population revealed conflicting results (52). Although significant data support the two collagen molecules as contributing to the pathogenesis of OPLL, lack of data congruency has made reliable conclusions difficult to make, likely due to disease heterogeneity.

Bone morphogenetic proteins and transforming growth factor-β have been studied extensively due to their role in physiological and pathological pathways of bone formation and metabolism. Several SNPs are associated with both of these proteins, specifically bone morphogenetic protein-2, bone morphogenetic protein-4, and transforming growth factor-β1 (53–58). Although fewer studies have focused on these molecules and replication studies still are needed, they present attractive targets for future research. Likewise, multiple other candidate genes have been investigated independently (**Table 1**) (43–51, 53–66). A full list of SNP associations is well summarized in other studies (1, 67, 68).

TABLE 1 | Genetics of ossification of the posterior longitudinal ligament.

Gene	Reference
Collagen VI	Tanaka et al. (49), Tsukahara et al. (51), Kong et al. (50)
Collagen XI	Koga et al. (59), Maeda et al. (48), Maeda et al. (60), Sakou et al. (47)
RXRβ	Numasawa et al. (61)
Vitamin D receptor	Shiigi et al. (62), Kobashi et al. (53)
ENPP1	Okawa et al. (43), Nakamura et al. (45), Koshizuka et al. (44), Tahara et al. (46)
BMRF	Ogata et al. (54)
CTGF/Hcs24	Yamamoto et al. (55)
BMP-2	Kawaguchi et al. (56), Tanaka et al. (57), Kawaguchi et al. (58), Wang et al. (63)
TGFβ	Kawaguchi et al. (56), Kamiya et al. (64), Horikoshi et al. (65)
Osteopontin	Aiba et al. (66)

RXRβ, retinoic X receptor-β; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; BMRF, bone metabolism regulatory factor; CTGF, connective tissue growth factor; Hcs24, hypertrophic chondrocyte-specific gene product 24; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β.

The first genome-wide association study for OPLL identified 26 SNPs on 3 chromosomes at 8p11.21, 8q23.1, 8q23.3, 12p11.22, 12p12.2, and 20p12.3 that are considered to be significantly associated with OPLL. Six of those SNPs were confirmed in a replication test as highly susceptible gene loci for OPLL (69). Interestingly, their comparison with previously reported gene loci from prior studies uncovered no significant associations. Two of the genes, radial spoke head 9 homolog, *RSPH9* (coding for a protein that composes cilia and plays a role in the hedgehog pathway of skeletal development), and serine/threonine kinase 38 like, *STK38L* (a protein kinase that inhibits cell cycle progression), are believed to have a part in the pathobiology of OPLL through membranous ossification (69). Hydroxyacid oxidase 1, *HAO1* (encodes hydroxyacid oxidase 1, which oxidizes 2-hydroxyacid), R-spondin 2, *RSPO2* (encodes R-spondin 2 protein that contributes to osteoblastogenesis through Wnt/β-catenin signaling pathways), and coiled-coil domain containing 91, *CCDC91* (encodes a trans-Golgi network protein) have putative roles in the endochondral ossification process (69). A follow-up study by the same group focused on *RSPO2* and further implicated it by evaluating the putative SNP *in vitro* and how it affected the binding of a vital transcription factor, CCAAT-enhancer-binding protein β (C/EBPβ) (70). Macroscopically, these associations remain speculative at this time; nevertheless, the cumulative findings of these studies open the door for investigation of multiple new gene targets and provide insight into the novel mechanisms of OPLL.

DEGENERATIVE LUMBAR SCOLIOSIS

DS is a disease that occurs after skeletal maturity, typically after the third decade of life, and is a distinct entity from idiopathic scoliosis. It is associated with severe back and leg pain, which leads to spinal dysfunction and debilitation. Pain may result from asymmetric muscular loading, facet joint arthritis, or nerve root impingement/traction (71). A Cobb angle of greater than 10° in the coronal plane is considered diagnostic (72). Although it has been recognized for many decades as a significant cause of pain and disability, increasing clinical awareness has yielded a growing body of literature on treatment and greatly improved clinical outcomes.

Although interest in outcomes and surgical treatments has gained ground, little is known about the pathogenesis of DS. Multiple studies have implicated osteoporosis in DS. There is a high degree of overlap of these two pathologies; however, a causal relationship has not been established, and definitive correlations are lacking (73). Other studies suggest that asymmetric IVD degeneration is the cause, resulting in uneven loading forces that perpetuate the rate of asymmetric degeneration. Recent evidence suggests that cytokines and growth factors are differentially expressed within various locations of the IVD, likely creating regional discrepancies in the rates of cellular apoptosis, inflammation, and angiogenesis (74–76). Whether or not these differences are the result of other causative etiologies or are the instigating impetus behind the disease remains to be seen. Investigations into other sources of asymmetric spinal degeneration, such as myopathy and mechanical instability, are lacking and provide future directions for research (76, 77).

Heritability of DS

Few studies have been performed to examine the heritability of DS to date. Twin–twin and family-based comparison studies should be conducted to help identify patterns of inheritance. The paucity of data likely is due to the relatively recent attention to this disease since the mid-1990s. Unlike OPLL, which is found at particularly high rates in specific parts of the world, DS appears to show less geographic variation.

Genetics of DS

For adolescent idiopathic scoliosis (AIS), a number of investigations have been conducted to examine the genetic basis of disease. In 2010, Ward et al. performed a genome-wide association study that identified 53 SNPs that correlated with scoliotic curvature in Caucasian females (78). Using this genotype information and an initial Cobb angle, they devised an algorithm that calculated the risk of curvature progression in selected patients, which they commercialized under the name *ScoliScore*. This DNA-based predictive calculator theoretically enabled clinicians to forecast which individual patients were at low likelihood of curve progression (78). However, replication studies failed to demonstrate the same SNP associations in different geographically diverse populations (79–82). Nevertheless, this attempt at personalized, genome-based, clinical and outcome prediction exemplifies methods by which genetic outcomes could guide future treatments for a multitude of diseases.

Comparatively, in DS, there have been fewer genetic incongruities identified than in other degenerative spine diseases. It is thought that distinct genetic characteristics define these seemingly similar, but quite clinically different syndromes. Our systematic review identified six works in the literature (**Table 2**) (52, 83–87) that identified genetic contributions for DS. The quality of the data is relatively limited (Level III studies) but provides some insight into potential genetic mechanisms for disease pathogenesis that could potentially be used in future studies.

Proteomic analyses of the sera of patients with DS have identified 11 proteins that are differentially expressed in such

TABLE 2 | Review of studies identifying genetic contributions to adult degenerative scoliosis.

Reference	Level of evidence	Genetic level of participation	Putative contributor(s)
Zhu et al. (83)	III	Proteomics	CLU, Ficolin-3
Han et al. (84)	III	Proteomics	PIAS2, NDUFA2, TRIM68
Shin et al. (85)	III	Copy number variation	TMEM163, ANKRD 11, NFATC1
Hwang et al. (86)	III	SNPs	rs2276454 of collagen type II alpha 1
Kim et al. (87)	III	SNPs	No SNPs of NMDA receptor genes associated
Kim et al. (52)	III	SNPs	RIMS2

CLU, clusterin; PIAS2, protein inhibitor of activated STAT 2; NDUFA2, NADH:ubiquinone oxidoreductase subunit A2; TRIM68, tripartite motif containing 68; TMEM163, transmembrane protein 163; ANKRD 11, ankyrin repeat domain 11; NFATC1, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1; SNPs, single nucleotide polymorphisms; NMDA, N-methyl-D-aspartate; RIMS2, regulating synaptic membrane exocytosis 2.

TABLE 3 | Summary of cervical spondylotic myelopathy (CSM), ossification of the posterior longitudinal ligament (OPLL), and lumbar degenerative scoliosis (DS).

CSM
- Complex disease with multiple degenerative processes contributing to underlying spondylosis
- Studies support an inherited predisposition to the disease
- Several genes have been implicated in CSM, but more studies are needed to confirm their genetic role in the disease

OPLL
- Data support the heritability of OPLL, and first-degree family members are at a much higher risk than others
- *Col6A1* and *Col11A2* are suggested by multiple studies to be associated with OPLL
- Multiple SNPs have been implicated in OPLL, along with several new genes from a genome-wide association study, but more work is needed to confirm their involvement

DS
- No studies have established any inherited predisposition to DS
- Fewer studies examining genetic associations have been performed for DS compared to CSM and OPLL, but there appear to be genetic contributions to DS
- More studies are required to identify participating genetic alterations in the disease pathogenesis

patients, 2 of which were secondarily confirmed with Western blot analyses, CLU (also known as apolipoprotein J, testosterone-repressed prostate message-2, SP 40-40, complement lysis inhibitor, gp80, glycoprotein III, and sulfate glycoprotein-2) and Ficolin-3 (also named Hakata antigen, thermolabile β-2 macroglycoprotein, thermolabile substance, and H-ficolin) (83). Both of these proteins have been suggested to have roles in autoimmunity, although they both likely have multiple roles in the human body and their association is non-specific. This protein expression analysis does not definitively implicate them in the disease pathogenesis but suggests that they may serve as potential future biomarkers and potentially raises the question of whether an autoimmune component of DS exists. The same group also compared proteomic expression in cultured mesenchymal stem cells of DS patients (84). This comparison revealed differential levels of three proteins, protein inhibitor of activated STAT 2 (PIAS2), NADH:ubiquinone oxidoreductase subunit A2 (NDUFA2), and tripartite motif containing 68 (TRIM68), none of which correspond to those elevated in the serum. All three of these proteins play various roles in biological processes and, therefore, pinpointing their roles may be difficult. Although the above proteomic analysis is helpful in identifying biomarkers of disease and drug targets, the proteins characterized in the analysis do not correlate exactly with genetic differences in the disease and are susceptible to environmental and other outside factors that change the genomic output *via* epigenetic influences. Consequently, much more work is required to tease out the meaning of these proteomic differences and reproduce these results with different patient populations.

One of the studies comparing genomic differences in DS examined copy number variations, which represent regional gene dosages of DNA segments 1 kb or larger (85). Of the 260 copy number variations identified by microarray analysis, quantitative polymerase chain reaction validation identified three genes with significant differences from the control group. These genes included transmembrane protein 163, *TMEM163*, a gene coding for a transmembrane protein of unknown function; ankyrin repeat domain 11, *ANKRD11*, an ankyrin repeating gene implicated in autism spectrum disorder and skeletal formation; and nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1, *NFATC1*, a gene reportedly involved in bone mineral density (85). This novel study provided evidence that DS could be predisposed by inherent genomic differences rather than resulting from external environmental forces causing asymmetric degeneration. Further work will be needed to expand on the biosignaling cascades by which these genes may affect disease pathogenesis.

Likewise, another area of gene-based research relates to SNPs associated with DS. Prior studies have given collagen molecules significant attention for similar diseases, including IVD degeneration and AIS (88). Collagen II has been investigated because of its structural role in stress-bearing of the spine (88). Investigators studied SNPs of *COL2A1* and found a significant association of SNP (rs2276454) in *COL2A1* to DS in Korean patients (86). Another study tested SNPs of glutamate receptors (*N*-methyl-D-aspartate receptors), given their role in controlling bone remodeling through stimulation, maturation, and differentiation of osteoblasts and osteoclasts (87). Interestingly, they found no association with any of the SNPs investigated. A similar study found that one of the SNPs in regulating synaptic membrane exocytosis 2, *RIMS2*, coding for a presynaptic active zone protein that regulates vesicle exocytosis of neurotransmitters, was significantly associated with DS (87). Therefore, glutamate, or other neurotransmitters, may still contribute to the disease progression of DS. The heterogeneity of these findings suggests that our insight into DS remains minimal; yet, these results lay the groundwork for further basic science in this area.

CONCLUSION

Adult degenerative spinal disease has tremendous health costs on a global level. CSM, OPLL, and DS have gained significant attention from medical providers and researchers as disease entities

that merit further focus and investigation (**Table 3**). Studies of families and twins suggest that there may be a significant component of heritability to CSM and OPLL, although few genetic studies of DS have been published. Thus, DS presents an opportunity for further research. Advances in genomic analysis and biostatistics continue to open new doors to finding genetic linkages with these degenerative spinal diseases, which ultimately could guide the course of future pathophysiological studies, clinical diagnoses, and treatments.

AUTHOR CONTRIBUTIONS

All the authors made substantial contributions to the conception or design of the work.

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REVIEW
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Genetic Alterations in Intervertebral Disc Disease

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Background: Intervertebral disc degeneration (IVDD) is considered a multifactorial disease that is influenced by both environmental and genetic factors. The last two decades of research strongly demonstrate that genetic factors contribute about 75% of the IVDD etiology. Recent total genome sequencing studies have shed light on the various single-nucleotide polymorphisms (SNPs) that are associated with IVDD.

Aim: This review presents comprehensive and updated information about the diversity of genetic factors in the inflammatory, degradative, homeostatic, and structural systems involved in the IVDD. An organized collection of information is provided regarding genetic polymorphisms that have been identified to influence the risk of developing IVDD. Understanding the proteins and signaling systems involved in IVDD can lead to improved understanding and targeting of therapeutics.

Materials and methods: An electronic literature search was performed using the National Library of Medicine for publications using the keywords genetics of IVDD, lumbar disc degeneration, degenerative disc disease, polymorphisms, SNPs, and disc disease. The articles were then screened based on inclusion criteria that included topics that covered the correlation of SNPs with developing IVDD. Sixty-five articles were identified as containing relevant information. Articles were excluded if they investigated lower back pain or just disc herniation without an analysis of disc degeneration. This study focuses on the chronic degeneration of IVDs.

Results: Various genes were identified to contain SNPs that influenced the risk of developing IVDD. Among these are genes contributing to structural proteins, such as *COL1A1*, *COL9A3*, *COL9A3*, *COL11A1*, and *COL11A2*, *ACAN*, and *CHST3*. Furthermore, various SNPs found in the vitamin-D receptor gene are also associated with IVDD. SNPs related to inflammatory cytokine imbalance are associated with IVDD, although some effects are limited by sex and certain populations. SNPs in genes that code for extracellular matrix-degrading enzymes, such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-14,

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Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; AF, annulus fibrosis; ECM, extracellular matrix; GDF5, growth differentiation factor 5; IL, interleukin; IVD, intervertebral disc; IVDD, intervertebral disc degeneration; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; NP, nucleus pulposus; SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor; VNTR, variable nucleotide tandem repeat.

ADAMTS-4, and ADAMTS-5 are also associated with IVDD. Apoptosis-mediating genes, such as caspase 9 gene (*CASP9*), *TRAIL*, and death receptor 4 (*DR4*), as well as those for growth factors, such as growth differentiation factor 5 and VEGF, are identified to have polymorphisms that influence the risk of developing IVDD.

Conclusion: Within the last 10 years, countless new SNPs have been identified in genes previously unknown to be associated with IVDD. Furthermore, the last decade has also revealed new SNPs identified in genes already known to be involved with increased risk of developing IVDD. Improved understanding of the numerous genetic variants behind various pathophysiological elements of IVDD could help advance personalized care and pharmacotherapeutic strategies for patients suffering from IVDD in the future.

Keywords: back pain, biomarker, degeneration, disc, gene expression, herniation, personalized care, single-nucleotide polymorphism

INTRODUCTION

Over 80% of all people will experience some form of lower back pain in their lifetime (1–3). Symptomatic intervertebral disc (IVD) degeneration (IVDD) is a common cause of lower back pain, yet the etiology and pathophysiology underlying IVDD remain poorly understood (4, 5). Although various environmental factors such as smoking, age, gender, and mechanical load increase the risk of IVDD, it is hypothesized that up to 74% of the etiology of IVDD is due to heritability (2, 6). With lower back pain costing over $100 billion/year in the United States, it is essential to investigate both the environmental and genetic predispositions to IVDD (5).

The normal IVD is composed of two parts: the outer annulus fibrosis (AF) region and the central nucleus pulposus (NP) (**Figure 1**). The AF consists of fibroblast-like cells with elongated nuclei placed between concentric layers of collagen fibers (3). The extracellular matrix (ECM) of the AF can be described as a fibrocartilaginous structure consisting of predominantly collagen-I fibers (60% of total dry weight), with low proteoglycan content (25%) and low water retention (5, 7). Its primary function is to provide structural integrity to the disc and hold the contents of the NP in the center (3, 5). The NP is a gelatinous structure with chondrocyte-like cells that secrete collagen II. Dispersed throughout the collagen fibers are an abundance of proteoglycans, predominantly aggrecan, which are responsible for facilitating water retention (3, 5, 8). The primary function of the NP is to create hydrostatic pressure to resist axial compression (5, 7).

Intervertebral disc degeneration seems to be an irreversible process that can begin as early as the second decade (5). The first molecular change that occurs at the beginning of degeneration is a reduced ability of the NP to retain water and consequently maintain a significant hydrostatic pressure (7). These changes result in decreased disc height and reduced ability of the spine to withstand compression. Over time, the collagen fibers and other ECM components of both the NP and AF are degraded and reduced in quantity (8). Upregulation of degradative systems such as apoptosis, inflammation, and matrix metalloproteinases (MMPs) further damage the existing (9–13). The past 20 years of genomic research has revealed an astounding number of genetic polymorphisms of various genes that are correlated with increased risk of developing IVDD. Polymorphisms in the genes coding for collagen, aggrecan, interleukins (ILs), apoptosis factors, vitamin D receptor (VDR), MMPs, and

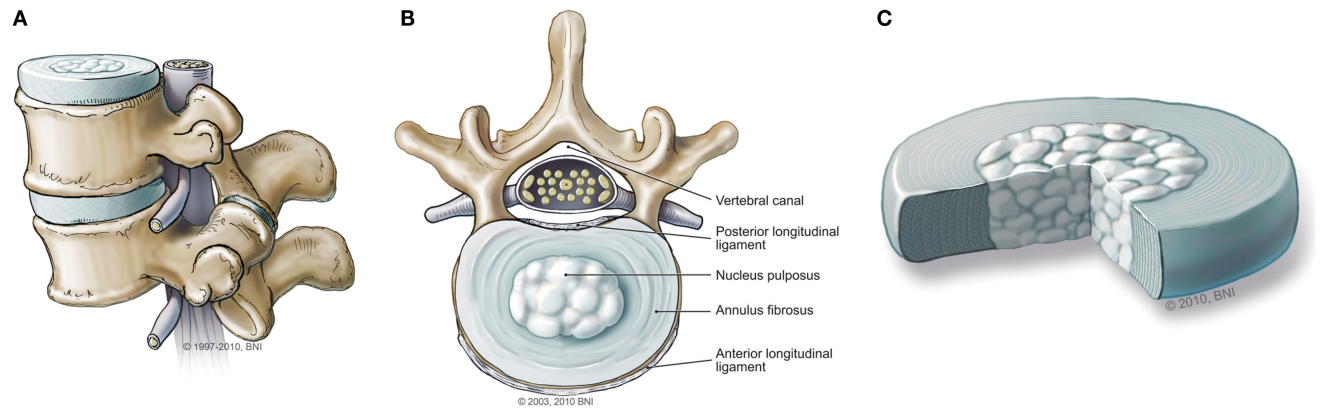

FIGURE 1 | **(A)** Normal intervertebral disc (IVD) from the sagittal view. **(B)** Normal IVD from the axial view. **(C)** Magnified illustration of an IVD. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

other critical proteins involved in IVDD are examined in this paper. Although previous reviews have documented the various single-nucleotide polymorphisms (SNPs) that are associated with IVDD, we aim to provide an up-to-date and comprehensive review of the subject (5, 7, 8).

With an improved understanding of the genetic variants associated with IVDD, we hope to help advance personalized care and pharmaceutical therapies for patients suffering from IVDD. Across various medical specialties, genome sequencing has begun to play a significant role in improving the care provided to patients (14). Human genome analysis allows physicians to obtain a deeper understanding of the pathophysiology of diseases to provide improved risk and prognostic assessments to patients. Furthermore, information regarding genetic variants provides insight into therapeutic options as physicians are better able to target the underlying disease-causing mechanisms (15). Throughout this paper, we will explore the complexity and diversity of the molecular and genetic factors involved in IVDD. Genetic variants from various molecular pathways are investigated including inflammatory, degradative, homeostatic, and structural systems. Clinical use of genome analysis allows physicians to pinpoint which systems and particular pathways are involved with the patient's unique case of IVDD and subsequently provide personalized and improved health care.

METHODS

An electronic literature search was performed using the National Library of Medicine for publications using these keywords: genetics of IVDD, lumbar disc degeneration, degenerative disc disease, polymorphisms, SNPs, and disc disease. The articles were then screened based on inclusion criteria that included topics that covered the correlation of SNPs with developing IVDD. Furthermore, articles containing supporting information regarding the treatment and diagnosis of IVDD were included. Sixty-five total articles were identified as containing relevant information. Articles were excluded if they investigated lower back pain or disc herniation without an analysis of disc degeneration or study of correlation with SNPs. This investigation focuses on the chronic degeneration of IVDs and the genetic factors that influence its development.

TREATMENT FOR IVDD

Diagnosis of IVDD requires a careful history, physical examination, and, most importantly for the experimental studies included in this review, magnetic resonance imaging (MRI) of the spine. The majority of studies that were included in this literature review used axial and/or sagittal T2-weighted MRIs to evaluate the lumbar spine of the patients (**Figure 2**). Once the patient has been accurately diagnosed with disc degeneration, limited approved therapeutics are available to abate the progression of the degeneration. Therapy to combat IVDD and the associated degeneration and pain is highly complex, and it can be difficult to predict its effectiveness. Recently, researchers have found success utilizing targeted molecular and gene therapies in an attempt to mitigate degradation and even promote anabolic processes. Injection of recombinant human bone morphogenetic protein 7 (BMP-7, also known as osteogenic protein 1, OP-1) has been successful in a rabbit model (16). BMP-7 injection restored the disc height and biomechanical properties of the damaged disc. Other growth factors such as rhGDF-5 have also shown great promise (17). In that study, a single injection was shown to increase disc height. Furthermore, rhGDF-5 injection has been shown to reduce the expression of ADAMTS-4 and ADAMTS-5 proteins for which, the genes have been identified to contain SNPs associated with altered risk of developing IVDD (18). This serves as an excellent example of the intersection of providing targeted therapy and gene analysis of patients with IVDD. RhGDF-5 injections may serve as the most effective therapy in a patient who has been screened for having high-risk IVDD due to SNPs in their ADAMTS-4 and -5 genes (18). Furthermore, injection of other molecules, such as TGF-β1 and BMP-2, has been shown to inhibit MMP-1 expression and increase expression of aggrecan protein. Genes for both MMP-1 and aggrecan protein are known to contain SNPs that predispose patients to develop IVDD (19). Combining the specific effects of these anabolic therapies with an understanding of the individualized molecular profile of each patient may yield a highly effective treatment. Therefore, it is essential that research efforts continue to progress in both targeted therapies and gene analysis of IVDD.

The most effective experimental approach in the treatment of IVDD is the use of viral vectors in gene therapy. *In vitro* bovine experimentation with the delivery of sex-determining region Y box 9 (*SOX9*) and *BMP7* through an adenovirus vector revealed increased expression of type II collagen and an increase in disc height (21). Another experiment showed that cells virally transduced with Ad-BMP-4 and -14 displayed an increase in collagen deposition, whereas cells transduced with Ad-BMP-2 and -7 displayed an increase in proteoglycan accumulation (19). The consistently positive results obtained from these experiments suggest a largely uncharted frontier exists in the use of personalized medicine for IVDD.

GRADING IVDD

Physicians utilize various grading systems to assist in diagnosing and measuring the severity of IVDD and to determine the most standardized and objective classification of disc degeneration. A popular and widely accepted scale is the Pfirrmann grading system. The system includes grades 1–5, where grade 1 signifies a normal disc with homogenous hyperintensity on MRI, and grade 5 signifies a collapsed disc space with a hypointense signal (**Figure 2**) (22). A common critique of the system is its subjectivity. It is often modified or combined with other grading systems such as Modic changes to create an objective, reproducible system (23). Some physicians and research groups opt to develop their own grading system, while others utilize classification systems such the one developed by Schneiderman et al. (13, 24–26). Once a patient's disc degeneration is objectively graded, a standard of care can be established.

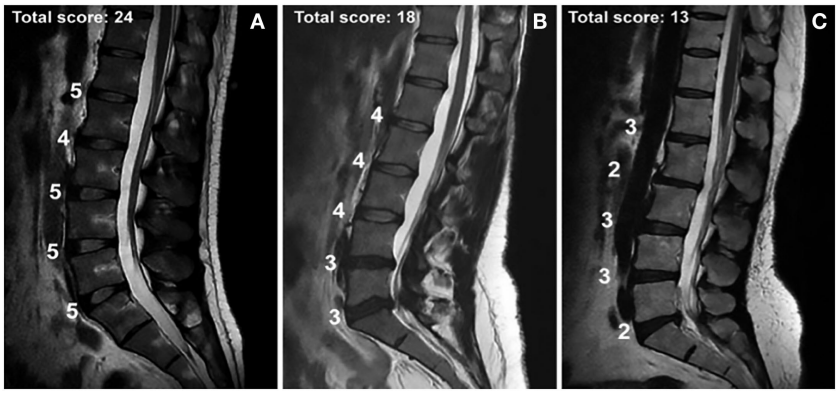

FIGURE 2 | MRIs of intervertebral disc disease in patients with total Pfirrmann scores of (A) 24, (B) 18, and (C) 13 and the assessed score for each lumbar disc. The Pfirrmann grading scale for disc degeneration classifies discs into 5 grades according to the amount of degeneration. Grade 1 corresponds to a hyperintense healthy disc, while grade 5 corresponds to a hypointense severely degenerated disc. The figure contains a point system corresponding to each intervertebral disc from L1 to S1. Five points were given for a grade 1 score, four points for a grade 2, three for a grade 3, two for a grade 4, and one for a grade 5. The highest possible total score is 25; the lowest possible score is 5. Used with permission from Toktas et al. (20).

GENE POLYMORPHISMS ASSOCIATED WITH IVDD

Table 1 (1, 4, 11–13, 20, 24, 25, 27–57) presents comprehensive information on the research studies that have investigated genes with SNPs associated with IVDD and their protein products. **Table 2** (1, 4, 11–13, 20, 24, 25, 27–58) summarizes the protein systems associated with such changes in the respective genes.

Collagens

Collagen is the most abundant protein found in the human body, with 28 different types. Throughout the body, the various collagen types are found in the ECM and have different structural support roles. Structurally, collagen fibers are composed of three polypeptide chains, referred to as α chains, that form one or more triple-helixes along their rod-shaped structure (59). When referring to the gene that produces a specific collagen type, the gene name and subunit name are given (e.g., collagen type IX alpha 2, *COL9A2*). The collagen types of interest to us are the ones found within IVDs: collagen I, II, IX, and XI.

The AF consists primarily of collagen I, a fibril-forming collagen. Fibrillar collagens – I, II, and III – are essential in defining the molecular and mechanical properties of a particular tissue (59). In the AF, collagen I is responsible for maintaining the tensile strength to withstand spinal compression, hydrostatic pressure, and keeping the NP contained. Collagen II is the primary collagen of the NP and is found as a loosely connected network (3). Various minor collagens such as collagen IX play an important supporting role in forming cross-links between different types of collagen, increasing structural strength. Collagen XI, although found in small amounts, is important in structural support of collagen II as well as forming connections between proteoglycans and collagen (5, 8). Considering the integral role of collagen in maintaining the structural integrity of the IVD, genetic polymorphisms affecting the function or abundance of collagen can predispose a patient to IVDD.

Collagen I

Collagen I, although found in both the NP and AF, is much more abundant within the AF of the IVD. Collagen I is made up of a helix consisting of two α1 chains, encoded by the collagen type I alpha 1 gene, *COL1A1*, and one α2 chain encoded by the collagen type I alpha 2 gene *COL1A2* (7). *COL1A1* contains a particular polymorphism that may be involved with increased risk of IVDD. Three noteworthy studies have established an association between the *COL1A1* Sp1-binding site SNP and IVDD (20, 27, 28). This particular SNP is a G to T substitution at position +1245, which is found within the first intron of the *COL1A1* gene (60). The change in nucleotides reportedly increases levels of *COL1A1* messenger RNA expression and subsequently COL1A1 protein expression (27). Investigators have hypothesized that the SNP leads to disequilibrium between COL1A1 and COL1A2 protein expression leading to instability of the collagen fibers (27, 28). Pluijm et al. examined 966 elderly (>65 years) Dutch individuals and reported that patients with the TT genotype had a 3.6-fold increased susceptibility to IVDD than patients with the GT or GG genotypes (27). The following year, Tilkeridis et al. examined the frequency of the Sp1-binding site polymorphism in 24 young Greek military recruits (28). The study reported that 33.3% of the patients with IVDD had the TT genotype while none of the control subjects did. Furthermore, the study indicated that 66.7% of the IVDD patients had the GT genotype while only 41.7% of the controls did. More recently, a 2015 study by Toktas et al. found that patients homozygous for the risk allele T had a significantly lower average Pfirrmann score (17.63) than patients without the allele (average score, 21.88) (20). They found a similar relationship between patients heterozygous for the allele compared with control patients. This study suggests that the *COL1A1* Sp1 polymorphism may not only be associated with an increased risk of developing IVDD but also associated with more severe forms of degeneration.

Collagen IX

Collagen IX is composed of three unique polypeptides, such as α1, α2, and α3, which are encoded by genes collagen type 9

TABLE 1 | Summary of research studies on single-nucleotide polymorphisms (SNPs) associated with intervertebral disc degeneration (IVDD).

Reference	Protein	SNP	Study population	Results
Pluijm et al. (27)	Collagen I	COL1A1 Sp1	966 Elderly Dutch subjects (>65 years old)	TT genotype odds ratio (OR) = 3.6 compared with GT or GG
Tilkeridis et al. (28)	Collagen I	COL1A1 Sp1	24 Greek military recruits (mean age 29 years old), 12 controls (mean age 25 years old)	TT genotype found in 33.3% of patients with IVDD and 0% of controls; GT genotype found in 66.7% of patients with IVDD and 41.7% of controls
Toktas et al. (20)	Collagen I	COL1A1 Sp1	75 Southern European men with IVDD, 25 controls (35–45 years old)	T allele associated with more severe IVDD based on Pfirrmann scores
Annunen et al. (29)	Collagen IX	COL9A2 Trp2	157 Finnish subjects (19–78 years old) with sciatic pain, 174 controls	Trp2 allele OR = 4.5 compared with patients without allele
Kales et al. (30)	Collagen IX	COL9A2 Trp2	105 Greek patients with IVDD, 102 controls (<60 years old)	No association between Trp2 and IVDD
Toktas et al. (20)	Collagen IX	COL9A2	75 Southern European men with IVDD, 25 controls (35–45 years old)	Did not find association between Trp2 and IVDD
Zhang et al. (31)	Collagen IX	COL9A2 rs12077871, rs12722877, rs7533552	Meta-analysis with 1522 lumbar disc disease (LDD) cases, 1646 controls	No association between the SNPs and IVDD
Paassilta et al. (32)	Collagen IX	COL9A3 Trp3	171 Finnish patients with sciatic pain, 321 controls (mean age 45 years old)	Trp3 allele OR = 2.7 for developing IVDD
Solovieva et al. (33)	Collagen IX	COL9A3 Trp3	135 Finnish male patients (40–45 years old)	Trp3 allele OR = 7.0 for developing dark nucleus pulposus; OR = 8.0 for degeneration of spine in absence of IL-1 βT3954 SNP allele
Toktas et al. (20)	Collagen IX	COL9A3 Trp3	75 Southern European men with IVDD, 25 controls (35–45 years old)	Trp3 allele associated with more severe degeneration based on Pfirrmann scores
Solovieva et al. (33)	Collagen XI	COL11A2 G to A SNP within intron 9	135 Finnish male patients (40–45 years old)	Risk allele OR = 2.1 for increased risk of disc bulges
Videman et al. (4)	Collagen XI	rs2072915, rs9277933, rs2076311, rs1337185, rs1463035	588 Finnish male twins (35–70 years old)	Some SNPs were significantly associated with reduced disc signal on MRI while others were associated with disc bulging
Rajasekaran et al. (34)	Collagen XI	rs1337185	308 Indian male patients with mild Total Disc Degenerative Score (mean age 29.6 years old), 387 Indian male patients with severe TDDS (mean age 31.7 years old)	SNP rs1337185 OR = 1.55 for developing IVDD
Virtanen et al. (35)	Interleukin-1a	-889C/T	150 Finnish men (38–56 years old), 61 control subjects	TT genotype OR = 7.87 for developing IVDD compared with patients with CC genotype
Eskola et al. (36)	Interleukin-1a	-889C/T	96 Danish adolescents with IVDD, 57 controls (mean age 13.1 years old at the beginning of the study)	In girls, the T-allele OR = 2.82 for disc degeneration
Noponen-Hietala et al. (37)	Interleukin-6	T15A within exon 5	155 Finnish subjects (17–78 years old), 179 controls (20–69 years old)	AA or AT genotypes OR = 4.4 for IVDD
Eskola et al. (13)	Interleukin-6	rs1800796, 572G/C	66 Children with LDD, 154 controls; total 352 children studied (mean age 13.1 years old at the beginning of the study)	C allele OR = 6.71 for IVDD in females
Eskola et al. (36)	Interleukin-6	rs1800797(Risk allele G), rs1800795 (Risk allele G)	96 Danish adolescents with IVDD, 57 controls (mean age 13.1 years old at the beginning of the study)	GA genotype of rs1800797 OR = 0.27 for IVDD; GC genotype of rs1800895 OR = 0.26 for IVDD in males
Dong et al. (38)	Matrix metalloproteinase (MMP)-2	-1306C/T	162 Chinese young adults with IVDD (mean age 25.4 years old), 318 controls (mean age 24.1 years old)	CC genotype OR = 3.08 for developing IVDD; CC genotype also associated with more severe forms of IVDD
Zhang et al. (39)	MMP-2	-735C/T	1008 Chinese Han patients with LDD (mean age 50.12 years old), 906 controls (mean age 49.54 years old)	Patients with TT or CT genotype OR = 0.413 for developing IVDD. CC genotype OR = 2.5 for developing IVDD compared with TT
Sun et al. (11)	MMP-9	-1562C/T	408 Northern Chinese young adults with IVDD (18–21 years old), 451 controls (16–30 years old)	TT and CT genotypes OR = 2.14 for developing IVDD

(Continued)

TABLE 1 | **Continued**

Reference	Protein	SNP	Study population	Results
Takahashi et al. (40)	MMP-3	5A Variant	54 Young Japanese (18–28 years old) and 49 elderly (64–94 years old) patients	5A/6A and 5A/5A genotypes associated with increased risk of IVDD in elderly
Yuan et al. (12)	MMP-3	5A Variant	178 Chinese patients with IVDD (mean age 48.5 years old), 284 controls (mean age 40.6 years old)	5A allele OR = 2.5 for developing IVDD; 5A allele also associated with more severe forms of IVDD
Zhang et al. (41)	MMP-14	-378T/C	908 Chinese Han IVDD patients with IVDD (mean age 51.12 years old), 906 controls (mean age 51.54 years old)	TT genotype OR = 1.59 for developing IVDD compared with CC genotype
Liu et al. (42)	A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-4	rs4233367: 1877C/T	482 Chinese Han patients (mean age 42.6 years old), 496 controls (mean age 41.4 years old)	TT genotype OR = 0.21 for developing IVDD compared with CC genotype
Rajasekaran et al. (34)	ADAMTS-5	rs162509	308 Indian male patients with mild Total Disc Degenerative Score (mean age 29.6 years old), 387 Indian male patients with severe TDDS (mean age 31.7 years old)	Risk allele OR = 1.281 for developing IVDD
Kawaguchi et al. (43)	Aggrecan	VNTR	64 Young women (20–29 years old), 32 cases, 32 controls	Patients with 18 or 21 repeats were at greater risk of developing IVDD than patients with longer alleles
Eser et al. (24)	Aggrecan	VNTR	150 Turkish young adults with IVDD, 150 controls (20–30 years old)	A13–26 length alleles associated with higher risk of IVDD than longer alleles
Xu et al. (44)	Aggrecan	VNTR	Meta-analysis	Repeats of <25 OR = 1.85 for developing IVDD
Gu et al. (45)	Aggrecan	VNTR	Meta-analysis with 965 cases and 982 controls	A13–25 repeats OR = 1.52 for developing IVDD. In Asian patients specifically, OR = 1.65
Solovieva et al. (46)	Aggrecan	VNTR	132 Finnish middle-aged men (41–46 years old)	A26 allele associated with increased risk of dark NP on MRI. A26/A26 genotype OR = 2.77 for dark NP compared with longer or shorter alleles
Song et al. (47)	Carbohydrate sulfotransferase 3 (CHST3)	rs4148941	4043 Patients with LDD; 28,599 controls	AA or AC genotype OR = 1.49 for developing IVDD
Videman et al. (48)	Vitamin D receptor	FokI	85 Pairs of Finnish twins (35–69 years old)	Ff and ff genotypes associated with reduced disc signal intensity on MRI
Eser et al. (24)	Vitamin D receptor	FokI	150 Turkish young adults with IVDD, 150 controls (20–30 years old)	ff Genotype associated with more severe grades of IVDD (grades III, IV)
Vieira et al. (49)	Vitamin D receptor	FokI	121 Brazilian patients with IVDD (mean male age 46.0 years old, female 45.2 years old), 131 Brazilian population controls (mean male age 33.8 years old, female 33.9 years old)	T allele OR = 1.58 for developing IVDD. Ff and ff genotypes OR = 1.742 for developing IVDD in Hispanics; OR = 1.293 in Asians
Videman et al. (48)	Vitamin D receptor	TaqI	85 Pairs of Finnish twins (35–69 years old)	tt Genotype associated with reduced disc signal intensity on MRI
Kawaguchi et al. (50)	Vitamin D receptor	TaqI	205 Japanese young adults (mean age 22)	Tt genotype associated with multilevel disc degeneration
Eser et al. (24)	Vitamin D receptor	TaqI	150 Turkish young adults with IVDD, 150 controls (20–30 years old)	TT genotype associated with milder forms of IVDD compared with tt genotype
Toktas et al. (20)	Vitamin D receptor	TaqI	75 Southern European men with IVDD, 25 controls (35–45 years old)	tt Genotype associated with more severe forms of IVDD based on Pfirrmann scores
Yuan et al. (12)	Vitamin D receptor	ApaI	178 Chinese patients with IVDD (mean age 48.5 years old), 284 controls (mean age 40.6 years old)	Risk allele OR = 1.70 for developing IVDD
Zawilla et al. (51)	Vitamin D receptor	ApaI	84 Egyptian patients with IVDD (mean age 44.2 years old) and 60 controls (mean age 43.3 years old)	Mutant T allele OR = 3.1 for developing IVDD; T allele also associated with more severe forms of IVDD
Guo et al. (1)	Caspase-9	rs4645978: -1262A/G	154 Patients with LDD (20–65 years old), 216 controls (20–65 years old)	GG genotype of rs4645978 OR = 2.76 for developing IVDD compared with AA genotype
Mu et al. (52)	Caspase-9	rs4645978: -1262A/G	892 Chinese male soldiers: 305 cases (mean age 21.94 years old), 587 controls (mean age 22.09 years old)	G allele OR = 2.059 for developing IVDD

(Continued)

TABLE 1 | **Continued**

Reference	Protein	SNP	Study population	Results
Xu et al. (53)	TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL)	1525A/G, 1595T/C	100 Chinese patients with IVDD (31–81 years old), 100 controls (34–70 years old)	GG genotype of 1525A/G and CC genotype of 1595T/C associated with increased risk of IVDD and more severe forms of IVDD (grade IV)
Tan et al. (25)	Death receptor 4 (DR4)	rs4871857: C626G	296 Chinese Han patients with IVDD (mean age 48.42 years old), 208 controls (mean age 47.90 years old)	Mutant G allele OR = 1.958 for developing IVDD; GG and GC genotypes associated with more severe forms of IVDD
Williams et al. (54)	Growth differentiation factor 5 (GDF5)	rs143383	Meta-analysis including 5295 Northern European women (19–90 years old)	T allele OR = 1.72 for disc space narrowing and osteophyte production
Mu et al. (52)	Growth differentiation factor 5 (GDF5)	rs143383	892 Chinese male soldiers: 305 cases (mean age 21.94 years old), 587 controls (mean age 22.09 years old)	T allele OR = 2.115 for low back pain
Han et al. (55)	Vascular endothelial growth factor (VEGF)	-2578C/A, -634CC	102 Young Koreans with IVDD (mean age 23.6 years old), 139 controls (mean age 23.4 years old)	SNPs -2568CA or AA genotype, -634CC genotype OR = 21 for developing IVDD
Williams et al. (56)	Parkin	rs926849	Meta-analysis of 4600 Northern Europeans (18–85 years old)	Mutant C allele associated with reduced risk of IVDD
Rajasekaran et al. (34)	Cyclooxygenase 2 (COX2)	rs5277, rs5275	308 Indian male patients with mild Total Disc Degenerative Score (TDDS, mean 29.6 years old), 387 Indian male patients with severe TDDS (mean age 31.7 years old)	SNPs rs5277 and rs5275 significantly associated with IVDD
Gruber et al. (57)	Catechol-*O*-methyltransferase (COMT)	rs165656, rs4633, rs2095019, rs4708592	40 Patients with disc degeneration	SNPs rs165656, rs4633, rs2095019, and rs4708592 significantly associated with IVDD

TABLE 2 | Summary of proteins influenced by changes due to SNPs in their respective genes.

System	Protein
Structural	Collagen I (20, 27, 28) Collagen IX (20, 29–33) Collagen XI (4, 33, 34) Aggrecan (24, 43–46)
Structural support	Carbohydrate sulfotransferase (47) Vitamin D receptor (12, 20, 48–51)
Cytokines	Interleukin-1a (35, 36) Interleukin-6 (13, 36, 37)
Extracellular matrix-degrading enzymes	Matrix metalloproteinase (MMP)-1 (58) MMP-2 (38, 39) MMP-3 (12, 40) MMP-9 (11) MMP-14 (41) A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-4 (42) ADAMTS-5 (34)
Apoptotic factors	TNF (tumor necrosis factor)-related apoptosis-inducing ligand (TRAIL) (53) Death receptor 4 (25) Caspase-9 (1, 52) Parkin (56)
Growth factors	Growth differentiation factor 5 (52, 54) Vascular endothelial growth factor (55)
Pain mediators	Cyclooxygenase 2 (34) Catechol-*O*-methyltransferase (57)

alpha 1 (*COL9A1*), collagen type 9 alpha 2 (*COL9A2*), and collagen type 9 alpha 3 (*COL9A3*), respectively (20). Collagen IX is thought to play a significant role in connecting various types of collagens together, particularly collagen II (8, 59). Various studies have found SNPs located on either *COL9A2* or *COL9A3* that may be associated with increased risk of IVDD.

Annunen et al. examined 157 unrelated Finnish subjects with IVDD-induced sciatica (29). The study characterized a *COL9A2* polymorphism named Trp2, which caused a substitution of Gln or Arg for Trp in the collagen molecule. This substitution is particularly interesting because there are no naturally occurring Trp residues in collagen because the *COL9* gene does not encode for the amino acid Trp. The statistical analysis showed that patients with the allele coding for Trp were at a 4.5-fold increased risk of developing IVDD than those without the allele (29). Their population analysis found that 6 of the 157 individuals with IVDD had the Trp allele while none of the 174 controls did. A few other investigators have attempted to establish a connection between the Trp2 allele and IVDD but failed. For instance, Toktas et al. (20), Kales et al. (30), and Zhang et al. (31) did not find a correlation between *COL9A2* polymorphisms and IVDD.

