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Commentary

Molecular targets for emerging anti-tumor therapies for neurofibromatosis type 1

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Abbreviations:
EGFR, epidermal growth factor receptor
FTI, farnesyl transferase inhibitor
GGTI, geranylgeranyl transferase inhibitor
GAP, GTPase activating protein
MPNST, malignant peripheral nerve sheath tumor
NF1, type 1 neurofibromatosis
PAK, p21-activated kinase
PAT, protein fatty acyltransferase

ABSTRACT

Neurofibromatosis type 1 (NF1) is the most common cancer predisposition syndrome. NF1 patients present with a constellation of clinical manifestations and have an increased risk of developing certain benign and malignant tumors. This disease results from mutation within the gene encoding neurofibromin, a GTPase activating protein (GAP) for Ras. Functional loss of this protein compromises Ras inactivation, which leads to the aberrant growth and proliferation of neural crest-derived cells and, ultimately, tumor formation. Current management of NF1-associated malignancy involves radiation, surgical excision, and cytotoxic drugs. The limited success of these strategies has fueled researchers to further elucidate the molecular changes that drive tumor formation and progression. This discussion will highlight how intracellular signaling molecules, cell-surface receptors, and the tumor microenvironment constitute potential therapeutic targets, which may be relevant not only to NF1-related malignancy but also to other human cancers.

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1. Neurofibromatosis type 1: introduction

Neurofibromatosis type 1 (NF1) is the most common inherited cancer predisposition syndrome, having a birth incidence of ~1:3000 [1]. NF1 is marked by the aberrant proliferation of tissues derived from the neural crest. Patients with this disease present with a constellation of clinical manifestations, including pigmentation lesions on the skin (café au lait macules), hamartomas of the iris (lisch nodules), learning disabilities, and malformations of the skeletal and cardiovascular systems [2]. NF1 patients are also at a higher risk of developing certain tumors, most notably optic pathway gliomas and neurofibromas. Neurofibromas comprise predominantly transformed Schwann cells and can involve any peripheral nerve. These tumors undergo malignant progression to malignant peripheral nerve sheath tumors (MPNSTs) in approximately 10% of all cases and remain the major source of morbidity and mortality [3]. Traditional management of NF1-associated tumors involves radiation, surgical excision, and cytotoxic drugs but provides limited long-term success [4]. The need for more specific approaches has fueled researchers to further elucidate the molecular changes that distinguish transformed Schwann cells from normal Schwann cells.

2. Loss of neurofibromin deregulates Ras signaling

NF1 is caused by mutation within the gene encoding the protein neurofibromin (Nf1). This protein plays a critical role in embryological development and remains important to the normal physiology of certain cell types, especially neural crest derivatives [5]. Germline mutation of one neurofibromin allele results in global haploinsufficiency that leads to cellular dysfunction in neural crest derivatives. The majority of clinical manifestations of this disease are thought to be a consequence of this haploinsufficiency. Disruption of both neurofibromin alleles (“loss of heterozygosity”) in Schwann cells deregulates critical cell signaling pathways and drives malignant tumor progression [1,2].