A common SNP that has been studied in *COL9A3* is Trp3. This SNP is similar to the one found in *COL9A2*; it is an Arg103 to Trp substitution. Paassilta et al. studied the occurrence of the Trp3 allele in 171 Finnish subjects (32). The statistical analysis showed that patients who had a copy of the Trp3 allele were at a 2.7-fold increased risk of developing IVDD compared with patients who did not have the allele. Evidence for the association between the Trp3 allele and IVDD grew with a 2006 study by Solovieva et al. (33). They examined 135 middle-aged Finnish men and found that patients who carried the Trp3 risk allele in the absence of the IL-1 βT^{3954} SNP allele were at a 7.0-fold increased risk of a dark NP on MRI. These men had an overall

8.0-fold increased risk of degenerative changes in the spine. Although this study qualified the association between Trp3 and IVDD as dependent on the absence of the IL-1 βT^{3954} SNP allele, it nonetheless established a connection between the two (33). More recently, a 2015 study by Toktas et al. established a connection between the Trp3 allele and increased severity of disc degeneration (20). The study showed that of the five cases with Trp3 alleles, the heterozygous patients with the allele had a significantly lower average Pfirrmann score (19.40) compared with the wild-type patients without the allele (average score, 21.07). This finding suggests that not only is the Trp3 allele associated with an increased risk of developing IVDD but also associated with more severe forms of degeneration.

Collagen XI

Collagen XI has a similar structure to collagen IX in that it is a heterotrimer. The three chains, such as α1, α2, and α3, are coded by collagen type XI alpha 1 (*COL11A1*), collagen type XI alpha 2 (*COL11A2*), and collagen type II alpha 1 (*COL2A1*), respectively (5). Collagen XI is found in both the AF and NP of IVDs and has an important role in connections between the different collagen molecules, particularly collagen II, as well as connections between proteoglycans and collagen (5, 8).

Solovieva et al. showed a relationship between a G to A substitution SNP within intron 9 of *COL11A2* and disc bulging (33). Patients who were carriers of the SNP allele had a 2.1-fold increased risk of disc bulging compared with patients who did not have the allele. The study also noted a 1.6-fold increased risk of signs of disc degeneration, but the SD was too large to be statistically significant. Nonetheless, it is worth noting that the G to An SNP of *COL11A2* was related to change associated with disc degeneration. A 2009 study by Videman et al. documented five different polymorphisms in collagen XI genes that were significantly associated with signs of disc degeneration such as reduced disc signal and disc bulging (4). This particular large-scale study enrolled 588 Finnish male twins ranging from 35 to 70 years of age. The rs2072915, rs9277933, and rs2076311 SNPs of *COL11A2* were significantly associated with reduced disc signal on MRI, whereas the rs1337185 and rs1463035 polymorphisms of *COL11A1* were significantly associated with increased risk of disc bulging. A 2015 study by Rajasekaran et al. supported these findings (34). The study revealed the rs1337185 SNP of *COL11A1* was associated with a 1.55-fold increased risk of developing IVDD. Research suggests that SNPs in both *COL11A2* and *COL11A1* could predispose an individual to an increased risk of developing IVDD.

Cytokines

Cytokines, such as IL-6, IL-1a, IL-1b, and tumor necrosis factor (TNF)-α, are some of the key pro-inflammatory mediators that are found and released at sites of tissue injury. IL-1 is naturally found within the IVD and is responsible for indirectly degrading ECM components through the production of degradative enzymes, upregulation of other cytokines, and preventing the production of ECM components (5). IL-1 has three different subtypes: IL-1a, IL-1b, and IL-1RN. The alpha and beta subtypes are pro-inflammatory, whereas IL-1RN is anti-inflammatory (7). Within the disc, a delicate homeostasis between the pro-inflammatory and anti-inflammatory subtypes exists that is easily disturbed by trauma to the spine and genetic polymorphisms.

A common SNP of interleukin 1 alpha (*IL1A*) was significantly associated with IVDD in a 2007 study by Virtanen et al. who examined 150 Finnish men (35). The SNP is an -889C/T substitution where the T allele is the risk allele. Patients in the study with the TT genotype were at a 7.87-fold increased risk of developing IVDD compared with patients with the CC genotype. These findings were supported by a 2012 study by Eskola et al. of Danish adolescents (36). The study found a 2.82-fold increased risk of developing IVDD among girls who were carriers of the T allele compared with the controls. The study also described the polymorphism as increasing IL-1a expression, and thus furthering its function as a cartilage destroyer (36). These two studies, along with a few others, established the -889C/T SNP of *IL1A* as a genetic risk factor for IVDD (7, 35, 36).

Interleukin-6 is an important mediator of inflammation and having involvement with lumbar disc herniation (36). Despite this information, the exact role of IL-6 in disc degeneration is not fully known (5). Noponen-Hietala et al. documented an SNP in the interleukin 6 gene (*IL6*) that was significantly associated with IVDD (37). A 15T/A substitution was located within exon 5 of *IL6*. Statistical analysis showed that patients with the AA or AT genotypes were at a 4.4-fold increased risk of IVDD than patients with the TT genotype. The study documented that the 15T/A SNP results in an exon 5 amino acid substitution that replaces Asp with Glu. The researchers hypothesized that this polymorphism led to disequilibrium of the pro-inflammatory cytokines and, therefore, accelerated inflammation (37).

Another SNP associated with *IL6* was described in a 2010 study by Eskola et al. (13). They identified SNP rs1800796, a 572G/C substitution, which was significantly associated with IVDD in Danish girls. The study found that female patients carrying the C allele were at a 6.71-fold increased risk of developing IVDD than those without the allele. This study did not find the same association in Danish boys (13). However, a 2012 study by Eskola et al. described two different SNPs of *IL6* that were found only in adolescent boys: rs1800797 and rs1800795. The G/A genotype (risk allele, G) of SNP rs1800797 was associated with a 0.27-fold decreased risk of developing IVDD, whereas the G/C genotype (risk allele, G) of SNP rs1800795 was associated with a 0.26-fold decreased risk of IVDD. Both polymorphisms were protective and potentially reduced the inflammatory tone of *IL6* (36). Overall, the research on *IL6* suggests that various polymorphisms may influence a patient's genetic risk of IVDD; however, this effect may be limited to certain genders or populations.

Matrix-Degrading Enzymes

Several types of matrix-degrading enzymes exist within the ECM of IVDs. Two of the major types of matrix-degrading enzymes that are involved in IVD degradation are MMPs and "a disintegrin and metalloproteinase with thrombospondin motif" (ADAMTS). The homeostasis of ongoing ECM turnover is managed by the balance between MMPs and tissue inhibitors of metalloproteinases (12). Various MMPs are responsible for degrading different substances. For example, collagen I, II, and III

are primarily degraded by MMP-1, -8, and -13 – the collagenases, whereas denatured collagen is the target of MMP-2 and MMP-9 (59). It is important to remember that increased expression of MMPs leads to accelerated destruction of the ECM. ADAMTS are also referred to as aggrecanases because their primary function within the IVDs is to digest aggrecan (34). Similarly, an increase in expression of ADAMTS results in accelerated IVDD.

Matrix Metalloproteinase

Song et al. examined 691 southern Chinese people between the ages of 18 and 55 years and found an SNP at position -1607 in the promoter of the matrix metalloproteinase 1 gene (*MMP-1*) (58). The SNP was significantly associated with IVDD, and of the two alleles, D and G, the D allele was the risk allele. The statistical analysis revealed that patients carrying the D allele had a 1.41-fold increased risk of IVDD compared with those without the allele. Further analysis showed an even stronger connection in patients over the age of 40 years. In patients over the age of 40 years carrying the D allele, there was a 1.445-fold increased risk of developing IVDD. This study was particularly interesting because previous studies have shown the G allele of the -1607 SNP as increasing MMP-1 expression. The researchers hypothesized that expression of the D allele might lead to disequilibrium between the MMPs, and thus, greater degradation of the AF and NP (58).

MMP-2, one of the two gelatinases, tends to target denatured collagen as its substrate (59). Dong et al. found that the -1306C/T polymorphism of the *MMP2* gene was a genetic risk factor for IVDD (38). The study examined 162 Chinese young adults with disc degeneration. The statistical analysis demonstrated that patients with the CC genotype had a 3.08-fold increased risk of developing IVDD than those with at least one T allele (CT or TT). The study also found that the CC genotype was associated with more severe forms of IVDD than the CT and TT genotypes. This study was exceptionally interesting because the SNP is a C to T substitution, where the T allele is the risk allele, and the C allele is the wild-type. The T allele reduces Sp1 transcription factor binding to the gene and thus reduces overall transcription. The polymorphism that leads to increased protein production is the most likely one associated with an increased genetic risk of IVDD; in this case, it happened to be the wild-type C allele (38). A later study in 2013 by Zhang et al. revealed a similar phenomenon in the -735C/T polymorphism of *MMP2* (39). The study found that patients with the TT or CT genotypes had a 0.413-fold reduced risk of developing IVDD, whereas patients carrying the CC genotype were at nearly a 2.5-fold increased risk of developing IVDD compared with patients with the TT genotype. Similar to the -1306C/T SNP, the T allele was associated with disrupting a Sp1-binding site (CCACC box) and reducing transcription, while the C allele was considered the wild-type and was associated with increased transcription (39). These studies reveal that multiple nearby Sp1-binding sites whose polymorphisms are connected to genetic risk of IVDD exist (38, 39).

MMP-9 is also a gelatinase with variable expression that has been linked to IVDD. A 2009 study by Sun et al. revealed a -1562C/T polymorphism that affected the protein expression of MMP-9 (11). Patients with the CT/TT genotypes were at a 2.14-fold increased risk of developing IVDD compared with patients with the CC genotype. The T allele is associated with increased MMP-9 expression, and thus an imbalance between MMPs and tissue inhibitors of metalloproteinases, leading to excessive degradation of the ECM (11).

MMP-3 is one of the three MMPs that are categorized as stromelysins (59). One of the main functions of stromelysins is to degrade proteoglycans, laminas, and other components of the IVD ECM as well as indirectly degrade the disc through activating other MMPs (40). Expression of MMP-3 has also been shown to rise in response to inflammation (51).

The most commonly studied SNP of *MMP3* is the 5A variant allele in the promoter region of the gene. A 2001 study by Takahashi et al. revealed that elderly patients who had the 5A/5A or 5A/6A genotype were at an increased risk of IVDD (40). However, the study did not find this association in younger patients. Yuan et al. investigated the same 5A polymorphism and found that patients who carried the shorter 5A allele were at a 1.96-fold increased risk of developing IVDD (12). More recently, Zawilla et al. found that the 5A allele was associated with a 2.5-fold greater risk of developing IVDD (51). The study also found a link between the 5A allele and increased severity of degradation. An abundance of evidence suggests that the shorter 5A polymorphism of *MMP3* is linked to an increased genetic risk of IVDD (51).

MMP-14 is a membrane-anchored MMP that is found at the cell surface and is involved in degrading small fragments of collagen and activating *MMP2* (41, 59). Researchers have hypothesized that overexpression of MMP-14 leads to overall disc degradation mainly through the activation of *MMP2* (41). In a 2015 study by Zhang, the -378T/C SNP in *MMP14* was a genetic risk factor associated with IVDD (41). Patients with the TT genotype had a 1.59-fold increased risk of IVDD compared with patients with the CC genotype.

Considering all the various SNPs associated with MMPs and their influence on patients' risk of developing IVDD, protein expression levels are a delicate and important aspect of ECM maintenance of IVDs. It is possible that genetic manipulation of MMPs is a significant factor in the etiology behind IVDD. Furthermore, MMPs are strong candidates for therapeutic options for mitigating or reversing IVD degradation.

A Disintegrin and Metalloproteinase with Thrombospondin Motif

A disintegrin and metalloproteinase with thrombospondin motif are enzymes that play a central role in disc degeneration *via* aggrecan turnover (42). In particular, ADAMTS-4 and ADAMTS-5 are found at the site of disc degeneration. Various genetic polymorphisms in the *ADAMTS* family of genes are linked to the risk of IVDD. Liu et al. were the first to investigate a polymorphism in *ADAMTS4* (42). They found that SNP rs4233367, an 1877C/T substitution, was associated with a reduced risk of IVDD. Patients with the TT genotype were at a 0.21-fold reduced risk of developing IVDD compared with those with the CC genotype. This strong connection suggests that *ADAMTS4* plays an important part in proteoglycan degradation within the IVD. Rajasekaran et al. investigated SNP rs162509 in *ADAMTS5* and found that the risk allele was associated with a 1.281-fold increased risk of

developing IVDD (34). Although this relationship is small, it supports the notion that ADAMTS proteins are essential for the maintenance of healthy, hydrated discs.

Aggrecan

Aggrecan is the most plentiful proteoglycan found within the IVD, and its primary function is to retain water. The core protein of aggrecan contains a large number of chondroitin sulfate and keratin sulfate chains that facilitate its ability to create an osmotic gradient. Furthermore, aggrecan binds to negatively charged glycosaminoglycans to increase the hydrostatic pressure of the NP (5, 7). One of the most investigated polymorphisms of aggrecan is the variable nucleotide tandem repeat (VNTR) in the chondroitin sulfate-1 encoding domain of the aggrecan gene (*ACAN*) (45). The chondroitin sulfate encoding allele has VNTRs ranging from 13 to 33 nucleotides, with the most common number being 26, 27, or 28 repeats. As aggrecan water-retention abilities are heavily reliant on the number and size of chondroitin sulfate chains, it makes sense that a reduced number of repeats would impair the ability of aggrecan to retain water (24, 43–46). One of the earliest studies published on this topic was in 1999 by Kawaguchi et al. (43). The study found patients with 18 or 21 repeats in the chondroitin sulfate encoding domain were at an increased risk of multilevel disc degeneration as well as more severe forms of degeneration when compared with patients with longer alleles. A 2010 study by Eser et al. supported these results (24). They found that patients with short alleles, consisting of VNTRs of A13 to A26, were at an increased risk of severe disc degeneration compared with those with longer VNTRs in their alleles. The study also found that patients with short, A13 to A26, or normal, A27, were at an increased risk of multilevel disc degeneration. These findings were further supported by a 2012 study by Xu et al. who found that patients with less than 23 VNTRs were at a 1.95-fold increased risk of IVDD compared with those with more than 23 repeats (44). The study also found that patients with less than 25 repeats were at a 1.85-fold increased risk of IVDD compared with those with more than 25 repeats. This study helped establish the dose-dependent nature of the VNTRs of the aggrecan gene. The risk associated with VNTRs seems to follow a continuous scale, as opposed to a Boolean, or "cut-off" pattern (44). A 2013 meta-analysis by Gu et al. revealed that patients with shorter alleles, A13 to A25, were at a 1.54-fold increased risk of IVDD compared with those with either normal, A26 to A27, or longer alleles, A28 to A32. This relationship was found to be even stronger in patients of Asian descent, who were at a 1.65-fold increased risk of IVDD (45). This study helped solidify the notion that shorter VNTRs are not only associated with increased risk of IVDD but also suggest that the magnitude of the effect may be associated with race.

In 2007, Solovieva et al. investigated the VNTRs for the aggrecan gene in 132 middle-aged Finnish men (46). Their analysis found that the A26 allele was associated with an increased risk of the patient's NP to be dark on an MRI scan, which is an indication of IVDD. The study also found that patients with A26/A26 genotype were at a 2.77-fold increased risk of a dark NP compared with patients who had longer or shorter VNTRs. This study is unique and did not follow the same trends as the previously mentioned studies. In previous studies, A26 was either considered within normal/typical range or even long (24, 43–45). This 2007 study helped support the notion that the effects of VNTRs may also be influenced by the race or ethnicity of the patient.

Various other genes that affect the aggrecan water-retention abilities or aggrecan expression have also been investigated. Carbohydrate sulfotransferase 3 (CHST-3) is an enzyme that is involved in sulfation of the aggrecan side-chains and is coded by *CHST3*. This function makes CHST-3 an important and indirect contributor to disc hydration. Song et al. identified the SNP rs4148941 that produced the risk allele A (47). They found that the allele A variant of *CHST3* had improved binding with micro RNA sequence miR-513a-5p. Their statistical analysis showed that patients with the AA or AC genotypes were at a 1.48-fold increased risk of developing IVDD. Further analysis revealed that the A allele was associated with reduced expression of the *CHST3* messenger RNA within the IVDs, suggesting reduced expression of CHST-3 protein (47). Overall, the study established the SNP of *CHST3* as a genetic risk factor for IVDD.

Vitamin D Receptor

The VDR is a nuclear receptor for a vitamin D metabolite, 1a,25-dihydroxyvitamin D3 (**Figure 3**). Previous studies have shown that *VDR* polymorphisms are associated with various bone disorders including osteoarthritis, osteoporosis, and cardiovascular disease (7, 44). VDR function in IVDs is hypothesized to be through an indirect pathway for chondrocyte proliferation and the effect of chondrocytes on proteoglycans (12). Over the past two decades, various polymorphisms affecting the expression and function of VDRs in IVDD have been identified. These SNPs include FokI (rs2228570), TaqI (rs731236), and ApaI (rs7975232) (12, 20, 24, 44, 48, 49, 51, 61, 62).

The FokI polymorphism of *VDR* is a C to T substitution found in exon 2 (49). This SNP leads to altered protein size, and subsequently, altered function. Research has shown that the shorter polypeptide of VDR is associated with the wild-type C variant. The F allele has a higher affinity for transcription factor II B. The wild-type alleles lead to normal functioning *VDR*, while the T substitution (risk allele f) is associated with reduced function (62). A 1998 study by Videman et al. of Finnish twins found that the ff genotype was associated with 9.3% reduced signal intensity within the T6–S1 region on an MRI compared with the FF genotype (48). They also found that the Ff genotype was associated with 4.3% reduced signal intensity within the same region. These results were supported by a 2010 study by Eser who found that the FF genotype was associated with milder grades of degradation (grades I and II), whereas the ff genotype was associated with more severe grades (grades III and IV) (24). The FokI SNP was not only associated with an increased severity of IVDD but also increased the risk of developing IVDD. Vieira et al. found that the T allele was associated with a 1.58-fold increased risk of developing IVDD compared with the C allele (49). These results were further supported by a recent 2016 study by Zhao et al. They found that Hispanic patients with the ff or Ff genotype (TT or TC alleles) were at a 1.742-fold higher risk of developing IVDD, whereas Asian patients with similar genotypes had a 1.293-fold increased risk (62). The data on the FokI SNP suggest that it is a genetic risk factor not only for IVDD but also for the

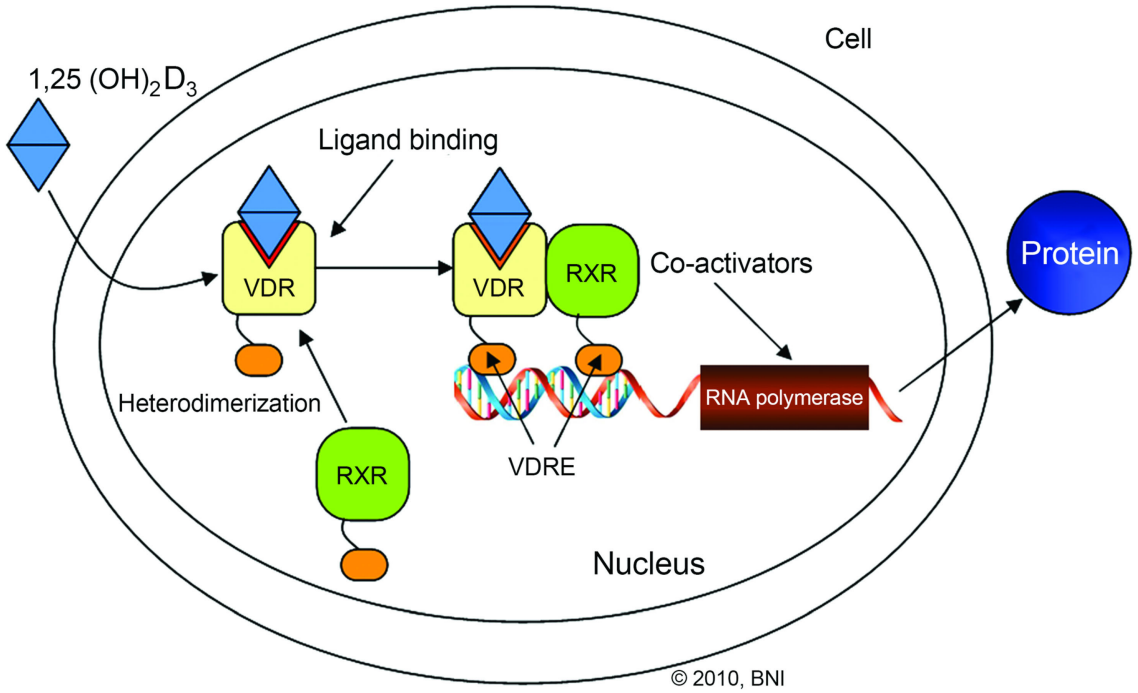

FIGURE 3 | Illustration outlining the vitamin D receptor (VDR) signaling pathway. VDR/retinoid X receptor (RXR) interaction with vitamin D response element (VDRE) stimulates gene transcription. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

severity of IVDD. Furthermore, these data suggest that the FokI polymorphism manifests differently in patients based on race or ethnicity.

Another significant SNP of *VDR* that has been the target of the most investigation among *VDR* polymorphisms is the TaqI variant (44). Interestingly, TaqI is a silent mutation in exon 9 of the *VDR* gene, yet it has a profound effect on a patient's genetic risk of developing IVDD (50). One of the earliest studies of the TaqI polymorphism was the 1998 study by Videman et al. (48). They found that the patients with the tt genotype displayed 12.6% reduced signal intensities in the T6–12 range on MRI compared with patients with the TT genotype. These findings were supported by a 2002 study by Kawaguchi et al. who investigated the incidence of the TaqI SNP in Japanese young adults (50). The study found that patients with the Tt genotype were at an increased risk of multilevel IVDD and more severe forms of degeneration. The study was unable to establish the same connection for the tt genotype because none of the subjects had the tt genotype. In 2010, Eser et al. found that patients with the TT genotype displayed significantly milder forms of IVDD than patients with the tt genotype (24). A study in 2015 by Toktas et al. supported the association of the TaqI SNP with increased severity of disc degeneration. They found that patients with the homozygous tt genotype had an average Pfirrmann score of 18.45, which was significantly lower than in those with wild-type genotypes (average score, 22.15) (20). The findings from these studies suggest that the TaqI SNP of *VDR* is associated with both increased risk of developing IVDD and severity of IVDD.

Another common polymorphism of *VDR* that has received much attention is the ApaI SNP. The ApaI SNP maps to intron 8 of *VDR* and is associated with increased risk of IVDD (50). Yuan et al. found that the risk allele of the ApaI SNP was associated with a 1.70-fold increased risk of developing IVDD (12). These findings are supported by a 2013 study by Zawilla et al. who found that the mutant T allele of *VDR* was associated with a 3.1-fold increased risk of developing IVDD (51). They also found that the mutant T allele was significantly associated with increased severity of IVDD. Although the ApaI polymorphism is associated with both severity and risk of developing IVDD, the exact mechanism and its impact on the VDR protein has not been thoroughly investigated (12, 50, 51). Despite this, ApaI is a well-established genetic risk factor of IVDD.

Apoptosis

Studies regarding the molecular mechanisms of IVDD have established that degenerated discs display much higher rates of apoptosis, programed cell death (3, 5). Although the exact cascade of molecules involved in apoptosis of IVD cells remains under investigation, there are a few significant genes whose polymorphisms have been associated with increased risk of IVDD. Among these are caspase-9 (*CASP9*), TNF-related apoptosis-inducing ligand (*TRAIL*), and death receptor-4, *DR4*, also known as *TRAIL* receptor 1 (*TRAILR1*) (1, 10).

Caspase-9 is an important activator of the intrinsic pathway of apoptosis. Its expression levels within the IVD have been reported to increase during disc degeneration (1). The first

study to report on *CASP9* polymorphisms and their relationship to IVDD was a 2011 study by Guo et al. (1). The study investigated two SNPs, rs4645978 (-1263A/G) and rs4645981 (-712C/T). They analyzed data from 154 patients with IVDD and found that the mutant GG genotype was associated with a 2.760-fold increase in the risk of IVDD compared with the AA genotype (1). Mu et al. investigated the same polymorphism, -1263A/G, and found that the G allele was associated with a 2.059-fold increase in the risk of developing lower back pain compared with the A allele (52). These studies suggest that SNPs affecting the expression and function of apoptosis factors may be another way in which genetic factors influence the progression of IVDD.

DR4 and DR5 are both receptors that bind to TRAIL and induce apoptosis within the target cell. Recent studies have shown that the TRAIL/DR4/DR5 system is important in mediating apoptosis within IVDs (10). Polymorphisms that influence the function and expression of either *TRAIL* or *DR4* can significantly impact the rate of apoptosis occurring within IVDs. Xu et al. identified two polymorphisms of *TRAIL* within the 3′-untranslated region, such as 1525A/G and 1595T/C, which are associated with IVDD (53). The mutant GG genotype at the 1525 locus and the mutant CC genotype of the 1595 locus were associated with increased risk of IVDD. The investigation found that both the GG1525 and CC1595 genotypes were associated with reduced TRAIL expression within the cells as well as more severe forms of IVDD (grade IV). Although reduced TRAIL expression has already been established in IVDD, the underlying pathophysiology remains under investigation (53).

The *TRAIL/DR4/DR5* system is also affected by polymorphisms in *DR4*. Tan et al. found that degenerating IVD cells had increased expression of DR4 (25). They investigated a Chinese Han population and found that SNP rs4871857 (626C/G) in exon 4 of *DR4* was associated with IVDD. Patients with the mutant G allele were at a 1.958-fold increased risk of developing IVDD. Furthermore, the GG and CG genotypes were associated with more severe grades of IVDD (25). The findings on *TRAIL* and *DR4* revealed another aspect of IVDD that may be controlled by genetic factors.

Growth Factors

Growth differentiation factor 5 (GDF5) is part of the transforming growth factor-β superfamily involved in bone, ligament, and soft tissue development (52, 54). Increased GDF5 expression is linked to increased collagen II and aggrecan production in human IVDs (63, 64). An investigation of polymorphisms in *GDF5* revealed that its variable expression and function are linked to osteoarthritis. Williams et al. investigated SNP rs143383 (a T to C substitution at position 104) located within the promoter region of the *GDF5* gene. Their analysis showed that the T allele was associated with 1.72-fold increased risk of disc space narrowing and osteophyte production in women (54). These findings are supported by a 2013 study by Mu et al. who investigated the same SNP (52). They found that the T allele of *GDF5* was associated with a 2.115-fold increased risk of lower back pain. Although the study revealed an association between the T allele and lower back pain, the findings still suggest the involvement of *GDF5* polymorphisms in IVDD.

Similar studies have investigated the influence of vascular endothelial growth factor (*VEGF*) gene polymorphisms and their link to IVDD (55). IVDs are some of the largest avascular structures within the human body. Consequently, they rely on small capillaries extending from the lumbar artery to help remove metabolic waste (5). One of the main features of a severely degenerated disc is neovascularization penetrating the AF, hence, the interest in VEGF, a key mediator of angiogenesis (55). Han et al. found that when a patient possessed multiple *VEGF* SNPs, there was a significant association with IVDD (55). For example, a patient with the genotype of -2578CA or AA, combined with -634CC genotype, was at a 21-fold increased risk of IVDD. With limited data, it is difficult to conclude with certainty that *VEGF* SNPs are associated with IVDD; however, Han et al. (55) have helped establish the preliminary data to warrant further investigation into *VEGF* polymorphisms.

Ubiquitin-Mediated Degradation

E3 ubiquitin-protein ligase is a multiprotein complex that functions in an ubiquitin–proteasome pathway, marking proteins for degradation. A key protein in this complex named Parkin is expressed in various organs and skeletal muscles. Parkin is coded by *PARK2*, which was recently associated with IVDD (8, 56). In a 2013 study of 4600 Northern Europeans, Williams et al. reported that the rs926849 SNP is a T to C substitution found within an intron of *PARK2* (56). Their statistical analysis revealed that the C allele was significantly associated with reduced risk of IVDD, suggesting that the C allele was protective. The underlying mechanism of how the C allele influences the expression of *PARK2* and the subsequent pathology remains under investigation (56). Nonetheless, this study adds another component to the etiology of IVDD as well as highlighting the complexity and continued discoveries associated with IVDD.

Cyclooxygenase

Cyclooxygenase 2 is an essential enzyme that is involved in the production of various prostaglandins and thromboxanes. The cyclooxygenase 2 gene *COX2* and its products participate in multiple pathways including inflammation and pain (8, 34, 65). In 2015, Rajasekaran et al. identified two SNPs, such as rs5277 and rs5275, in *COX2* that are significantly associated with severe IVDD (34).

Catechol-*O*-Methyltransferase

Catechol-*O*-methyltransferase is an enzyme that is involved in the degradation and processing of catechol neurotransmitters such as dopamine. Previous clinical studies showed a relationship between certain polymorphisms in the catechol-*O*-methyltransferase (*COMT)* gene and pain. The IVDD researchers believed that variable catechol-*O*-methyltransferase expression led to increased pain in IVDD. Gruber et al. identified four *COMT* SNPs, such as rs4633, rs165656, rs2095019, and rs4708592, significantly associated with IVDD (57). Their findings supported results that were previously published regarding the association of rs4633 and IVDD. Although rs165656 has previously been

associated with mental retardation, Gruber et al. were the first to show its significant association with IVDD (57). The rs2095019 and rs4708592 polymorphs are novel SNPs that have not been reported previously (57). The study is a strong indicator of the complexity of the acute and chronic changes that occur with IVDD as well as highlighting the ongoing research that has revealed new aspects of its etiology.

Personalized Medicine

The ultimate goal in reviewing the medical literature about the genetic polymorphisms associated with IVDD is to provide patients with personalized and targeted therapeutics. When a patient enters a clinic with lower back pain and degenerative disc disease is suspected, an MRI can provide a conclusive diagnosis. To provide targeted treatment for the specific patient, the physician must understand the patient's unique molecular profile. Through gene sequencing and screening for SNPs, physicians can obtain a better understanding of the imbalances that led to the patient's disc degeneration. Some patients may primarily have imbalances with ECM degrading enzymes, whereas others may have overexpression of proapoptotic factors. With this information, unique to each patient, specific therapies can be selected to provide the best long-term outcome.

CONCLUSION

Despite continued research, the etiology and pathophysiology underlying IVDD remain poorly understood (34). Nonetheless, a significant shift in the understanding of IVDD has occurred over the past two decades, and we now understand that roughly 75% of the etiology behind IVDD is genetic (2, 6). One of the crucial techniques that have helped researchers to realize this understanding is the advent of large-scale DNA arrays and computational analysis software to analyze polymorphisms quickly (34). These techniques have helped bring to light new proteins and associations within systems that were previously thought not to be linked to IVDD. With a better understanding of the pathophysiology of IVDD and improved technology for scanning entire genomes for SNPs than in the past, we expect to produce innovative, new therapeutic approaches.

Two important aspects of genetic polymorphisms that have come to light are variations in race and ethnicity. Some polymorphisms tend to have stronger, or even no effect, on certain races. For example, Hispanics with the FokI SNP of *VDR* were at a much higher risk of IVDD than their Asian counterparts. Furthermore, the same meta-analysis found that the FokI SNP was not associated with IVDD in people of Caucasian decent (62). These racial variations add a new aspect and complexity to the understanding of the genetic factors underlying IVDD.

With more than 20 unique polymorphisms associated with IVDD, the molecular changes in the associated proteins or pathology of the disc are not yet fully understood. In the coming years, research targeted toward fully understanding the protein changes due to the already identified SNPs is crucial. If we can fully understand the molecular changes involved in IVDD then creating targeted therapeutics based on genetic profiling becomes a possibility.

With improved understanding of the genetic variants associated with IVDD, and rapid genomic analysis available through next-generation genotype sequencing, the possibility of providing effective personalized medicine can become a reality (14, 15). This comprehensive literature review regarding the genetic variants associated with IVDD not only affords a better understanding of the molecular mechanisms behind IVDD but also allows physicians the possibility of providing targeted treatments. For instance, if an IVDD patient were identified to have genetic variants resulting in the overexpression of apoptotic factors, physicians would be able to refine their therapy and target the specific underlying IVDD-causing mechanism unique to that patient. Furthermore, genomic screening for the known variants associated with IVDD can help predict disease progression and severity. This knowledge can help provide more effective treatments personalized to the unique phenotypic presentation of the patient. Considering the majority of the etiology underlying IVDD is genetic, it is essential that researchers and clinicians have a keen understanding of this underlying etiology to optimize treatment (2). Consequently, DNA screening for the genetic variants explaining the pathophysiology of the patient's IVDD should be the standard of care.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work.

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REVIEW
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The Role of microRNA Markers in the Diagnosis, Treatment, and Outcome Prediction of Spinal Cord Injury

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Spinal cord injury (SCI) is a devastating condition that affects many people worldwide. Treatment focuses on controlling secondary injury cascade and improving regeneration. It has recently been suggested that both the secondary injury cascade and the regenerative process are heavily regulated by microRNAs (miRNAs). The measurement of specific biomarkers could improve our understanding of the disease processes, and thereby provide clinicians with the opportunity to guide treatment and predict clinical outcomes after SCI. A variety of miRNAs exhibit important roles in processes of inflammation, cell death, and regeneration. These miRNAs can be used as diagnostic tools for predicting outcome after SCI. In addition, miRNAs can be used in the treatment of SCI and its symptoms. Significant laboratory and clinical evidence exist to show that miRNAs could be used as robust diagnostic and therapeutic tools for the treatment of patients with SCI. Further clinical studies are warranted to clarify the importance of each subtype of miRNA in SCI management.

Keywords: biomarker, gene expression, microRNA, miRNA, regeneration, spinal cord injury

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INTRODUCTION

Acute spinal cord injury (SCI) is a devastating condition that affects mostly young and active individuals. It is estimated that approximately 180,000 persons around the world will experience some form of traumatic SCI every year (1–3). These injuries are particularly devastating because they can result in physically, socially, and financially burdensome consequences, such as quadriplegia and paraplegia. Over the past decade, we have gained a much better understanding of the biological mechanisms underlying the damage caused by acute SCI. This damage can be divided into two phases: primary and secondary (4, 5).

The primary phase of damage after SCI is characterized by the immediate loss of sensory, motor, and autonomic functions after a sheer, lacerating, or impact injury to the spinal cord or sudden compression of the spinal cord. The initial mechanical damage to the spinal cord primarily disrupts gray matter and microvasculature. In contrast, white matter, although damaged in the primary phase, is primarily damaged in the secondary phase (4). The secondary phase of damage begins seconds

Abbreviations: BBB, blood–brain barrier; BMP, bone morphogenic protein; CNS, central nervous system; GFAP, glial fibrillary acidic protein; JAK/STAT, Janus-associated kinase/signal transducer and activator of transcription; miRNA, microRNA; ROS, reactive oxygen species; SCI, spinal cord injury; SOD, superoxide dismutase.

after SCI and continues for months after injury. Within seconds to minutes of injury, vascular and metabolic disturbances occur in the spinal cord. In a matter of minutes to hours, biochemical changes lead to altered lipid peroxidation and neurotransmitter accumulation. Within hours to weeks, cascades of inflammatory cells and evidence of apoptosis occur, and, in weeks to months, fiber tract disturbances occur due to demyelination and glial scar formation (4–6). The secondary phase of damage is the primary target for investigation and clinical therapeutics.

Despite advances in our understanding of the pathways activated and responsible for secondary injury after SCI, therapeutic advances for patients with SCI have lagged behind. So far, the "one-size-fits-all" approach to treating SCI has repeatedly come up short. Gaps in our knowledge are partially responsible for the lack of improvement in therapy. In addition, the inability to identify a *bona fide* biomarker that predicts which patients are likely to have a good or poor outcome after SCI has hindered delivery and implementation of new therapies. Because no two SCIs are alike, treatment approaches should be tailored to the nature, quality, and duration of the injury. Novel approaches to SCI treatment will thus involve a shift toward personalized medicine.

In recent years, investigators have begun to document the potential effects that microRNA (miRNA) sequences may have on the regulation of the processes involved in SCI (7–9). The miRNA sequences are small, unique, non-coding RNA fragments that form hairpin structures averaging 22 nucleotides in length. They are produced in the nucleus by RNA polymerase II and processed by a variety of proteins before entering the cytoplasm as pre-miRNA. In the cytoplasm, the enzyme coded for by the gene, dicer 1 ribonuclease type III, or *DICER1*, processes the pre-miRNA duplex into a single-stranded miRNA sequence. This single-stranded miRNA sequence is incorporated into an RNA silencing complex that then binds to an mRNA sequence to carry out a negative regulatory effect by either degrading or blocking translation in the targeted mRNA (8, 10, 11). This regulatory system has a powerful effect on mRNA levels, and, as a result, it influences protein expression levels (12). Because miRNAs do not need a perfectly complementary sequence to act upon an mRNA, investigations have found that a single miRNA sequence is able to target up to 200 mRNA transcripts (11).

The miRNAs have a powerful and important influence on the protein profile of a given cell. Since the discovery of the first miRNA sequence in 1993, thousands of unique miRNA sequences that play key regulatory roles in the human body have been identified (8). In particular, miRNAs have been identified as playing an important role in regulating neurogenesis and cortical development (13). The management of SCI will be dramatically improved if novel pathophysiological mechanisms of SCI and sensitive biomarkers to monitor them can be determined. Alterations in extracellular RNAs could be used to identify regulators of secondary injury cascades, and overall changes in RNA concentrations could be used to stratify patients into risk categories for secondary injury and could be predictive of patient outcomes – a form of personalized medicine for patients suffering from the effects of SCI.

In this article, we review the regulatory role of miRNAs in the cascade of events, after an acute SCI. We also discuss their temporal and spatial expression, as well as their potential role as therapeutic agents in the personalized treatment of patients with SCI.

SCI PATHOPHYSIOLOGY AND miRNAs

Astrogliosis

Normally, astrocytes regulate central nervous system (CNS) homeostasis by maintaining the blood–brain barrier (BBB) and the blood–spine barrier, directing neuronal migration, differentiation, and development, and providing materials for axonal growth or regeneration (14). When a patient suffers a significant injury to the spinal cord and suffers the effects of secondary trauma, the area near the injury undergoes astrogliosis. In the acute stages of astrogliosis (at approximately the third day after injury), hypertrophic astrocytes expressing glial fibrillary acidic protein (GFAP) and vimentin (*VIM*) surround the lesion site, where they keep out inflammatory leukocytes, release antioxidants, and initiate repair of the blood–spinal cord barrier. These effects are both protective and beneficial. In later stages of astrogliosis (at approximately 4–6 weeks after injury), astrocytes change from hypertrophic to hyperplastic, forming a glial scar by expressing chondroitin sulfate proteoglycans. This is a detrimental process that compresses the entire lesion site and prevents the beneficial self-rehabilitating and protective actions that were present in the acute hypertrophic phase (**Table 1**) (7, 14–19).

The process of astrogliosis has been shown to be associated with miRNAs, which play a pivotal role in the shift from hypertrophy to hyperplasia (7, 14–19). The miRNA miR-21 is highly expressed in astrocytes near and at the lesion site during astrogliosis, but not elsewhere (7). In addition, miR-21 is directly responsible for the shift from hypertrophy to hyperplasia, where it suppresses *GFAP* and *VIM* (**Figure 1**). The miR-21 is governed by bone morphogenic protein (BMP) signaling *via* the signal transducer and activator of the transcription 3 gene, *STAT3* (14–16, 19). Specifically, the BMP receptor type 1A and 1B genes, *BMPR1A* and *BMPR1B*, control astrogliosis by regulating miR-21 in opposing directions. *BMPR1A* downregulates miR-21 signaling, while *BMPR1B* upregulates it (7, 15, 16). These regulatory genes have become a target of interest for developing therapeutics. Knockout mice with suppressed miR-21 signaling maintain astrocyte hypertrophy, correlating with smaller lesion sites, less demyelination, greater axon regeneration, and an overall lower inflammatory response (7, 15, 16). Future treatment modalities could be geared toward preventing the shift to hyperplastic astrogliosis. The targets of these potential treatment modalities include the final processing of pre-miR-21 to its mature form, the formation of chondroitin sulfate proteoglycans, RNases that could suppress miR-21, and the suppression of *BMPR1B*.