Nf1 contains a central domain (the Nf1-GAP-related domain or Nf1-GRD) that shares extensive sequence homology with other GAPs that regulate Ras [6]. Ras is a small GTPase and plays a central role in cell survival, proliferation, and differentiation by transducing responses to growth stimuli initiated at the cell surface to several intracellular signaling molecules [7]. Ras functions as a molecular switch: it toggles between an inactive (GDP-bound) state and an active (GTP-bound) state. Transition from one state to the other is regulated by guanine nucleotide exchange factors (GEFs), which mediate the replacement of GDP with GTP, and by GAPs, which catalyze the hydrolysis of GTP. Nf1 accelerates the rate of Ras-GTP hydrolysis ~10^5-fold more quickly, compared to the intrinsic rate of Ras-GTP hydrolysis [8]; therefore, Nf1 serves as a potent negative regulator of Ras. Activating point mutations in codons 12,13, or 61 of Ras lead to forms that are resistant to GAP-mediated hydrolysis. These constitutively active (oncogenic) mutants drive aberrant cell division and contribute to over 30% of all human cancers [9,10]. Ras is structurally normal in NF1 tumor cells; however, as diagrammed in Fig. 1, functional loss of Nf1 compromises Ras inactivation and drives deregulated Ras-dependent signaling pathways [11]. Studies have confirmed the prediction that Nf1-deficient Schwann cells maintain an accumulation of active Ras (up to 15 times higher than normal counterparts) and an increase in Ras-dependent signaling [12,13]. This aberrant Ras signaling occurs despite the continued expression of other proteins with Ras-GAP activity, which suggests a unique ability of Nf1 to regulate Ras at specific subcellular locations [12]. Nf1 likely serves functions outside its role as a Ras-GAP; for example, Nf1 can associate with microtubules and regulate cellular cyclic AMP levels [14–16]. Additional genetic or epigenetic events involving loci outside the Nf1 gene (inactivation of pRb, p53, p15 (INK4b), p16 (INK4a), p14 (ARF), p27 (Kip1), cyclin D1, MDM2, etc.) may also contribute to NF1-related malignancy [17–19]. However, introduction of the Nf1-GRD alone restores normal growth to Nf1-deficient Schwann cells [20]. In addition, a specific point mutation within the Nf1-GRD, which specifically abolishes Ras-GAP activity but does not affect the secondary or tertiary structure of the protein (conserving other putative functional domains within the protein), results in classical expression of the disease [21]. These observations underline the importance of Ras activation in the disease process and highlight Ras as a plausible pharmacological target.

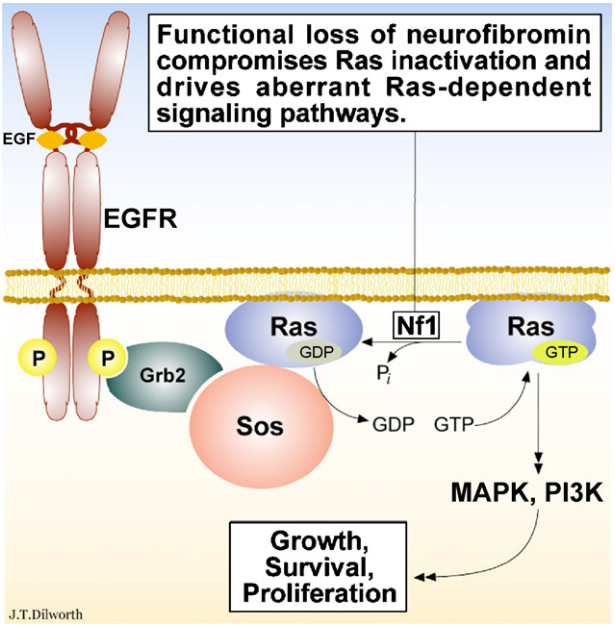

Fig. 1 – Loss of neurofibromin deregulates Ras signaling. In response to growth factors, the EGFR recruits a guanine nucleotide exchange factor Sos, which activates Ras. In its active, GTP-bound, form, Ras initiates several signaling cascades, including the MAPK and PI3K pathways, in order to regulate cell growth, survival, and proliferation. Functional loss of neurofibromin compromises Ras inactivation and drives aberrant Ras-dependent signaling, which contributes to tumor formation and progression.