Additional miRNAs involved in astrogliosis include miR-145, which is expressed in gray matter astrocytes during acute astrogliosis but which increases expression in astrocytes during the shift to hyperplasia. It has been shown to control the astrocytic cytoskeleton, where it affects cell growth and migration, in addition to negatively regulating *GFAP* and the cell growth gene, *MYC* (c-myc) (**Table 1**; **Figure 1**) (17). Strickland et al. have shown that

TABLE 1 | Functions of miRNAs in spinal cord injury pathophysiology[a].

miRNA	4 h	12 h	1 day	3 days	4 days	7 days	14 days	≥30 days	Regulation	Target genes	Function
miR-9 (20)		↓	↓	↓		↑			↑ miR-9	↓ *MCPIP1*	↓ Inflammation, ↓ apoptosis
miR-17-5 (21)									↑ miR-17-5p	↓ *CDKN1A*, ↓ RB1	↑ Astrocyte proliferation
miR-20 (7, 22)	↑	↑	↑	↑	↑				↑ miR-20a	↓ *NEUROG1*	↑ Oxidative stress, ↑ functional recovery
miR-21 (8, 9, 15, 16, 18, 23–31)	↑		↑	↑	↑	↑	↓	↑	↑ miR-21	↓ *GFAP*	↓ Astrogliosis
									↑ miR-21	↓ *FASLG*, ↓ *PTEN*	↓ Apoptosis
									↑ miR-21	↑ *MTOR*	↑ Axon sprouting
									↑ miR-21	↓ CACNA1C, ↓ CACNA2D1, ↓ CACNAB1	↓ Calcium signaling, ↓ neurotransmission
									↑ miR-21		↑ Neuropathic pain
									↑ miR-21		↑ Functional recovery
miR-29b (22)	↓	↓	↓	↓	↓				↓ miR-29b	↑ *BH3*	↑ Apoptosis
miR-103 (23, 24, 32–34)	↑					↑			↑ miR-103	↓ CACNA1C, ↓ CACNA2D1, ↓ CACNAB1	↓ Neuropathic pain
									↓ miR-103	↑ CACNA1C, ↑ CACNA2D1, ↑ CACNAB1	↑ Neuropathic pain
									↓ miR-103		↑ Inflammation
miR-124 (35–39)		↓	↓	↓	↓	↓			↑ miR-124	↓ *PTBP1*	↑ Neuronal expression, ↑ functional recovery, ↓ apoptosis
miR-125b (9, 40)									↑ miR-125b		↑ Axonal regeneration
miR-126 (41)			↓	↓		↓			↑ miR-126	↓ *VCAM1*	↓ Immune cell infiltration
										↓ *PIK3R2*	↑ Apoptosis
										↓ *SPRED1*	↑ Cell growth, ↓ angiogenesis
miR-145 (17, 24, 42)	↑	↑	↑	↑		↓		↓	↑ miR-145	↓ *GFAP*, ↓ *MYC*	↓ Astrogliosis
									↓ miR-145	↑ *SOD2*	↓ Oxidative stress
miR-146 (7, 18, 24, 43, 44)						↑	↑		↑ miR-146	↓ *IRAK1*, *TRAF6*	↓ Inflammation
									↑ miR-146		↓ Astrogliosis
miR-146a (7, 18, 43, 44)	↓		↑			↑			↑ miR-146a	↓ *IRAK1*	↓ Astrogliosis
									↑ miR-146a	↓ *CASP3*, ↓ *FAS/CD95*	↓ Apoptosis
									↑ miR-146a	↓ *IL6*, ↓ *COX2*	↓ Inflammation
									↓ miR-146a	↑ *IL6*, ↑ *COX2*	↑ Inflammation
									↓ miR-146a	↑ *CASP3*, ↑ *FAS/CD95*	↑ Apoptosis
miR-155 (45, 46)				↑	↑	↑	↑		↑ miR-155 ↓ miR-155	↓ *SOCS1* ↑ *GAP43*, ↑ *NEFH*	↑ IL-17 Expression, ↑ Th17 differentiation
miR-181 (47, 48)									↓ miR-181	↑ *TNF*	↑ Inflammation
									↓ miR-181		↑ Astrogliosis

(Continued)

TABLE 1 | **Continued**

miRNA	4 h	12 h	1 day	3 days	4 days	7 days	14 days	≥30 days	Regulation	Target genes	Function
miR-195 (49)									↑ miR-195	↑ *IL1B*; ↑ *TNF*	↑ Neuropathic pain
									↓ miR-195	↓ *IL1B*; ↓ *TNF*	↓ Neuropathic pain
miR-199a-3p (27)									↓ miR-199a-3p	↑ *MTOR*	↑ Axon regeneration
miR-200c (50)				↑		↑	↑		↑ miR-200c	↓ *FAP-1*	↑ Apoptosis
miR-210 (51)									↑ miR-210		↑ Axon growth, ↑ remyelination, ↑ angiogenesis, ↑ functional recovery
miR-218 (52)			↑	↑		↑	↑		↓ miR-218	↓ *JAK*, *STAT3*, *SOCS3*	↓ Pain behavior, ↓ inflammation
miR-219 (53)									↓ miR-219	↑ *ELOVL7*	↑ Demyelination
miR-223 (7, 54)			↑	↑		↑			↑ miR-223	↓ *GRIA2*, ↑ *BAX*, *CASP3*, ↓ *BCL2*	↑ Apoptosis, ↓ angiogenesis, ↓ functional recovery
									↑ miR-223		↑ Inflammation
miR-320 (55)									↓ miR-320	↑ *HSP20*	↑ Neuroprotection, ↑ functional recovery
miR-381 (56)									↑ miR-381	↑ *HES1*	↑ Neuronal differentiation, ↓ glial cell differentiation

[a]*An up arrow (↑) indicates upregulation; a down arrow (↓) indicates downregulation.*

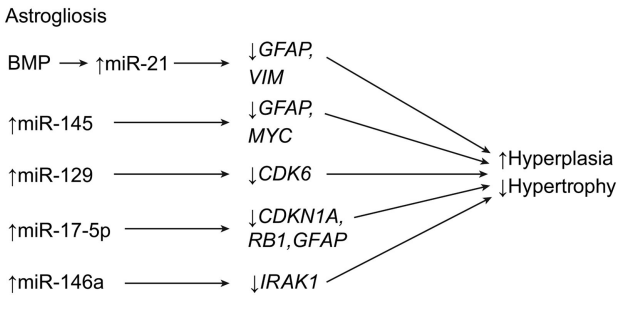

FIGURE 1 | **Schematic for astrogliotic effects of miRNA expression after SCI**. The end result is a downregulation of astroglial swelling, and upregulation of cell division. *CDK6*, cyclin-dependent kinase 6; *CDKN1A* (*P21*), cycline-dependent kinase inhibitor 1A; *MYC*, V-myc avian myelocytomatosis viral oncogene homolog; *GFAP*, glial fibrillary acidic protein; *IRAK1*, interleukin-1 receptor-associated kinase-1; *MBP*, myelin basic protein; *RB1*, ginsenoside Rb1; *VIM*, vimentin. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

approximately 30 miRNAs are altered by SCI (18). They report that miR-146a works with miR-21 to drive astrocyte hyperplasia, while miR 129-1 and miR 129-2 both inhibit the cyclin-dependent kinase gene, *CDK6*, and therefore prevent cell growth (**Figure 1**). The knockdown of these genes in SCI suggests a more conducive environment for hyperplastic astrogliosis (18).

In 2014, Hong et al. speculated that miR-17-5p is somehow involved in *p21 (CDKN1A)* regulation and promotes astroglial cell proliferation after injury by way of *DICER1* (**Table 1**) (21). Knocking out *DICER1* in mice caused miR-17-p5 to decrease *GFAP* expression while maintaining cell proliferation (**Figure 1**). This effect suggests that *DICER1* and miR-17-5p are directly involved in the maturation and proliferation of astrocytes. The knockdown of these components delayed astrocyte maturation and ultimately caused a failure to respond to the SCI cascade. These data further support the idea that selective manipulation of the astrogliotic response to SCI may be a key therapeutic strategy for SCI (21).

Apoptosis

Apoptosis, or programed cell death, is a hallmark of SCI. Apoptosis can affect all cell types in the spinal cord, including glial cells. This is important when considering that SCI induces miRNA expression to either upregulate or downregulate apoptotic genes, depending on the target (**Table 1**) (7, 14, 23–25, 57–63). Among the miRNAs involved in this process, miR-21 has been shown to be one of the most dysregulated miRNAs after SCI (24, 25). As mentioned above, the shift from hypertrophy to hyperplasia in astrogliosis is heavily governed by miR-21, and the suppression of miR-21 is known to cause apoptosis. The miR-21 is a downregulator of the Fas ligand gene, *FASLG*, and the phosphatase and tensin homolog gene, *PTEN*, both of which promote apoptosis (**Table 1**; **Figure 2**) (7, 25, 58, 63). Strickland et al. demonstrated that miR-21 was significantly upregulated 4 days after SCI, only to be downregulated, relatively, by day 14 (18). This effect explains the transition from hypertrophy to hyperplasia that occurs 4–10 days after injury. This trend parallels that described by Liu et al., who reported that miR-21 levels were not significantly elevated at 10 days after SCI, but were still somewhat elevated (63). Although the suppression of miR-21

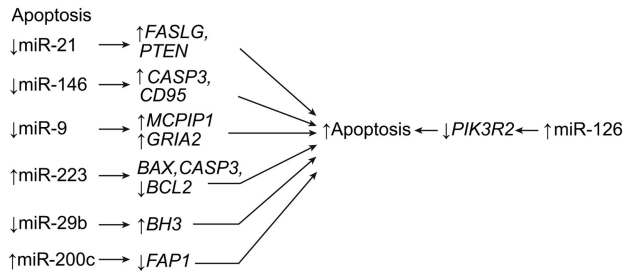

FIGURE 2 | Apoptotic effects of miRNA expression after SCI. The result is apoptotic signaling. *BAX*, *BCL2*-associated X, apoptosis regulator; *BCL2*, B-cell chronic lymphocytic leukemia/lymphoma 2; *BH3*, *BCL2* homology domain 3; CASP3, caspase 3; *CD95*, also known as *FAS* receptor (cell surface death receptor), apoptosis antigen 1, and tumor necrosis factor receptor superfamily member 6; *FAP1*, Fas-associated phosphatase-1; *GRIA2*, glutamate ionotropic receptor AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) type subunit 2; *MCPIP1*, monocyte chemotactic protein-induced protein 1; *PIK3R2*, phosphoinositide-3-kinase regulatory subunit 2; *PTEN*, phosphatase and tensin homolog. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

appears to have many beneficial effects related to astrogliosis, neuronal cell death has the opposite effect. Suppressing miR-21 using antagomir-21 increases neuronal deficits and lesion size in spinal cord tissue at 28 days after SCI (25). This effect is in contrast to findings of the previously mentioned study that found that the inhibition of miR-21 causes smaller lesion sizes and more effective recovery at 1–2 weeks after injury. However, both sets of data may be correct within their respective time frames.

Like miR-21, miR-146a is antiapoptotic as well as anti-inflammatory. However, it appears that, while miR-21 decreases in expression after 14 days, miR-146a remains constant in upregulation (**Table 1**) (18). This constant upregulation may indicate that miR-146a maintains the antiapoptotic state after the acutely reacting miR-21 has downregulated. In addition to miR-21 and miR-146a, miR-9 controls apoptosis by directly regulating the monocyte chemotactic protein-induced protein 1 gene, or *MCPIP1*, which is a known proapoptotic and macrophage-activating gene (**Figure 2**) (20). This postulated mi-9-controlled apoptosis is supported by the observation that, between days 1 and 7 after SCI, miR-9 was significantly downregulated in ventral horn motor neurons whereas *MCPIP1* was upregulated at the lesion site. However, by day 7, the miR-9 expression had increased, suppressing the expression of *MCPIP1*. Interestingly, *MCPIP1* also promotes GFAP, which is expressed by reactive astrocytes during astrogliosis (20). Thus, miR-9 appears to have a bimodal effect on SCI, such that its downregulation during acute stages allows for the expression of *GFAP* and the activation of astrocytes, while its upregulation at day 7 suggests a neuroprotective role of ventral motor horn cells. Considering that miR-21 plays a strong antiapoptotic role during acute SCI, miR-9 may work the opposite of miR-21, such that the downregulation of one is countered by the upregulation of the other. Further research is needed to observe the expression of miR-9 beyond 7 days in order to elucidate the relationship between these two miRNAs.

Other studies have shown that miR-223 is expressed in human and mouse hematopoietic systems (64). In SCI, miR-223 is temporally expressed, with increased peaks of expression at 1, 3, 7, and 14 days after SCI. Antagomir-223 treatment after SCI resulted in significantly lowered apoptotic cells, coinciding with downregulated *BAX* and *CASP3* and upregulated *BCL2*. This treatment preserved spinal cord tissue and significantly increased scores on the Basso, Beattie, and Bresnahan locomotor scale as early as 3 days after SCI (**Table 1**; **Figure 2**) (64).

In general, apoptosis after SCI is caused by either downregulation of miRNAs that target proapoptotic genes, such as caspase family genes, or upregulation of miRNAs that target antiapoptotic genes, such as *BCL2* or *MYC* (**Table 1**) (7, 23, 60–63). These regulatory effects provide multiple avenues for potential therapeutic strategies, which involve managing the balance of proapoptotic and antiapoptotic miRNAs. One such strategy was described by Liu et al. in 2010; in their study, a 5-day cycling exercise regimen within the first 10 days of injury significantly altered miRNA expression in mice (63). Their study showed that, with exercise, the antiapoptotic agents, miR-21 and *BCL2*, are upregulated, while proapoptotic agents miR-15b, *CASP7*, and *CASP9* are downregulated. These authors posit that exercise after SCI may stimulate beneficial antiapoptotic effects through the influence of miR-21 on the v-akt murine thymoma viral oncogene homolog 1 gene, *AKT1*, and on phosphatidylinositol triphosphate (**Table 1**) (63). Another potential therapy strategy is posttraumatic hypothermia and antisense silencing. Truettner et al. demonstrated that specific miRNAs are sensitive to post-traumatic hypothermia in brain lesions, which downregulates their apoptotic effects (59).

Endogenous Antioxidant Systems and Neuroprotection

Another secondary effect of SCI, traumatic brain injury, or ischemia is the presence of reactive oxygen and nitrogen species (65). These molecules function to destroy cell and DNA structure, interfere with important cellular processes, and ultimately cause unintended cell death. The production of free radicals is caused by cytotoxic concentrations of glutamate (65, 66). During astrogliosis (the hypertrophic state), astrocytes build barriers against these reactive species, releasing antioxidants by way of the superoxide dismutase (SOD) family of genes and diverting glutamate excitotoxicity away from oligodendrocytes and neurons (14). However, during an oxidative crisis, miRNAs appear to exhibit functions that hinder the body's protective response. For example, it has been observed that miR-21 strongly influences reactive oxygen species (ROS)-induced apoptosis during oxidative stress (67). When miR-21 was silenced, ROS-induced cell death was reduced in spinal cord neurons. The effect on spinal cord cells by miR-21 was analogous to that of free radicals (**Table 1**) (67). These data suggest that the antiapoptotic effects of miR-21 overexpression do not apply to neurons.

One example is miR-486, which targets the neuronal differentiation 6 gene, *NEUROD6*, a trigger for heat shock proteins. When miR-486 is knocked down, the expression of *NEUROD6* caused increased clearance of ROS and lower levels of proinflammatory agents (**Figure 3**) (7, 68). The neurogenin 1 gene,

NEUROG1, is a differentiation factor in embryogenesis whose overexpression in progenitor cells heavily favors neuronal differentiation. It is generated from the same precursor factor that produces *NEUROD6* (**Table 1**) (7, 69, 70). The miRNA miR-20a, which is overexpressed in SCI, targets *NEUROG1* and prevents neuronal regrowth in the lesion site, presumably contributing to the motor neuron degeneration and apoptosis that follows spinal cord trauma (**Figure 3**) (22). A therapy that blocked miR-20a or that introduced exogenous *NEUROG1* would result in regeneration of neurons and improved functional deficit, making miR-20a a prime candidate for therapy (7, 68). Conversely, miR-29b is implicated in having antiapoptotic effects in ischemia by repressing the apoptotic *BH3* gene (**Table 1**) (22). A treatment strategy could take advantage of these effects by manipulating miR-20a and miR-29b in tandem to treat SCI (22).

Dharap et al. demonstrated that miR-145 targets *SOD2* after experiments with antagomir-145 led to higher levels of *SOD2* expression in mice with cortical ischemia (**Table 1**; **Figure 3**) (42). A similar pattern of expression has been observed in cortical ischemia and in plexus root avulsion, such that neurons ipsilateral to the lesion site express the highest levels of miRNA (71). This relationship suggests a unilateral miRNA response to SCI injuries. However, the contralateral side shows microglial activation in what is hypothesized to be a spillover of the changes observed on the opposite side (42, 71). Recent research has shown that miR-200c is another miRNA significantly altered by apoptotic events after SCI, as miR-200c upregulation was observed alongside the downregulation of its target, Fas-associated phosphatase-1 (*FAP1*), and, ultimately, the induction of *FAS* signaling (50).

Inflammation

Although inflammation is part of the natural healing process, much of medicine is geared toward minimizing it or eliminating it, altogether. Inflammation is constantly regulated by the body's innate immune system (43). In the setting of SCI, the role of inflammation is that of deleterious effects causing tissue compression and excessive cell death. One of the main functions of astrogliosis is to minimize inflammatory reactions in and around the lesion site to minimize the spread of secondary damage beyond the initial point of trauma. In SCI, the expressions of the various miRNAs that help regulate the body's inflammatory processes are altered and are thus an important target for potential therapies.

The miRNA miR-146a is described above as being intimately cooperative with miR-21 and involved in the shift from astroglial hypertrophy to hyperplasia. Studies have shown that miR-146a is highly expressed in spinal astrocytes during SCI. It targets the proinflammatory enzyme cyclooxygenase-2 (COX-2) and the proteins encoded by the genes, *IL1B* and *IL6* (**Table 1**; **Figure 4**) (7, 18, 43, 44). In previous studies on temporal lobe epilepsy, astrocytes were highly expressed during latent periods, in relation to high levels of miR-146a (43, 44). Furthermore, these studies showed that miR-146a (in macrophages) works in cyclic feedback with the transcription factor NF-κB, *via* interleukin 1 receptor-associated kinase 1 gene (*IRAK1*) and the TNF receptor-associated factor 6 gene (*TRAF6*). Activation of the NF-κB pathway in macrophages upregulates miR-146a, resulting in the downregulation of *IRAK1* and *TRAF6* pathway constituents (**Table 1**) (43, 44). A correlation can be drawn between these phenomena and SCI, in which miR-146a levels peak and astrocytes increase anti-inflammatory activity. However, although some research on hyperplastic glial scars attributes functional deficit to miR-146a overexpression, other research deems miR-146a valuable in preventing the deleterious effects of inflammation (43, 44). Therefore, while miR-146a is beneficial in the schema of acute anti-inflammatory treatment, overexpression of miR-146a beyond the subacute stages of SCI seems to become deleterious, as seen with miR-21. The Notch-1 pathway may exhibit a potential therapeutic role in SCI. Notch-1 can lead to malignant astrocyte proliferation, but it is inhibited by miR-146 (44). This relationship is important, considering that previous research has implicated miR-146a in the negative effects of glial scar formation, which supports the idea that miR-146a attenuates the hyperplastic effects of miR-21 (44). Manipulation of miR-146a

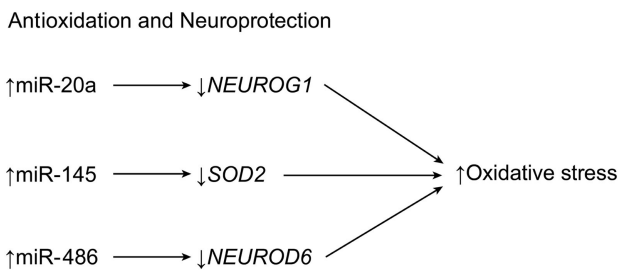

FIGURE 3 | Antioxidation and neuroprotection of central nervous tissue. After SCI, increased expression of three miRNAs results in a loss of neuroprotective metabolites, causing reactive oxygen species-induced apoptosis. *NEUROD6*, neurogenic differentiation 6; *NEUROG1*, neurogenin 1; *SOD2*, superoxide dismutase 2. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

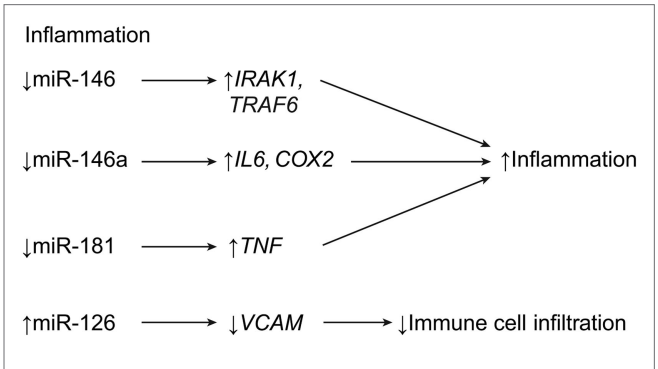

FIGURE 4 | Inflammatory effects of miRNA expression post SCI. While downregulation of miR-146 and miR-181 leads to increased inflammation, upregulation of miR-126 leads to decreased extravasation of immune cells into the CNS. *COX2*, cyclooxygenase 2; *IL6*, interleukin 6; *IRAK1*, IL-1R-associated kinase 1; *TRAF6*, TNFR-associated factor 6; *TNF*, tumor necrosis factor; *VCAM1*, vascular cell adhesion molecule 1. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

after SCI may allow for an ideal balance of anti-inflammatory and antihyperplastic effects, which may lead to improved healing and reduced scarring.

The miRNA miR-181 is expressed in macrophages, monocytes, and astrocytes (47, 48). It is a well-known anti-inflammatory agent because its overexpression leads to the suppression of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNFα. Furthermore, miR-181 suppresses HMGA-1, a proinflammatory factor that is regulated by *COX2* and *STAT3* (**Table 1**; **Figure 4**) (47, 48). However, miR-181 expression is decreased in SCI, which would allow for an increased inflammatory response after SCI. This situation is confounding in light of the strong anti-inflammatory signals that astrocytes send out during acute astrogliosis, which may mean that miR-181 is less functional in SCI than it is in Alzheimer's disease or cortical ischemia, as research suggests (47). Another miRNA, miR-223, is shown to be overexpressed near areas of increased neutrophil aggregation, which suggests that miR-223 is implicated in neutrophil homing. This process counters that of hypertrophic astrocytes, which attempt to rid the central lesion of inflammatory cells during the subacute phase of astrogliosis (7, 54). The expression of miR-223 is time-dependent. It peaks twice at 12 h and 3 days after SCI and significantly decreases after that. This coincides with peak neutrophil expression 1 day after SCI, followed by downregulation 5 days after SCI (**Table 1**) (7).

In 2015, Hu et al. demonstrated that miR-126 is highly involved in attenuating inflammation, alongside angiogenesis and functional recovery (41). An agonist method showed that, in mice treated with exogenous miR-126, the expression of biomarkers for extravasated leukocytes and macrophages (CD45 and CD68, respectively) were downregulated. This observation implicates miR-126 as a potential therapeutic strategy for SCI, because it is downregulated in SCI. The miR-126 targets *VCAM1*, which is a receptor on endothelial cells for leukocyte homing. *VCAM1* was shown to be downregulated in agomir-126-treated mice (**Table 1**; **Figure 4**) (41).

PROCESSES OF SCI RECOVERY REGULATED BY miRNA

Neuroplasticity

One of the main clinical concerns after SCI is the potential for functional recovery. Chances for recovery after complete transverse spinal cord lesions are slim. When spinal cord lesions are incomplete, the chances of recovery are greater, in part due to the neuroplasticity of the cortical and subcortical neurons and glial cells (72). Chronic SCI can cause regional changes in glucose metabolism, revealed on positron-emission tomography as larger areas of activation in somatosensory regions for SCI patients compared with normal patients (72). Certain miRNAs are expressed in the brain, which indicates that miRNAs could function in both tissue development and higher brain function. In fact, researchers have compiled data that explain the array of morphological functions that miRNAs perform in the CNS (73, 74). An example of the functions of miRNAs in neuroplasticity is miR-133b in stroke. Xin et al. conducted an experiment in which mice with induced middle cerebral artery occlusion were infused with three types of modified murine mesenchymal stem cells: naïve, miR-133b(+), and miR-133b(−) (74). Their results showed that the greatest functional recovery occurred in subjects with the miR-133b(+), while no recovery occurred in miR-133b(−) subjects. Furthermore, it was determined that the miR-133b(+) mesenchymal stem cell group also exhibited the greatest increase in neuronal plasticity and neurite remodeling in the ischemic zone. Exosome-mediated transfer of mesenchymal stem cells occurred in greatest numbers to neurons and astrocytes, where miR-133 downregulated connective tissue growth factor expression (74). Connective tissue growth factor is colocalized with GFAP, which is highly expressed during hypertrophic astrogliosis. Selective expression of GFAP and not connective tissue growth factor during astrogliosis could potentially function to maximize the beneficial effects of hypertrophic astrocytes.

Axon Regeneration and Remyelination

In mammals, regeneration is the main difference in recovery between a peripheral nervous system injury and a CNS injury. Peripheral nervous system injuries are more likely to self-repair, whereas CNS injuries do not self-repair. Peripheral nervous system axons have one Schwann cell per myelin sheath (equaling many Schwann cells per single axon), but CNS axons have one oligodendrocyte per several sheaths. Thus, oligodendrocytes are far more indispensable than Schwann cells. Therefore, the CNS is capable of regeneration, so long as the oligodendrocytes remain intact, which is often not the case in SCI, when trauma, ROS, inflammation, and other factors destroy any neuron or glial cell in their path.

Recent research shows that it may be possible to rebuild oligodendrocytes and to repair axonal damage after SCI using miRNAs. Park et al. regenerated axons after optic nerve injury by deleting the phosphatase and tensin homolog gene, *PTEN,* and thereby upregulating the mechanistic target of rapamycin gene, *MTOR*, in the adult retinal ganglion cells of adult mice (26). As a follow-up to this article, Liu et al. extrapolated on their earlier work, in which early exercise after SCI correlated with upregulation of miR-21 and downregulation of miR199a-3p (**Table 1**) (27). According to Liu et al., the upregulation of miR-21 and the downregulation of miR199a-3p with exercise lead to the subsequent downregulation of *PTEN* and the upregulation of *MTOR*, the respective targets of miR-21 and miR-199a-3p (**Figure 5**) (27). This evidence strengthens the idea that bimodal control of neuronal apoptosis and axon degeneration can be achieved through these two miRNAs (**Table 1**) (26, 27).

Letzen et al. have shown that there are many miRNAs involved in the growth and proliferation of oligodendrocytes (28). Multiple oligodendrocyte-related miRNAs can be regulated by an oligodendrocyte-specific dicer. Research shows that mutant mice lacking the oligodendrocyte-specific dicer suffer brain demyelination and axonal impairment (**Table 1**) (53). These traits were found in addition to severe astrogliosis, inadequate antioxidant systems, and increased inflammation in the brain. Of the three major miRNAs downregulated by oligodendrocyte-specific dicer

inhibition, miR-219 is observed to have the greatest contribution to astrogliosis, oxidative stress, and inflammation. Downregulating miR-219 leads to the dysregulation of its target gene, elongation of very long chain fatty acids protein 7 gene, *ELOVL7*, causing lipid accumulation in the white matter of the brain (**Figure 5**) (53). If miR-219 can be identified as playing an important role in the spinal cord, a potential therapy may be found in upregulating its expression after SCI.

The expression patterns of miRNA-125b help explain the discrepancies between the regenerative powers of reptiles and mammals (40). Reptiles are capable of regenerating CNS tissue after excision, whereas mammals are not. In the axolotl salamander, expression of miR-125 is precisely controlled, so that excess expression causes erratic axonal growth with incomplete reconnection, and reduced expression causes inhibition of axonal regeneration. In addition, glial scars are not seen after SCI in the axolotl, as they are in rats. This study shows that mammals could potentially benefit from increased miR-125 expression after SCI (40).

As mentioned above, the miRNA miR-133 was found to have a beneficial effect on neuroplasticity. Yu et al. demonstrated that miR-133 exhibits axonal regenerative properties in zebrafish, where it targets the ras homolog family member A gene, *RHOA*, whose product is a GTPase that inhibits neural regeneration and functional recovery in mammals and fish (**Figure 5**) (75). Another miRNA, miR-210, has been implicated as a possible therapeutic strategy for SCI, because it has been correlated with axon growth, in addition to neovascularization, astrogliosis, and myelination. The miR-210 suppresses the protein tyrosine phosphatase, non-receptor type 1 gene, *PTPN1*, and the ephrin-A3 gene, *EFNA3*, which has been shown to provide these benefits, leading to functional recovery in mice (**Table 1**) (51). As mentioned above, the miRNA miR-9 controls apoptotic factors during acute and subacute SCI (see Apoptosis). In addition to these effects, miR-9 has also been shown to play a role in suppressing Schwann cell migration, a critical step in neuroregeneration. Because of this ability, Xu et al. suggest that lower levels of miR-9 are needed during the acute stage of SCI to allow for adequate axon regeneration and remyelination (**Table 1**) (20).

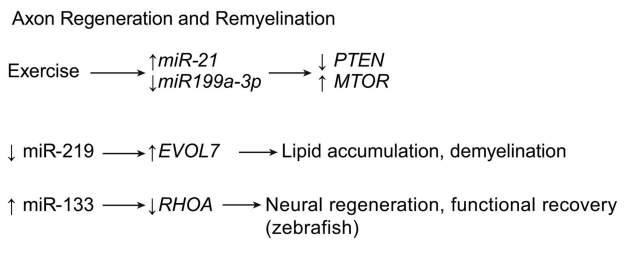

FIGURE 5 | Axon regeneration and remyelination by miRNA expression. Exercise is known to decrease inflammation and thus increase regeneration of axon structures. Meanwhile, downregulation of miR-219 showed to induce demyelination after SCI. *ELOVL7*, elongation of very long chain fatty acids protein 7; *MTOR*, mechanistic target of rapamycin; *PTEN*, phosphatase and tensin homolog; *RHOA*, ras homolog gene family, member A. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

Neuron Regeneration

Even though the CNS is restorable after injury if oligodendrocytes are intact, neuron degeneration is a problem after SCI, because, so far, neurons cannot be brought back. However, miRNA therapy offers an avenue to achieve neuron regeneration.

A bioinformatics study by Liu et al. (76) demonstrated that the body attempts to preserve neurons and to stimulate neuron growth, regeneration, and remyelination through the expression of a handful of genes through the action of miRNAs. Genes such as the brain-derived neurotrophic factor gene, *BDNF*, and the cell division cycle 42 gene, *CDC42*, are genes positively influencing SCI self-repair. The expression of these genes is inversely related to a large list of miRNAs that are thought to target them (*BDNF* can be influenced by miR-183, miR-195, miR-30a, miR-182, miR-381, miR-300-3p, and miR-325-5p; and CDC42 can be influenced by miR-185, miR-329, miR-340-5p, miR-381, and miR-383) (**Table 1**; **Figure 6**). Additionally, several miRNAs are thought to promote these two genes, and a balance between these two sets of miRNAs may play a critical role in self-repair (76). miR-124 restores

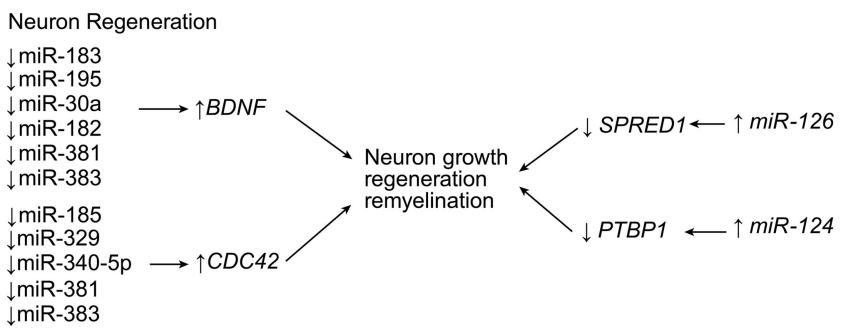

FIGURE 6 | Neuroregenerative effects of miRNA expression. Multiple miRNA are responsible for neuron growth, regeneration, and remyelination. Upregulation of miR-126 and miR-124 leads to downregulation of negative regulators of growth signaling, while the downregulation of many other miRNA induce the expression of pro-growth factors. *BDNF*, brain-derived neurotrophic factor; *CDC42*, cell division protein 42; *PTBP1*, polypyrimidine tract-binding protein 1; *SPRED1*, Sprouty-related *EVH1* (enabled/vasodilator-stimulated phosphoprotein homology 1) domain containing 1. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

neurons and recovers function (35). In SCI, miR-124 is downregulated continuously through the first 7 days after SCI at and around the lesion site (**Table 1**; **Figure 6**) (36, 37). Zou et al. observed that mesenchymal stem cells derived from bone marrow do not naturally express adequate levels of miR-124, so they transfected mesenchymal stem cells with miR-124, transplanted those cells into injured rat spinal cords, and observed that the transfected mesenchymal stem cells had significant neuronal expression (35). The transplanted rats had higher scores on the Basso, Beattie, and Bresnahan locomotor scale, and their functional recovery was higher, but their apoptosis was lower. These data suggest that the overexpression of miR-124 is linked with neural cell development and regeneration in SCI (35). miR-124 acts by targeting the polypyrimidine tract-binding protein 1 gene, *PTBP1*, which is a regulatory gene for neural precursor cell differentiation (36, 37). Xu et al. conducted a similar experiment; (38) however, they used neural stem cells from bone marrow-derived mesenchymal stem cells. After conducting a very similar experiment to that of Zou et al. (35), Xu et al. also observed significantly greater motor function outcomes in rats with SCI (38). It might be of interest for future studies to evaluate the time-dependence of therapy with miR-124 in order to understand its effects on astrogliosis since miR-124 selectively proliferates neurons over glial cells. Other studies have implicated miR-124 in reducing CNS inflammation by downregulating macrophage/microglia expression, while maintaining astrocytic expression, thus maintaining the anti-inflammatory effects of astroglial scarring (39).

Hu et al. made similar observations with miR-126, when they demonstrated that the injection of miR-126 increased protection of spinal cord motor axons (41). Typically, miR-126 is significantly downregulated by SCI, but upregulation therapy reversed this expression pattern, causing a decrease in apoptosis of motor neurons and an increase in functional motor recovery as long as 28 days after SCI (41). One of the main targets of miR-126, the phosphoinositide-3-kinase regulatory subunit 2 gene (*PIK3R2*) is a negative regulator of the phosphoinositide 3-kinase (PI3K) pathway, and thus, the apoptotic pathway. Another target is the sprouty-related, EVH1 domain-containing 1 gene (*SPRED1*), which is a negative regulator of growth factor signaling (**Table 1**; **Figure 6**) (41).

Neural stem cells have been a therapeutic strategy focus. Shi et al. demonstrated that miR-381 regulates neural stem cell differentiation (56). They found that overexpression of miR-381 in neural stem cells causes the expression of the hes family bHLH transcription factor 1 gene, *HES1*, which triggers proliferation and differentiation into neurons, but which inhibits differentiation into astrocytes (**Table 1**). These results suggest that manipulating neural cell differentiation should be done in a time-dependent manner, depending on the length of time since the patient's injury. Introducing miR-381-infused neural stem cells and inhibiting astrocyte proliferation during acute stages of SCI may inhibit the beneficial effects of astrogliosis during acute stages. Conversely, infusing miR-381 during subacute or chronic stages may be of great benefit in reversing the cell death seen later.

Functional Recovery

The main objective in researching SCI is ultimately to help patients achieve superior functional recovery. Importantly, investigators often achieve functional recovery in experiments on miRNAs in SCIs (7, 15, 16, 25, 29, 30, 35, 38, 47, 51, 72, 74, 75, 77, 78). Recent evidence shows miRNAs may be helpful for repairing hind limb functionality in murine SCI (55). In this study, inhibitory treatment of miR-320 markedly improved motor scores, while upregulating the expression of phosphorylated *HSP20*, a gene that protects against ischemia-reperfusion injury (**Table 1**; **Figure 7**) (55).

A view not previously mentioned is observed with vimentin in scar formation. GFAP and vimentin are factors expressed in hypertrophying astrocytes, and their expressions are downregulated by miR-21 during the shift toward hyperplastic astrogliosis (14–16). In contrast, Qian et al. demonstrated highly expressed vimentin levels in spinal cord scar tissue as a result of astrocytic hyperplasia. In fact, the knockdown of vimentin in scar tissue showed significant improvement in locomotor function, while overexpression of vimentin showed the opposite (**Table 1**; **Figure 7**) (77). This result suggests that vimentin might play a detrimental role in astrogliosis, as opposed to our previous understanding.

Research on miR-155 has provided insight into another possibility for functional recovery from SCI. The miR-155 is normally upregulated in most leukocytes; it is also upregulated during SCI, contributing to the inflammatory destruction of the spinal cord. However, in miR-155 knockout mice, Basso Mouse Scale locomotor scores have been found to be higher 6 weeks after SCI compared with control groups. In addition, miR-155 knockout mice also expressed significantly lower levels of IL-17–expressing T cells, suggesting that miR-155 has a direct effect on Th17 cell proliferation after SCI *via* the regulation of Janus-associated kinase/signal transducer and activator of transcription (JAK/STAT) signaling by the suppressor of cytokine signaling 1 gene, *SOCS1* (**Table 1**; **Figure 7**) (45, 46).

Spinal cord injury is often associated with severe pain syndrome. Therefore, careful consideration should be given to optimal pain treatment of patients with SCI, as 1 mode in particular leads to decreased motor recovery and increased chronic pain (30). In their study of morphine delivery and SCI, Strickland

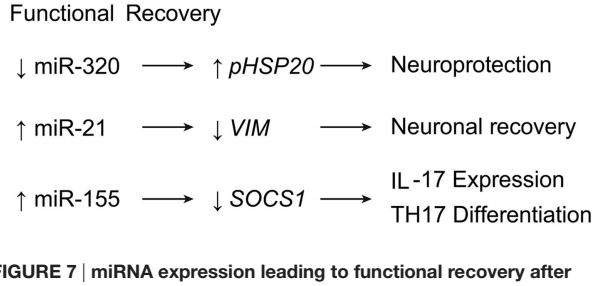

FIGURE 7 | miRNA expression leading to functional recovery after SCI. Downregulation of miR-320 leads to neuroprotective effects, while upregulation of miR-21 and miR-155 induce neuronal recovery and T-cell expression, respectively. *IL17*, interleukin 17; *HSP20*, heat shock protein 20; *SOCS1*, supressor of cytokine signaling 1; Th17, T helper cell 17. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

et al. noticed that acute administration of morphine correlated to these effects, potentially *via* the expression of miRNA, including miR-21 and 146 (30). The miR-21 and 146 expressions were elevated by morphine. However, the rats experienced significant dysregulation of miR-21 and decreased motor function up to 15 days after SCI recovery. These results may be attributable to morphine-induced inflammation. They further suggest that the effects of miR-21 on SCI recovery are time-sensitive and must be regulated beyond the acute stages of SCI, both to optimize the beneficial effects of miR-21 and to minimize its deleterious effects.

Pain

Increasing evidence over the past decade has suggested that miRNAs play a significant role in regulating both inflammatory and neuropathic pain following SCI (18, 29, 32–34, 49, 79–87). One of the major objectives in identifying miRNA sequences that influence pain modulation is to manipulate them for therapeutic uses. Although many miRNA sequences have already been found to influence chronic pain at the site of the lesion and in higher cortical structures, only a few miRNA sequences have been modulated and have been confirmed to have a therapeutic effect (33, 34, 49, 83, 86, 87).

In 2016, Li and Zhao demonstrated that miR-218 expression is consistent with neuropathic pain symptoms in rats with compressive spinal cord injuries (52). When miR-218 was downregulated in rats, pain behavior and inflammation decreased. The authors hypothesize that miR-218 acts by inhibiting the JAK/STAT3 pathway by influencing the expression of the suppressor of the cytokine signaling 3 gene, *SOCS3* (**Table 1**; **Figure 8**) (52). In 2011, Favereaux et al. identified miR-103 as regulating three subunits of a L-type voltage-gated calcium channel named CaV1.2 (subunits alpha-1C, alpha-2 delta-1, and beta-1 are encoded by *CACNA1C*, *CACNA2D1*, and *CACNB1,* respectively) in dorsal horn neurons. This regulation was found to be bidirectional: upregulation of miR-103 led to downregulation of each subunit, and vice versa (**Table 1**; **Figure 8**) (33). This finding was significant because an earlier study had already established a strong connection between the CaV1.2 protein and chronic pain, showing that a knockout of CaV1.2 protein would lead to complete reversal of long-term sensitization (32, 34). Favereaux et al. also showed that the intrathecal application of miR-103 significantly relieved pain, thus establishing miR-103 as a strong candidate for the treatment of chronic pain after SCI (33).

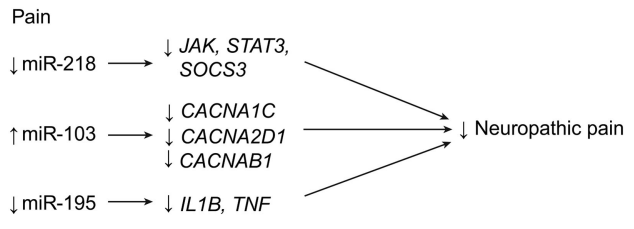

FIGURE 8 | Pain regulation with miRNA expression after SCI. Downregulation of miR-218 and miR-195, in addition to upregulation of miR-103, lead to decreases in neuropathic pain after SCI. *CACNA1C*, calcium channel, voltage gated, L-type, alpha-1 C; *CACNA2D1,* calcium channel voltage gated, L-type, alpha-2 delta-1; *CACNB1*, calcium channel, voltage gated, L-type, beta-1; *IL1B*, interleukin 1 beta; *JAK*, Janus kinase; *STAT3*, signal transducer and activators of transcription 3; *SOCS3*, supressor of cytokine signaling 3; *TNF*, tumor necrosis factor. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

Another miRNA that has been linked to inflammatory pain after SCI is miR-195 (49). It was found to increase significantly after spinal nerve ligation, and it was also associated with the continuous release of proinflammatory cytokines, such as IL-1β and TNF-α (**Figure 8**). These researchers proposed that upregulation of miR-195 leads to decreased autophagic activity and thus to increased neuroinflammation. In addition, upregulation of miR-195 was also positively linked with increased mechanical and cold sensitivity (49).

Both miR-103 and miR-195 have been shown to modulate chronic pain at the lesion site (33, 34, 49). Substantial evidence also shows that changes in miRNA expression in higher cortical structures are associated with inflammatory and neuropathic pain, indicating that pain relief therapies for SCI may not be restricted to the site of injury and nearby structures (83–87). For instance, miRNA expression changes substantially in both the prefrontal cortex and the hippocampus when stimulated by chronic pain (83, 86, 87).

Although the role of miRNAs in pain regulation is substantial, the exact mechanisms involved in pain sensation are very complex and require much further study (32). However, preliminary data suggest a promising role for the use of miRNAs as therapeutic agents for pain relief in many pathologic conditions, including SCI (32).

CONCLUSION

Spinal cord injury is a serious and debilitating injury with limited treatment resources. After the initial injury to the spinal cord, numerous secondary pathophysiological events occur that contribute to a major part of the total damage. Such secondary events include inflammation, apoptosis, ROS formation, and astrogliosis. In recent years, many studies have identified miRNAs as contributors and regulators of secondary injury, with most of the research providing specific mRNA targets for the miRNA involved. Not all miRNAs affect SCIs negatively, however. Some miRNAs appear to promote the beneficial aspects of the healing mechanisms of the body. For instance, miRNAs have been shown to promote neuroplasticity, axon regeneration and remyelination, neuron cell regeneration, and functional recovery. However, the underlying problem is that, in most cases, SCI causes both the overexpression of harmful miRNAs and the inhibition of beneficial miRNAs. Manipulating the expression of miRNAs after SCI might be a new therapeutic strategy for overcoming the lasting and detrimental effects of SCI, thereby giving clinicians better diagnostic tools and giving patients better outcomes. Overall, miRNAs may lead to an era of personalized medicine for individuals with SCIs. More research is mandated, and the expected results should provide new hope for better treatment of patients with SCIs.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work.

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Network Plasticity and Intraoperative Mapping for Personalized Multimodal Management of Diffuse Low-Grade Gliomas

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Gliomas are the most frequent primary brain tumors and include a variety of different histological tumor types and malignancy grades. Recent achievements in terms of molecular and imaging fields have created an unprecedented opportunity to perform a comprehensive interdisciplinary assessment of the glioma pathophysiology, with direct implications in terms of the medical and surgical treatment strategies available for patients. The current paradigm shift considers glioma management in a comprehensive perspective that takes into account the intricate connectivity of the cerebral networks. This allowed significant improvement in the outcome of patients with lesions previously considered inoperable. The current review summarizes the current theoretical framework integrating the adult human brain plasticity and functional reorganization within a dynamic individualized treatment strategy for patients affected by diffuse low-grade gliomas. The concept of neuro-oncology as a brain network surgery has major implications in terms of the clinical management and ensuing outcomes, as indexed by the increased survival and quality of life of patients managed using such an approach.

Keywords: awake surgery, functional brain mapping, intraoperative mapping, anatomofunctional connectivity, low-grade gliomas, neuroplasticity, direct electrical stimulation

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INTRODUCTION

Neurosurgical resection remains the standard of care for gliomas, and the extent of resection (EOR) is one of the most important factors affecting the patients' survival and quality of life for both high- and low-grade gliomas (1–9). The diffuse low-grade gliomas (DLGGs) portray a distinct clinical and radiological behavior and display particular gene expression signatures. DLGG is thought to represent a chronic invasive lesion that migrates along the white matter pathways, and eventually undergoes malignant transformation leading ultimately to death (10).