3. Ras as a therapeutic target

Ras is translated on free ribosomes and then undergoes a series of post-translational modifications to achieve membrane attachment (Fig. 2). This post-translational processing is directed at its four most C-terminal residues: its ''CaaX'' box (''C'' = cysteine, ''a'' = aliphatic, and ''X'' = any amino acid, usually, methionine, serine, or glutamine) [22]. First, a 15-carbon farnesyl group is covalently attached to the cysteine. Second, the last three residues are cleaved. Third, the prenylated cysteine is methylated. In addition to these steps, N- and H-Ras isoforms are further modified with the attachment of a palmitate group. These modifications (for K-Ras, a poly-lysine stretch of amino acids takes the place of palmitoylation) facilitate association with lipid membranes and govern subcellular localization as well as protein–protein interactions [23]; moreover, these modifications are essential not only for the normal function of Ras but also for the transforming activity of Ras [24,25].

In an attempt to antagonize Ras activity in cancer cells, researchers have employed drugs that inhibit the various enzymes responsible for CaaX-box alterations with the rationale that improper CaaX processing leads to mislocalized and thus non-functional protein [24,26,27]. Mislocalized constitutively active Ras may recruit and sequester signaling molecules to inappropriate subcellular locations [28]. This

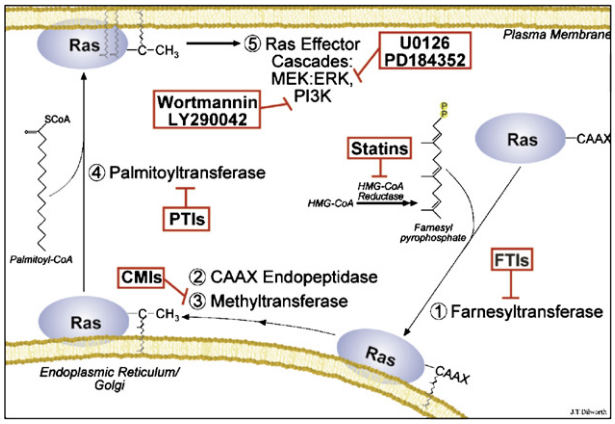

Fig. 2 – Ras and Ras-dependent signaling molecules are pharmacological targets. Ras is translated on free ribosomes and then undergoes a series of post-translational modifications in order to achieve membrane attachment. This post-translational processing is directed at the C-terminal CaaX box and includes farnesylation, cleavage, and methylation. N- and H-Ras are, subsequently, palmitoylated at cysteine residues that neighbor the CaaX box. The attachment of these lipid moieties governs subcellular localization and protein:protein interactions and is crucial for both the physiological and transforming ability of Ras. Pharmacologically targeting the enzymes involved with Ras processing or signaling molecules downstream of Ras provides numerous therapeutic strategies. *Abbreviations*: **FTIs = farnesyl transferase inhibitors, CMIs = carboxymethylase inhibitors, PTIs = palmitoyltransferase inhibitors.**

action may limit the amount of substrate available for properly located Ras proteins. There exist at least two different approaches to blocking the prenylation step. The lipid group donor, farnesyl pyrophosphate, is an intermediate in the biosynthesis of both prenylated proteins and cholesterol, and its production depends on the enzyme HMG-CoA reductase. Inhibiting this enzyme with a member of the statin family of drugs reduces the formation of lipid substrate and interferes with CaaX processing [26,27]. Blocking the attachment of farnesyl to Ras accomplishes the same goal. The latter approach is accomplished by inhibiting the enzyme farnesyl protein transferase, using farnesyl protein transferase inhibitors (FTIs) [28]. It has been proposed that statins' ability to regulate protein prenylation allows these drugs not only to lower cholesterol but also to influence multiple cellular functions important in tumor formation and metastasis. These functions include cell proliferation, motility, adhesion, and invasion [29]. Regarding NF1, statins may also serve a therapeutic role separate from that in tumor formation. One report describes the ability of lovastatin to rescue neuronal long-term potentiation (LTP) and learning deficits in Nf1 conditional knockout mice [30].