The concept of individualized surgery in neuro-oncological treatment of glial tumors is based on the goal of achieving a maximal tumor resection without inducing new neurological deficits. Analogously, for tumors located in proximity to critical functional areas, the use of intraoperative cortical and subcortical electro stimulation mapping (IEM) during awake craniotomy evolved over time and allows a substantial increase in the survival and quality of life of patients (1, 6, 9, 11–14).

The joint efforts of neuroscientists, researchers, and clinicians have provided an unprecedented ability to localize lesions and to assess the human brain function at the microscopic, mesoscopic,

and macroscopic scales (15). The resultant array of invasive and non-invasive measures allowed surgeons to push the boundaries of safe surgical resection with subsequently improved clinical outcomes. As depicted by the extensive work performed by several research groups, the concepts of brain connectome and brain plasticity represent promising notions that advanced the neurosurgical treatments available for neurosurgical patients affected by DLGG.

A Dynamic Concept: Tumor Growth and Functional Neuroplasticity

The survival benefit associated with an increased EOR has been demonstrated for both high- and low-grade gliomas; however, such oncological benefits need to take into account the risks of inducing neurological deficits. Although it is acknowledged that in DLGG, tumor infiltration follows the white matter tracts beyond the boundaries visualized on standard neuroimaging techniques, current treatment thresholds are still based on static radiological perceived boundaries. For instance, conventional radiotherapy protocols target a 2 cm conventional Euclidean distance around the macroscopically visible tumor, without taking into account the infiltrative and dynamic growth patterns of the lesion, thus equally radiating "non-cancerous brain tissue that could not only cause neurological deficits but also restrict the residual plasticity potential while leaving alive cancerous cells in other areas" (15).

One of glioma's hallmark properties is the ability of cancer cells to invade healthy tissue, extensive attempts having been made both on the microscopic and macroscopic scales in order to determine the underlying pathophysiology. The different mechanisms involved in the plasticity of tumor cell motility have already been summarized by Taddei et al. and Cuddapah et al. (16, 17). Among the different structural and cellular adaptive strategies displayed by cancer cells, enhanced cell motility as well as resistance to hypoxia and acidity represent some of the key factors allowing tumors to elude antineoplastic drugs and radiotherapy treatments. From a biological perspective, the migration and invasion of tumor cells requires an increase in cellular motility, which involves formation of actin-based dynamic protrusions of the plasma membrane (18–20). Actin represents one of the key cytoskeletal filaments and its instability caused by hypoxia or tissue injury can facilitate entry of the cell into mitosis, thereby acting as an epigenetic determinant of the cell fate (21). Also, tumor cell motility can be modulated by acidity as the assembly of actin filaments in migrating cells increases with an intracellular pH higher than 7.2 (16). Moreover, *in vivo* imaging of membrane tube development over time revealed that the microtube-connected astrocytoma cells create a multicellular anatomical network that serve as routes for brain invasion, proliferation, and communication over long distances (18, 19). Disconnection of astrocytoma cells by targeting their tumor microtubes was already proposed as a possible new therapeutic strategy against cancer (22). Ion channels and transporters also appear to play a major role in the invasion strategies by mediating the hydrodynamic shape and volume changes displayed by tumor cells (17, 23–26). For instance, K+ and Cl− ions are thought to function as osmotically active ions that facilitate the dynamic cytoplasmic volume regulation occurring in tumor cells as they migrate and invade the surrounding tissue (25, 26).

The diffuse invasion exhibited by cancer cells can follow the same "extracellular routes of migration that are traveled by immature neurons and stem cells," which similarly migrate along extracellular routes such as intracranial vasculature and white matter tracts (17, 27). Although the origin of gliomas is still unknown, it likely represents a complex phenomenon involving both genetic and epigenetic factors with a suspected cellular origin from a neural stem cell or an oligodendrocyte precursor cell (27–29). In addition, tumor recurrence occurs predominantly at the primary location of the tumor for both low- and high-grade gliomas. Tumor relapses might be linked to the presence of a cell subpopulation with stem cell characteristics, labeled as glioma stem cells (29, 30). While multiple studies assessed the presence of tumorigenic stem cells in high-grade lesions, the occurrence of those cells has equally been reported in patients harboring LGG (30). These cells are highly resistant to conventional chemotherapeutic drugs and could equally mediate tumor recurrence following radiation therapy (31–33). Tumor cell dissemination and heterogeneity represent important aspects that should be taken into account in order to improve the medical and surgical therapeutic regimens (34). Computational models attempt to simulate the functional consequences associated with brain tumor growth by incorporating the tumor-induced plastic compensatory mechanism along with the structural and biological heterogeneity of gliomas (35).

Delineating the extent of tumor infiltration has been subject to intense research, as the boundaries between tumor and healthy tissue are difficult to detect macroscopically with current imaging techniques like functional MRI (fMRI), positron emission tomography, spectroscopy, and diffusion tensor imaging (36–38). In the case of tumor-related epilepsy, such techniques allowed to establish a link between the peritumoral tissue and the tumor-related epileptogenesis, which can explain both the antiepileptic effects of oncological treatments (39–41) and the increase in seizure frequency as tumors progress (42). As both infiltrated peritumoral tissue and connectivity changes have been related to the development of seizures, understanding brain reorganization mechanisms has important clinical implications for controlling refractory seizures (43, 44). Recent studies investigated the role of functional network synchronizability to predict spread of seizures before they begin and also described control regions that strongly synchronize or desynchronize network dynamics (45). By investigating time-varying functional networks, the dynamic changes in the topographical organization of different functional networks could have wide applicability in mapping the plastic reorganization occurring in other diseases such as stroke and trauma (46–51). Similarly, brain tumors may also induce changes in large-scale functional connectivity (FC) that should be taken into account by the surgical approach (52). For instance, the complex language network reorganization occurring in the setting of a dominant left hemisphere DLGG infiltrating classical "Broca" and "Wernicke" areas (53–55) allow tumor resection with no functional consequences as depicted in the case illustrated in **Figure 1**. Thus, understanding the underlying neuromodulation principles governing the

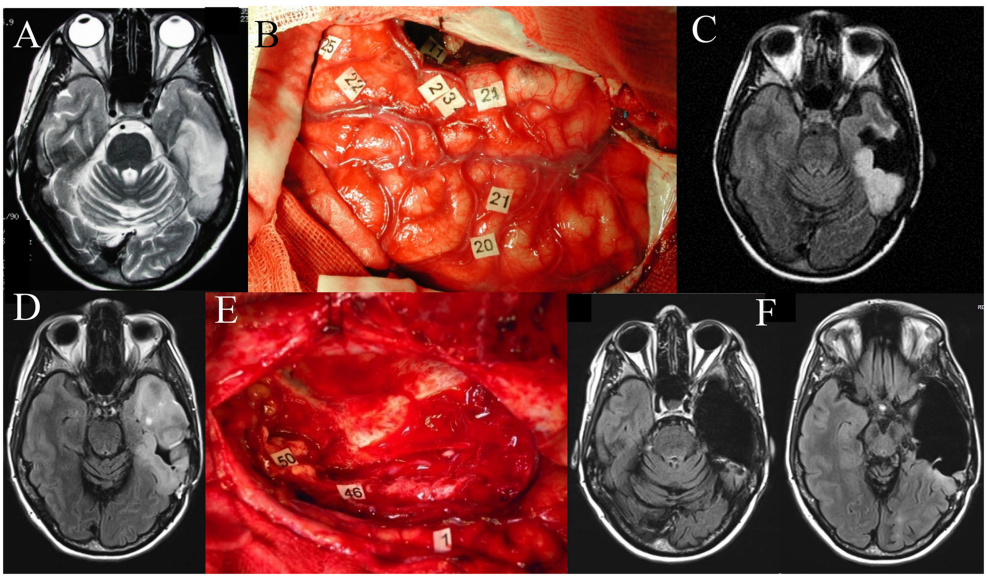

FIGURE 1 | **Left temporal diffuse low-grade glioma (DLGG)**. Axial FLAIR-weighted MRI **(A)** showing a left temporal DLGG in a 36-year-old patient who presented with isolated seizures and no neurological deficits. Intraoperative photograph during the first awake surgery **(B)**, after resection was performed according to individual functional boundaries. Stimulation mapping demonstrated the persistence of eloquent cortical areas in the temporal lobe (tags 22, 23, 24, 25) as well as subcortical fibers (tag 11, corresponding to the inferior longitudinal fascicle) still critical for language function. Postoperative axial FLAIR-weighted MRI **(C)** revealing a partial resection, with a posterior residual tumor voluntarily left for functional reasons. The diagnosis of DLGG was confirmed, and the patient resumed a normal familial, social, and professional life. Ten years later, epileptic seizures recurred concomitantly with an imaging progression as demonstrated on the axial FLAIR-weighted MRI **(D)**. Reoperation was proposed to the patient. Intraoperative photograph **(E)** during the second awake surgery, after resection was performed according to the new individual functional boundaries. Electrocortical stimulation mapping revealed brain reorganization, allowing the achievement of a significantly wider resection compared to the first surgery. Of note, at the subcortical level, stimulation of the left inferior fronto-occipital fascicle (IFOF) (46 and 50) elicited semantic paraphasia when stimulated at the end of surgery. Thus, resection of the anterior part of the inferior longitudinal fascicle was possible given the compensation provided by the direct ventral pathway represented by the left IFOF. Postoperative axial FLAIR-weighted MRI **(F)** performed 3 months after the second surgery showing a complete resection, made possible due to mechanisms of neuroplasticity, in a patient who returned to a normal life with no permanent neurological deficit.

neurosynaptic networks could lead to new methods for functional restoration (48, 49, 53).

Cerebral plasticity represents the "continuous process allowing short-term, middle-term, and long-term remodeling of neurosynaptic maps, to optimize the functioning of brain networks" (56). The concept of adult neuroplasticity exemplifies the strong interplay between the cortex and other structures provided by the myriad of cortical and subcortical connections. The underlying mechanisms for this functional reorganization and brain plasticity are not fully elucidated, and multiple theories have been proposed such as modulation of synaptic efficacy, neurogenesis, cortical hyperexcitability, redistribution, unmasking of latent networks, and establishment of new functional connections (51, 57–61). Although mounting evidence depict functional reorganization in the setting of a surgical intervention, the concept of brain plasticity in the context of DLGG is still controversial (62). Nonetheless, our current understanding of the morphological, biochemical, and connectivity changes occurring in the setting of a tumor is still in its infancy and long-term large multicenter studies incorporating longitudinal multimodal investigations will likely allow us to gather more objective evidence and improve our understanding of the underlying mechanisms.

This approach facilitates the concepts of "functional neuro-oncology" and of "preventive glioma surgery" in order to achieve earlier and more complete resections, while giving the patients the opportunity to enjoy a normal life. Understanding the individual organization of the cortical and subcortical connectivity is essential to optimize the risk–benefit ratio of glioma surgery (63). Prominent experts in this field suggest an integration of the conceptual achievements in the neuroscience, neuroimaging, and genetic fields, in order to create a holistic personalized treatment strategy incorporating "the course of this chronic disease, reaction brain remapping, and oncofunctional modulation elicited by serial treatments" (10).

Connectomics and Glioma Surgery

Functional connectivity is a measure used to express the degree of communication between brain areas and thus to describe brain networks (64). One of the main proposed mechanisms of adult plasticity reposes on the connectome concept where the brain processing relies on "dynamic large-scale, parallel subcircuits able to interact and to compensate themselves following cerebral injury" (65, 66). The concept of brain connectome depicts the dynamical structural and functional neural networks that form at multiple spatial and temporal scales (67). While it is possible

to portray structural networks delineating anatomical connectivity with deterministic tractography-derived fiber tracts (68), "functional networks" are derived from statistical estimates of time series data such as resting-state fMRI (69). For instance, using multimodal magnetic resonance images derived from the Human Connectome Project, Glasser and colleagues performed a multimodal parcelation of distinct cortical areas using an objective, semiautomated neuroanatomical approach and a robust machine-learning classifier (70). Although such non-invasive imaging studies outline a detailed non-invasive mapping of the macroscopic functional connectome, it provides just one view of the "complete" brain connectome and cannot provide the direct neuronal activity flow available through electrophysiological techniques (67, 71).

Brain tumors alter the normal structural and FC of the brain, consequently impacting the normal functioning of the brain. Altered FC in patients with brain tumors affects not only the tumor area but also other brain areas, as demonstrated through different imaging modalities (72–77). For instance, changes in resting-state networks in patients with tumors localized in the left hemisphere were observed in the contralateral side and correlated with alterations in some cognitive functions even before the onset of major symptoms (74). Intrinsic FC measures can also predict surgical outcome, and thereby could "provide information regarding the residual presence of function and also could define the extent of brain tumor invasion that may not be evident on structural MRI" (78).

As described by De Benedictis and Duffau, the classical "topological" representation ought to be replaced by a "hodotopical" framework, which takes into account the changes occurring in the large-scale networks of the brain (65). Only by acknowledging the complex cortico-subcortical network of the brain, the clinicians could further understand and take into account the dynamical neural processes occurring at distinct spatial and temporal scales (79). The functional connectome framework refined our understanding of functional localization as evidenced through the contemporary concepts of language organization, namely, that neuronal groups participate as components of a network allowing reorganization and recruitment of parallel circuits in the setting of injury (80–83).

Considering glioma surgery as "brain networks surgery" has led not only to a dramatic decrease of permanent neurologic impairment (<2% in series using intraoperative cortico-subcortical mapping) but also to improvement of higher order functions such as working memory, neurocognitive functions, and emotions and behavior, as evidenced by postoperative neuropsychological assessments following surgery (84). Therefore, the concept of neuro-oncology as a brain network surgery has major implications in terms of the clinical management and ensuing outcomes, as indexed by the increased survival and quality of life of patients managed using such an approach.

Awake Craniotomy and Intraoperative Mapping

Although the art and science of brain mapping was once the purview of epilepsy surgeons, the use of this technique in the neuro-oncological field had seen an exponential increase over the last decades. Proper choice and execution of brain mapping techniques has improved the precision and safety of the surgical treatment for some of the most challenging cases and can currently allow a more radical surgical resection than indicated by presumed preoperative functional localization. This entails an optimization of the intraoperative tests' selection based on the functional cortico-subcortical networks expected to be encountered as well as on the specific preoperative neurological and neuropsychological assessments of each patient (85–87).

Although many promising brain mapping techniques are currently being refined, the large interindividual differences between healthy and diseased brain preclude the ability to reliably identify standard imaging-based biomarkers for functional connectomics (88, 89). As such, cortical and subcortical mapping via direct electrical stimulation continues to remain the most reliable approach for accurate localization of highly functional centers specific to each patient; the usefulness of this technique being described even for children (90). Furthermore, the continuous assessment of cognitive and neurophysiological parameters provides the neurosurgeon with immediate feedback on the impact of his/her intervention.

The concept of "eloquent" cortex depends on the view that, although all cortical areas are "capable of being engaged in useful function, some brain regions are clearly more critical than others" (91), causing some degree of functional decline if resected or disconnected. This framework shift has direct implications in the clinical practice as the presumed eloquence represents a modifiable risk factor for survival (92, 93). Although a detailed knowledge of both cortical and subcortical anatomical structures represents the cornerstone of neurosurgery, understanding the underlying functional correlations provide the fine details of the relationship between the lesion to be managed and the healthy brain (94). **Figure 1** shows an illustrative case depicting the importance of performing intraoperative mapping of cortical and subcortical fibers in a patient with a left temporal DLGG. As portrayed, the respect of functional boundaries during the first surgery allowed the patient to enjoy a normal life for 10 years, whereas the language network reorganization occurring in the setting of a slowly growing tumor allowed subsequent resection of tumoral tissue at sites where functionality prevented tumor resection initially (**Figure 1**).

Despite the fact that the precise influence on the electrophysiological state of brain's networks and the biophysical modeling of the electrode–tissue interface is not well elucidated (95), direct electrical stimulation represents a highly reliable and reproducible technique (58, 95–101). IEM has equally allowed to increase the quality of life for patients affected by a glioma in the non-dominant hemisphere by testing functions such as spatial awareness (102) or even mentalizing (103) to avoid injuring the networks involved in those functions. Furthermore, IEM during awake craniotomy allows the unique opportunity to assess and validate the anatomo-functional connectivity for multimodal systems such as sensorimotor, language, visuospatial, and socio-cognitive systems (82) providing a real-time understanding of the individual organization of both cortical epicenters and subcortical connectivity (104). We can envisage the future development of platforms allowing neurosurgeons to link the intraoperative

cortical stimulation results with macroscopic neuronal network models and use connectivity-based modeling to predict functional changes.

Several manuscripts provide a comprehensive overview of the methods and technical nuances proposed for a maximal safe resection during awake brain tumor surgery (105–107). There is increasing evidence that this technique allows to improve the outcomes by maximizing the EOR while preserving functional cortex in both low- and high-grade gliomas (1, 6, 9, 108, 109). Other benefits associated with this technique are shorter hospital stay, less blood loss, shorter operative time, reduced pain and anxiety, cost effectiveness, as well as lower complications and morbidity (110, 111). Nonetheless, careful preoperative planning by a dedicated multidisciplinary team with an informed patient remains an important prerequisite for a successful awake craniotomy (90).

CONCLUSION

The paradigm shift encouraging the translation of the most recent findings in the field of neurological science to the clinical setting allowed a better understanding of the interactions between the infiltrative and dynamic growth patterns of DLGG and brain adaptation mechanisms (such as neuroplasticity and network reorganization). Using multimodal imaging studies and different neurophysiological tools does not take the place of a meticulous surgical technique and an extensive knowledge of the functional–structural anatomy in order to protect the cortical and subcortical FC. The concept of awake craniotomy as a brain network surgery allows neurosurgeons to adequately assess the dichotomy between the neurological and oncological risk management. It also highlights the delicate function–oncological balance that needs to be maintained, as it will ultimately reflect on the quality of life and overall survival rate of the patients. A joint multidisciplinary approach where the emerging advancements from different fields are integrated in clinical practice in a personalized dynamic approach using ongoing feedback from clinical–radiological monitoring could provide more effective treatment options for patients affected by DLGG as already demonstrated by the increased survival and quality of life of patients treated using such a treatment strategy.

AUTHOR CONTRIBUTIONS

All authors have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Intraoperative Fluorescence Imaging for Personalized Brain Tumor Resection: Current State and Future Directions

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Introduction: Fluorescence-guided surgery is one of the rapidly emerging methods of surgical "theranostics." In this review, we summarize current fluorescence techniques used in neurosurgical practice for brain tumor patients as well as future applications of recent laboratory and translational studies.

Methods: Review of the literature.

Results: A wide spectrum of fluorophores that have been tested for brain surgery is reviewed. Beginning with a fluorescein sodium application in 1948 by Moore, fluorescence-guided brain tumor surgery is either routinely applied in some centers or is under active study in clinical trials. Besides the trinity of commonly used drugs (fluorescein sodium, 5-aminolevulinic acid, and indocyanine green), less studied fluorescent stains, such as tetracyclines, cancer-selective alkylphosphocholine analogs, cresyl violet, acridine orange, and acriflavine, can be used for rapid tumor detection and pathological tissue examination. Other emerging agents, such as activity-based probes and targeted molecular probes that can provide biomolecular specificity for surgical visualization and treatment, are reviewed. Furthermore, we review available engineering and optical solutions for fluorescent surgical visualization. Instruments for fluorescent-guided surgery are divided into wide-field imaging systems and hand-held probes. Recent advancements in quantitative fluorescence-guided surgery are discussed.

Conclusion: We are standing on the threshold of the era of marker-assisted tumor management. Innovations in the fields of surgical optics, computer image analysis, and molecular bioengineering are advancing fluorescence-guided tumor resection paradigms, leading to cell-level approaches to visualization and resection of brain tumors.

Keywords: 5-ALA, confocal, endomicroscopy, fluorescein, fluorescence-guided surgery, fluorescent probe, glioma, ICG

Abbreviations: 5-ALA, 5-aminolevulenic acid; BBB, blood–brain barrier; EGFR, epidermal growth factor receptor; FITC, fluorescein isothiocyanate; GTR, gross total resection; ICG, indocyanine green; NIR, near-infrared; PDT, photodynamic therapy; PpIX, protoporphyrin IX; ROS, reactive oxygen species.

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INTRODUCTION

Malignant glioma is a highly invasive, heterogeneous, complex, and fatal tumor type, the extent of which is not precisely identifiable by modern imaging techniques. Despite all of the current treatment modalities for malignant gliomas, such as microsurgery, chemotherapy, and radiotherapy, there is no definitive treatment. Nonetheless, the maximum extent of surgical resection is associated with a longer recurrence-free period and overall survival of patients with glioblastomas (1, 2), low-grade gliomas (3), meningiomas (4), and other intracranial malignancies. Therefore, the initial treatment goal should be the maximal removal of the tumor mass. Tumor mass resection is guided intraoperatively by anatomically registered images (usually CT and MRI) incorporated into a stereotactically based image-guided surgery platform. Such a surgical strategy becomes a balance of aggressive tumor removal while producing no new or further permanent neurological deficit. Although there are characteristics of images from CT and MRI that indicate what tumor, necrosis, or edematous cortex is, the main focus of surgery is achieving maximal resection of the invading tumor front. In light of this, researchers have endeavored to make any invisible part of the tumor visible using advanced imaging techniques.

Advances in imaging began with the philosophies of cerebral localization and function, while techniques for improving precision and the customization of brain tumor surgery can be traced to the late nineteenth century. The evolution of imaging techniques in neurosurgery began with the first attempts at craniometric localization of intracranial lesions (5). The introduction of X-rays in neurosurgery in 1896 by Krause and Cushing (6), pneumoencephalography in 1919 by Dandy (7, 8), and cerebral angiography specifically for brain tumors by Moniz in 1927 (9, 10) were the first steps in preoperative imaging diagnosis of brain tumors, which was previously possible only by clinical neurological examination. Intraoperative stimulation in awake patients to increase the safety of tumor resection was performed by Thomas and Cushing (11). This stimulation was possible due to Cushing's previous experience in mapping the motor cortex of primates in 1902 in the physiology laboratory of Sherrington (12). However, the origins of intraoperative neurophysiology for functional localization have roots in the works of Betz (13), Ferrier (14), Fritsch and Hitzig (15), and Clark and Horsley (16). Since the beginning of the twentieth century, several neurosurgeons, most notably Penfield in 1928, have used intraoperative brain stimulation extensively to map the cortex to guide brain tumor resection and surgical treatment of epilepsy (17). Building on earlier work, intraoperative electrophysiological monitoring and cortical and subcortical mapping performed with the patient conscious remain state-of-the-art methods to elicit functions of brain areas and define and personalize safe boundaries of tumor resection (18, 19).

Techniques for visually identifying the tumor mass began in the mid-twentieth century. The application of the fluorescent dye fluorescein sodium to highlight tumor tissue during its removal was introduced in neurosurgery by Moore et al. in 1948 (20), decades before computed tomographic (CT) scanning was introduced into broad clinical practice (1973) (21–23). Fluorescein sodium was in use even earlier than the first operative microscopes used by neurosurgeons and was pioneered by Kurze in 1957 (24). However, fluorescent dye technology did not gain widespread acceptance due to the high rate of background fluorescence from normal brain tissue and the shortcomings of visualization technologies (25). Fluorescein injection for cerebrovascular and tumor surgery was studied in detail by Feindel in the 1960s (26). A major step after the introduction of CT with contrast injection (23) was gadolinium-enhanced MRI, introduced around 1987, that allowed even more precise tumor mass visualization and precise anatomical co-registration for planning surgery (27). Infrared frameless neuronavigation systems resulting from developments in stereotactic and computer technologies were rapidly adopted in neurosurgical operating rooms in the late 1980s (28–30). The virtual linkage of neuroimaging and intraoperative anatomy allowed a precision of nearly 2 mm, selection of the best approach trajectory (31), and radically improved the surgeon's intraoperative orientation. The main drawback of neuronavigation remains brain shift [1 cm on average (32)] as a consequence of opening the cranium, which significantly limits the accuracy of determining an infiltrative tumor border (33). Despite software advances (32), intraoperative ultrasound (34, 35), and intraoperative MRI corrections (36), the current (2015) technologies do not provide the desired accuracy for consistent, precise, and extensive resection (37). The main drawback continues to be that MR and CT image characteristics are not directly indicative of regional tissue type and cannot provide clinically applicable imaging at or near cellular resolution.

Accurate visualization of brain tumors marked by fluorescent probes and even residual tumor cells is possible with emerging new technologies. These emerging technologies are expected to become state-of-the-art tools to maximize customized brain tumor treatment. These technologies are the logical extension of the evolution of the search for precision in brain tumor surgery. Such technologies will allow real-time imaging interrogation of the brain during surgery at the cellular resolution to maximize or tailor brain tumor resection.

This review summarizes recent achievements and future perspectives of clinical, laboratory, and translational studies that bring fluorescence-guided neurosurgery to the cellular level, thereby allowing for individualized brain tumor resections, representing a crucial breakthrough in this field.

FLUORESCENT DYES IN NEUROSURGERY

In the last decade (2006–2016), the number of fluorescent stains and cellular tags used in preclinical studies has increased significantly, with many novel fluorophores awaiting approval for clinical use. The fluorescent probes and dyes discussed in this review are summarized in **Table 1** (25, 38–74). Three fluorescent contrast agents that have been studied and used in human neurosurgical procedures are fluorescein sodium, indocyanine green (ICG), and 5-aminolevulinic acid (5-ALA), although not all are approved by regulatory committees in all countries. Other fluorophores (including acridine orange, acriflavine, cresyl

TABLE 1 | Summary of published preclinical and early clinical data on probes and imaging equipment for potential personalized fluorescence-guided brain tumor surgery.

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
Targeted probes							
IRDye 800CW-labeled VEGF (38) (Bevacizumab)	675 and 745 nm (*in vivo*)	800 nm (*in vivo*)	1. IVIS Spectrum (PerkinElmer, Inc.) 2. Multispectral Fluorescence Camera System (Institute for Biological and Medical Imaging, Technical University, Munich, Germany and SurgOptix Inc., Redwood Shores, CA, USA), *in vivo* 3. Olympus Fluoview 300 Confocal Scan Box mounted on an Olympus IX 71 inverted microscope (Olympus America Inc.), *ex vivo* 4. Pearl Imaging System (LI-COR Biosciences) *in vivo*	Xenograft mice model (human ovarian, breast, and gastric cancers)	Distinguish submillimeter lesions intraoperatively. Longer lasting and more accurate signal for VEGF and EGFR2 than ICG alone. Bevacizumab-800CW fluorescence detection in extracellular matrix, trastuzumab-800CW fluorescence detection on tumor cell surface	Long half-life for detecting tumors. Long elimination time	IV, 6 days (optimal time)
IRDye 800CW-labeled human EGFR 2 [Trastuzumab (38); Erbitux (39)]	675 and 745 nm (*in vivo*); 685 and 785 nm	800 nm (*in vivo*); 720 and 820 nm	1. IVIS Spectrum (PerkinElmer, Inc.) 2. Multispectral Fluorescence Camera System (Institute for Biological and Medical Imaging, Technical University and SurgOptix Inc.), *in vivo* 3. Olympus Fluoview 300 Confocal Scan Box mounted on an Olympus IX 71 inverted microscope (Olympus America Inc.), *ex vivo* 4. Pearl Imaging System (LI-COR Biosciences) *in vivo*	Xenograft mice model (human ovarian, breast, and gastric cancers); Xenograft mice model (human breast cancer lymph metastasis)	Distinguish submillimeter lesions intraoperatively. Longer lasting and more accurate signal for VEGF and EGFR2 than ICG alone. Bevacizumab-800CW fluorescence detection in extracellular matrix, trastuzumab-800CW fluorescence detection on tumor cell surface	Long half-life for detecting tumors. Long elimination time	IV, 3–6 days (optimal time); 3 h for lymph node visualization
IRDye 800CW-labeled anti-EGFR nanobody 7D12 (40, 41)	760 nm; 656–678 nm; 745–779 nm	774 nm; 700 nm; 800 nm	1. IVIS Lumina System (PerkinElmer, Inc.) with ICG filter sets 2. FLARE imaging system (Beth Israel Deaconess Medical Center) 3. IVIS Spectrum (PerkinElmer, Inc.)	Xenograft mice (human epidermoid carcinoma); xenograft mice (human metastatic oral squamous cell carcinoma)	Better tumor penetration and distribution of nanobody probe *in vivo* (vs. cetuximab full antibody). Significantly higher tumor to background fluorescence (vs. cetuximab full antibody)	Not mentioned	IV, 30 min (earliest); 2 h (optimal); or 24 h (optimal)
IRDye 680RD labeled EGFR inhibitor (cetuximab) (42)	620 nm	650–800 nm	Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska), *ex vivo*	Xenograft mice (human U251 glioma)	Higher affinity for tumor than anti-EGFR targeted affibody used in same study	Concentration of antibody in tumor focused primarily in the center	IV, 1 h
IRDye 800CW-labeled anti-EGFR targeted affibody (42)	720 nm	730–900 nm	Odyssey Infrared Imaging System (LI-COR Biosciences), *ex vivo*	Xenograft mice (human U251 glioma)	Smaller size molecule results in better penetration of BBB. Higher concentration in outer tumor than antibody	30 times lower affinity than antibody and a shorter plasma half-life	IV, 1 h

(Continued)

TABLE 1 | **Continued**

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
IRDye 800CW-labeled chemokine stromal cell derived factor-1 (SDF-1) (43)	685 and 785 nm	702 or 789 nm	Pearl Imaging System (LI-COR Biosciences), *in vivo*	Xenograft mice (A764 human glioma, MCF-7 human breast cancer)	Detected as low as 500 cells *in vitro*. Specific for tumor cells. Signal persisted for days	Labeled bone marrow, transient non-specific labeling during first 24 h was observed in the liver and skull	IV, 1-h visualization of tumors and background structures; 24–92 h background fluorescence diminished, tumors remained clearly visible
IRDye 800CW-labeled anti-CD105 monoclonal antibody (angiogenesis related) (44)	778 nm	806 nm	Pearl Imaging System (LI-COR Biosciences) *in vivo* and *in vitro*	Mice with 4T1 mouse breast cancer; human MCF-7 breast cancer cells in cultures	Tumor could be visualized as early as 30 min post-injection; may be used in the clinic for imaging tumor angiogenesis	CD105 expression is observed only on actively proliferating tumor endothelial cells	IV, 30 min (early); 16 h (optimal)
Cy5.5-labeled EGFR inhibitor (cetuximab) (45)	683 nm (max); 630–670 nm (range used in experiment)	707 nm (max); 685–735 nm (range used in experiment)	1. Leica MZFL3 stereo research microscope (Leica Microsystems, Bannockburn, IL, USA) fitted with a GFP and Cy5.5 filter and an ORCA ER charge-coupled device camera (Hamamatsu, Bridgewater, NJ, USA) 2. eXplore Optix time-domain fluorescence imaging system (ART/GE Healthcare, Princeton, NJ, USA)	Cell cultures: UM-SCC-1, FaDu, CAL 27, and AB12; xenograft mice model (human head and neck squamous cell carcinoma cell lines SCC-1, FaDu, CAL 27); mice with mouse mesothelioma	Can be used to detect tumors *in vivo*	EGFR expression did not correlate with the fluorescent intensity	IV, 48–72 h (optimal)
Alexa-680 labeled insulin-like growth factor 1 receptor (IGF1 R) (AVE-1642-conjugated Alexa 680) (46)	575–605 nm	645–850 nm	1. Maestro Imaging System (CRI), *in vivo* 2. Olympus Fluoview FV500 laser scanning confocal system (Olympus America Inc.)	Xenograft mice model (MCF-7 human breast cancer cells)	Can detect the downregulation of IGF1R after treatment with a monoclonal antibody	Further studies required to determine the amount of background fluorescence produced by IGF1R	1 day (earliest); 2 days (clear imaging)
Folate–fluorescein isothiocyanate probe (for folate receptor) (47)	495 nm	520 nm	Intraoperative Multispectral Fluorescence Camera System (Institute for Biological and Medical Imaging, Technical University)	Humans with ovarian cancer	High specificity for labeling FR-alpha expressing cells. Real-time image-guided excision of fluorescent tumor deposits of size <1 mm was feasible	Four patients experienced mild discomfort in the upper abdominal region after injection	Imaging completed 2–8 h after injection

(Continued)

TABLE 1 | **Continued**

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
BODIPY FL-labeled PARP inhibitor (Olaparib) (48)	503 nm	515 nm	Maestro Imaging System (CRI)	Xenograft mice model (U87 MG and U251 MG human glioblastomas)	High specificity for the DNA repair enzyme PARP1 with therapeutic effect. Promising new targeted antitumor drug, which is already in clinical trials. High tumor-background fluorescent ratio. Toxicity profile is known and similar to Olaparib	Not mentioned	60–180 min (optimal)
Liposomes with RGD peptide and the neuropeptide SP, gadolinium, Indium-111, Rhodamine-B (49)	554 nm	576 nm	Zeiss LSM 510 Microscope (Carl Zeiss Meditec AG, Jena, Germany)	Cultured mouse fibroblast cells with U87 MG human glioblastoma and M21 human melanoma tumor cells (*in vitro*)	Combination of radioactive, fluorescent, and magnetic resonance imaging signaling; multifunctionality of liposomes as a carrier of different probes	Moderate tumor uptake	*In vitro* fluorescence microscopy was completed after tumor cells were grown in mouse fibroblast culture
ZW800-1 zwitterionic NIR fluorophore (50, 51)	750 ± 25 nm; 773 nm	810 ± 20 nm; 790 nm	1. FLARE Imaging System (Beth Israel Deaconess Medical Center) 2. FLARE Imaging System (Beth Israel Deaconess Medical Center) 3. Pearl Small Animal Imaging System (LI-COR Biosciences)	Xenograft mice model (M21 human melanoma, Lewis lung carcinoma, HT-29 human colorectal adenocarcinoma)	Higher tumor-to-background ratio than IRDye800-CW and Cy5.5	Wash-out of dye from tumors started occurring at 4 h (dye still present at 24 h)	4 h, low visibility at 4 h, highest visibility from 24 to 72 h
M13-stabilized single-walled carbon nanotubes (SBP-M13-SWNTs) (52)	808 nm	950–1400 nm	Liquid nitrogen-cooled OMA V 2D InGaAs array detector with a 256 × 320 pixel array (Princeton Instruments) coupled with SWIR-25 NIR camera lens (Navitar, Rochester, NY, USA)	Xenograft mice model (OVCAR8 human ovarian epithelial carcinoma)	Stable and showed 10 times more selective fluorescent staining of ovarian tumor cells than same construct without targeting peptide. Nanotube fluorescence intensity relative to background (5.5 ± 1.2) was superior to same construct labeled with other NIR AlexaFluor750 dye (3.1 ± 0.42) or FITC (0.96 ± 0.10)	Study did not assess possible penetration of the probe into the brain	24 h
Fluorescent gold nanoparticles conjugated with diatrizoic acid and AS1411 aptamer (53)	400 nm	620 nm (max)	1. Ultra-VIEW RS Confocal System (PerkinElmer, Inc., Waltham, MA, USA) 2. IVIS (PerkinElmer, Inc.)	Xenograft mice model (human lung adenocarcinoma) separate MCF-7 cell assay	Specific binding to tumor cells due to AS1411 aptamer, which targets nucleolin. Allowed X-ray visualization due to high electron density of gold nanoparticles	Small sample size ($n = 6$)	30 min

(Continued)

TABLE 1 | **Continued**

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
Lymphoma-specific fluorescent (Alex488) switchable TD05 aptamer (54)	489 nm	505–535 nm	Zeiss 710 laser Scanning Confocal Microscope (Carl Zeiss Meditec AG) equipped with a 40×/1.2NA water emersion objective (*ex vivo*)	Xenograft rat model (U251 human glioma and Ramos human CNS lymphoma)	Probe could rapidly and specifically identify human B cell lymphoma in biopsies. System would be useful for discriminating non-operative CNS B-cell lymphoma from malignant glioma rapidly after biopsy	*Ex vivo* only study	Total antibody staining time was 24 h and aptamer staining time was 1 h (*ex vivo*)
Chlorotoxin (CTX) conjugated to ICG (BLZ-100) (55)	785 nm	Near-infrared spectrum	Custom imaging system: 16-mm VIS-NIR Compact Fixed Focal Length Lens (Edmund Optics, Barrington, NJ, USA) coupled 785-nm StopLine single-notch filter, NF03–785E-25 (Semrock, Rochester, NY, USA)	Xenograft mice model (LN229 human glioblastoma)	High affinity to human gliomas	Not mentioned	48 h
5-Carboxyfluorescein (FAM)-labeled fluorescent probe consisting of tLyP-1 small peptide targeted to the neuropilin receptors (FAM-tLyP-1) (56)	Blue light	Not given	Kodak *In Vivo* Imaging System F (Kodak, Rochester, NY, USA)	Xenograft mice model (U87MG human glioblastoma)	Selective uptake. May have advantages over CTX-Cy5.5 probe due smaller size	Fluorescein labeling was less than ideal, could be exchanged for more intense fluorophore	1 h
Activity-based probes							
Modified hydroxymethyl rhodamine green (gGlu-HMRG) (57)	488 nm	505–530 nm	1. In-house-made portable fluorescence camera for *ex vivo* tumor specimens 2. Zeiss LSM510 Microscope (Carl Zeiss Meditec AG)	Human breast cancer tissue samples; breast cancer cell culture	High sensitivity and spatial resolution Fast processing: evaluation can be done within 5 min after probe application	In breast cancer, this method cannot distinguish malignant and benign regions	5 min
MMPSense 750 FAST (MMP-750) (58)	749 nm	775 nm	Surgical Navigation System (Institute of Automation, Chinese Academy of Sciences, Beijing, China) (59)	Mice with 4T1-luc breast cancer tumors	Imaging method offered precise detection of the orthotopic breast tumors and metastases intraoperatively in real time	Not mentioned	IV, 6 h (fluorescent signal observed); 24–36 h (optimal fluorescent signal)
Caspase-sensitive nano-aggregation fluorescent probe (C-SNAF) (60)	635 ± 25 nm	670–900 nm	Maestro Hyperspectral Fluorescent Imaging System (CRI)	Xenograft mice model (subcutaneous HeLa tumors)	Highly feasible for imaging of drug-induced tumor apoptosis *in vivo*, signal strengthens as tumor cells die	Not mentioned	IV, 1 h

(Continued)

TABLE 1 | **Continued**

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
Nanoparticles							
Polyacrylamide-based nanoparticles loaded with ICG or Coomassie blue dye (61)	647 nm	675–725 nm	1. Olympus IX70 confocal microscope (Olympus America, Inc.) 2. Ultra-VIEW Confocal Laser Scanning Microscope (PerkinElmer, Inc.)	Cell cultures: 9L rat gliosarcoma, MDA-MB-435 human melanoma, MCF-7 human breast cancer	Produced visible color change in tumor cell lines	Significant non-specific binding was observed	Imaged after 2 h of incubation
Iron oxide magnetic NH_2-CLIO nanoparticles labeled with Cy5.5 (Cy5.5-CLIO) (62)	Not given	Not given	1. Custom-built surface reflectance imaging system (Siemens Medical Systems, Erlangen, Germany), *in vivo* 2. Zeiss LSM 5 Pascal (Carl Zeiss Meditec AG, Jena, Germany), *ex vivo*	Rat 9L gliosarcoma tumor model	Clear tumor border demarcation Co-localization based on MRI imaging Elimination more predictable than other nanoprobes	Not as accurate as target probes for *in vivo* tumor cell visualization	IV, 24 h
Cyto647 labeled anti-EGFR antibody-conjugated SERS-tagged gold nanoparticles (antibody-Panitumumab) (63)	642 nm (Olympus); 785 nm (Raman)	700–775 nm	1. Olympus IX81 inverted fluorescence microscope (Olympus America, Inc.) 2. Hamamatsu Back-Thinned EM-CCD camera, 9100-13 (Hamamatsu, Bridgewater, NJ, USA) 3. Spinning Disk Confocal Scanning Raman Microscope (Renishaw, Wotton-under-Edge, UK)	*In vitro* cell cultures: rat gliosarcoma cell line 9L; rat C6 glioma; human GBM cells U87, A172, U251, U373; normal fetal human astrocytes; primary oligodendroglioma tumor cells BT2012036; and GBM adherent stem cell line GLINS1	Selective uptake by tumor cells; unlike other fluorescent dyes, SERS nanoparticles have enhanced photostability	Not mentioned	Not applicable
Others							
5-ALA that metabolically converts into fluorescent PpIX	400–410 nm violet	620–720 nm red	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH)	Studies in human (25) and in animals (64)	Studies have shown increased extent of tumor resection with PpXI guided surgery; useful for brain tumor biopsy	Disruption of BBB necessary for fluorophore accumulation (can decrease/vary contrast) Low fluorescence intensity in low-grade gliomas	Oral, IV, 2 h (65)
Indocyanine green (ICG)	780 nm	>795 nm	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical, GmbH) 3. Zeiss LSM710 (Carl Zeiss Surgical, GmbH)	Mice with GL261 mouse glioma (66) Human (67)	Extensively studied; hand-held confocal endomicroscope and LSM showed ICG selectively stained glioma cells in mouse model (66) Intraoperative administration at end of 5-ALA guided resection may show additional tumor tissue (67)	ICG visualization can only be displayed on a monitor	IV, 15 min

(Continued)

TABLE 1 | **Continued**

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
Fluorescein sodium (68)	494 nm	521 nm	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH) 3. LSM710 (Carl Zeiss Surgical, GmbH)	Human	Convenience for surgeon, surrounding tissue has more natural color	Rapid photobleaching, non-specific accumulation of fluorescein along the margins of resection. Possible extravasation along with edema	IV, 5 min (65)
CLR1501 (69)	500 nm	517 nm	Nikon A1RSi Confocal Microscope (Nikon, Minato, Tokyo, Japan); IVIS Spectrum system (PerkinElmer, Inc.)	Xenograft mouse model (U251 human glioblastoma, 22T, 22CSC, 33CSC, 105CSC patient derived glioblastoma)	Tumor-to-brain fluorescence ratio similar to 5-ALA	Tumor must be visualized on separate monitor	IV, >4 days
CLR1502 (69)	760 nm	778 nm	1. IVIS Spectrum system (PerkinElmer, Inc.) 2. Fluobeam 800 (Fluoptics, Grenoble, France) 3. Leica OH4 intraoperative microscope with FL800 attachment (Leica Microsystems, Bannockburn, IL, USA)	Xenograft mouse model (U251 human glioblastoma, 22T, 22CSC, 33CSC, 105CSC patient derived glioblastoma)	Tumor-to-brain fluorescence ratio superior to 5-ALA	Tumor must be visualized on separate monitor	IV, >4 days
CH1055 (70)	~750 nm	1055 nm	In-house-built NIR spectroscopy instrument with Acton SP2300i spectrometer (Princeton Instruments, Trenton, NJ, USA) and Princeton OMA-V liquid-nitrogen-cooled InGaAs linear array detector (Princeton Instruments)	Xenograft mice model (U87MG human glioblastoma)	High tumor-to-background signal ratio Possibility for precise image-guided tumor removal in the model 90% excreted through the kidneys within 24 h	Tumor must be visualized on separate monitor	IV, 6 h (tumor is clearly visible); 72 h (optimal)
Acridine orange (64)	488 nm	505–700 nm (LSM); 505–585 (VWCE)	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH) 3. LSM710 (Carl Zeiss GmbH)	Mice with GL261 glioma; swine normal brain	Suitable for rapid intraoperative *ex vivo* analysis of glioma tissue	Cannot be used in the brain due to toxicity profile	Topical application, immediately
Acriflavine (64)	405 nm (LSM); 488 (VWCE)	505–585 nm	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH) 3. LSM710 (Carl Zeiss GmbH)	Mice with GL261 glioma	Suitable for rapid intraoperative *ex vivo* analysis of glioma tissue	Cannot be used in the brain due to toxicity profile	Topical application, immediately
Cresyl violet (64)	561 nm (LSM); 488 nm (VWCE)	620–655 nm (LSM); 505–585 nm (VWCE)	1. VWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH) 3. LSM710 (Carl Zeiss GmbH)	Mice with GL261 glioma	Highlights tumor boundaries *ex vivo*	No current *in vivo* brain application Low signal-to-noise ratio	Topical application, 10 min

(Continued)

TABLE 1 | Continued

Name of probe	Reported excitation wavelength	Reported reading emission wavelength	Used equipment	Species tested	Advantages	Disadvantages	Mode of administration and time to imaging (unless noted otherwise)
Sulforhodamine 101SR101 (64)	561 nm (LSM); 488 nm (WWCE)	585–615 nm (LSM); 505–750 nm (WWCE)	1. WWCE 2. Zeiss Pentero Microscope (Carl Zeiss Surgical GmbH) 3. LSM710 (Carl Zeiss GmbH)	Xenograft rat model (U251 human glioma)	Strongly labeled cells within the tumor and astrocytes within normal brain	Non-specific	1 h
Demeclocycline (72)	402 nm	~520 nm	Custom confocal laser scanning microscope	Human low- and high-grade glioma tissues	Highlights tumor cells *ex vivo* and correlates with histology. Limited data suggest specificity for tumor cells (73)	Non-specific	Topical application, timing not reported
Methylene blue (74)	642 nm	~690 nm	Custom confocal laser scanning microscope	Human meningioma, glioma, and adenocarcinoma tissues	Highlights tumor cells *ex vivo*	Non-specific	Topical application, timing not reported

BBB, blood–brain barrier; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; SERS, surface-enhanced Raman scattering, a nanoparticle tagging method to increase signal detection; FMI, fluorescence molecular imaging; BCS, breast cancer surgery; LSM, laser scanning microscope; VWCE, visible wavelength confocal endomicroscope (Optiscan 5.1) (71).

violet, and sulforhodamine 101) have been used for pulmonary, gastrointestinal, or gynecologic procedures and in *ex vivo* brain biopsies. They have not been used directly in the human brain. Fluorescent probes and labels are classified based on the actual fluorescent molecule (i.e., intrinsic and extrinsic endogenous fluorophores) and excitation/emission profile and can be further categorized by their mechanism of action:

1. Passive fluorescent probes (ICG, fluorescein sodium, and other stains);
2. Metabolic probes (5-ALA, activatable probes); and
3. Targeted probes.