The FTIs lonafarnib (SCH 66336), tipifarnib (R115777), BMS-214662, FTI-277, and L-744,832 have produced promising results in preclinical trials [31,32]. They have been shown to inhibit the growth of numerous human tumor cell lines in vitro and have resulted in either tumor growth inhibition or tumor regression in a spectrum of xenograft models involving hematological, head and neck, ovarian, lung, colon, breast, bladder, melanoma, and prostate cancers [24,33]. The literature regarding the use of FTIs with NF1-derived cell lines is more limited. Yan et al. showed that the treatment with FTIs led to growth inhibition and morphological reversion of an MPNST cell line [27]. Weiss et al. showed that L-744,832 inhibited H-Ras prenylation in primary Nf1-null hematopoietic cells [34] and Mahgoub et al. demonstrated that the same drug blocked the proliferation of Nf1-null myeloid progenitor colonies in response to granulocyte/macrophage-colony stimulating factor (GM-CSF) [35]. The FTI L-739,749 proved to reverse the proliferation of Nf1-null Schwann cells but had no effect on the angiogenic or invasive quality of these cells [36]. The use of lonafarnib and tipifarnib to treat non-NF1 cancers in phase II and phase III clinical trials has achieved limited success thus far compared to that in placebo groups [24]. While there is an on-going phase II clinical trial evaluating the effects of the FTI R115,777 on progressive plexiform neurofibromas (National Cancer Institute, NLM#: NCT00021541), research has shifted to alternative strategies.

A number of factors may explain the limited usefulness of FTIs in the clinic. Of the four Ras isoforms (N-, H-, and two K-Ras), N- and K-Ras can, alternatively, become geranylgeranylated in the presence of FTIs [22,37]. This substituted lipid group allows N- and K-Ras to signal from membranes and, thus, circumvent the action of FTIs. With N- and K-Ras more commonly mutated than H-Ras in human cancers, FTIs may only be useful in a small subset of cases. NF1 seems to follow this theme: we have shown that N- and K-Ras are the predominant isoforms expressed and active in human NF1 cell lines [12]. Recognizing the potential limitation of FTIs, researchers have investigated the effects of inhibiting geranylgeranyl transferase, the protease (Ras converting enzyme)

responsible for cleavage of the CaaX box, or the carboxymethylase responsible for methylation of the CaaX box [22,24] (Fig. 2). While these approaches enable manipulation of N- and K-Ras, another confounding issue exists: CaaX boxes are not exclusive to Ras. About 0.5% of all proteins are prenylated, including Ras, Rho proteins, the nuclear lamins, and certain kinetechores [38,39]. Thus, the effects of CaaX-box processing inhibitors on cell cycle, morphology, motility, etc. are not attributed solely to blocking Ras function. For example, our data show that the anti-proliferative effects of novel pro-drug FTIs, especially in combination with Lovastatin, on vascular smooth muscle cells [40] and on NF1 and non-NF1 MPNST cells[1] may at least in part be due to an effective block of RhoB prenylation. The fact that several tumors without Ras mutations respond to FTIs and geranylgeranyltransferase inhibitors (GGTIs) also supports the notion that the regulation of non-Ras targets at least contributes to the anti-cancer effects of these drugs [41].

Ras-directed therapeutic approaches may require the identification of enzymes that show more selective action on Ras proteins. One candidate may be the enzyme(s) involved with Ras palmitoylation. Palmitate modification is a dynamic (reversible) process necessary for proper function of numerous proteins, including Ras and Rho proteins, non-receptor tyrosine kinases, G-protein-coupled receptors, and components of the plasma membrane SNARE complex [42]. Soon after the identification of the putative protein fatty acyltransferase domain DHHC-CRD [43], Swarthout et al. described a protein complex composed of DHHC9 and a golgi-localized protein that possesses protein fatty acyltransferase (PAT) activity for H- and N-Ras [44]. Palmitoylated cysteine residues exist in a wide variety of sequence contexts and may mandate a large family of PATs with different substrate specificities. At least 23 DHHC-CRD-containing proteins have emerged from human genomic sequencing [43]. Identification of PATs exclusive for Ras isoforms may provide avenues for more specific drug design.