One of the most important characteristics of the probes is their ability to accumulate in tumor tissues in high concentrations. In the case of brain tumors, the blood–brain barrier (BBB) influences the delivery of probes that are not lipophilic or have a molecular weight more than 400–600 kDa (75). Based on their physical properties, photons with longer wavelengths in the near-infrared (NIR) spectrum have greater tissue penetration and thus are advantageous for visualizing obscure residual tumor tissue or cells (**Figure 1**). However, fluorophore phototoxicity caused by the generation of reactive oxygen species (ROS) may be harmful to healthy cells. The principle of phototoxicity is also used in combination with fluorescence-guided tumor resection and photodynamic therapy (PDT). Most fluorescence is associated with the production of some ROS and PDT effects (76). The combination of fluorescence-guided resection and post-resection cavitary PDT with strong photosensitizers may have a synergistic effect and has already shown promising results in several clinical trials (77, 78). Nonetheless, this approach and the exact methodology regarding the choice of a photosensitizer, excitation wavelengths, dosages, and other parameters remain to be defined.

In this section, we discuss fluorescent agents that are used or could potentially be used for fluorescence-guided resection and intraoperative diagnosis of brain tumors.

Indocyanine Green

Characteristics

Indocyanine green is a small water-soluble molecule with molecular weight of 744.96 Da. ICG is excited at the wavelength of about 780 nm, and it emits fluorescence in the 700- to 850-nm range, which is not visible to the naked eye. After IV administration, ICG binds to plasma proteins and is cleared by the liver. In brain tumor surgery studies, 5- to 25-mg ICG concentrations were used (79, 80), and the observed duration of fluorescence was limited, with a peak at about 10 min.

Applications in Brain Tumor Surgery

Indocyanine green video angiography is a widely used method for intraoperative assessment of blood flow and vessel patency in tissue flap pedicles (81) and for assessment of intestinal perfusion near an anastomosis (82). ICG has been extensively studied for detection of sentinel lymph nodes in gastrointestinal oncology. Lymphatic drainage has been traced after subcutaneous ICG administration (83).

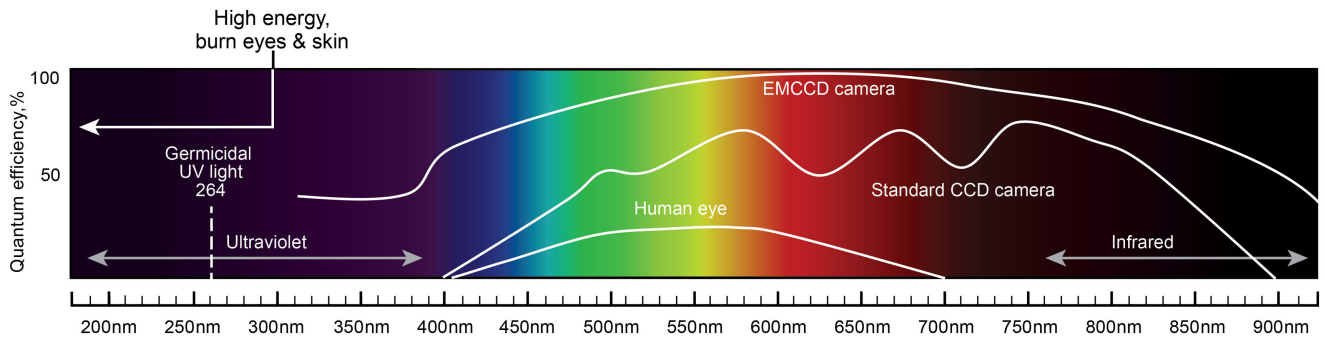

FIGURE 1 | A schematic diagram of the light spectrum and corresponding wavelengths. Quantum efficiency of the human eye, standard CCD camera, and EMCCD camera are plotted together to show the differences in the covered wavelengths and the sensitivity to light. Light with shorter wavelengths has higher energy than light with longer wavelengths. Light wavelengths below 300 nm may burn eyes and skin. UV light of 264 nm is germicidal. Longer wavelengths (infrared) have greater tissue penetration properties. EMCCD, electron multiplying charge-coupled device; CCD, charge-coupled device. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

Although we do not discuss ICG angiography for vascular neurosurgery here, assessment of arterial and venous anatomy during some tumor resections may be necessary (84). Confirmation of the distal circulation with ICG angiography and test occlusion may be used when arterial sacrifice is required during tumor removal (85).

During a glial tumor resection using the operating microscope, ICG injection shows increased blood flow in the tumor tissue and pathology-induced alteration in the surrounding brain circulation (86). Hansen initially showed that ICG was able to highlight glioma tissue using an *in vivo* rat model (87), but therapeutically adequate doses did not produce sufficient fluorescence and required enhanced imaging technologies beyond the standard operating microscope (88, 89). Prior administration of bradykinin analog reportedly increased ICG extravasation and staining of tumor tissue in a glioma model (90) but not in the clinical setting. Simultaneous application of ICG during 5-ALA-guided glioma resection permitted detection of hypervascularized, angiogenic hotspots at the edge of resection potentially increasing the extent of resection (67). Although ICG produces an NIR signal with deeper penetration, it may not be specific for glial tumor tissue. ICG follows proteins leaked from the disrupted BBB and may diffuse into surrounding tissues. A unique liposomal formulated phospholipid-conjugated ICG has a particular brain-to-tumor biodistribution that may allow more accurate imaging guidance during surgery than ICG alone (91). Using a hand-held confocal endomicroscope, we observed that ICG selectively stained glioma cells *in vivo* (66).

Hemangioblastomas are highly vascularized tumors, and ICG fluorescence helps to identify hidden arterial feeders and vessels en passage (80, 92, 93). ICG has shown some usefulness in meningioma surgery. In cases of an occluded superior sagittal sinus, ICG was helpful in guiding the dural opening, tumor resection, and venous management, although multiple ICG injections were necessary (94). Additionally, ICG was used to highlight pituitary adenoma tissue through a microsurgical approach using the operative microscope (95, 96). ICG imaging using an endoscope was also recently reported to assist in visualization of tumors that infiltrated the sphenoid sinus (97) and to assess blood flow to the optic nerves and normal pituitary tissue during transsphenoidal surgery (98). ICG has also been used to guide transventricular endoscopic biopsies but not all areas of tumor dissemination were visible (99). ICG was further applied to visualize the facial nerve during temporal bone resection (100). After the facial canal was drilled to make it thin, ICG was injected and the fluorescence from the nerve blood supply guided further bony dissection to the internal acoustic meatus. Thus, the technique allowed visualization and preservation of the facial nerve (100).

One of the drawbacks of ICG visualization is that the image can only be displayed on a monitor, and technical refinements are needed to increase the comfort and ergonomics of ICG imaging instrumentation. Recent advances in this method include an overlay of fluorescence video angiography with a white-light field transmitted from the conventional operating microscope (101).

5-Aminolevulinic Acid

Characteristics

5-Aminolevulinic acid is a drug that is an intermediate metabolite of the heme synthesis pathway. 5-ALA is converted to protoporphyrin IX (PpIX), which is an endogenous fluorophore. PpIX peaks in 6 h after 5-ALA administration (102). Established correlation of gadolinium, a marker of BBB breakdown, with PpIX concentrations in glioma tissues, suggests (103) that BBB disruption is the leading cause of increased 5-ALA accumulation in malignant cells. However, increased 5-ALA-induced PpIX fluorescence was demonstrated within the areas with preserved BBB (104). PpIX has an excitation peak in the violet–blue light range (405 nm). Under blue light illumination, normal brain tissue reflects the light, whereas tumor tissue with accumulated PpIX emits a bright red fluorescence with two peaks, a large peak at 635 nm and a small peak at 710 nm.

Interest in 5-ALA application in neuro-oncology has been stimulated by promising PDT results with 5-ALA as the photosensitizer for the treatment of other types of cancers. PDT is recognized as a treatment modality mainly for tumors of hollow organs such as the stomach, colon, rectum (105), and most

successfully, for skin malignancies (106). Its success is mainly related to a tumor location close to the surface that allows for sufficient depth of penetration by the irradiation.

Applications in Brain Tumor Surgery

For wide-field fluorescence, 5-ALA is usually administered 3 h before surgery so that the peak of PpIX production corresponds to the intraoperative tumor removal stage. Fluorescence observed in glioblastomas is often patchy and varies in intensity. Low-grade gliomas may not be visualized with wide-field techniques, although confocal endomicroscopy may detect 5-ALA in such tumors (107). In meningiomas, the observed fluorescence is usually high in intensity and is homogeneous (108). Tumor-specific fluorescence suffers from photobleaching. Natural fading occurs about 9 h after administration. 5-ALA-induced fluorescence decays to 36% of the peak within 25 min in light filtered to match the excitation wavelength of 405 nm; in contrast, with unfiltered wide-field illumination, 87 min was required to reach the same level of decay (25).

Most studies on glioma surgery with 5-ALA fluorescence for guidance have documented increases in tumor resection area (109, 110). The results of a phase III study indicated a 1.5-month increase in progression-free survival with 5-ALA fluorescence-guided surgery (111). In patients older than 55 years, regardless of tumor location, progression-free survival increased an additional 6 months. 5-ALA was also successfully used in brain tumor biopsy to obtain specimens of higher quality and to make a preliminary photodynamic diagnosis in a situation of primary central nervous system lymphoma (112).

Several approaches have advanced 5-ALA technology. One approach is to calculate the severity of the malignancy based on the fluorescence intensity. The emission spectrum must be analyzed accurately to calculate the ratio of peak emission intensity to the reflected excitation intensity (i.e., fluorescence intensity ratio). This ratio can then be used to predict the proliferative activity of the tumor (113). However, investigation of this characteristic was done in *ex vivo* tissue and requires technical improvement for intraoperative use. Other potential significant advancements for the use of 5-ALA involve the intraoperative use of high-magnification imaging optical technologies, such as confocal endomicroscopy, which may bring detection of fluorescence to the cellular level (107). The results of conventional histopathological methods correlated with confocal endomicroscopic imaging during 5-ALA-guided tumor resection (107). However, image quality was poor, and *in vivo* visualization of 5-ALA using blue laser confocal endomicroscopy in animal models could not confirm the findings (64, 107). Results from various clinical trials using intraoperative 5-ALA for brain tumor resection are ongoing (114).

5-Aminolevulinic acid, like all fluorophores, has drawbacks. Disruption of the BBB is necessary for fluorophore accumulation. In some low-grade gliomas, this may decrease or vary contrast accumulation. However, recent quantitative measurement studies suggest that diagnostic concentrations of PpIX do accumulate in low-grade tumors, but the concentration is below the detection threshold of current wide-field systems (115). Blood and overlying soft tissues can decrease visible fluorescence and hide the residual tumor. 5-ALA consumption and PpIX production may be highly variable (116) and depend on the several factors such as cell type (117), glucose concentration (118), pH (119), and other factors.

Fluorescein

Characteristics

Fluorescein is an orange–red powder with the molecular formula $C_{20}H_{12}O_5$ and a molecular weight of 332.31 Da. It is widely used in the scientific and medical industries as fluorescein isothiocyanate 1 (FITC), Alexa 488 fluorophore, and other variants. In medicine, the fluorescein sodium salt is used, but for brevity, we refer to it here as fluorescein. Fluorescein as a marker of BBB disruption demonstrated perilesional edema in a cortical cold lesion model in rats (120). Tumor boundaries observed using fluorescein fluorescence correlate well with preoperative gadolinium contrast-enhanced boundaries (68). However, fluorescein has no particular interaction with the tumor cells and may not show fluorescence in diffuse, low-density tumor cell infiltrates (68, 121, 122).

Application in Brain Tumor Surgery

Although the first clinical use of fluorescein for glioma surgery was in 1948 (20), fluorescein use in brain tumor surgery is not currently an FDA-approved use. Thus, it is restricted to clinical research studies. Its application is reported at several dosages. High doses of 15–20 mg/kg have been used for naked eye guidance without creating any permanent adverse effects (123). Yellow staining of the sclera, skin, and urine after high doses disappeared in approximately 24 h (124). Lower doses of 5–10 mg/kg for fluorescein-guided surgery using a special operative microscope module with excitation and observation filters were typically safe and effective in clinical trials (125), although one case of intraoperative anaphylaxis has been reported (126). The timing of fluorescein injection also varied across the studies. Some researchers have injected fluorescein intravenously after induction of anesthesia (127) at a dose of 3–4 mg/kg and waited for 10 min or 1 h, whereas others have injected it into a central venous line and waited for 20 min before resection (20, 124, 128–130). The half-life of fluorescein glucuronide, the main metabolite of fluorescein, is 264 min (131), and urinary clearance requires 24–32 h.

Fluorescein accumulates in glioma tissue homogenously and may be observed by the naked eye as bright to dark yellow staining of the tumor (123). Fluorescein-guided resection using operative microscopy without a special fluorescence detection module was reported by Shinoda et al. They achieved a gross total resection (GTR) in 84% of patients (129). Koc et al. produced GTR in 83% versus 55% of controls (132), and Chen et al. achieved an 80% GTR rate (123). The study by Koc et al. did not show a difference in survival (43.9 weeks in the patients given fluorescein and 41.8 weeks in the control patients) (132) while others did not assess survival.

A custom microscope for fluorescein-guided surgery was described in 1998 that increased fluorescent enhancement and contrast of intravenously injected fluorescein (8 mg/kg) during tumor removal (128), although there was diminished fluorescence

in gadolinium unenhanced areas (65, 131, 133). The introduction of special filters to neurosurgical operative microscopes has stimulated interest in fluorescein-guided surgery, despite the dispute over fluorescein specificity for identifying tumor tissue.

Fluorescein has been used with success to guide removal of skull base tumors such as pituitary adenomas, craniopharyngiomas, meningiomas, and schwannomas (130). da Silva et al. reported enhancement of a meningioma dural tail by fluorescein (134). However, not all brain metastases and not all tumor areas were selectively highlighted by fluorescein, and some residual non-fluorescent tumor tissue was confirmed on postoperative enhanced MRI (135, 136).

Disruption of the BBB is an essential factor determining fluorescein extravasation, and several other factors may also confound fluorescein-guided glioma surgery. Variations in dose and timing of fluorescein administration may result in a variable degree of fluorescence in line with other factors such as fluorescein extravasation in surgically perturbed tissues, brain swelling, and unknown fluorescein distribution (127). Simultaneous administration of 5-ALA and fluorescein has shown that fluorescein was visible in normal brain and not detected in some areas highlighted by PpIX fluorescence (137). Thus, the benefit of fluorescein in guiding resection of malignancies is openly questioned and actively discussed. Some researchers warned that fluorescein application outside of clinical studies is premature and emphasized possible false positive and false negative staining during surgery (135). Nonetheless, confocal endomicroscopy with fluorescein in patients with brain tumors has revealed promising results on par with frozen section pathologic examination as a means of optically interrogating tissue (121).

Other Fluorophores

Various new fluorophores and smart-targeted fluorescent probes are in different stages of preclinical development. Here, we review new fluorescent labels and activity-based and targeted bioengineered fluorescent probes.

Cresyl violet, acridine orange, and acriflavine are fluorescent dyes that were investigated for *ex vivo* use for rapid brain tumor tissue diagnosis using confocal endomicroscopy (64, 138). Methylene blue was used as a dye to color insulinomas and parathyroid glands to a blue hue. When diluted, methylene blue also acts as a 700-nm fluorophore and was studied for use in parathyroid (139) and breast tumor surgery (140). Methylene blue was administered at a dose of 1.0 mg/kg over 5 min and imaged with the Mini-Fluorescence-Assisted Resection and Exploration (FLARE)™ system. Methylene blue is excreted by the kidneys and therefore was investigated as an NIR fluorophore to visualize the ureters intraoperatively (141). NIR imaging of meningiomas and low- and high-grade gliomas topically stained with 0.05 mg/ml methylene blue provided good, but not specific, delineation of tumor cells (74).

Demeclocycline (excitation/emission peaks at 458/529 nm) is a tetracycline antibiotic with phototoxic effects. It has been used to demarcate tumor cells when used as an *ex vivo* stain on various human cancer tissues including gliomas (72, 142).

Novel cancer-selective alkylphosphocholine analog fluorophores CLR1501 (green with excitation/emission peaks 500/517 nm) and CLR1502 (NIR with excitation/emission peaks 760/778 nm) were reported to have higher tumor-to-normal brain fluorescence than 5-ALA (7.23 ± 1.63 and 9.28 ± 1.08 vs. 4.81 ± 0.92, respectively) in a mouse xenograft glioblastoma model (69). Another new fluorophore, a synthetic organic molecule CH1055 (970 Da), has a superior depth of penetration of almost 4 mm due to the higher emitted wavelength of about 1050 nm (70). High probe uptake by brain tumors in mice, the possibility of conjugation with anti-epidermal growth factor receptor (EGFR), and a high tumor-to-background ratio are reported. Combining the fluorophore with a radioactive probe is another promising surgical method for finding sentinel lymph nodes or residual tumor tissue that are deep and do not produce visual fluorescence (143), although it may not be useful for brain imaging because scintigraphy does not have the precision required for brain surgery.

Activity-Based Probes

Several new types of probes referred to as activity-based probes, "activatable" probes, fluorescence-quenched probes, or substrate-based probes were recently designed and investigated in preclinical studies (144–154) and recently reviewed in detail (155). Such probes contain a fluorophore that is "quenched" until the probe is activated (unquenched) by the given local environment. In caged-type fluorophores, some modified hydroxymethyl rhodamine green (Ac-HMRG; emission peak, 521 nm) probes are highly fluorescent when the quenching part of the probe is cleaved by its specific enzyme (57). One tumor-labeling strategy is to use quenching agents that are reconfigured by tumor-associated proteases, which are highly expressed in malignant tumor cells aiding invasion. For example, the matrix metalloproteinase-750 probe is activated by the broad range of matrix metalloproteinase family enzymes and facilitates accurate detection and complete removal of breast cancer tissue (58). One of the major drawbacks of untargeted probes, including activity-based probes, is their susceptibility to washout (active or passive removal from the site). One probe designed to be unquenched by cathepsin L and further covalently bound to protease reduced this limitation (152). Other limitations such as topical application of probes with inherent waiting time for binding, unknown biodistribution, and possibly uneven penetration await investigation. The practicality of fluorescence-guided surgery dictates that fluorescent tags should penetrate or bind to the cancer cells or remain in proximity to them for long enough to detect them.

One interesting approach is the design of activity-based probes such as caspase-sensitive nano-aggregation fluorescent probe (C-SNAF) that microaggregate after cleavage by caspase-3 and -7 by intramolecular cyclization (60). Another exciting approach is the complex activity-based probe. The construct is synthesized by combining a cell-penetrating peptide that may be activated and a nanoparticle labeled with gadolinium and Cy5 fluorophore. This complex probe is taken up by the tumor cells after matrix metalloproteinases 2 and 9 cleave the peptide and activate its cell-penetrating domain. In a murine model, gadolinium allowed *in vivo* visualization of the tumor using MRI and Cy5 allowed fluorescence detection (156). The advantages of such probes are the ability to carry several labels for various types of detection,

high contrast to the background, and applicability to a wide range of tumors. However, this model was not tested for application in brain tumors.

Molecular Targeted Probes

Molecular targeted probes are also known as affinity-based probes. Targeting molecules with colored and fluorescent dyes has revolutionized microscopy. Application of this method of visual guidance for tumor resection is under investigation in cell cultures and animals by several research groups (149–154, 157, 158). Many known tumor targets such as EGFR, HER2, CD105, VEGFR, and folate receptors have been tested for fluorescence visualization of tumors. Additionally, targeting and highlighting of normal peripheral nerves have been investigated to prevent nerve injury during surgery (159).

Molecular targeted probes may be classified based on the fluorophore, targeted molecule, and other components. The majority of the targeting molecules fall into three categories:

1. Antibodies;
2. Recombinant antibody mimicking binders:
   a. Affibodies: small (6.5-kDa) single domain engineered proteins that bind target proteins, imitating antibodies (160).
   b. Nanobodies: a single variable domain of an antibody, which is capable of specific binding (161).
3. Aptamers: short single strands of nucleic acids, which are capable of specific binding (162, 163).

The rapid growth of targeted molecular probes has occurred because of the development of new fluorophores that may be conjugated to a variety of specific targeting molecules. Numerous possible combinations, including the possibility of adding a second (or more) label, significantly increase this potential. Many fluorophores have become commercially available and are being investigated in numerous preclinical and several clinical trials. Two clinical trials of IRD 800CW-labeled probes for visualization of breast cancer and familial adenomatous polyposis have been completed (164, 165), and other trials are recruiting patients (166–172) for the use of the Cy 5.5-labeled probe (173). However, none of these trials involve brain tumors. A promising fluorescent label, zwitterionic NIR fluorophore, ZW800-1, was recently described (50). ZW800-1 has great promise as it shows a higher tumor-to-background ratio than IRDye800-CW and Cy5.5 *in vitro* and *in vivo* (17.2 vs. 5.1 and 2.7, respectively) (50, 51).

The major drawbacks of targeted molecular probes are uneven passive distribution and non-specific binding. Dual-labeled probes were designed to address this limitation (174). This approach uses targeted probes that bind, and untargeted probes that do not bind, to the target. Differentiation in the fluorescence intensity allows a quantitative assessment of the binding potential of the probe (175, 176). Another drawback is that tumor regions with an undisrupted BBB can decrease the accumulation of the large probes. Sexton et al. addressed this problem by designing a small targeted fluorescent affibody peptide (about 7 kDa vs. 150 kDa for a full antibody) and demonstrated in a mouse xenograft GBM model almost two times increased fluorescence at the tumor edge compared to the full anti-EGFR antibody probe (42). Gong et al. showed that both anti-EGFR-specific affibody and the therapeutic antibody panitumumab labeled with IRDye 800CW could be used as imaging agents for both wild-type EGFR and EGFRvIII glioblastoma cells in cell culture studies (177).

In a 2014 report, Ghosh et al. described a novel targeted probe construct containing a single-walled carbon nanotube as a fluorescent tag (52). It consists of an M13 bacteriophage as a scaffold, a targeting protein, and the fluorescent nanotube. The single-walled carbon nanotubes have emission within the NIR range (950–1400 nm), resulting in less optical scattering and deeper tissue penetration. This setup is also less susceptible to photobleaching or quenching effects. The construct was stable and showed 10 times more selective fluorescent staining of ovarian tumor cells than the same construct without the targeting peptide. The nanotube fluorescence intensity ratio relative to the background (5.5 ± 1.2) was superior to the same construct labeled with other NIR AlexaFluor750 dye (3.1 ± 0.42) or FITC (0.96 ± 0.10). However, this study did not assess the possible penetration of the probe into the brain (52).

A targeted probe consisting of fluorescent gold nanoparticles conjugated with diatrizoic acid and AS1411 aptamer (53) has an absorption band of 300–400 nm and orange–red emission (maximum 620 nm), which can be observed by the naked eye. This probe showed specific binding to tumor cells due to the AS1411 aptamer, which targets nucleolin. The probe allowed X-ray visualization due to the high electron density of the gold nanoparticles. A novel lymphoma-specific fluorescent (Alex488) switchable TD05 aptamer in a human brain tumor xenograft model has been described (54). This probe rapidly and precisely identified human B cell lymphoma in biopsies. Such a system would be useful for rapidly discriminating non-operative CNS B-cell lymphoma from malignant glioma based on the biopsy.

Another agent, BLZ-100, a tumor ligand chlorotoxin conjugated to ICG, was shown to have high affinity to human gliomas in mice (55). Chlorotoxin was extensively studied in preclinical *in vivo* studies as a conjugate with Cy 5.5 (178) and IRDye 800CW (179). Chlorotoxin is a new drug that binds to chloride channel–MMP-2 membrane complexes. It reduced the invasiveness of glioma cells (180), inhibited glioma cell growth and metastasis, and accelerated tumor apoptosis (181). The advantage of the probe is its small size and ability to penetrate the BBB.

Another 5-carboxyfluorescein-labeled fluorescent probe consisting of tLyP-1 small peptide targeting neuropilin receptors was recently described. Neuropilin receptors are co-receptors for vascular endothelial growth factor and play a role in tumor-mediated angiogenesis. They are overexpressed in most gliomas. The probe has selective uptake and may have advantages over the CTX-Cy5.5 probe due to its small size. However, the fluorescein labeling was less than ideal and could be exchanged for a more intense fluorophore for use in intraoperative imaging (56).

IRDye800CW-labeled anti-EGFR nanobody 7D12 was compared to the full antibody cetuximab and showed better penetration and distribution of the nanobody probe *in vivo* in a preclinical study (40). Another study of the same nanobody for orthotopic tongue tumors showed significantly higher tumor to background fluorescence (2.00 ± 0.34 in the FLARE imaging

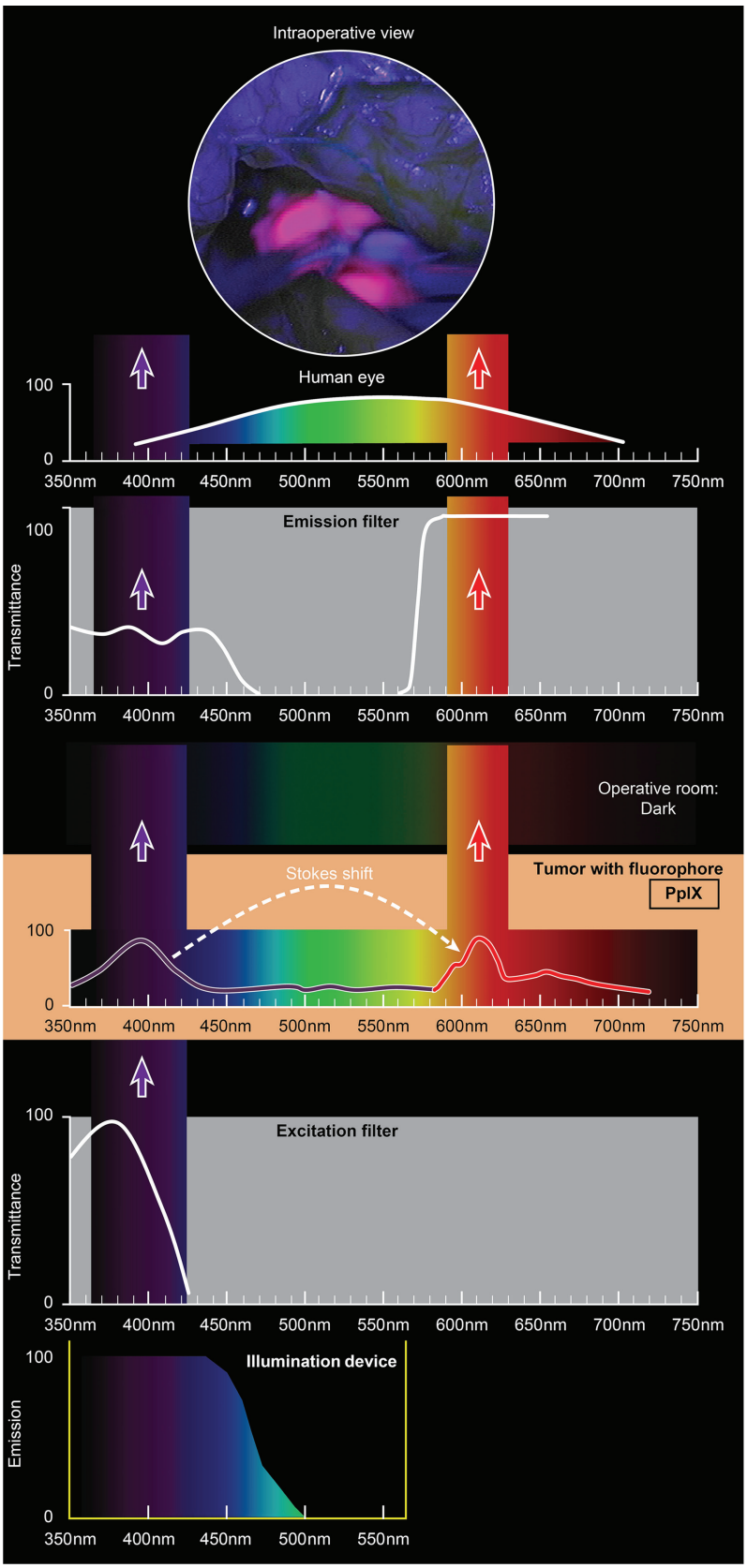

FIGURE 2 | Continued

FIGURE 2 | Continued
Schematic view of the concept of PpIX-guided tumor visualization using a wide-field operative microscope with appropriate filters. Wavelength scales are in the same position in the figure. The illumination device emits light in the wavelength band less than 470 nm. The excitation filter then transmits light with the peak of about 405 nm. PpIX, which is accumulated in the tumor cells, absorbs photons in the spectrum band around 405 nm and then emits photons of lower energy at a wavelength of about 630 nm. The blue light from the illumination device and the emitted red fluorescence band are observed through the operative microscope optics equipped with an emission (observation) filter. This filter has a cut-off transmittance at about 450 nm and cut-on transmittance at about 570 nm. The two bands of light observed fall into the visible spectrum (with the naked eye) and are perceived as a violet–blue background and "pink-to-red" fluorescence. The light in between those two bands is blocked; therefore green, yellow, and orange colors are not visible. PpIX, protoporphyrin IX. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

system) than in the group with the same non-targeted fluorescent probe (41).

Polyacrylamide nanoparticles have been coated with the F3 protein that binds to nucleolin and loaded with methylene blue, Coomassie blue, or ICG. F3-coated constructs increased the color change in glioma cells *in vitro* (61). Magnetic NH_2-cross-linked iron oxide nanoparticles labeled with Cy5.5 (32 nm in diameter) produced clear tumor border demarcation and co-localization on MRI imaging in a rat gliosarcoma model (62). As a non-targeted construct, it produced demarcation mainly due to BBB disruption and was designed as a magneto-optical probe. Additionally, iron oxide particles are eliminated by reticuloendothelial cell endocytosis (182), suggesting that their elimination is more predictable than that of other nanoprobes. However, this magnetic nanoparticle design is less attractive than that of targeted probes, which aim to increase the accuracy of tumor cell visualization *in vivo*.

Summarizing fluorophore use in neurosurgery, 5-ALA-guided brain tumor surgery may improve the gross tumor resection rate and is approved in Europe but is available only in clinical trials in the US. Fluorescein-guided resection has emerged as an alternative due to its safety profile, although fluorescein is not tumor cell-specific. ICG shows promise for vascular tumors, such as hemangioblastomas, but may also have the potential to define malignant gliomas. Many new targeted and activatable fluorescent probes are awaiting full assessment to be used in clinical studies. Although molecular targeting probes are attractive and technologically advanced, their benefit and cost compared to already existing 5-ALA and fluorescein for fluorescence-guided resection are yet to be proven. Assessing the advantages of the many probes being designed is a difficult and time-consuming task considering the emerging improved, quantitative fluorescent detection methods. Combining the probes with molecules for secondary goals such as chemotherapy, photosensitization, and others may be advantageous.

INSTRUMENTATION FOR FLUORESCENCE-GUIDED RESECTION

Several different technologies are applied in fluorescence-guided resection of brain tumors. These technologies are classified into several categories (183–187):

1. Wide-field fluorescence imaging:
   a. Commercial operative microscopes with built-in fluorescence channels;
   b. Custom modified surgical microscopes;
   c. Surgical endoscopes equipped with fluorescence modules;
   d. Non-microscope fluorescent excitation systems with emission detecting devices.
2. Quantitative fluorescence systems:
   a. Spectroscopic tools for imaging one region at a time;
   b. Laboratory grade stand-alone systems;
   c. Combination systems that integrate fluorescence with spatial imaging.
3. Intraoperative high-resolution endomicroscopy.

Wide-Field Fluorescence Imaging

Wide-field fluorescence imaging refers to non-microscopic, endoscopic, or microsurgery in which full fields of view are seen continuously through the eyepieces or on the screen during image acquisition at a rapid frame rate with a digital detector array (CMOS or CCD cameras) (184). Several instrument solutions exist for wild-field fluorescence imaging. By definition, such systems have a magnification of 5× to 40× and resolution of less than cellular level. Fluorescence-guided surgery is undergoing revitalization as advancements in optics are allowing improved visual perception of fluorescence. Numerous neurosurgical studies highlight the benefits of wide-field fluorescence-guided glial tumor resection, mainly to increase the GTR rate and progression-free survival (2, 111, 188). Some studies even showed increased overall survival (189).

Instruments

The use of custom operative microscopes with modules to measure fluorescein (128) and PpIX (190) fluorescence was reported in 1998. There are three fluorescence detection modules, for use on commercially available operative microscopes for 5-ALA- (**Figure 2**), ICG- (**Figure 3**), and fluorescein-guided (**Figure 4**) tumor resection in the brain. The modules consist of three components: (1) a set of optical filters for selective wavelength separation, (2) a broad-spectrum illumination device, and (3) optional CCD cameras for detection of visible and invisible (NIR) light (**Figure 3**). New optical filters for selection of bandpass width and blocking intensity are now available due to progress in optical engineering. A combination of these new filters permits the best possible fluorescence intensity and contrast to the background ratio. One example is the new Yellow 560 Module for the Zeiss operative microscope that reintroduced fluorescein in brain tumor surgery. A set of filters facilitates excitation of the fluorescein with the maximum intensity while preserving illumination of the background with another visible spectral band of lower intensity (**Figure 4**). The resulting bright yellow fluorescence of tumors is observed and contrasted with the natural colored background (191).

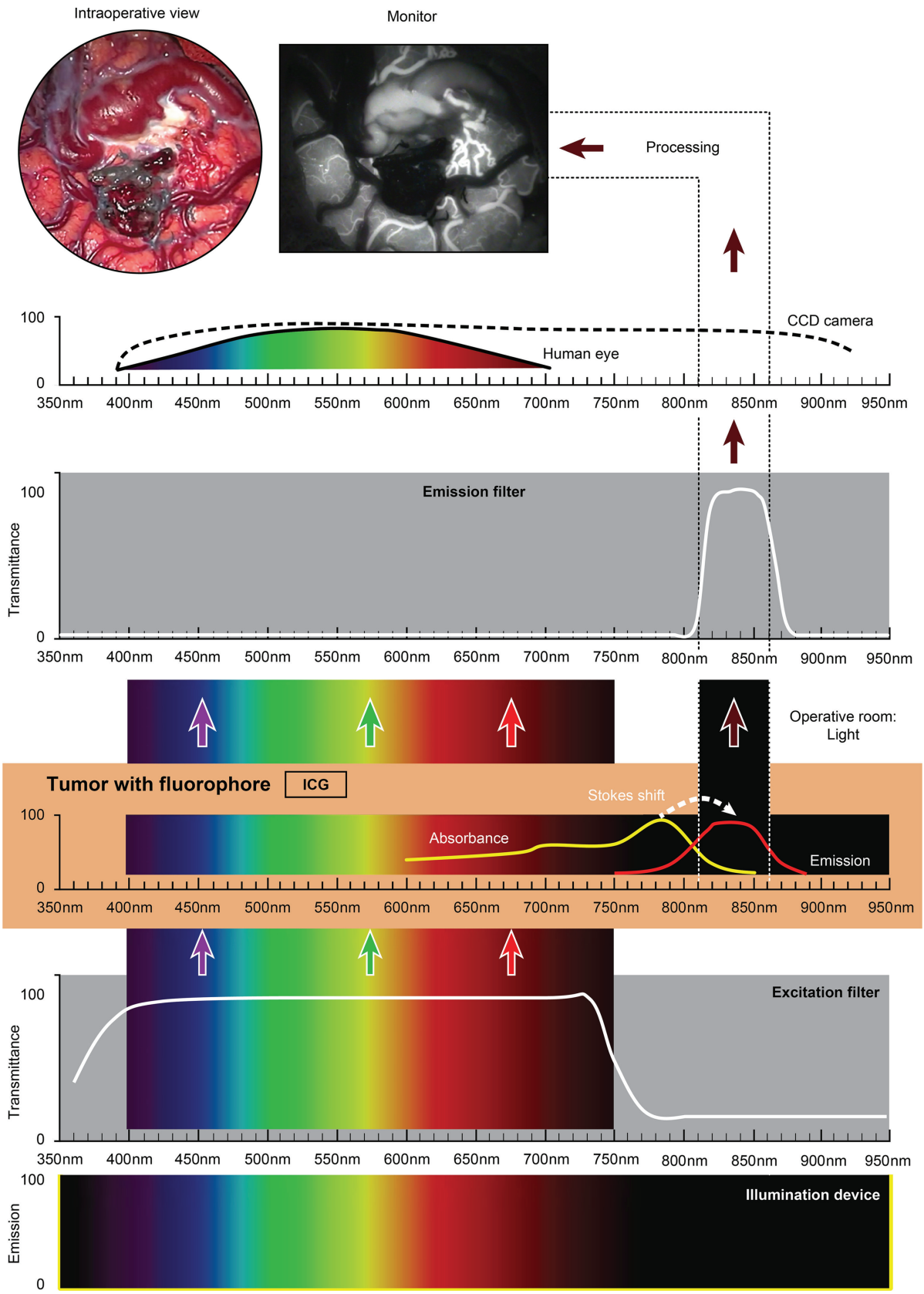

FIGURE 3 | Continued

FIGURE 3 | Continued
Schematic view of the concept of ICG fluorescence visualization using a wide-field surgical microscope with appropriate filters. Wavelength scales are in the same position in the figure. The illumination device (xenon lamp) emits light in a wide range of wavelengths. The excitation filter cuts off the light longer than about 750 nm. ICG present in the tissue (vessels) absorbs photons in the available spectrum band below 750 nm and then emits photons in a NIR spectrum around 820 nm, invisible to the naked eye. The emission filter then transmits this NIR light to the CCD camera and blocks the light with other wavelengths. The CCD camera records the images during the desired period. After image processing, the resultant surgical picture is displayed on the monitor of the neurosurgical microscope in the grayscale as a short movie fragment. ICG, indocyanine green. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

Fluorophores that emit in the NIR spectrum require CCD cameras or other detection technologies. An ICG (NIR) module does not require an operative microscope *per se* because the resultant fluorescence is not perceived by the eye and is observed on an ancillary screen. The fact that NIR probes are in abundance and are either commercially available or awaiting approval by the FDA (ICG, Cy 5.5, IRDye800-CW, and BLZ-100) has stimulated the field of computer engineering to develop wide-field systems that overlay an NIR signal on the surgical field of view. Such an overlay is desirable in real time and with measurable specificity. Non-microscopic, mobile, wide-field video imaging systems for open, laparoscopic, thoracoscopic, and robotic surgery are in development and clinical trials (192, 193).

Work in intraoperative NIR imaging technologies in neurosurgery shows potential for advantageous applications. A novel proof-of-concept NIR imaging system consists of a narrow-band laser at 785 nm, a notch filter, and a standard 2-CCD camera for wide-field visualization. This system has been tested with an ICG-conjugated targeted BLZ-100 probe in a murine brain tumor model (55).

A new endoscopic technology, scanning fiber endoscopy (194), also holds great promise due to its ultrathin probe and increased resolution. A color image is acquired by combining red, blue, and green laser lights through a spiral actuated optical fiber. Laser induced fluorescent imaging of 5-ALA-induced PpIX fluorescence on tumor cell phantoms (195) and a murine tumor model (work in progress) allowed detection of the fluorescence with greater sensitivity than through the operative microscope. We found this optical imaging technology very convenient for potential intraoperative use due to its small size with a field of view of 2–30 mm and high spatial resolution of up to 15 μm.

A concept for a low-cost fluorescein detection system for glioma surgery (196) consists of a xenon light source, fiber optic light cable, a set of glass interference filters (neutral, 490 nm, 465 nm), and yellow photographic filters for oculars or UV yellow glasses. In clinical trials, the system showed great potential due to its low cost, especially beneficial for low-income countries, although limitations of the custom hardware and fluorescein usefulness in glioma surgery itself still require confirmation (127).

Limitations of wide-field visualization technologies in fluorescein-guided surgery are similar to those of 5-ALA studies. Wide-field, fluorescence-guided surgery limitations include (184) ambiguity at the margins where fluorescence intensity decays and difficulty of visualization on the sides of a resection cavity and shaded areas in the surgical wound. Further limitations include fluorescence absorbance by blood and tissue layers (197), insufficient fluorescence intensity in >95% of low-grade gliomas (107, 198, 199), and lack of quantitative assessment of fluorescence intensity. In PpIX fluorescence visualization, PpIX is quantitatively related at the microscopic level to increasing malignancy in both low- and high-grade gliomas (117). Such works emphasize the limitations of fluorescence detection by the current wide-field technologies at low concentrations of the fluorophore or when few cells are labeled. New approaches that increase the sensitivity of visualization systems include the use of a quantitative spectrophotometer, an additional camera for quantitative image processing (200), and new endoscopic and confocal endomicroscopy probes.

Intraoperative Quantification of Fluorescence

Absorption, scatter, anisotropy, and autofluorescence of the tumor and background tissue play important roles in the detection of the fluorophore signal, especially at low signal levels. Thus corrections for the optical properties of the tissues provide qualitative information about the fluorescence intensity in the area of interest (193). For this reason, researchers have studied the utility of spectrophotometric quantification of PpIX emission spectra. Visual light spectroscopy for the calculation of the background fluorescence intensity ratio has been investigated in postoperative astrocytoma samples (113). The fluorescence intensity correlated with the MIB-1 proliferation index, a prognostic indicator for tumor progression.

A spectrally resolved quantitative fluorescence imaging system with submillimeter spatial resolution (214–125 μm) has been integrated with a conventional operative microscope (200). This system provides a colored digital overlay of the quantitative fluorescence intensity map over the surgical field of view. A pilot study of human glioblastoma surgery showed that the system showed a signal from histologically confirmed residual tumor tissue when the standard wide-field BLUE400 filter image was negative (200). Quantitative PpIX detection elevated the diagnostic sensitivity of low-grade gliomas (67% in 12 cases) to the level of qualitative wide-field detection of high-grade gliomas (115). Improved sensitivity with PpIX fluorescence was confirmed using an *ex vivo* animal tumor model with a similar system that included a CCD camera attached to the operative microscope (201). Quantification using the intraoperative contact optical probe demonstrated increased accuracy in detecting neoplastic meningioma tissue with a 90% diagnostic accuracy for differentiating tumor from the normal dura in 10 grade 1 meningiomas (108).

Intraoperative Confocal Endomicroscopy

The rationale for high-resolution intraoperative imaging is the inherent limitations of wide-field fluorescence microscopy

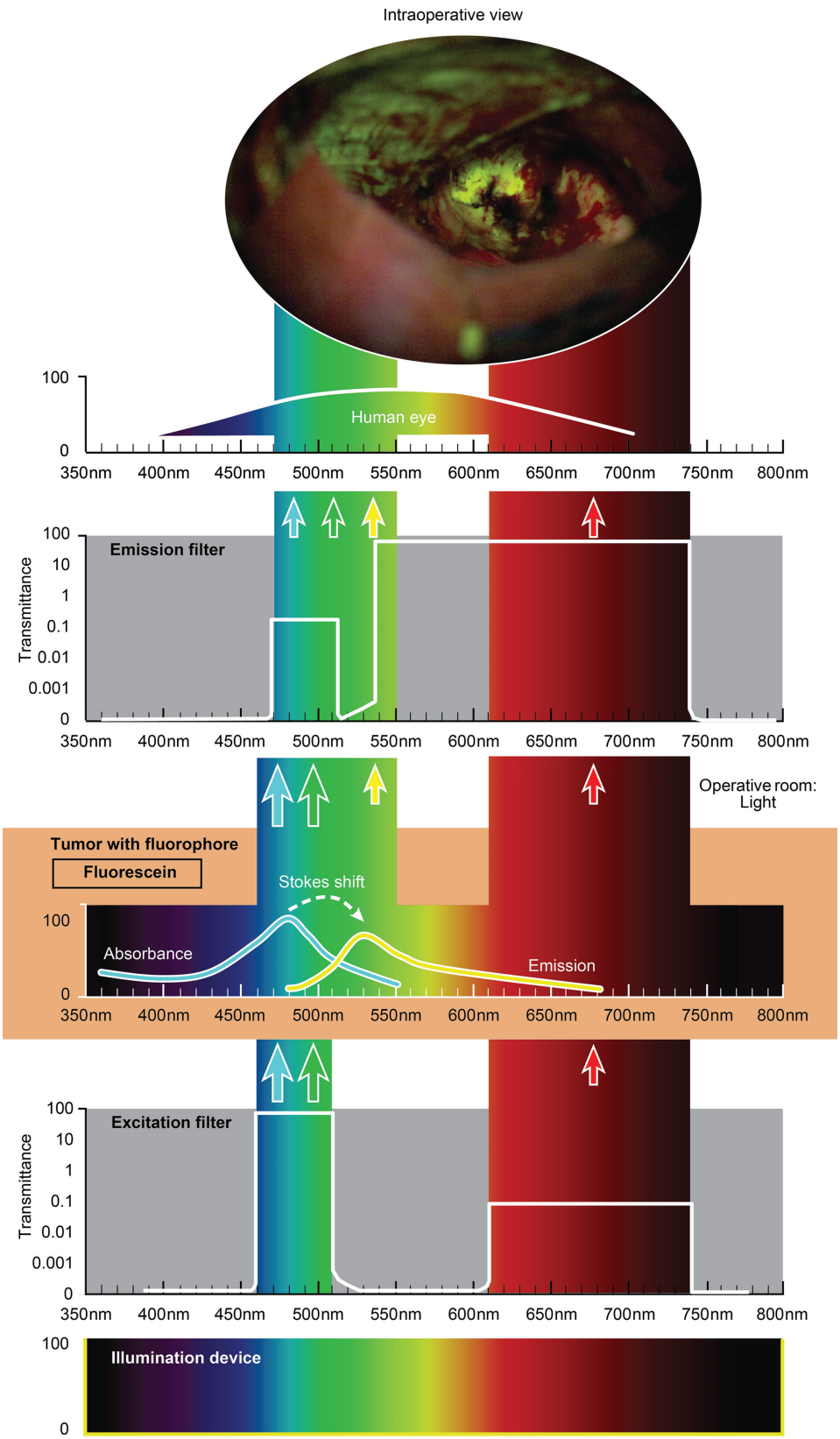

FIGURE 4 | Continued

FIGURE 4 | Continued
Schematic view of the concept of fluorescein-guided tumor visualization using a wide-field operative microscope with appropriate filters (https://www.google.ch/patents/US8730601). Wavelength scales are in the same position in the figure. The illumination device (xenon lamp) emits light in a broad range of wavelengths. The excitation filter then transmits the light as narrow bands at about 450–520 nm and about 600–750 nm. The first (blue–green) transmittance band is significantly more intense (see log scale on the side of the filters in the figure) than the second (red) band of light. Fluorescein, which is accumulated in the tumor tissue, absorbs photons in the spectrum band around 485 nm (high-intensity band) and then emits photons with a wavelength around 514 nm (yellow) with a lower energy (new low-intensity yellow band). Blue–green and red bands of light from the illumination device, as well as the new yellow (around 514 nm) fluorescence band, are observed through the operative microscope optics equipped with an emission (observation) filter. This emission filter has a transmittance in two bands: first in the range of 475–515 nm with significantly lower transmittance (see log scale in the figure) and the second in the range of 530–700 nm with the maximum transmittance. The three bands of light, the blue–green emission band, red band, and emitted yellow band, all fall into the naked-eye-visible spectrum for observation. The transmittance of all filters together results in the uniform intensity of all bands, with a higher possible intensity of emitted yellow light. A portion of the spectrum between the bands could be blocked by the filters, but the remaining three primary color bands allow the surgeon to see the intraoperative picture with almost the full spectrum of colors. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

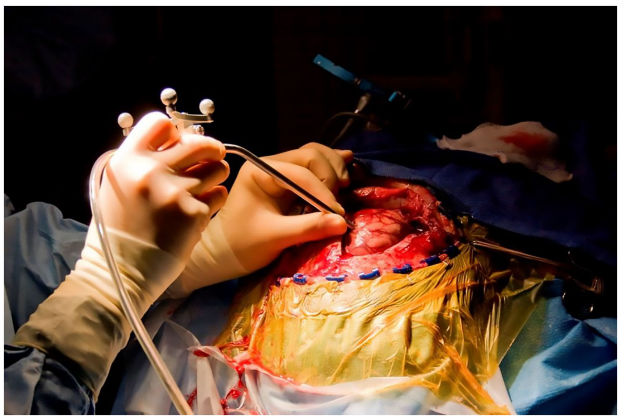

FIGURE 5 | Intraoperative use of a hand-held confocal endomicroscopy probe co-registered with a StealthStation neuronavigation system during brain tumor surgery. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

and the desire for precise tissue visualization at the cellular level. Fiber optic confocal microscopy was invented in 1988 (202), commercialized in 1994, and the first results of use in neurosurgery were published in 2010 (122). Such systems consist of a miniature handheld probe and movable workstation with an LCD screen (**Figure 5**). Excitation and emission light is transmitted through a single optic fiber. The system provides non-invasive real-time imaging through optical sectioning at a known depth. Most importantly, it appears to provide real-time images for histopathological analysis without the laborious process of tissue preparation, although this development is still being validated (**Figure 6**) (203). Two commercially available systems include the Optiscan FIVE 1[1] and Cellvizio.[2] Both systems have been reviewed for neurosurgical applications (186). The Optiscan has a 475 μm × 475 μm field of view with a focal plane to a depth of 250 μm, and the Cellvizio has nine objectives covering fields of view ranging from 300 to 600 μm and 15 to 70 μm optical sectioning depth. The Cellvizio and Optiscan currently use a 488-nm excitation light, and the Cellvizio also has a 660-nm single-band excitation light. The first feasibility study of intraoperative confocal endomicroscopy was reported for a variety of brain tumor pathologies in 33 patients with intravenous fluorescein injection for tumor visualization (204). Intraoperative imaging permitted the neuropathologist to make a diagnosis, but this diagnosis was not compared with standard histological staining for accuracy (205). Another study using confocal endomicroscopy enabled the correct diagnoses based on intraoperative images (fluorescein) in 26/28 of cases (206). A clinical series of 74 patients who underwent intraoperative confocal endomicroscopy showed diagnostic specificity and sensitivity for gliomas of 94 and 91%, respectively, compared to the interpretation of frozen section and permanent histologic diagnoses (121). Ongoing studies of this technology aim to improve these indices.

Confocal endomicroscopy may also be employed as a rapid diagnostic tool for biopsy specimens in *ex vivo* tissue analysis within the operating room. The utility of fluorescein, 5-ALA, acridine orange (stains DNA/RNA/lysosome), acriflavine (topical application, stains membrane/DNA), cresyl violet (topical application, stains ER/cytoplasm), and sulforhodamine 101 (topical application, stains glial cell cytoplasm) for visualization of tumor cells was demonstrated with the Optiscan 5.1 system (64, 122).

Although 5-ALA visualization was not optimal due to the limitation of the probe excitation profile, the other fluorescent stains clearly showed the histological features of the tumor cells and margins in a murine brain tumor model. Normal morphology in various brain regions was also clearly discernible in a large animal model (pig) using confocal endomicroscopy with topical acridine orange. Selective detection of ICG in a murine glioblastoma model was also shown using a clinical-grade, NIR confocal endomicroscopic system (64).

The initial experience with the Cellvizio confocal endomicroscope for immediate *ex vivo* imaging of human intracranial tumors after fluorescein-guided resection combined with topical acriflavine staining shows practical potential (138). Although rapid histopathological diagnoses were possible for a wide variety of brain neoplasms, this application is pending comparison with standard histological staining in validation assessments. Clinical trials of the Cellvizio system for brain tumors are underway in Europe to assess the neuropathological diagnostic agreement and completeness of tumor removal (207–209). Furthermore, the confocal endomicroscope was successfully used to visualize

[1] www.optiscan.com
[2] www.maunakeatech.com

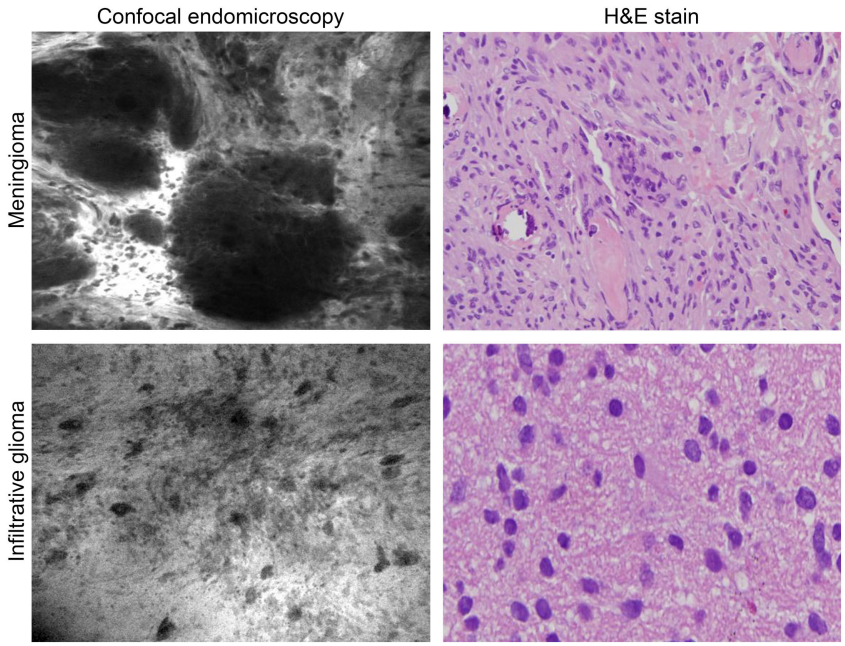

FIGURE 6 | Intraoperative images of meningioma and glioma after intravenous fluorescein sodium injection taken with the confocal endomicroscopy probe and shown with corresponding histopathological pictures. Used with permission from Barrow Neurological Institute, Phoenix, AZ, USA.

targeted probes consisting of two tyrosinase-related protein antibodies labeled with Alexa Fluor 488 fluorescent dye (210). In a murine brain tumor model, these probes correctly identified tumor cells with high specificity, confirming in principle that the targeted probes could be used along with the confocal endomicroscope to increase the extent of resection in a variety of brain tumors (211).

The inherent limitations of the intraoperative confocal endomicroscope are a narrow field of view, the image appearing on a separate display, and the necessity of non-standard image analysis and interpretation, along with limited resolution, laser excitation spectrum, and corresponding detection power. Several computer image processing methods have been proposed to improve the diagnostic value of these small-field-of-view systems. For example, an image stitching technique has been applied to create panoramic wide-field images (212–214). Multiple histogram operations provide image contrast enhancement (215). Image quality and its diagnostic value, as well as the surgeon's knowledge of histopathology, are important factors in the practical application of intraoperative confocal endomicroscopy because the resultant images differ from the stained histopathological slides and require additional training for interpretation. Additionally, the probe should be in a stable position during image acquisition, although the surgeon may acquire good images in the free-hand mode with practice.

Approval of targeted fluorescent probes for clinical use will likely stimulate the refinement of confocal endomicroscopy and its broad clinical use in neurosurgery and tumor pathology. These two technologies are complimentary and allow tailored, tumor-specific resections for personalized patient treatment and, certainly, precision tumor surgery. The ability to interrogate the tumor border optically is of significant advantage in the acquisition of selective biopsies of higher diagnostic yield. Such a situation could improve the neurosurgery–neuropathology workflow for increased efficiency.

FUTURE DIRECTIONS

Several studies in optics, bioengineering, biotechnology, experimental oncology, and biochemistry have advanced the field of fluorescence-guided surgery in the preclinical arena (216). Because many fluorophores emit light in the NIR band, outside of the visible spectrum, improvements in overlay imaging technology are expected. Pharmacological and toxicological restrictions stimulate the application of "microdoses" of a fluorophore, which, in turn, may allow for approval for clinical use. Moreover, the fluorophores in use still require more sensitive detectors. The need for these features drives the focus of future system developments in fluorescence-guided surgical imaging and overlay techniques (216).

Pulsed-light imaging is a technology that exploits pulsed excitation light and time-gated detection. It allows fluorescence imaging under normal operating room light conditions with high detection sensitivity (217). This technology is more sensitive to lower concentrations of PpIX than surgical microscopy (217).

A novel type of fluorophore, quantum dots, appears to be a relevant nanotechnology for fluorescence. The quantum dot is a 5- to 20-nm nanocrystal made from a semiconductor material that acts like a traditional fluorophore but works by a different

mechanism. The emitted wavelength of the quantum dot depends on the size of the crystal. Fluorescent probes with the desired emission band may be designed. The main advantages of the quantum dot are much longer excitation life leading to photostability. The color of the emitted light may be tuned to the size of the probe. However, the safety of quantum dots is significant because larger quantum dots may not be well cleared, and the long-term effects of accumulation are unknown (218, 219). Quantum dots conjugated with transferrin have been used as a fluorescent probe to target transferrin receptors in glioblastoma cells (220).

Another important parameter of future fluorophore probes is the size of the molecule, in which small targeted molecules, even with lower affinity, show better delineation of tumor boundaries most likely due to crossing the BBB more easily (42). In another approach, the BBB is reversely disrupted to allow more intense binding to the tumor tissue. Several methods to bypass the BBB were developed (221, 222) to enhance targeted fluorescent probe binding to brain tumor tissue and were tested in animals (63).

Some other emerging technologies may help in differentiating normal tissue from brain tumor tissue. For example, optical coherence tomography does not require any targeting agent. The technology utilizes differences in the optical signatures of the tissues to differentiate brain tumor from normal tissue, as shown in an animal study (223).

Intraoperative fluorescence imaging is capable of maximizing tumor tissue resection, providing rapid histopathological diagnoses based on innovative fluorophore probes and tools for intraoperative visualization. What is clear is that we sit on the threshold of technology that will enable neurosurgeons to see tumor cells in groups or individually in real time, which will allow tailoring or personalization of neurosurgery in terms of tumor resection. The term "theranostics" was coined to define ongoing efforts to develop precise, specific, individualized diagnostics and therapeutics for various diseases. For neurosurgery, we are adapting true precision modalities or biomarker techniques into diagnosis, including the imaging techniques described here, and this facilitates precise approaches to surgery. Cell-specific visualization will make possible the optimal surgical treatment of invading tumors such as gliomas that are composed of heterogeneous tissue with various genetic and metabolic characteristics. Therefore, the previously impossible may become routinely possible. If invading tumor cells are discovered in eloquent cortex, which is not normally resected, the neurosurgeon might be able to proceed on a cell-by-cell basis, targeting only tumor cells. Improved imaging technologies will bring about novel techniques to target or remove tissue or even individual cells. The advantages of such techniques are better surgical outcomes as nearly "cell-by-cell" or precision surgery becomes possible. Such surgical advancements will undoubtedly come with additional responsibilities, decisions, and challenges to be faced by both the neurosurgeon and patient.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work.

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Advancing Treatment of Pituitary Adenomas through Targeted Molecular Therapies: The Acromegaly and Cushing Disease Paradigms

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The current treatment of pituitary adenomas requires a balance of conservative management, surgical resection, and in select tumor types, molecular therapy. Acromegaly treatment is an evolving field where our understanding of molecular targets and drug therapies has improved treatment options for patients with excess growth hormone levels. We highlight the use of molecular therapies in this disease process and advances in this field, which may represent a paradigm shift for the future of pituitary adenoma treatment.

Keywords: acromegaly, Cushing disease, endonasal, pituitary adenoma, transsphenoidal

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INTRODUCTION

Pituitary adenomas make up more than 90% of all pituitary tumors and are the second most commonly diagnosed non-malignant brain tumors (1). The clinical presentation of patients with pituitary adenomas is highly variable and often depends on the endocrinologic function of the tumor, the size of the tumor, or a combination of both. Given the increased use of neuroimaging studies over the past decade, a significant number of pituitary lesions are incidentally found, and the prevalence of pituitary tumors in the general population is estimated to be around 17% (2). The heterogeneity of clinical presentations combined with the relatively high prevalence of "incidentalomas" poses a diagnostic challenge to providers treating these patients, and multidisciplinary teams consisting of endocrinologists, neuro-ophthalmologists, and neurosurgeons have proven essential for delivering the highest quality of care.

Distinguishing a functional (i.e., hormone-secreting) from a non-functional adenoma is crucial for guiding subsequent treatment strategies. Although surgical resection remains the mainstay of therapy for macroadenomas causing compression of neurovascular structures, as well as for many functional microadenomas, pharmacotherapy can play a crucial role in adenoma treatment. Recent advances in genetic and molecular analysis of pituitary adenomas have provided new insights into the growth patterns and secretory functions of these tumors and have allowed for a more precise characterization of individual adenomas. These advances have led to the development of targeted molecular therapies for several subtypes of pituitary adenoma and the development of a

Abbreviations: ACTH, adrenocorticotropic hormone; DR, dopamine receptor; GH, growth hormone; IGF, insulin-like growth factor; OGTT, oral glucose tolerance test.

"personalized" approach to pharmacotherapy for some patients with adenomas.

Acromegaly is one pituitary disease where recent and ongoing research has changed the standard treatment paradigm. Although pharmacotherapy has not replaced surgical resection as the mainstay of treatment, exciting advances in targeted molecular therapies have developed in recent years. We can currently implement individualized treatment options for patients with acromegaly, and the potential of this strategy is immense as our understanding of the molecular pathology of these tumors progresses. We believe that the combined surgical, targeted pharmacotherapeutic, and radiosurgical approach that is employed in acromegaly represents a paradigm that will continue to improve the treatment of not only growth hormone (GH)-secreting adenomas but also other functional and nonfunctional adenomas.

In this review, we highlight the current literature on the diagnosis and treatment of acromegaly with an emphasis on current targeted molecular therapies. We also review emerging treatment paradigms for Cushing disease that parallel this approach, and we discuss future directions for this exciting field.

GROWTH HORMONE-SECRETING ADENOMAS

Overview

Growth hormone-secreting pituitary adenomas manifest as the clinical syndrome acromegaly, which is a chronic disorder that results in acral overgrowth, cardiovascular disease, insulin resistance, arthritis, and sleep apnea, among other conditions (3). In children harboring a GH-secreting adenoma, excess GH production before closure of the epiphyseal plates leads to gigantism. Given the widespread effects of GH overproduction, as well as the often indolent physiologic changes in an individual patient, the diagnosis of acromegaly is often delayed. If untreated, acromegaly results in significant morbidity and increased rates of mortality for these patients (4).

The current diagnosis of acromegaly is dependent upon both the oral glucose tolerance test (OGTT) and serum levels of insulin-like growth factor-1 (IGF-1). A decline in GH production after an oral glucose load is present in normal patients, and this decrease is diminished in patients with acromegaly; detailed criteria have been established for utilizing this diagnostic tool to help identify patients with acromegaly (5). Additionally, serum IGF-1 levels are elevated in patients with acromegaly because of increased production from the liver. Circulating GH binds GH receptors on hepatocytes and activates a signaling cascade, resulting in increased in *IGF1* transcription, translation, and IGF-1 secretion (3). IGF-1 levels adequate for diagnosis are dependent on sex and age, and established values have been outlined (5–9). IGF-1 exerts effects on numerous target tissues throughout the body, and stimulation *via* this growth factor contributes to the increased morbidity and mortality encountered in patients with acromegaly (4).

Surgical resection is the mainstay of treatment for acromegaly caused by a GH-secreting adenoma. However, not all patients are candidates for surgery, and not all adenomas are amenable to complete resection. Since surgical treatment is not always an option, a large role for both pharmacotherapy and stereotactic radiosurgery has developed in this population of patients. An improved molecular understanding of pituitary adenomas has advanced pharmacologic options for acromegaly patients, and we hypothesize that this is the start of a paradigm shift in the treatment of acromegaly. In this article, we review the literature on surgical success rates and targeted molecular therapies in acromegaly. Radiosurgery success rates and expert opinions on the implementation of this strategy have been reviewed elsewhere (10–12).

Surgical Treatment

Surgical success rates in the literature vary widely depending on tumor size, the degree of invasion, surgeon experience, adjuvant therapies, and definition of success (i.e., laboratory values defined as curative). When examining the success of surgery alone, the largest series as of 2016 examined 688 patients with acromegaly treated at a single center (13). Criteria required to define a cure included normalization of basal GH to <2.5 ng/L, suppression of GH to <1 ng/L during the OGTT, and IGF-I normal for age and sex, which are the current standard definitions for biochemical remission. The overall remission rate for all tumors treated *via* the transsphenoidal approach was 57.3% at the 3-month follow-up in this study. Of note, success varied widely based on tumor size and invasion characteristics, with 75.3% of microadenomas surgically in remission versus 41.5% of macroadenomas with parasellar or sphenoidal extension. In this series, only two patients with surgical remission developed recurrent acromegaly within a mean follow-up of 10 or more years.

Numerous smaller series in the literature largely support these values, with surgical remission rates ranging to 60% (14–20). Reported recurrence rates in the literature to date vary widely due to the different criteria for biochemical remission and varying years of follow-up; recurrence rates ranging from 0.4 to 19% (7, 13, 17, 21–23) are reported, with one 2012 meta-analysis citing a mean 6% recurrence rate within 10 years (20).

Targeted Molecular Therapies

For the subset of acromegaly patients without biochemical remission after surgery, or for those patients who are unable or unwilling to undergo surgery, pharmacotherapy takes on an essential role. Pharmacotherapy for acromegaly was first used in the 1970s, and our understanding of GH-secreting adenomas has significantly advanced since that time (24). With a better understanding of the molecular biology of GH-secreting cells, the introduction of more targeted therapies has been possible, and we can now better tailor pharmacotherapy regimens for individual patients with acromegaly.

The population of cells within the anterior pituitary gland that secrete GH were identified in the early twentieth century in association with acromegaly and became known as somatotroph cells (24). Like other cell types of the anterior pituitary gland, somatotroph cells typically remain under tight physiologic control through positive and negative feedback from the hypothalamus.

Somatotroph cells express two classes of receptors that mediate negative feedback – dopamine receptors (DRs) and somatostatin receptors. Both pathways have been successfully targeted pharmacologically and with a resultant decrease in GH secretion in patients with acromegaly. A third pathway, the GH receptor pathway, has also been successfully targeted for acromegaly pharmacotherapy. All three pathways are reviewed here.

Dopamine receptors are encoded by five separate genes (*DRD1–DRD5*). However, *DRD2* and *DRD4* are the two genes predominantly expressed in the normal pituitary gland (25). *DRD2* is strongly expressed in both somatotrophs and lactotrophs, and binding of dopamine (or dopamine agonist medications) to DRD2 triggers an inhibitory signaling cascade to decrease prolactin secretion. DRs were first targeted in the 1970s with the dopamine agonist bromocriptine; however, the dopamine agonist cabergoline has since proven to be more effective due to its increased DR2 selectivity and longer half life (26, 27). Interestingly, DR2 expression levels in somatotrophs are correlated with dopamine agonist response rates both *in vitro* and *in vivo*, and analysis of prolactin and DR2 expression patterns within GH-secreting adenomas has been proposed as a guide for pharmacotherapy strategies in acromegaly patients (28–31). Furthermore, dopamine agonists are recommended for adenomas that secrete both GH and prolactin if pharmacotherapy is needed after surgery because both expression pathways are targeted by these agents (32).

Somatostatin receptors are also encoded by five separate genes (*SSTR1–SSTR5*), and the *SSTR2* and *SSTR5* subtypes make up 90–95% of receptor expression in GH-secreting adenomas (33). *SSTR* expression is found within normal pituitary cells including corticotrophs and lactotrophs, and binding of somatostatin to SSTRs triggers a G-protein-mediated signal cascade that inhibits secretory function in these cells. The two standard somatostatin analogs in use today are octreotide and lanreotide, which activate this signaling pathway to inhibit hormone production in functional adenomas. There is significant heterogeneity in clinical responsiveness to these agents, and recent research suggests this may be due to heterogeneous *SSTR* subtype expression between patients (34, 35). More recently, the somatostatin analog pasireotide was developed, which has increased binding affinity for SSTR2 and SSTR5 compared to octreotide and lanreotide. Pasireotide has shown superior efficacy for biochemical control in some studies of patients with acromegaly (36). This drug class is one example of how an improved molecular understanding of somatotrophs may provide more efficacious treatment options for patients with acromegaly; however, further studies are required before receptor expression profiles can be used to guide clinical practice.

The class of GH-receptor antagonists is the third and final example of successful targeted molecular therapy in acromegaly. GH receptors are found primarily in the liver and cartilage where activation triggers the JAK–STAT (Janus kinase/signal transducers and activators of transcription) pathway and ultimately leads to upregulation in cell proliferation and antiapoptotic proteins, including IGF-1 (3). Pegvisomant is currently the only GH-receptor antagonist approved by the U.S. Food and Drug Administration that is available for treatment of acromegaly, and it is a pegylated analog of human GH, which directly competes for receptor binding with plasma GH (37). Binding of pegvisomant prevents dimerization of the GH-receptor and thereby blocks the signaling cascade, resulting in decreased IGF-1 production. Of note, this mechanism is significantly different from that of the dopamine and somatostatin analogs, because it blocks the downstream effects of a GH-secreting adenoma, independent of tumor receptor expression patterns (3, 38). Use of pegvisomant is typically reserved for patients in whom treatment with somatostatin analogs fails or in patients with diabetes mellitus (32, 39).

Current and Future Clinical Practice

Complete surgical resection of a GH-secreting adenoma remains the first-line treatment option for acromegaly today. Surgical cure rates are high, with low morbidity and mortality when surgery is performed at a center with an experienced neurosurgical team as the first-line treatment for eligible patients. In patients with persistent or recurrent disease after surgery, or those unable to or unwilling to undergo surgery, pharmacotherapy and stereotactic radiosurgery remain excellent treatment options. Several pathways of pharmacotherapy for acromegaly have been evaluated, including use as adjuvant therapy (following surgery), as neoadjuvant therapy (before surgery), and as primary therapy (in place of surgery). The efficacy and implementation of stereotactic radiosurgery for functional pituitary adenomas have been extensively reviewed elsewhere (10–12).

Numerous studies have evaluated the efficacy of pharmacotherapy for persistent or recurrent disease after surgical resection, and somatostatin analogs are considered first-line therapy for these patients (40). It is estimated that approximately 30–60% of patients with persistent disease after surgical resection achieve biochemical remission with the addition of a somatostatin analog (41–44). An additional percentage of patients achieve biochemical remission with dopamine agonists, pegvisomant, or combination therapy with these agents. Radiological follow-up in these patients must be interpreted with caution. Tumor shrinkage is often observed with postoperative somatostatin analog treatment, but, this does not reliably correlate with biochemical remission (42, 45). Notably, some studies have noted correlations in somatostatin and dopamine expression patterns in the adenoma with treatment response, which may allow for a more individualized approach to pharmacotherapy strategies in these patients in the future (2, 29, 30, 34, 35, 46–49). GH and IGF-1 must be closely monitored in patients with known residual tumor undergoing adjuvant treatment, and treatment strategies for recurrent disease must be made on a case-by-case basis.

Neoadjuvant therapy with somatostatin analogs has been attempted in patients with large GH-secreting adenomas with some success. Preoperative treatment with somatostatin analogs was investigated in multiple studies of macroadenomas secreting GH, and this regimen was consistently shown to decrease tumor volume and GH secretion levels in patients prior to surgery (50). Additionally, short-term biochemical remission rates (3–4 months postoperatively) were consistently improved with neoadjuvant therapy. However, this effect was not clearly

demonstrated for long-term remission rates, and further studies on this subject remain to be performed. Although preoperative somatostatin analogs may decrease tumor volume, they do not convert unresectable, invasive tumors into resectable lesions. We believe this may limit the success of this strategy going forward, and preoperative somatostatin analogs should be given only to a small subset of carefully selected patients.

Since their introduction, the success of monotherapy with somatostatin analogs for some patients has been the most impressive contribution of pharmacotherapy for acromegaly. Recent studies have demonstrated good biochemical control with such treatment (36, 51–53). When interpreting biochemical control data, it is important to consider whether the patient population was preselected for somatostatin responsiveness and what other treatments the patients have received. The published remission rates for somatostatin analogs have declined as more experience is gained with the drugs because of the increased recognition of these two factors. Although most studies to date have focused on octreotide or lanreotide monotherapy, we hypothesize that future studies investigating new-generation somatostatin analogs, such as pasireotide, could demonstrate superior results. Preliminary studies with pasireotide show significantly higher rates of biochemical control compared with octreotide (36, 54).

At our institution, we attempt complete resection as the first-line treatment. In patients with residual disease not amenable to further resection (and with elevated GH and IGF-1 levels postoperatively), adjuvant somatostatin analog therapy is initiated, and patients are monitored for their biochemical response. Concurrent adjuvant radiosurgery with somatostatin analog treatment is provided on a case-by-case basis considering the location and volume of the residual tumor.

As our molecular understanding of somatotrophs advances and drugs are developed to target new sites, the role for pharmacotherapy in acromegaly will continue to expand. Although surgical resection remains the mainstay of treatment today, the future likely holds a shift in our treatment paradigm to one that emphasizes pharmacotherapy (**Figure 1**). Personalized approaches to pharmacotherapy for acromegaly may emerge based on molecular expression profiles of individual patient tumors; however, future research on this subject is required before it can be used to guide treatment for patients with acromegaly.

OTHER FUNCTIONAL ADENOMAS

Cushing Disease

Adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas manifest as the clinical syndrome of Cushing disease. Excessive secretion of ACTH leads to hypercortisolemia, and these patients present with widespread clinical symptoms including central obesity, facial plethora, amenorrhea, and skin changes, among many others. Surgical resection of the ACTH-secreting adenoma is currently the mainstay of treatment for patients with Cushing disease. However, as with acromegaly, the role for pharmacotherapy in treated Cushing disease is growing. As our molecular understanding of this disease progresses, drug development continues to produce new treatment options for patients with persistent or recurrent disease after surgery, as well as for those patients unable or unwilling to undergo surgery.

Today, the rate of postoperative biochemical remission following surgical resection of microadenomas is approximately 75% while remission of macroadenomas is about 43% (55–57). For the remainder of patients and for those who do not undergo surgery, the addition of pharmacotherapy plays a crucial role in treatment. Pharmacotherapy of Cushing disease targets three main pathways: central secretory action at the level of the pituitary, steroidogenesis, and end-target action at the glucocorticoid receptor (58). As with acromegaly, increasing knowledge of corticotroph receptor expression has guided medical treatment options for this disease.

Corticotrophs express high levels of *SSTR5* and *DRD2*, similar to the expression seen in somatotrophs (59). Pharmacotherapy targets these receptors using somatostatin ligands and dopamine agonists, respectively, to decrease ACTH production by corticotroph adenomas. Pasireotide, in particular, has demonstrated efficacy in patients with Cushing disease due to its relatively high

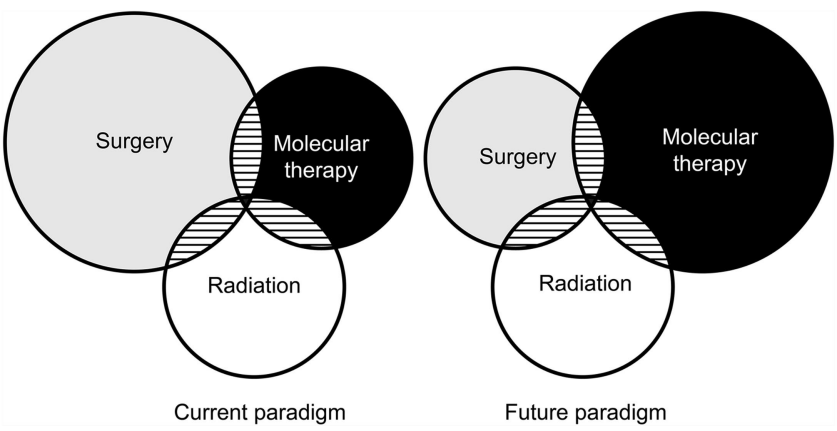

FIGURE 1 | Venn diagram illustrating the relative impact of antitumor treatments in acromegaly as practiced today and what it might look like in the future. Used with permission from Barrow Neurological Institute, Phoenix, Arizona.

binding affinity for SSTR5 (60). Phase II and III clinical trials utilizing pasireotide in patients who have not undergone surgery have demonstrated a significant reduction in urinary free cortisol levels, as well as improvement in symptoms of hypercortisolemia (61). A large, randomized, double-blind, multicenter, phase III study is currently underway to evaluate pasireotide as monotherapy for this group of patients. Cabergoline is also used in the medical management of Cushing disease by targeting corticotroph secretory function. Several trials have demonstrated its efficacy both *in vitro* and *in vivo* (62–64). In non-responders or partial responders to a single agent, combination therapy with pasireotide and cabergoline, was shown to be effective in decreasing urinary free cortisol levels (65). Other somatostatin analogs and dopamine agonists (octreotide, lanreotide, and bromocriptine) are not as effective in Cushing disease as they are in acromegaly, and these agents are not routinely used in clinical practice today.

Ketoconazole and metyrapone are the most widely used steroidogenesis inhibitors prescribed today for refractory Cushing syndrome; however, no prospective studies have evaluated these agents in Cushing disease, and their use is currently off-label (66). Investigations into alternative steroidogenesis targets are ongoing and may hold future promise (67). Mifepristone is the only current glucocorticoid receptor antagonist available for use in Cushing disease, and it is Food and Drug Administration approved for treatment of hyperglycemia in Cushing syndrome (61). It has demonstrated efficacy in long-term symptom resolution in a multicenter trial (68); however, its use is contraindicated in pregnant women, and it may be associated with adenoma enlargement (69). Serial magnetic resonance imaging is warranted to monitor for such enlargement in patients with Cushing disease treated with mifepristone.

Although numerous pharmacologic targets exist in Cushing disease, medical management has yet to reach the efficacy and safety of surgery; transsphenoidal resection remains the treatment of choice for eligible patients with an ACTH-secreting adenoma. Similar to acromegaly, an improved understanding of the molecular basis of corticotroph cells and end-target receptors will continue to spur drug development and improve medical treatment options for this challenging disease.

CONCLUSION AND FUTURE DIRECTIONS

Pituitary adenomas are relatively common tumors, and transsphenoidal resection is a safe and effective treatment option for many of these lesions. Surgical resection by an experienced pituitary surgeon remains the mainstay of therapy for both acromegaly and Cushing disease. However, a significant percentage of patients have persistent or recurrent disease after surgery or are not surgical candidates. An improved understanding of the molecular biology of these diseases has evolved since the mid-1970s, and targeted molecular therapies that limit the growth, secretory function, and end-organ effects of these tumors continue to be developed. The greatest success has come with the class of somatostatin analogs, and new knowledge regarding receptor subtype expression in pituitary adenomas has helped guide treatment strategies. Further research into this domain may allow for more individualized treatment strategies for patients harboring tumors with expression patterns that can be characterized. Although some research has supported this approach to date, further studies are required before this paradigm can be applied outside of academic pituitary practices. Characterization of tumor expression patterns is a challenging task, but we believe that targeted pharmacotherapy could approach, and eventually surpass, the efficacy of surgical resection for the treatment of these lesions.

AUTHOR CONTRIBUTIONS

All authors have made substantial, direct, and intellectual contributions to the work and approved it for publication.

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frontiers in Surgery

MINI REVIEW
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Meningioma Genomics: Diagnostic, Prognostic, and Therapeutic Applications

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There has been a recent revolution in our understanding of the genetic factors that drive meningioma, punctuating an equilibrium that has existed since Cushing's germinal studies nearly a century ago. A growing appreciation that meningiomas share similar biologic features with other malignancies has allowed extrapolation of management strategies and lessons from intra-axial central nervous system neoplasms and systemic cancers to meningiomas. These features include a natural proclivity for invasion, frequent intratumoral heterogeneity, and correlation between biologic profile and clinical behavior. Next-generation sequencing has characterized recurrent somatic mutations in *NF2*, *TRAF7*, *KLF4*, *AKT1*, *SMO*, and *PIK3CA*, which are collectively present in ~80% of sporadic meningiomas. Genomic features of meningioma further associate with tumor location, histologic subtype, and possibly clinical behavior. Such genomic decryption, along with advances in targeted pharmacotherapy, provides a maturing integrated view of meningiomas. We review recent advances in meningioma genomics and probe their potential applications in diagnostic, therapeutic, and prognostic frontiers.

Keywords: meningioma, genomics, molecular taxonomy, targeted therapy, precision medicine

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Meningioma genetics are undergoing a revolution in taxonomy and molecular stratification, punctuating an equilibrium that has existed since Cushing's germinal studies nearly a century ago (1). The understanding of meningiomas rests on a growing appreciation that these tumors share similar features with other intra-axial central nervous system (CNS) neoplasms as well as systemic cancers. Moreover, maturing technologies in genomics and immunotherapy are increasingly intersecting to provide an integrated view on meningioma biology. We review recent advances in meningioma genomics and probe their potential applications in diagnostic, therapeutic, and prognostic frontiers.

MENINGIOMA HISTOPATHOLOGIC CLASSIFICATION

Meningiomas account for over a third of all primary CNS tumors diagnosed in the United States, with ~18,000 new cases diagnosed annually and a prevalence of 97.5/100,000 individuals, making them the most common primary intracranial neoplasms in adults (2, 3). Most meningiomas are considered benign. A small, but growing, proportion display aggressive behavior characterized by invasive growth patterns and higher rates of recurrence (4).

Meningiomas are classified by the World Health Organization (WHO) system as grades I, II, and III, with higher grades associated with greater rates of morbidity and mortality (**Figure 1**) (5). Grade I meningiomas display a broad range of morphologic features and are considered histologically benign, with fewer than 4 mitoses/10 microscopic high-power fields (HPF). Nine subtypes of benign meningiomas are recognized by the WHO: meningothelial, fibroblastic, transitional (containing both

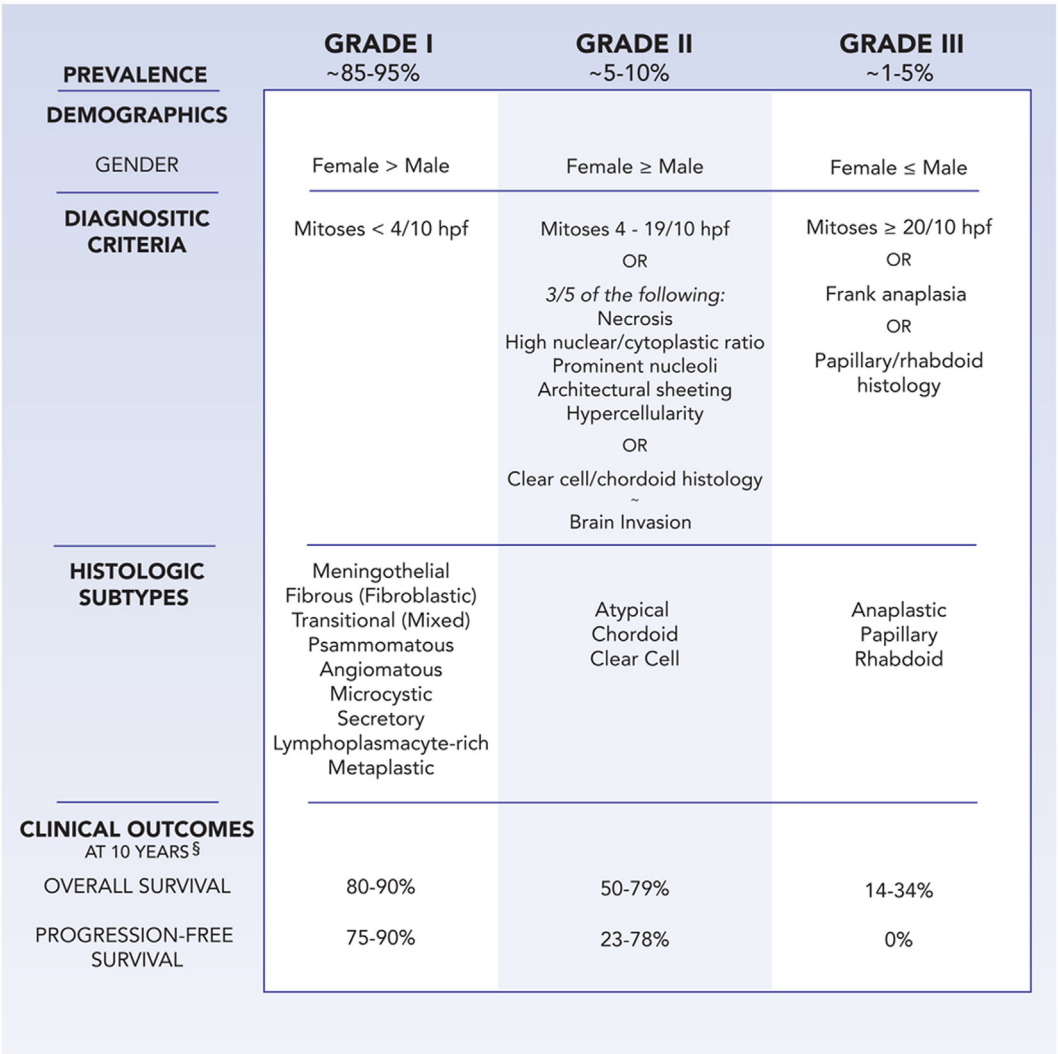

FIGURE 1 | **Demographics, WHO diagnostic criteria, histologic subtypes, and clinical outcomes at 10 years follow-up for meningioma, as stratified by grade**. §Clinical outcomes are influenced by age, comorbidities, extent of resection, adjuvant therapy, and tumor location.

meningothelial and fibroblastic components), psammomatous, angiomatous, microcystic, secretory, lymphoplasmacyte-rich, and metaplastic.

Grade II, also known as atypical, meningiomas are defined by the presence of 4–19 mitoses/10 HPF or 3 of 5 criteria: sheet-like growth, spontaneous necrosis, high nuclear to cytoplasmic ratio, prominent nucleoli, and increased cellularity. Meningiomas with two or less of the five atypical features are classified as grade I meningiomas with atypical features, and incur a higher risk of recurrence than benign meningiomas without atypical features (6). Two distinct histologic variants, clear cell and chordoid, are considered grade II meningiomas as well. In addition, the presence of brain invasion implies a similar recurrence rate and risk of mortality as atypical meningiomas (7).

Grade III meningioma is synonymous with anaplastic or malignant meningioma. Morphologically, they can resemble sarcoma or carcinoma, challenging pathologic diagnosis, and also include the papillary and rhabdoid histologic variants. Grade III meningiomas harbor a mitotic index of 20 or greater per 10 HPF, and classically lose markers of differentiation, such as epithelial membrane antigen. Patients with anaplastic meningiomas observe an aggressive clinical course of tumor recurrence and premature mortality.

CHALLENGES IN MENINGIOMA MANAGEMENT

The histopathologic classification of meningioma provides a powerful harbinger for its natural history. However, clinical outcome in a subset of patients belies the designated pathologic grade for the tumor. Improved understanding of the genomic underpinnings of meningioma offers new strategies for molecular

stratification and rationally guided therapies. We first highlight some of the challenges facing meningioma management, then review recent advances in meningioma genomics, and draw upon lessons learned from other cancers.

Limitations of Diagnostic Criteria

On initial detection of an extra-axial mass lesion consistent with meningioma on imaging, no reliable parameters exist to date for predicting tumor grade, and ultimately, biologic course. A number of radiographic metrics are under investigation, including nature of the tumor–brain interface, intratumor heterogeneity, lesion irregularity, intrinsic tumor diffusion and perfusion characteristics, and peritumoral edema, but all merit further validation. Furthermore, tumor location, such as parasagittal and falcine, may portend a more aggressive nature to the meningioma. Ultimately, mass effect leading to existing or impending symptoms, steadfast radiographic growth over a period of observation, and patient preference dictate the decision to intervene on a suspected meningioma.

Variations in operative philosophy, operative technique, and choice and timing of radiation permeate clinical practice. In general, maximal surgical resection without compromise of neurologic function imparts the most optimal prognosis for the patient. Standard of care typically invokes adjuvant radiation therapy for malignant meningiomas, with greater variability in the administration and timing of radiation for atypical meningiomas. This variability in management strategy for intermediate grade meningiomas is further complicated by shifts in diagnostic criteria over time (5, 8–10).

For example, application of the 2000 instead of the 1993 WHO guidelines results in a change in classification in up to 30% of high-grade meningiomas, often from a higher grade to lower grade (11). The 2007 WHO guidelines introduced less of a paradigm shift, but brain invasion remained ambiguous as a marker for atypical meningioma (12). The evolution of WHO grading scales associates with an improved correlation between grade and survival (13). However, inter-observer differences and representative sampling of select sections from large tumors may bias the final grading and, therefore, prediction of natural history. As with other CNS tumors, unbiased criteria for diagnostic arbitration, such as molecular signatures, can abet definitive stratification of tumor class. Furthermore, an association between such molecular signatures, tumor phenotype, and, ultimately, prognosis would improve initial planning for treatment interventions.

Meningioma as an Invasive Tumor

Independent of tumor classification, the clinical course of meningiomas following surgical resection highlights its biologic proclivity for invasiveness. In Simpson's classic analysis of symptomatic recurrence following resection of meningiomas, residual dural attachment and juxtaposition to venous sinuses – which serve as a potential haven for neoplastic cells in the absence of bulk tumor – were associated with significantly higher rates of recurrence (14). Furthermore, meningiomas with benign histopathologic features that invade the brain exhibit a similar likelihood of recurrence as higher grade, atypical, meningiomas. Thus, despite being the quintessential icon of CNS extra-axial tumors, the invasive potential of meningioma cells highlights an inherent limitation to debulking strategies and should be accounted for in therapeutic strategies.

Intratumoral Heterogeneity

Surgical resection aside, radiation serves as a common adjuvant treatment for meningioma, especially in high-grade and recurrent tumors. A few biological agents, such as hydroxyurea and somatostatin inhibitors, have been trialed with limited success in meningiomas refractory to standard treatment modalities (15). These treatments rely upon the biologic response of non-senescent tumor cells. Additionally, the development of targeted pharmacologic inhibitors, as widely studied for systemic cancers and discussed below for meningioma, presumes a global distribution of the oncogenic driver or modulator target. The presence of intratumoral heterogeneity poses a fundamental impediment to the efficacy of these therapeutic strategies.

The observation of meningioma heterogeneity stems from a number of potential etiologies, including intratumoral necrosis, cystic degeneration, heterogeneous tumor cell expansion, imbalances in cell density, and hemorrhage. In particular, subclonal expansion within an admixture of functionally distinct cancer cells has been posited to account for incomplete treatment response, acquired and innate treatment resistance, and disease relapse for malignancies, such as glioblastoma and systemic cancers. Similarly, molecular and cellular heterogeneity is increasingly appreciated in meningioma (16), and may present a similar challenge to the development of therapeutic strategies.

These characteristics of meningiomas echo challenges posed by other tumors, some of which serve as exemplars in decrypting the molecular code toward a more unified front in diagnosis and treatment, as discussed below.

GENOMICS OF MENINGIOMA

Meningioma represents one of the first tumors associated with a genomic driver, with the initial identification of *neurofibromin* (*NF2*), the causative gene for neurofibromatosis 2 (NF2), in which 50–75% of patients develop one or more meningiomas. Sporadic low- and high-grade meningiomas are also observed to harbor mutations, allelic inactivation, and loss of the *NF2* in ~40–60% of tumors, resulting in alteration of its protein derivative, Merlin (17–20). The development of meningiomas in *NF2*-knockout mice corroborates its role as an early oncogenic driver in meningioma tumorigenesis (21, 22).

More recently, several additional recurrent somatic mutations have been identified through next-generation sequencing approaches, which are collectively present in ~40% of sporadic meningiomas (**Figure 2A**) (19, 20, 23). These genes are the pro-apoptotic E3 ubiquitin ligase *TNF receptor-associated factor 7* (*TRAF7*), the pluripotency transcription factor *Kruppel-like factor 4* (*KLF4*), the proto-oncogene *v-Akt murine thymoma viral oncogene homolog 1* (*AKT1*), the Hedgehog pathway *signaling member smoothened* (*SMO*), and the oncogene *PIK3CA*. Notably, mutations of these genes in meningiomas occur to large degree without concurrent alteration of *NF2* or loss of chromosome 22.

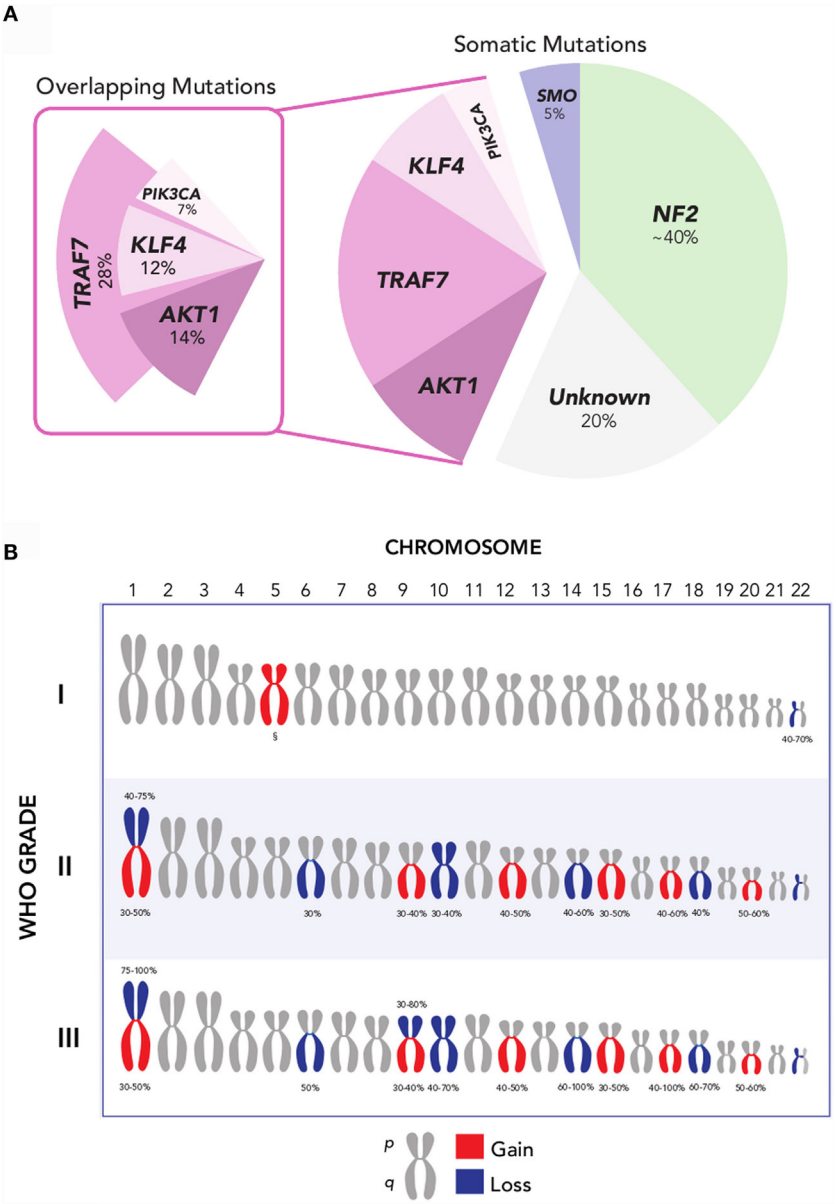

FIGURE 2 | **(A)** Recurrent *NF2*, *AKT1*, *SMO*, *TRAF7*, *KLF4*, and *PIK3CA* mutations are collectively present in over 80% of grade I meningiomas. Mutations in *AKT1*, *KLF4*, and *PIK3CA* overlap with *TRAF7*, but not with each other, and largely occur in a mutually exclusive pattern with *NF2* and *SMO*. Oncogenic driver mutations remain unclear for ~20% of meningiomas [Data aggregated from Ref. (19, 20, 23, 41)]. **(B)** Recurrent chromosomal copy number alterations in meningioma. Chromosomal arm-level gains (red) and losses (blue) are observed with increasing frequency in higher-grade meningiomas, compared to grade I meningiomas. §Polysomy 5 is observed in angiomatous subtype of grade I meningiomas [Data adapted from Ref. (5, 32)].

The most common of these is *TRAF7*, located on chromosome 16p13, which harbors a mutation in 12–25% of meningiomas (20). *TRAF7* mutations frequently co-occur with mutations in *KLF4*, *AKT1*, or *PIK3CA*, and are mutually exclusive with *SMO* and *NF2* mutations (20, 23, 24). A recurrent mutation in $KLF4^{K409Q}$, located on chromosome 9q31 and resulting in a lysine to glutamine substitution at codon 409 (K409Q), represents the next most frequent somatic alteration observed to date – affecting ~15% of benign meningiomas. This may recapitulate embryologic mechanisms to spur tumor formation, given the role of *KLF4* as a transcription factor that promotes reprograming of differentiated somatic cells back to a pluripotent state in normal development (25). Another recurrent mutation in *AKT1*, located on chromosome 14q32, is observed in 6.8% of meningiomas and produces a glutamic acid to lysine substitution at codon 17 (E17K) (20, 26). $AKT1^{E17K}$ mutation results in downstream activation of the *PI3K/AKT/mTOR* oncogenic pathway, rendering it targetable by selective AKT inhibitors, several of which are currently under investigation for the treatment of cancers of the breast, lung, and colon, among others (27). Oncogenic mutations in *PIK3CA* are

observed in ~7% of non-*NF2*-mutant meningiomas, and occur mutually exclusive of *AKT1* and *SMO* mutations, although they frequently co-occur with TRAF7 mutations (23). Lastly, ~5.5% of benign meningiomas, or more than 10% of meningiomas without *NF2* alteration, express mutations in *SMO* (19, 20). These *SMO* alterations result in activation of Hedgehog signaling, another well-characterized pathway in cancer that is notably dysregulated in basal-cell carcinoma and medulloblastoma (28, 29). In basal-cell carcinoma, where over 90% of tumors have mutations in either *SMO* or *PTCH*, SMO inhibition has been particularly effective in the setting of locally advanced and metastatic disease (30). Inhibitors of SMO, AKT1, and PIK3CA hold promise as molecularly targeted pharmacotherapy in meningioma.

Collectively, these somatic mutations hold significant promise for advancing the molecular taxonomy of meningioma. However, ~20% of meningiomas remain without an identifiable oncogenic driver mutation to date (31). Beyond mutations, insertions, and deletions at the single nucleotide level, meningiomas harbor a classic constellation of chromosomal copy number alterations (**Figure 2B**). Monosomy 22 is the most common chromosomal change, observed in 40–70% of meningiomas, across all grades (7). Aside from loss of chromosome 22, the copy number landscape of benign meningiomas is typically neutral. One exception is the angiomatous subtype of grade I meningiomas, which notably express multiple polysomies across the genome, most commonly of chromosome 5 (32). In comparison, higher-grade meningiomas express a markedly higher burden of chromosomal losses and gain. These include frequent loss of chromosomes 1p, 6q, 10, 14q, and 18q, as well as gain of chromosomes 1q, 9q, 12q, 15q, 17q, and 20q (7, 33, 34). Among these, loss of chromosomes 1p and 14q is the most frequent cytogenetic abnormality observed in meningiomas after chromosome 22, affecting half of grade II and nearly all grade III meningiomas (33). Investigations into candidate oncogenes on these chromosomal arms have yet to elucidate clear drivers for meningioma tumorigenesis.

In addition to mutations and copy number alterations, epigenomic changes may provide another complementary biologic mechanism in meningioma development and progression (35). Overall, all existing evidence suggests a progression in genomic complexity in high-grade meningiomas.

APPLICATIONS OF MOLECULAR TAXONOMY IN MENINGIOMA

These significant advances in our understanding of meningiomas provide an expanding toolbox to formulate a molecular taxonomy and explore novel therapeutic options for this surprisingly diverse tumor entity. This paradigm shift toward molecular taxonomy is inspired by examples from several tumor types, including glioblastoma, medulloblastoma, and ependymoma, where molecular stratification has transformed their diagnosis and management (36–38). Similarly, associations between molecular signatures with characteristic phenotypes, intracranial locations, tumor subclass, and clinical prognosis have begun to emerge as an increasing number of meningiomas are systematically characterized.

Genetic Hallmarks of Meningioma Subtypes

The histologic subtype and location of meningioma associates with its molecular profile (Table S1 in Supplementary Material). Grade II and III meningiomas harbor an incremental complement of chromosomal alterations, as discussed above. Copy number gains, especially polysomy 5, are also characteristic of angiomatous meningiomas, a grade I subtype (32).

Focally, inactivation of *NF2*, through copy loss or mutation, occurs in 70–80% of fibroblastic and transitional meningiomas. By contrast, secretory meningiomas almost uniformly harbor mutations in both *TRAF7* and $KLF4^{K409Q}$ but not *NF2* (24), while meningothelial meningiomas are associated with *AKT1* mutations (26). Additionally, clear cell meningiomas are associated with loss-of-function mutations of *SMARCE1* in the hereditary multiple spinal meningioma syndrome and some cranial locations (39, 40).

Interestingly, genetic alterations also correlate with anatomic location in some meningiomas. Mutations in *SMO* or *AKT1/TRAF7* are most frequently observed in meningiomas of the anterior cranial base (19, 20). In comparison, convexity meningiomas are more likely to express *NF2* mutations and loss of heterozygosity of chromosome 22. The association between tumor location and genotype may aid candidate selection in future clinical trials that target specific oncogenic mutations.

Predicting Clinical Outcome

Aside from the role of molecular biomarkers in abetting the diagnosis of meningioma, one fundamental question in the clinical management of meningioma patients is the risk of recurrence following surgical resection. There is particular ambiguity among grade II meningiomas, for which no consensus exists regards appropriate adjuvant treatment modality and timing. Recently, analysis of a cohort of atypical meningiomas following gross total resection revealed an association between increased chromosomal copy number alterations and risk of recurrence (41). By summing the incidence of broad copy number events across an aggregate pool of common chromosomal aberrations in meningiomas, this strategy bypasses the limitations of assessing isolated molecular candidates in meningioma oncogenesis and offers a rapid molecular appraisal of potential outcome through routine clinical cytogenetic testing. In other words, patients harboring grade II meningiomas with high chromosomal disruption, which may have a higher risk of recurrence, may benefit from closer surveillance or adjuvant therapies.

The validity of such molecular prognostication strategies remains to be proven in future studies. If corroborated, they may serve a powerful tool in counseling patients, guiding management decisions, and stratifying clinical trials.

Designing Rational Strategies in Meningioma Treatment

Elucidation of critical oncogenic drivers in a number of cancers (e.g., *BRAF* in melanoma or *KIT* in gastrointestinal stromal tumors) has enabled targeted therapies in the so-called "mutation-to-drug" paradigm (42, 43). Such an approach is now feasible in

meningioma with the recent identification of *AKT1*, *SMO*, and *PIK3CA* mutations, which opens the door for targeted pharmacotherapeutics in ~20% of grade I meningiomas. A clinical trial targeting AKT1 and SMO is currently underway for progressive meningiomas (NCT02523014).

While this genomically stratified trial augurs an exciting direction for refractory meningiomas that progress after standard therapy, other meningiomas that do not express these mutations, including most high-grade tumors, remain devoid of effective pharmacologic options. Furthermore, recognition of intratumoral cellular and molecular heterogeneity, which may foster resistant subclonal growth following targeted therapies, encourages investigation of alternative treatment strategies – such as immunotherapy (44).

Deployment of the innate and adaptive immune response offers an attractive option for genomically complex tumors, where presumably a higher neoantigen load is available for immune targeting (45, 46). Suppression of inhibitors of T-cell activation, known as immune checkpoints, has achieved durable clinical responses in several advanced systemic cancers (47). In grade II and III meningiomas that progress after surgery and standard radiation, a phase 2 clinical trial evaluating checkpoint blockade with nivolumab is anticipated to initiate (NCT02648997).

CONCLUSION

Contemporary advances in molecular, genomic, epigenetic, and immune profiling has ushered a renaissance in the study of meningiomas. These systematic approaches suggest a molecular taxonomy that promises to influence diagnosis, disease classification, and, ultimately, clinical management. Furthermore, appreciation of shared biological characteristics between meningiomas and other CNS cancers – including invasiveness and intratumoral heterogeneity – may lead to an expansion of the therapeutic arsenal in the treatment of this increasingly disparate tumor.

AUTHOR CONTRIBUTIONS

WLB and IFD drafted the manuscript and supervised the study. MZ, WW, and YM contributed to data collection. All authors critically revised the manuscript and approved the final submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fsurg.2016.00040

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REVIEW
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Personalized Medicine for Nervous System Manifestations of von Hippel–Lindau Disease

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von Hippel–Lindau disease (VHL) is a familial neoplasia syndrome associated with multisystem tumor development. Depending on tumor type and location, current treatments for VHL-associated tumors can include a combination of chemotherapy, radiation therapy, and/or surgery. Central nervous system (CNS) manifestations of VHL include craniospinal hemangioblastomas and endolymphatic sac tumors (ELSTs). While the first-line treatment for both types of VHL-associated CNS tumors is surgery, the indications for treatment are patient specific and different for each tumor type. Although early sign/symptom formation is the primary indication for resection of craniospinal hemangioblastomas, radiographic discovery (asymptomatic and symptomatic) of ELSTs can be an indication for resection of ELSTs in VHL patients. Recently, research has revealed that specific *VHL* germline mutations may permit targeted medical treatments of not only CNS manifestations of VHL-associated tumors but also visceral tumors. Specifically, missense mutations can result in the translation of functional VHL protein (pVHL) that is rapidly degraded resulting in functional loss of the pVHL, and inhibitors of pVHL degradation may slow protein degradation and restore pVHL function. Emerging research will investigate the safety and practicality of using potential targeted therapies.

Keywords: von Hippel–Lindau, personalized medicine, hemangioblastoma, histone deacetylase inhibitor, endolymphatic sac tumor

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INTRODUCTION

von Hippel–Lindau disease (VHL) is an autosomal dominant inherited genetic disorder caused by a germline mutation of chromosome 3 (*VHL gene*). Patients affected with VHL develop multiple central nervous system (CNS) lesions, including retinal and craniospinal hemangioblastomas, as well as endolymphatic sac tumors (ELSTs). Visceral VHL-associated lesions frequently include renal cell carcinomas, renal cysts, pheochromocytomas, extra-adrenal paragangliomas, pancreatic microcystic adenomas, pancreatic cysts, and pancreatic neuroendocrine tumors, as well as cystadenomas of the epididymis and broad ligament (1–3) (**Figure 1**). VHL has an incidence of approximately 1 in 36,000–39,000 live births (4, 5). Penetrance is nearly complete and most (over 90%) patients display evidence of the disease by the age of 65 years (6).

Treatment of VHL is complex due to the multisystem involvement, the location of tumors, and the immense variability of symptoms that can be produced related to various tumor locations and systems involved. Recent studies have elucidated new germline-based targets for treatment. Existing treatments include combinations of chemotherapy, radiation therapy, and surgical resection. We describe the current personalized management of the CNS manifestations of VHL and

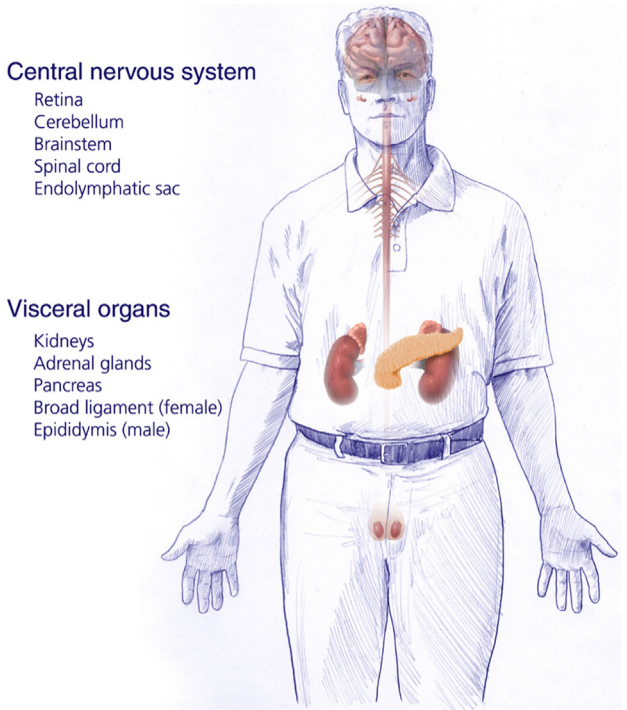

FIGURE 1 | Central nervous system and systemic locations and types of tumors of patients harboring germline protein VHL mutations and von Hippel–Lindau disease [adapted from Lonser et al. (7)].

the potential putative therapeutics that are based on germline mutation types.

GENETICS AND PATHOGENESIS

Mutations in the *VHL gene* lead to the development of the manifestations of VHL. The *VHL gene* is located on the short arm of chromosome 3 (3p) and is a tumor suppressor gene (8). Germline mutations of *VHL* account for more than 95% of the patients affected by VHL (5% have somatic inactivation of the *VHL* gene in sporadically occurring hemangioblastomas and renal cell carcinomas) (9). VHL patients inherit a *VHL* germline mutation from the VHL-affected parent and a normal (wild-type) gene from the non-affected parent. Tumorigenesis occurs when the wild-type *VHL* allele is inactivated (loss of heterozygosity) in certain susceptible target organs that include the viscera (kidneys, pancreas, adrenal glands, and adnexal organs), as well as the CNS (7).

The *VHL gene* encodes VHL protein (pVHL), a protein that is part of the E3 ubiquitin ligase, which is involved in proteasomal degradation. It targets hypoxia inducible factor (HIF)-1/2α (10) transcription factors that are activated in hypoxic conditions to upregulate genes, including vascular endothelial growth factor (VEGF), transcription growth factor (TGF), erythropoietin (EPO), EPO receptor, transferrin, and angiopoietin (11). These factors are involved in angiogenesis, erythropoiesis, cell proliferation, and/or tumorigenesis/metastasis. HIF-2α is a known oncogene that contributes to cell proliferation and tumorigenesis (11). pVHL participates in degradation of HIF-1/2-α by binding the transcription factors to the proteasome complex (**Figure 2**). When the *VHL gene* is mutated and its function is reduced/lost, HIF-1/2α is upregulated (even in the absence of hypoxic conditions) due to its reduced degradation by the VHL ubiquitin–proteasome complex (7).

Multiple VHL germline mutations have been discovered, ranging from deletions to missense mutations. Germline VHL missense mutations are the most common and underlie 60–70% of all VHL-associated mutations (4). Recent studies have shown that the proteins translated from the missense mutated *VHL gene* are highly unstable and rapidly degraded (10), but retain the functional capacity of wild-type protein. Consequently, treatment strategies that extend the half-life of pVHL in this circumstance could lead to normalization (reversal) of VHL-related pathobiologic features.

VHL-ASSOCIATED TUMORS

Hemangioblastomas

Hemangioblastomas are highly vascular tumors that arise in the CNS. They are the most common tumor presentation of VHL patients. Previously, studies have estimated that 60–90% of VHL patients will develop multiple hemangioblastomas in their lifetime (12, 13). Cerebellar lesions are the most common, followed by spinal cord, brainstem, and supratentorial tumors (**Figure 3**) (3, 9). CNS hemangioblastomas are histologically benign but cause a multitude of symptoms and can result in death depending on their location and size. Symptomatic CNS hemangioblastomas are most frequently associated with peritumoral cysts, although symptoms can be caused by solid tumors and are location dependent (1, 14, 15).

Recent natural history studies have provided a better understanding of the growth and development of hemangioblastomas in VHL. We prospectively studied 250 VHL disease patients with a total of 1921 CNS hemangioblastomas (9). At the end of the study, mean number of craniospinal hemangioblastomas had increased from 7 to 8 per person over a mean follow up of 6.9 years (new hemangioblastoma development was inversely associated with age). When observed out to 5 years, 49% of known hemangioblastomas progressed in size in a linear, saltatory, or exponential pattern. Brainstem and cerebellar hemangioblastomas grew significantly faster than the spinal or cauda equina hemangioblastomas. Male sex was associated with a significantly faster growth rate than females. Most VHL patients will develop multiple hemangioblastomas over time. Because hemangioblastomas grow at different rates, in multiple locations, and exhibit irregular growth patterns, symptom formation can be unpredictable. Surgical resection is often reserved until the first onset of signs/symptoms that correlate with the location of the hemangioblastoma. This management paradigm attempts to ensure that unnecessary surgical intervention and associated possible complications are avoided. This can often result in maintenance of baseline neurologic function for most patients (9).

Peritumoral cysts are a frequent cause of hemangioblastoma-associated signs/symptoms, and they form by a plasma ultrafiltrate

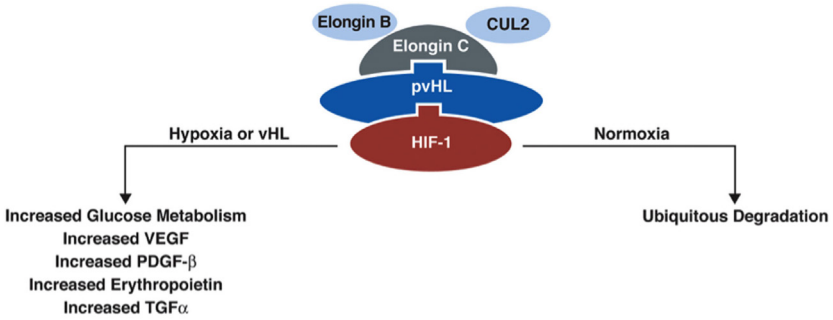

FIGURE 2 | **Function of protein VHL in the proteasome**. pVHL is thought to function as an E3 ubiquitin ligase in the proteasome complex and bind HIF-1α, which results in ubiquitination of HIF-1α and leads to degradation. In normoxic conditions, HIF-1α is degraded, but in conditions of hypoxia, HIF-1α is upregulated. In the absence of pVHL, HIF-1α is not ubiquitinated and degraded [adapted from Lonser et al. (7)].

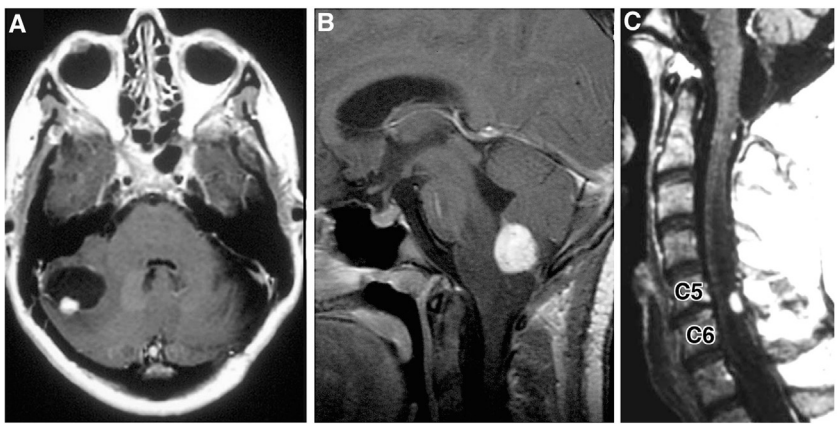

FIGURE 3 | **Radiographic images of hemangioblastomas**. **(A)** Axial, contrasted, T1-weighted MRI showing cerebellar hemangiolastoma with contrast enhancing mural nodule and peritumoral cyst. **(B)** Sagittal, contrasted, T1-weighted MRI revealing contrast enhancing medullary hemangioblastoma with surrounding vasogenic edema. **(C)** Sagittal, contrasted, T1-weighted MRI with contrast enhancing posterior/dorsal hemangioblastoma with associated syrinx [adapted from Lonser et al. (7)].

passing into tissue surrounding the hemangioblastoma from permeable tumor blood vessels (1). Previously, we prospectively followed 225 patients with VHL disease, of which 132 patients had 292 peritumoral cysts (14). Approximately 75% of peritumoral cysts progressed within 3 years. Cysts grew faster if located in the cerebellum, in patients under 35 years of age, and if they were associated with symptoms. Peritumoral cysts appeared to grow in three patterns, including saltatory (phases of growth followed by stability), linear, or exponential. Overall, a majority of the cysts grew in a saltatory manner (41.7%). However, of the 60 symptomatic peritumoral cysts, 45% grew exponentially (9). Risk of an increased total number of tumors was significantly associated with a partial deletion germline mutation and male sex, while new cyst development was associated with a greater number of cysts at the time of initial evaluation for the study and age younger than 35 years.

Radiation therapy [most frequently stereotactic radiosurgery (SRS)] has been utilized for treatment of CNS hemangioblastomas in VHL. Asthagiri and colleagues (16) prospectively evaluated the effect of SRS on craniospinal hemangioblastomas in 20 VHL patients (11 symptomatic, 9 asymptomatic) with 44 hemangioblastomas. Fourteen tumors (32%) progressed after SRS treatment, and four of these tumors required surgical resection. Local control rates decreased over time with 91, 83, 70, 61, and 51% at 2, 5, 8, 10, and 15 years, respectively, and were similar to rates of progression in untreated hemangioblastomas (9). These data indicate that SRS should be reserved for treating hemangioblastomas that are not surgically resectable or in patients who cannot tolerate surgical resection (16).

ENDOLYMPHATIC SAC TUMORS

Endolymphatic sac tumors are vascular, low-grade papillary adenocarcinomas affecting up to 11% of VHL patients. Mean age of diagnosis is 22 years, and bilateral ELSTs are found in approximately 30% of patients with VHL (17). The majority of

the patients had associated audiovestibular symptoms, including sensorineural hearing loss (84% of ears), tinnitus (73%), and vertigo (68%) that did not correlate with tumor size (18). The audiovestibular findings associated with ELSTs are thought to be due to intralabyrinthine hemorrhage, endolymphatic hydrops, and/or direct invasion of the otic capsule by tumor (19). Sudden hearing loss (43%) has been correlated with intralabyrinthine hemorrhage. Gradual hearing loss (47%) is most often related to endolymphatic hydrops.

Regular screening of VHL patients for ELSTs is recommended, with surgical intervention in selected patients before morbidity develops. Surgery is curative for completely excised tumors. Kim and colleagues found that hearing was stabilized postoperatively in 90% of patients after ELST resection (18). Current indications for ELST resection in VHL patients include imaging evidence of an ELST with serviceable hearing (and/or audiovestibular signs/symptoms), evidence of ELST-associated intralabyrinthine hemorrhage, ELST-associated hydrops, or mass effect by the ELST (19, 20). Contrast-enhanced delayed FLAIR MRI has been found to be an efficacious, non-invasive method of detecting ELST-associated hydrops (21). The role of adjuvant therapy, including chemotherapy, fractionated radiotherapy, or stereotactic radiosurgery, is not established.

EMERGING TARGETS FOR TREATMENT OF VHL

Recent investigations into the pathogenesis of VHL tumors have revealed new potential targets for treatment. Metelo and colleagues (22) studied VHL models in zebrafish using $vhl^{-/-}$ embryos. Treatment with HIF-2α inhibitors decreased expression of HIF-2α targeted genes. The effect was dose dependent in these studies. It improved new angiogenic sprouting that was seen in $vhl^{-/-}$ embryos and returned abnormal cardiac function to baseline, suggesting that HIF-2α could lead to potential targeted treatments for systemic VHL tumors.

The most frequent mutations in VHL are missense mutations. VHL patients that harbor a missense germline mutation have a quantitative reduction of missense mutant VHL protein (pVHL), but still maintain physiologic pVHL mRNA expression. Recent data indicate that mutant pVHL is highly unstable and is quickly degraded after translation. Interestingly, missense mutant pVHL retains its E3 ligase function, including HIF degradation. The premature pVHL degradation is due to misfolding and imbalance of chaperonin binding (23).

Histone deacetylase inhibitors (HDACis) can modulate the pVHL degradation pathway by inhibiting the HDAC6–Hsp90 chaperone axis, stabilizing pVHL, and restoring activity comparable to wild-type protein *in vitro* and in mouse VHL models. HDACi-mediated stabilization of missense pVHL significantly attenuates the growth of mouse VHL tumors (23). These findings provide direct insight into the pathobiology of VHL-associated tumors and elucidate a new treatment paradigm for personalized therapy in those individuals with missense VHL mutations.

PERSONALIZED APPROACHES

von Hippel–Lindau disease is a complex and progressive process involving multisystem tumor formation. Individuals afflicted with VHL disease require a personalized approach for therapy, as tumors are neither uniform in their locations nor identical in their symptomatology. Currently, successful systemic treatments are lacking. The first choice of therapy for hemangioblastomas and ELSTs is surgery, and the decision to proceed with surgery is personalized to the individual. Surgery is reserved for hemangioblastomas based on symptom development and progression in tumor or cyst size. Surgery for ELSTs can also be based upon symptomatology, but indications also include radiographic findings of ELSTs and serviceable hearing. Care is taken to tailor the exact treatment to each individual patient and their tumor burden.

Recent studies revealed missense mutations in the *VHL gene* actually allow for transcription of the protein, albeit an unstable one that is rapidly degraded due to misfolding and chaperonin binding. The protein is then unable to function and degrade its target, HIF-2α as part of the proteasome. HDACis interfere with chaperonin pathways and, in result, stabilize the protein and allow return to function *in vitro* and in VHL mouse models. This offers a unique opportunity for a new treatment modality that in theory would be able to treat the underlying mechanism of tumorigenesis and affect the whole body, multiple systems, and possibly all of the differing tumor types in the body.

Further investigation and Phase I clinical trials would need to be conducted to assess the feasibility of developing HDACis as possible treatments. Currently, vorinostat, an HDACi, is undergoing Phase I trials for VHL patients with hemangioblastomas and missense mutations, trial #NCT02108002. With the potential of new therapies on the horizon, this could permit further treatment of the complex manifestations of VHL disease tailored to the individuals with this most common VHL germline mutation.

CONCLUSION

von Hippel–Lindau disease is a complex disorder, and patients develop a wide constellation of symptoms related to the varying locations and types of tumors present. Currently, therapies are tailored toward the individual tumors and patient findings. Discovery of pVHL stabilization with use of HDACis in missense mutated pVHL provides a potential for new treatment directed toward the underlying mechanisms of tumorigenesis that could further tailor therapy toward the individual with VHL and offer a uniform treatment for various tumors associated with the disease.

AUTHOR CONTRIBUTION

All three authors participated in the research, composition, drafting, and editing of the manuscript.

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MINI REVIEW
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The Current and Future Treatment of Brain Metastases

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Brain metastases are the most common intracranial malignancy, accounting for significant morbidity and mortality in oncology patients. The current treatment paradigm for brain metastasis depends on the patient's overall health status, the primary tumor pathology, and the number and location of brain lesions. Herein, we review the modern management options for these tumors, including surgical resection, radiotherapy, and chemotherapy. Recent operative advances, such as fluorescence, confocal microscopy, and brachytherapy, are highlighted. With an increased understanding of the pathophysiology of brain metastasis come increased future therapeutic options. Therapy targeted to specific tumor molecular pathways, such as those involved in blood–brain barrier transgression, cell–cell adhesion, and angiogenesis, are also reviewed. A personalized plan for each patient, based on molecular characterizations of the tumor that are used to better target radiotherapy and chemotherapy, is undoubtedly the future of brain metastasis treatment.

Keywords: brain metastasis, chemotherapy, personalized medicine, radiotherapy

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INTRODUCTION

Nearly 200,000 patients are newly diagnosed with brain metastases annually in the United States, and metastases of the lung, skin, kidney, breast, and gastrointestinal tract are the most common intracranial malignancies (1, 2). Historically, overall survival after diagnosis is poor; however, in the last 30 years, improved systemic disease therapies and multimodality brain metastasis treatment have substantially increased survival. This increase in the *quantity* of life after diagnosis allows clinicians to minimize morbidity and focus on the patient's *quality* of life. Choosing an appropriate personalized treatment plan for patients with brain metastasis maximizes survival and minimizes morbidity from unnecessary or futile treatments. The wide variety of tumor types, treatment strategies, and constant innovations within the field requires close collaboration among neurosurgeons, medical oncologists, radiation oncologists, and other specialists. Current treatment paradigms for brain metastases employ several treatment modalities, including open surgical resection, Gamma Knife or CyberKnife stereotactic radiosurgery, focused external beam radiotherapy, whole-brain radiotherapy (WBRT), traditional chemotherapy, and newer targeted biological agents personalized for tumor type. We review the current standards of care for brain metastases and summarize modern advances in their intraoperative diagnosis and treatment (**Table 1**). Lastly, we provide an overview of recent basic science and translational research leading to better understanding of the personalized biology of brain metastasis through modern genomic, transcriptomic, and proteomic techniques.

Abbreviations: 5-ALA, 5-aminolevulinic acid; CNS, central nervous system; LITT, laser interstitial thermal therapy; WBRT, whole-brain radiotherapy.

TABLE 1 | Modern challenges in the multimodality management of brain metastasis.

Challenge in brain metastasis management	Potential consequence to patient	Modern treatment solution(s)
Identification of microscopic brain–tumor interface at time of surgery	Residual micrometastasis left behind despite resection	Intraoperative fluorescence, handheld confocal microscopy
Inability to target tumor bed with external beam radiation due to radiosensitive structures, prior radiation, etc.	Out-of-field recurrence, or conversely, radiation toxicity	Intracavitary brachytherapy, improved modern stereotactic radiosurgery-targeting software
Inaccessible tumor or patient unable/unwilling to tolerate open surgery	Inability to achieve cytoreduction and tissue diagnosis	Stereotactic biopsy with MRI-guided laser interstitial thermal therapy (LITT)
Negative neurocognitive effects of whole-brain radiation	Decreased patient quality of life	Stereotactic radiosurgery and other WBRT-sparing paradigms

CURRENT TREATMENT PARADIGMS

Key elements driving decision-making for brain metastasis care are patient factors and tumor factors. Patient factors include the patient's overall age, condition, and systemic disease burden, summarized as life expectancy independent of central nervous system (CNS) disease. Tumor factors include histological type, number, and location of lesions, and, more recently, the biology of the tumor based on molecular and genetic testing. Patients with poor life expectancy independent of CNS disease may reasonably be offered palliative care or no treatment for the CNS disease, regardless of the nature of the brain involvement. Conversely, patients in good medical condition with a low systemic disease burden, and hence a good survival chance independent of the brain metastases, may warrant aggressive treatment. Similarly, certain histological types of tumors (e.g., small cell lung cancer, breast cancer) are more likely to respond to adjuvant treatment with irradiation or chemotherapy, which can make their use beneficial even for numerous or poorly located lesions. Additionally, the more numerous the brain metastases, the poorer the prognosis is, irrespective of treatment. Lesions in eloquent parts of the brain (i.e., those that subserve a discrete function, such as speech or movement) or in parts of the brain less accessible *via* open neurosurgery also connote a poorer prognosis.

Neurosurgical resection of individual symptomatic brain metastases remains the standard of care. Lesions causing deficits due to local mass effect and cerebral edema should almost always undergo surgical extirpation once diagnosed, particularly if the lesion is a new diagnosis and tissue is required for pathology. Modern advances in microneurosurgical techniques and intraoperative magnetic resonance imaging-based neuronavigation allow for safe resection of lesions almost anywhere in the cerebrum. For single metastases, Patchell et al.'s landmark randomized clinical trial strongly supports surgical excision (3). Patients with a single brain metastasis underwent surgical excision followed by radiation or biopsy and radiation alone. Local control, overall survival, and quality of life were all significantly improved with surgical resection plus radiation. This study comprised mostly patients with lung cancer metastases who had high function status. Despite its lack of generalizability to all tumor patients, it remains one of the best randomized trials supporting neurosurgical intervention for brain metastases.

Traditionally, WBRT has been used after surgical resection of a single lesion or when there are multiple small asymptomatic lesions. However, WBRT carries a risk of significant cognitive morbidity, and WBRT-sparing strategies are increasingly used (4, 5). Both the American Society for Radiation Oncology and the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology have published consensus statements supporting stereotactic radiosurgery after surgical resection of a single metastasis, instead of WBRT, in patients with a single lesion and good systemic disease control (6, 7). This WBRT-sparing alternative is not supported by Level 1 randomized trial data, but rather by significant lesser strength evidence (8–12).

Lastly, depending on tumor histology and the organ of origin, standard chemotherapy is implemented at the discretion of the medical oncologist after the surgical site heals.

INTRAOPERATIVE ADVANCES IN SURGICAL TREATMENT

Neurosurgical Resection and Tumor Visualization

Nests of tumor cells exist for several millimeters outside the confines of the distinct metastatic brain lesion and its gliotic capsule (13). Aggressive resection of this microscopic margin, when feasible, can reduce the local recurrence of brain metastases (14). Therefore, the intraoperative ability to visualize and resect these microscopic margins using fluorescence-guided surgery is of considerable interest. Fluorescence-guided neurosurgery has become commonplace in glioma surgery; various agents exploit either a degraded blood–brain barrier (e.g., fluorescein) or unique metabolism [e.g., 5-aminolevulinic acid (5-ALA)], with the goal of improving the extent of resection in infiltrative processes (15–19). Within vascular neurosurgery, indocyanine green video angiography is used in cerebral aneurysm, arteriovenous malformation, and dural arteriovenous fistula surgery as an alternative or adjuvant to traditional angiography (20–24). Multiple authors have described intraoperative fluorescence for resection of metastatic tumors, albeit with less robust support than in glioma surgery or neurovascular surgery. Schebesch and colleagues published results from a series of 30 patients with brain metastases who underwent fluorescein-guided resection using a Zeiss microscope filter system (25). Most tumors (90%) avidly expressed fluorescein, and no patients suffered complications attributable to the intravenous dye. No control group was reported, but the gross-total resection rate of 83% and permanent neurological complication rate of 6.7% are similar to reported surgical results (3, 25). Therefore, the use of fluorescein requires additional study before definitive recommendations can be made about its efficacy in improving the safe extent of resection. Some authors have noted that the area of intraoperative fluorescence seems to extend well beyond the gross tumor margins, possibly

because of breakdown of the blood–brain barrier induced by the tumor. A prospective trial planned by Schebesch and colleagues will validate the usefulness of fluorescein in this setting. An alternative fluorescent agent is 5-ALA, which has found significant use in glioma surgery. Kamp et al. reported results from a retrospective series of 52 patients undergoing brain metastasis surgery using 5-ALA (26). As with fluorescein, most tumors (62%) expressed 5-ALA positivity. Residual cavity fluorescence was detected in most patients (75%) after gross-total resection of the distinct metastasis. Unfortunately, only one-third of those patients with available histological tissue samples were found to harbor microscopic disease at these 5-ALA-positive margins. Therefore, in non-eloquent areas, the use of 5-ALA seems to drive "supra-maximal" resection of surrounding reactive tissue, which means that caution is required in eloquent areas because 5-ALA positivity was not particularly sensitive for residual micrometastasis. These studies and others demonstrate the pressing need for additional research into novel fluorescent compounds to better define microscopic tumor margins in brain metastases.

Intraoperative Diagnosis

Preoperative diagnosis of a metastatic brain tumor is not always obvious, especially in patients with no known primary malignancy and an isolated lesion. Rapid intraoperative diagnosis *via* confocal microscopy is now a viable alternative to traditional frozen sectioning with light microscopy. Given the relatively limited literature surrounding fluorescence to date, the *in vivo* application of intraoperative confocal microscopy is particularly appealing for inspection of the microscopic edges of metastatic lesions within the resection cavity itself. Our group has had success with the use of *in vivo*, real-time, handheld confocal microscopy for diagnosis of various brain tumor types and visualization of the brain–tumor interface (27–30). Further technological refinement is required for handheld confocal microscopy to be widely adopted, but it remains an appealing method to detect residual tumor.

Brachytherapy

Radioactive brachytherapy seeds used in neurosurgery have had mixed results for a half-century (31–33). Brachytherapy enables delivery of high doses of radiation with quick dose fall-off and custom dosing to areas of residual tumor while sparing nearby radiosensitive structures *via* selective seed placement within the resection cavity. Isotypes used for intracranial brachytherapy have evolved significantly since the 1960s, with cesium-131 and iodine-125 now replacing older gold- and iridium-based therapies. Modern intracranial brachytherapy has been studied in atypical and anaplastic meningiomas, low- and high-grade gliomas, and metastases (34, 35). Most recently, the use of cesium-131 in brain metastases was reported by Wernicke et al. from a Phase I/II trial (36). Twenty-four patients underwent first-time gross-total resection of a brain metastasis and intraoperative placement of a permanent cesium-131 source with a planned dose of 80 Gy to a surface depth of 5 mm beyond the resection cavity. The patients had no local recurrences, no incidents of symptomatic radiation necrosis, and minimal surgical morbidity. This study was limited by its small size, limited follow-up, and the confounding variable of gross-total resection, which is associated with lower rates of recurrence and progression. Future studies will likely confirm these promising preliminary results with cesium-based brachytherapy for treatment of brain metastases.

Laser Interstitial Thermal Therapy

The use of MR-guided laser interstitial thermal therapy (LITT) for metastasis has been reported in the neurosurgical literature. MR-guided thermal ablation is not a new technology, but recent advances in materials and methods have significantly improved the ability to ablate lesion tissue accurately and safely while sparing nearby brain tissue. Two series have had good results (albeit in small samples with short follow-up) for tumors that failed to respond to traditional radiotherapy and subsequently underwent LITT. The technology allows biopsy and subsequent laser ablation from a small (approximately 4 mm) access port inserted in the operating room. Carpentier et al. described four patients with six tumors treated with LITT without complications; no tumors recurred within the 90-day follow-up (37). Hawasli and colleagues demonstrated similar results using LITT for various lesions, including five metastases (38). Two patients suffered transient neurological morbidity (one aphasia and one hemiparesis), and two had progression of CNS disease 2.2 and 3.5 months after LITT. Nevertheless, LITT has good prospects as a means to extend quantity and quality of life for patients with radiation-resistant brain metastases in eloquent or deep locations who are left with few options. Furthermore, LITT can ablate radiation treatment effect found on biopsy. Further study is warranted.

PERSONALIZED METASTASIS TREATMENT

As our technological ability to successfully treat brain metastases has grown in recent decades, so too has our knowledge of the intricate biology of tumorigenesis. The CNS is different from other organs, as blood-borne metastatic cells must first overcome the blood–brain barrier after escaping their primary site of origin. Once these cells pass this barrier, they must establish themselves in a biological niche with a milieu of cytosolic growth factors unlike those of their site of origin. Lastly, once the cells have grown into a macroscopic tumor, different metastatic brain tumors have variable responses to irradiation and chemotherapy due to genetic and epigenetic alterations and poor penetration of the blood–brain barrier by some targeted chemotherapies. Each stage offers the potential for personalized, targeted intervention or, at least, better prognostication based on molecular (vs. histological) disease stratification. Many surgical clinical trials have grouped all metastatic tumors when evaluating new treatment strategies, but these lesions clearly have numerous biological differences despite their commonality as "brain metastases."

Molecular Initiation of Distal Metastases

The molecular pathophysiology of brain metastasis has been the focus of extensive research. The ability to predict, *via* primary tumor tissue, which cancer patients will suffer brain metastasis would facilitate prognostication and focus metastasis screening efforts. Numerous lung cancer researchers have attempted to

correlate single gene mutations and chromosomal translocations with the development of brain metastases. For example, Lee et al. found that chromosomal amplifications of regions 5q35, 10q23, and 17q23–24 were associated with early development of brain metastases within 3 months of initial tumor diagnosis (39). The exact mechanisms by which these amplifications lead to lung-to-brain metastasis are not yet understood. Genes associated with the development of brain metastases in lung cancer include *PLGF*, *VEGFR1*, *c-MET*, and *CXCR4*, all of which are targets for further investigation (40–42). *HER2*-positivity predisposes patients with breast cancer to the development of brain metastases. This predisposition to brain metastases is probably caused by a combination of increased general *HER2*-positive tumor aggressive behavior as well as *HER2*-specific neural tropism *via* downstream pathways, such as TGF-β (43–45). Lastly, the pathophysiology of metastasis is not limited to protein-coding genes. Long non-coding RNA MALAT1 is associated with numerous cancer types and aggressive tumor behavior, despite a relatively poor understanding of the exact function of this highly preserved non-coding RNA. Regardless, high tumor levels of MALAT1 RNA are correlated with poor overall survival in patients with lung-to-brain metastasis, and MALAT1 promotes brain metastasis *via* the induction of epithelial to mesenchymal transitions (46). Further research is warranted on this long non-coding RNA, which represents an interesting non-protein target for personalized lung metastasis therapy.

Breaching the Blood–Brain Barrier

Once individual tumor cells have hematologically spread to the cerebral microvasculature, they must exit into the perivascular space across the blood–brain barrier to propagate macroscopic tumors. Pharmacological blockade of this transgression is a highly appealing strategy to prevent the formation of brain metastases. Research on multiple tumor types has elucidated the mechanics of this process, although much work remains. An elegant murine model by Kienast and colleagues characterized the individual steps of metastasis formation as tumor cells reach the brain (47). First, individual tumor cells arrest in tiny vessel branches. Next, cells that go on to form macroscopic tumors transmigrate across the vasculature wall within 72 h after being lodged into the capillary. After transmigration, formation of a macroscopic tumor requires that tumor cells proliferate in direct contact with endothelial cells of the brain capillary akin to a pericyte. Cells that do not maintain proximity to the vessel wall regress. Lastly, vessel co-option and angiogenesis must allow for sufficient nutrient delivery to propagate the macrometastasis (47). Each step is driven by complex molecular interactions between the tumor cell and its surroundings, and all these interactions are potential targets for more directed, individualized therapies. The process of cellular transmigration out of the capillary is regulated by complex junctional adhesion molecules, and proteases that degrade these junctional adhesions are implicated in brain metastasis. For example, high levels of cathepsin S are negatively associated with overall brain-metastasis-free survival in patients with breast cancer (48). Depletion of cathepsin S in a murine model reduced *in vivo* experimental brain metastases, thus identifying another potential personalized target for those patients with high tumor cathepsin S expression (48). The degradation of the blood–brain barrier by tumor cells is regulated not only by protein-protein interactions but also by non-canonical means. Tominaga et al. demonstrated that breast cancer cells release extracellular vesicles, including microRNA such as miR-181C, which promotes the local destruction of the blood–brain barrier *via* actin fiber delocalization in a *PDPK1*-mediated fashion (49). Other exosomal microRNAs, such as miR-105, have also been implicated in the loss of cell–cell adhesion at tight junctions (50). The blockade of microRNA signaling pathways is not yet clinically practical but may represent future targeted therapies.

Metastatic Evolution

Once established within the brain parenchyma, metastatic tumor cells continue to evolve (**Table 2**). Excellent genomic studies have demonstrated that brain metastases harbor gene alterations distinct from the primary tumor. These alterations have widespread ramifications, especially for patients with inoperative brain metastases whose primary lesion is the only tissue available for molecular profiling for additional therapy selection. Brastianos et al. found that in 53% of tumors, clinically relevant alterations occurred in the brain metastasis but not in the primary tumor (51). Many of these mutations arose in the *PI3K*/*AKT*/*mTOR*, *CDK*, and *HER2*/*EGFR* pathways, all of which have inhibitors available for clinical use. In this same cohort, multiple distinct brain lesions were genetically homogeneous compared to the extracranial metastases (51). This genetic homogeneity has significant practical implications because personalized targeted therapies for multiple brain lesions are best chosen on the basis of molecular data from any single brain metastasis rather than from the more divergent primary tumor or extracranial metastatic disease. Similar results demonstrate significant genetic divergence between brain metastasis and primary tumor tissue, specifically for squamous cell lung cancer (52). Further DNA- and RNA-based high-throughput sequencing comparing primary tissue and brain metastases will shed additional light on the metastatic process (and subsequent potential therapies) in coming years.

Targeted Drug Delivery

Personalization of metastatic cancer treatment aims to improve treatment by using select therapies chosen *via* molecular profiling

TABLE 2 | Examples of metastatic events, their molecular processes, and potential targeted chemotherapeutics.

Cellular event	Pathway(s) implicated	Potential personalized treatments
Migration across BBB	cathepsin S	Inhibitors in development
	miR-181C	None to date
	miR-105	None to date
Survival in CNS microenvironment	mTOR	Everolimus, temsirolimus
	CDK	Palbociclib, others in development
	VEGF	Bevacizumab
	EGFR	Erlotinib
Establishment of radioresistance	Chk1	Inhibitors in development
	c-Met	Cabozantinib

to benefit the patient while sparing the patient from biologically irrelevant therapies with potential toxicity. In brain metastases, this strategy is limited because of poor penetration of most novel chemotherapeutics across the blood–brain barrier. Earlier research has used mannitol as a non-specific agent for blood–brain barrier permeation with limited success (53). Researchers have since demonstrated the ability to permeate the blood–brain barrier selectively in murine models at the site of metastases using intravenous tumor necrosis factor (54). Although in its infancy, MR-focused ultrasound combined with microbubbles represents another method of blood–brain barrier disruption but requires significant dedicated infrastructure (55). Many novel targeted delivery strategies are in development in multiple centers, making it likely that more options will become available in the future.

Advances in Radiotherapy

At times, the molecular biology of radiation resistance can be overcome, allowing for more effective delivery of radiotherapy. Xenograft brain metastasis in murine models have demonstrated improved survival and response to external beam radiation after inhibition of Chk1 (DNA damage checkpoint protein) and c-Met (receptor tyrosine kinase with downstream oncogenes) (56–59). Clinical trials are required to demonstrate a benefit in human patients.

SUMMARY AND FUTURE DIRECTIONS

Brain metastases represent a common source of morbidity and mortality for cancer patients. Current treatment paradigms include surgical resection, radiotherapy, and chemotherapy. Recent advances in intraoperative surgical technology (i.e., fluorescence, confocal microscopy, and brachytherapy) hold promise for improved outcomes for brain metastasis resection. The future of brain metastasis management is predicated on personalized therapy targeted to specific tumor molecular pathways, such as those involved in blood–brain barrier transgression, cell–cell adhesion, and angiogenesis. Brain metastases are often biologically distinct lesions compared to the primary tumor. Personalized therapies should therefore be chosen on the basis of brain metastasis tissue whenever available. The multidisciplinary management of patients with brain metastases by neurosurgeons, medical oncologists, and radiation oncologists is essential as therapies become increasingly complex and individualized.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Immune Evasion Strategies of Glioblastoma

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Glioblastoma (GBM) is the most devastating brain tumor, with associated poor prognosis. Despite advances in surgery and chemoradiation, the survival of afflicted patients has not improved significantly in the past three decades. Immunotherapy has been heralded as a promising approach in treatment of various cancers; however, the immune privileged environment of the brain usually curbs the optimal expected response in central nervous system malignancies. In addition, GBM cells create an immunosuppressive microenvironment and employ various methods to escape immune surveillance. The purpose of this review is to highlight the strategies by which GBM cells evade the host immune system. Further understanding of these strategies and the biology of this tumor will pave the way for developing novel immunotherapeutic approaches for treatment of GBM.

Keywords: glioblastoma, immune system, immunosuppression, immune evasion, cancer immunotherapy

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INTRODUCTION

One of the challenges scientists face in the treatment of glioblastoma (GBM) is suboptimal responses to immunotherapy (1, 2). GBM is the most common adult brain tumor and patients usually succumb to the disease in <2 years. Despite significant improvement in chemo- and radiotherapy approaches for treatment of GBM, the median survival of one and a half years has not seen a significant change in the past few years (3, 4). Stagnation in the treatment of GBM is attributable to different challenges in therapy and our poor understating of both tumor biology and interactions with its microenvironment. Due to infiltrative growth, local microscopic metastases, and sometimes presence of multiple lesions at the time of diagnosis (5), complete surgical excision of the tumor is practically impossible and there is a strong need for new and effective therapies. With the introduction of immunotherapy as a novel and promising approach to cancer treatment, new hopes are raised for the management of brain tumors. However, as far as GBM is concerned, immunotherapeutic strategies so far have not been able to prompt a great change in survival. This article aims to review the mechanisms employed by GBM cells to suppress and evade the body's immune responses. The collection of different molecules and mechanisms discussed in this review are summarized in **Table 1** and a schematic representation of the GBM tumor cell interaction with the surrounding immune environment can be found in **Figure 1**.

CENTRAL NERVOUS SYSTEM AND THE IMMUNE SYSTEM

The central nervous system (CNS), and more specifically the brain, has been historically presumed as the "immune privileged" organ of the body due to an intact blood–brain barrier (BBB). Absence of a usual lymphatic system and paucity of antigen-presenting cells (APCs) in brain tissue have also fueled

TABLE 1 | Summary of mechanisms employed by GBM to evade the immune system.

Category[a]	Molecule/ mechanism	Major source	Effect	Reference
Central nervous system	Blood–brain barrier	CNS anatomy	Prevents entry of immune cells	(6, 7)
	Glymphatic system	CNS anatomy	Carries immune cells and macromolecules	(8, 9)
	FasL/CD95L	Astrocytes	Induces T-cell apoptosis	(10, 11)
Microenvironment	IL-6	Microglia/TAMs	Suppresses immune effector cells	(12–14)
	IL-10	Microglia/TAMs	Enhances tumor growth, inhibits production of IFN-γ and TNF-α, down-regulates expression of MHC class II in monocytes, induces anergy in infiltrating T-cells	(15–20)
	TGF-β	Microglia/TAMs	Blocks T-cell activation and proliferation, inhibits IL-2 production, suppresses natural killer cell activity, promotes Treg activity, promotes tumor growth and invasion	(21–24)
	PGE2	Microglia/TAMs	Transforms DCs into regulatory phenotype	(25–29)
	IL-1	Microglia/TAMs	Promote tumorigenesis	(12–14)
	bFGF	Microglia/TAMs	Promote tumorigenesis	(12–14)
	CD70	GBM cells	Mediates T-cell apoptosis through interaction with CD27	(30, 31)
	Gangliosides	GBM cells	Induces T-cell apoptosis	(30, 31)
	FasL	Microglia/TAMs	Induces cytotoxic T-cell compromise and apoptosis	(32, 33)
	Hypoxia	Inappropriate vascularization/ excessive oxygen consumption by GBM cells	Activation of Tregs through STAT3	(34–36)
Immune checkpoints	PD-L1	GBM cells, microglia/ TAMs	Suppresses cytotoxic T-cell proliferation and function and activated Tregs by binding to PD-1	(37–43)
	CTLA-4	GBM cells	Modulates T-cell activation	(44, 45)
Regulatory T-cells	CCL22	GBM cells	Attracts Tregs to the tumor site by binding to CCR4	(46–48)
	CCL2	GBM cells	Attracts Tregs to the tumor site by weakly binding to CCR4	(46–48)
Tumor-associated macrophages	CSF-1	Microglia/TAMs	Polarizes TAMs toward M2 phenotype	(36, 49, 50)
	TGF-β1	Microglia/TAMs	Polarizes TAMs toward M2 phenotype	(36)
	MIC-1	Microglia/TAMs	Polarizes TAMs toward M2 phenotype	(36)
	IL-10	Microglia/TAMs	Polarizes TAMs toward M2 phenotype	(36)
	S100B	GBM cells	Inhibits production of pro-inflammatory cytokines by TAMs through STAT3 pathway	(51)
	EGF	Microglia/TAMs	Promotes tumor invasion and migration	(49, 52, 53)
	IL-6	Microglia/TAMs	Promotes tumor invasion and migration	(54)
	Metalloproteinases	Microglia/TAMs	Promotes tumor invasion and migration	(55)
	VEGF	Microglia/TAMs	Promotes tumor growth and vascularity	(56–58)
Human cytomegalovirus	cmvIL-10	Infected GBM cells	Impairs mononuclear cell proliferation, inhibits DC maturation and antigen presentation, suppresses inflammatory cytokine production, promotes TGF-β production, down-regulates MHC expression, prompts monocytes differentiation into M2 macrophages, upregulates PD-L1 on tumor cells	(59–63)

[a]*The section on antigen presentation is not given a separate category as the respective pieces of information are represented in other sections of the table.*

CNS, central nervous system; IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor beta; PGE2, prostaglandin E_2; bFGF, basic fibroblast growth factor; DCs, dendritic cells; TAMs, tumor-associated macrophages; Tregs, regulatory T-cells; STAT3, signal transducer and activator of transcription 3; PD-L1, programed cell death ligand-a; PD-1, programed cell death protein-1; CTLA-4, cytotoxic T-lymphocyte antigen 4; CCL, CC chemokine ligand; CCR4, CC chemokine receptor 4; CSF-1, colony-stimulating factor-1; MIC-1, macrophage inhibitory cytokine-1; EGF, endothelial growth factor; VEGF, vascular endothelial growth factor.

this notion (6, 7). This assumption has been questioned in light of recent discoveries. The CNS possesses a functional "glymphatic system" located within the walls of dural sinuses and connected to the deep cervical lymph nodes capable of carrying immune cells and macromolecules (6, 8, 9). Immune cells can migrate into the brain parenchyma by chemotaxis, in which interferon-gamma (IFN-γ) and integrins play a major role (64, 65). Antigens can pass through walls of cerebral arteries and enter cervical lymph nodes through the Virchow–Robin perivascular spaces (66). By attaching to FcRn, a receptor found on a variety of body tissues, immunoglobulins are also able to cross the BBB via carrier-mediated transport (67, 68). APCs are present in many areas of the brain, including leptomeninges, ventricles, and perivascular spaces (69, 70). Via the rostral migratory stream, dendritic cells (DCs) can travel outside the brain and present antigens to T-cells located in the cervical lymph nodes (71). Peripheral immune cells can migrate to the CNS perivascular spaces but not into the brain parenchyma, thanks to the BBB. Tight junctions between foot processes of astrocytes form the physical BBB between perivascular spaces and parenchyma, while FasL/CD95L, expressed on these processes, induces apoptosis of T-cells that express the Fas receptor (10, 11). In disease states however, the integrity of the barrier is compromised, enabling immune cells to migrate past the BBB (72). During clinical trials for DC vaccines in patients with brain tumors, tumor-infiltrating lymphocytes have been observed in GBM samples (73, 74).

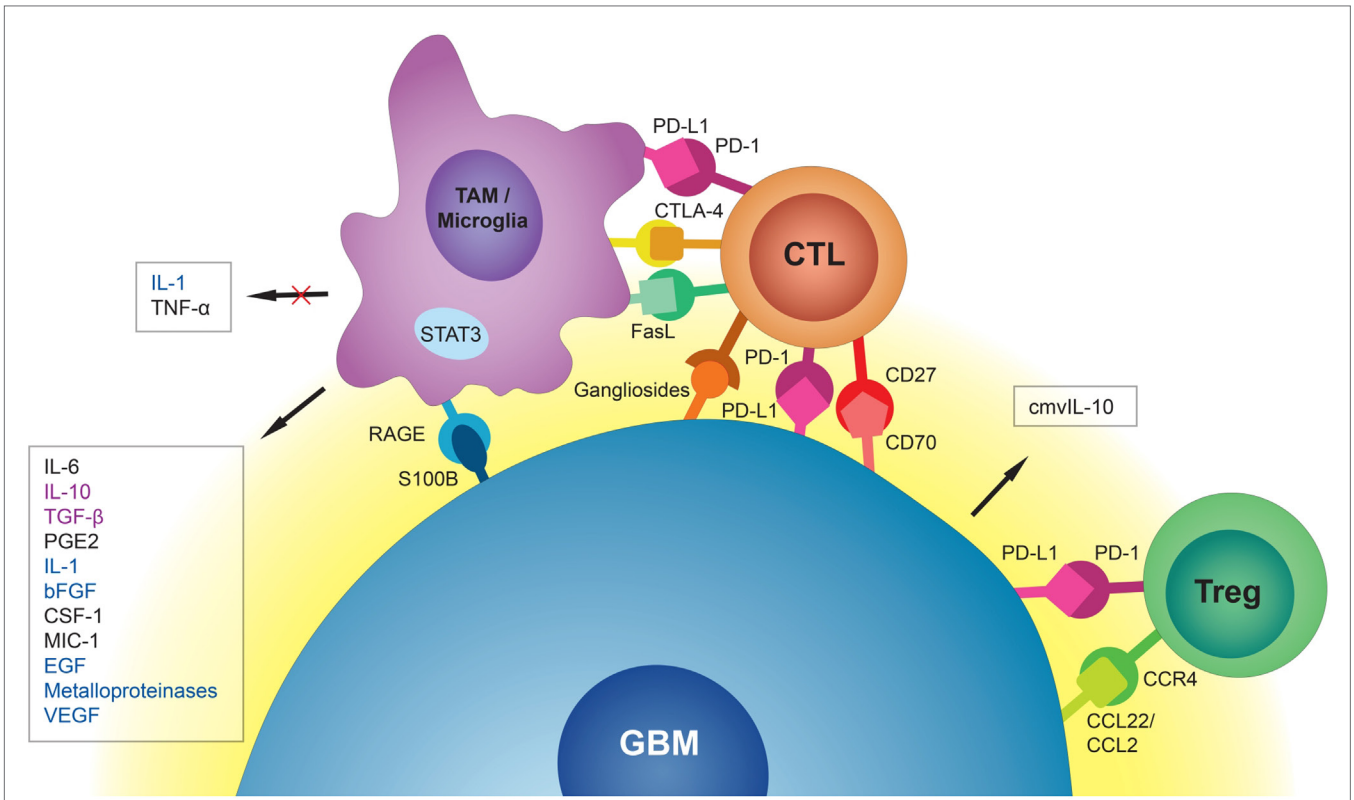

FIGURE 1 | Schematic representation of the GBM tumor cell interaction with surrounding immune environment. Tumor-associated macrophages (TAMs) and microglia release immunosuppressive and pro-tumorigenic cytokines into the GBM microenvironment. They also induce cytotoxic T-cell (CTL) apoptosis via PD-L1, CTLA-4, and FasL. GBM cells, through interaction of S100B protein with receptor for advanced glycation end products (RAGE), inhibit production of immunostimulatory cytokines by TAMs and microglia. CMV-infected GBM cells secrete cmvIL-10 into their microenvironment with a range of immunosuppressive properties. Through interaction of CC chemokine ligand 22 (CCL22) and the weaker CC chemokine ligand 2 (CCL2) with CC chemokine receptor 4 (CCR4), GBM cells attract regulatory T-cells (Tregs) to the tumor site. Interaction of PD-L1 on GBM cells with PD-1 on Tregs promotes immunoregulatory functions of these cells. Immunosuppressive signals (black) could be distinguished from tumorigenic signals (blue) and signals that are both immunosuppressive and tumorigenic (purple).

MICROENVIRONMENT

Functional immunosuppression in the GBM microenvironment is characterized by production of immunosuppressive cytokines, inhibition of T-cell proliferation and effector responses, activation of FoxP3+ regulatory T-cells (Tregs), and tissue hypoxia. Immunosuppressive cytokines, including interleukin (IL)-6, IL-10, transforming growth factor-beta (TGF-β), and prostaglandin E_2 (PGE2), as well as tumor-promoting cytokines, IL-1, and basic fibroblast growth factor (bFGF), are present in the GBM microenvironment and dampen the antitumor immune response (12–14). TGF-β promotes immunosuppression in GBM by blocking T-cell activation and proliferation, inhibiting IL-2 production, suppressing natural killer cell activity, and promoting Tregs (21). In addition, TGF-β has been shown to promote tumor growth and invasion by supporting GBM stem cells and enhancing angiogenesis (22–24).

Generally known as an immunosuppressive cytokine, IL-10 is found at high levels in a variety of neoplasms (15, 16). This cytokine is secreted by various immune cells (mainly macrophages, but also helper and cytotoxic T-cells, DCs, B-cells, monocytes, and mast cells) as well as GBM cells (16, 17). IL-10 associated with GBM is shown to enhance tumor growth (18), inhibit production of IFN-γ and tumor necrosis factor-alpha (TNF-α) by the immune system, downregulate expression of MHC class II in monocytes, and, via the co-stimulatory CD28-CD80/86 pathway, induce anergy in infiltrating T-cells (19, 20).

PGE2 is known to promote regulatory immune response in cancers and stimulate tumor cell growth (25). Together with TGF-β, it transforms DCs into a regulatory phenotype that suppresses T-cell proliferation (26, 27). In the GBM microenvironment, however, the concentration of PGE2 is not found to be high enough to suppress T-cell functions on its own (28, 29).

The GBM microenvironment also mediates immunosuppression via mechanisms that increase T-cell propensity to apoptosis through a cooperative interaction between CD70 and gangliosides (30, 31). CD70, through interaction with CD27, a member of TNF receptor family proteins, mediates apoptosis in T-cells. Inhibition of gangliosides, components of the plasma membrane that modulate signal transduction events, causes GBM cells to be significantly less efficient at inducing T-cell apoptosis. It has been shown that blocking both CD70 and ganglioside function

produces an additive effect on provoking T-cell apoptosis (31). Programed cell death protein-1 ligand (PD-L1, B7-H1, or CD274), a potent immunosuppressive molecule, is expressed on microglia. The expression of PD-L1 on microglial cells is increased when in proximity to GBM cells that can induce T-cell apoptosis (37–39). The role of PD-L1 as an immune checkpoint is discussed further in the respective section. Another immunoinhibitory molecule expressed on tumor-associated microglia is FasL, which can induce cytotoxic T-cell compromise and apoptosis. Inhibition of FasL has resulted in an increased number of immune cells within the tumor (32, 33).

Lack of oxygen in the GBM microenvironment is the result of morphologically inappropriate neovascularization, irregular blood flow, and excessive consumption of oxygen from rapidly proliferating tumor cells. Hypoxia is a strong stimulus for expression of genes involved in tumor cell growth and angiogenesis (34). Specifically, the hypoxic GBM microenvironment activates signal transducer and activator of transcription 3 (STAT3), an immunosuppressive pathway and potent regulator of anti-inflammatory responses, which triggers the synthesis of hypoxia-inducible factor-1α (HIF-1α) that subsequently induces activation of Tregs and production of vascular endothelial growth factor (VEGF) (34). Tregs are modulators of the immune response, and VEGF is known for its immunosuppressive effects. Additionally, the hypoxic microenvironment triggers CNS macrophages to transform into tumor-associated macrophages (TAMs), which then adopt immunosuppressive and tumor-supportive phenotypes (M2). This transformation, via the STAT3 pathway, induces TAMs to promote angiogenesis and tumor cell invasion (35). Additionally, it has been shown that TAMs are modulated by GBM cancer stem cells (gCSCs) through induction of an immunosuppressive phenotype via the STAT3 pathway (36). Furthermore, since HIF-1α promotes gCSCs, hypoxia likely causes a feed-forward mechanism in tumor-mediated immunosuppression.

ANTIGEN PRESENTATION

Despite tremendous research, the mechanisms involved in developing tumor-sensitized immune effector cells are not well understood. Antigens from dead tumor cells are collected and processed by APCs and "cross-presented" on MHC class I to cytotoxic T-cells (75). Whether this antigen presentation for GBM occurs mainly in the brain or in the periphery is a subject of ongoing research (76). Microglia are the major myeloid immunocompetent cells of the brain, and scientists have elaborated their ability to present antigens to cytotoxic T-cells within the CNS (77, 78). However, the immunosuppressive microenvironment of GBM down-regulates MHC expression and compromises the antigen-presenting ability of microglia (79–83). GBM cells also stimulate secretion of IL-10 and inhibit production of TNF-α by microglia, further promoting suppression of the immune response (84). In fact, studies suggest that tumor-infiltrating DCs have a bigger part in GBM antigen presentation. In a 2008 study, Beauvillain et al. discovered that tumor-infiltrating DCs were more efficient than neonatal microglia in priming cytotoxic T-cells with exogenous antigens and could trigger higher levels of IL-2 and IFN-γ secretion by these cells (85). Presence of tumor-infiltrating DCs in the brain alongside microglia would prompt a better immune response in the CNS (77). Both glioma-associated antigen-pulsed and tumor-lysate-pulsed DCs have been successful in eliciting T-cell response in GBM patients (73, 74, 86). Wilms' tumor 1 (WT1)-pulsed DC vaccine could improve neurological findings and shrink the tumor in a recent study (87). Nonetheless, tumor microenvironments would also blunt the action of tumor-infiltrating DCs and further investigation is needed to optimize this therapeutic technique (14, 20).

Macrophages are the major population of immune cells infiltrating solid tumors and GBM (88, 89). These cells are involved in antigen presentation, immune induction, cytotoxicity, removal of debris, regulation of inflammatory response, and thrombosis. Macrophages derived from monocyte precursors polarize into two distinct categories based on signals from the environment: M1, with a pro-inflammatory cytokine profile, and M2, with overall anti-inflammatory properties. Exposure to IFN-γ or bacterial lipopolysaccharide polarizes monocytes toward M1 macrophages. An alternate activation process happens by exposure to IL-4, resulting in the M2 category (90, 91). TAMs are believed to be of the latter population as they share many functions and surface proteins with M2 macrophages. While TAMs are known to be capable of cross-presenting tumor antigens to T-cells and prime antitumor immune response (92) due to limitations in histologic differentiation of TAMs from microglia, there is no definite answer to their importance in tumor antigen presentation in the brain (93, 94).

While mainly involved in humoral immune response, B-cells can also act as APCs and directly present antigens to T-cells via both MHC class I and II (95–97). Interaction of GBM cells with tumor-infiltrating B-cells has not been thoroughly investigated. Candolfi et al. studied the role of B-cells in a GBM murine model. After treatment of mice with intratumoral adenovector and immunostimulatory cytokines, B-cells were found to have remnants of tumor antigens in their cytoplasm and the ability to stimulate T-cell proliferation *in vitro* (98).

Tumor antigen presentation can also occur in peripheral lymph nodes. Activated T-cells have been found in the cervical lymph nodes of murine GBM models (99). Evidence exists that CNS antigens can move out of the CNS through perivascular spaces and be collected by resident DCs in cervical lymph nodes (100). Immunosuppressive cytokines secreted by GBM cells do not have a high enough systemic concentration to justify impairment of peripheral immune cell functions (101, 102). Engineered CTLs targeting IL-13 receptor 2 have shown promise in GBM *in vivo* models (103). Regardless of the underlying cause, vitiated cell-mediated immunity in GBM patients can compromise antigen presentation and T-cell activation even in the peripheral lymphatic tissue, adding to the challenges of immunotherapeutic efforts.

IMMUNE CHECKPOINTS

Immune checkpoint molecules, a group of co-stimulatory and co-inhibitory pathways that limit the function of immune system, have recently been targets for extensive research. By inhibition of immune checkpoints, researchers were able to reverse

immunoresistance of cancer cells and activate the immune cells against tumors (104).

A major immune checkpoint molecule implicated in GBM immune evasion is PD-L1. Modulated by the PI(3)K–Akt–mTOR pathway (38), PD-L1 suppresses proliferation and function of cytotoxic T-cells and promotes Tregs activity by binding to programed cell death-1 (PD-1) (40). Expression of PD-L1 on tumor cells and T-cells is correlated with tumor grade (41) and poor survival of GBM patients (42). Microglia and TAMs are also known to express PD-L1 on their surface and at the same time promote PD-L1 expression on GBM cells (37, 43, 105). Collectively, these findings have made this immune checkpoint a prime target for GBM immunotherapy. Pre-clinical studies have been promising (106, 107) with plans for clinical trials on GBM patients currently under way.

Another immune checkpoint molecule, cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on activated T-cells and Tregs could play a role in GBM immune evasion. Targeting CTLA-4 in GBM models might be able to enhance antitumor activity by T-cells (44, 45). Immune checkpoint inhibitors as targeted cancer therapeutics have shown promise in recent years with researchers trying to find new checkpoints as immunotherapeutic targets.

REGULATORY T-CELLS

Tregs, a small population of CD4+ T-cells that specifically express FoxP3 transcription factor, are a group of circulating lymphocytes with suppressive effects on various immune cells (108, 109). Other markers that help distinguish Treg subpopulations are CD25 (high-affinity IL-2 receptor), CTLA-4, and glucocorticoid-induced tumor necrosis factor receptor (110). Tregs can be divided into two major subpopulations based on their origin. Thymus-derived Tregs, developed from naïve CD4+ cells after antigen presentation in the thymus, express high levels of FoxP3. By contrast, under IL-10 and TGF-β signaling in the periphery, conventional CD4+ T-cells differentiate into peripherally induced Tregs with negligible FoxP3 expression (109). Tregs are commonly known to regulate immune response against tumor cells and to shift the tumor cytokine milieu toward immunosuppression. The presence of Tregs in GBM patients was described years ago (111), but their intricate function and interaction with other cells is a matter of ongoing investigation. A higher population of Tregs is demonstrated in GBM patients, reported to comprise up to 25% of tumor-infiltrating lymphocytes, and their abundance is associated with poor prognosis (112–114). Studies have revealed that glioma-associated Tregs are mostly of thymic origin rather than tumor-derived (115), suggesting that the abundance of Tregs in GBM is a result of chemotactic attraction of the thymus-derived subpopulation rather than local differentiation in the tumor (116). The CC chemokine ligand 22 (CCL22) and the weaker CC chemokine ligand 2 (CCL2) are among the first molecules revealed to attract Tregs to the tumor site by binding to CC chemokine receptor 4 (CCR4) (46, 47). Further studies revealed that blocking this receptor cannot completely abrogate Treg infiltration into GBM tumor mass, suggesting involvement of other secretory molecules in Treg chemoattraction (48). Peripherally derived Tregs are not believed to be the major population of Tregs in GBM, but presence of IL-10 and TGF-β at high levels in the GBM microenvironment suggests the possibly noticeable role of these cells in immune evasion of the tumor (14, 109). Further studies are needed to reveal the holistic picture of Tregs recruitment mechanisms into GBM.

TUMOR-ASSOCIATED MACROPHAGES

Involvement of macrophages in GBM progression is a question to be further investigated. Recent studies provide significant evidence in contextual response of macrophages in tumor progression, highly modulated by the tumor microenvironment and tumor response to conventional treatments. Distinguishing TAMs from microglia in the brain is still a challenge for researchers. While TAMs are found to have a high expression of CD11b and CD45 compared to microglia, which have high expression of CD11b but low expression of CD45, there is still disagreement over a universally accepted histological marker that distinguishes the two cell types (117, 118).

Tumor-associated macrophages are usually linked to accelerated disease progression and poor outcome in cancer patients (119–121). Recently, several approaches have been investigated to abrogate tumor progression through ablating TAMs. Modulating the routes involved in macrophage polarization has provided insight into the regulatory effect of these cells in the GBM microenvironment (122).

Innate immunosuppressive properties of gliomas are derived from the regulatory cross-talk between M2 phenotype macrophages and tumor cells (93). Macrophages and microglia as dominant populations of tumor-infiltrating immune cells are, to a great extent, regulated by glioma initiating cells. Upon chemoattraction into the tumor environment (47, 49, 123, 124) with a high concentration of colony-stimulating factor-1 (CSF-1), TGF-β1, macrophage inhibitory cytokine-1 (MIC-1), and IL-10, TAMs are polarized toward the M2 phenotype, subsequently inhibiting their phagocytic ability and enhancing their capacity to inhibit cytotoxic T-cell proliferation and increase the effect of Tregs (36). Inhibiting the CSF-1 receptor can shift the polarization of TAMs away from M2, hinder their tumor-promoting functions, and increase survival of the GBM-bearing mice (50). Another protein recently found on GBM cells to induce innate immune suppression is S100B. Through interaction of S100B with receptor for advanced glycation end products (RAGE) on macrophages, GBM cells induce the STAT3 pathway in TAMs and inhibit the production of IL-1β, TNF-α, and other pro-inflammatory cytokines by these cells (51).

Tumor-associated macrophages and microglia can also play a role in GBM growth, invasion, and angiogenesis. Endothelial growth factor (EGF), CSF-1, TGF-β1, IL-6, and metalloproteinases originating from TAMs and microglia are instrumental for glioma invasion and migration (49, 52, 54, 55, 125). Inhibition of the EGF receptor (EGFR) on GBM cells has been associated with antiangiogenic and proapoptotic effects on the tumor (53). Inhibition of VEGF signaling in TAMs and microglia leads to decreased GBM growth and vascularity (56), but addition of anti-VEGF-A antibody to standard treatment has not improved

patient survival (57, 58). Other populations of cells from myeloid lineage have been found in gliomas, including tumor-associated neutrophils, angiogenic monocytes, and immunosuppressive myelomonocytic cells, the importance of which is yet to be elucidated (126).

HUMAN CYTOMEGALOVIRUS INFECTION

Human cytomegalovirus (HCMV) is a β-herpesvirus implicated in GBM pathogenesis. Different studies have found HCMV genome in most tested GBM samples with no trace of the virus in surrounding brain tissue (59, 60). The role of HCMV in GBM development and pathogenesis is not yet clarified. What is clear though is that HCMV infection could play a role in immunosuppression in the context of GBM microenvironment.

Human cytomegalovirus genome encodes an IL-10 homolog (cmvIL-10) – a product of UL111A gene – that could impair mononuclear cell proliferation, inhibit DC maturation and antigen presentation, suppress inflammatory cytokine production, and down-regulate MHC expression (61, 62). Moreover, it has been demonstrated that cmvIL-10 prompts monocytes to differentiate into M2 macrophages and up-regulates the immunoinhibitory PD-L1 protein on GBM cells. Additionally, monocytes treated with cmvIL-10 produce TGF-β, augmenting the immunosuppressive microenvironment (63).

SUMMARY AND FUTURE PROSPECTS

The interaction of GBM with the immune system is intricate at every level. Any of the various mechanisms employed by this tumor to evade and suppress the immune response could be targeted with immunotherapy. To date, trials of immunotherapeutic modalities for GBM have not been as successful as promised. As different mechanisms of GBM immune resistance are revealed, scientists could have a better understanding of the pitfalls in GBM immunotherapy. GBM strategies for immune evasion are diverse and the key to successful immunotherapeutic treatment seems to be in targeting several pathways at the same time.

AUTHOR CONTRIBUTIONS

SMR organized and wrote major parts of the article. KL helped with writing the article. BJ helped with writing the article. PA helped with designing the figure. SG helped with writing the article.

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