4. Ras: downstream effectors

Dasgupta et al. showed that blocking K-Ras by expressing a dominant negative K-Ras protein reversed the proliferative advantage and abnormal actin cytoskeletal-mediated processes in Nf1-null astrocytes [45]. These results demonstrate the advantage of reducing the activity of specific Ras isoforms, yet this is not currently pharmacologically possible. As a result, researchers have looked downstream of Ras. Ras produces its effects on the cell via several effector molecules, including those that constitute the Raf–MAPK–ERK and PI3K–Akt axes [46] (Fig. 2). These signaling cascades mediate promitotic and pro-survival effects, and an upregulation of both is correlated with the increase of Ras-GTP subsequent to loss of Nf1 [12,27,47]. Multiple lines of evidence support the importance of these pathways in NF1-related tumorigenesis. While Schwann cells are normally quiescent, they re-enter the cell cycle to proliferate and rebuild the myelin sheath following nerve damage [48]. Regeneration involves de-differentiation, proliferation, and re-differentiation. This repair response is governed by the MAPK–ERK pathway [48] and may parallel the process of tumorigenesis in Nf1-deficient cells. If increased activation of the Ras–ERK pathway is responsible for the proliferative capacity of Nf1-deficient Schwann cells, blocking ERK activation should inhibit cell division. Our group has shown that treatment of NF1 cell lines with the MEK1, 2 inhibitors U0126, PD184352, and PD98059 resulted in concentration-dependent suppression of proliferation [12]. Chadee and Kyriakis showed that blocking ERK activation (along with blocking JNK and p38 activation) using RNAi against mixed lineage factor 3 (MLK3) had anti-proliferative results in MPNST cells [49]. Another target, p21-activated kinase (PAK), may be relevant since it is activated downstream of Ras (via Rac and CDC42) and permits Ras-dependent activation of ERK [50,51]. Using a dominant negative PAK vector to block ERK activation, Tang et al. blocked Ras-mediated transformation of Schwann cells [51]. Hirokawa and co-workers also supported the role of PAK in Schwann cell transformation by using the histone deacetylase inhibitor FK228 to activate gelsolin and p21(WAF) and to inhibit PAK, which led to regression of a MPNST xenograft in nude mice [52].

PAK also promotes the PI3K–Akt pathway. This pathway is upregulated in response to increased Ras-GTP levels and, by protecting cancer cells from programmed cell death, may enhance tumor growth [53]. The rationale for inhibiting proteins within the MEK–ERK pathway extends to those that constitute the PI3K–Akt pathway. The PI3K inhibtor LY294002 was found to reduce EGF-stimulated cell growth of MPNST cells [54]. Johannessen et al. proposed that increased PI3K activation results in constitutive activation of mTOR (by inactivating the protein TSC2); they found that inhibiting mTOR with rapamycin also blocked the proliferation of MPNST cells [55]. Further support of these pathways in tumorigenesis comes from work done by Huang et al., which shows that inhibiting either the PI3K or the MAPK pathway decreased the migration of Nf1-null mouse Schwann cells [56].

5. Ras: upstream activators

Due to crosstalk within protein networks, additional intracellular signaling molecules will likely emerge as therapeutic targets, yet promising candidates exist on the surface of cancer cells as well. One feature of MPNST cells is their expression of the epidermal growth factor receptor (EGFR), which is absent from normal Schwann cells [57]. In response to soluble growth factors, the EGFR activates Ras, which in the context of Nf1-deficiency is greatly potentiated. The acquisition of the EGFR is correlated with the loss of Nf1 and may confer a growth advantage to Schwann cells [57]. Our data suggest that the increase in EGFR is a result downstream of activated N- and K-Ras. This reciprocal interaction between the EGFR and Ras (the receptor activates a signaling molecule, which, in turn, increases expression of the receptor) establishes a positive feedback loop and may drive cell proliferation and tumor formation. Considering that normal Schwann cells do not

[1] [71].

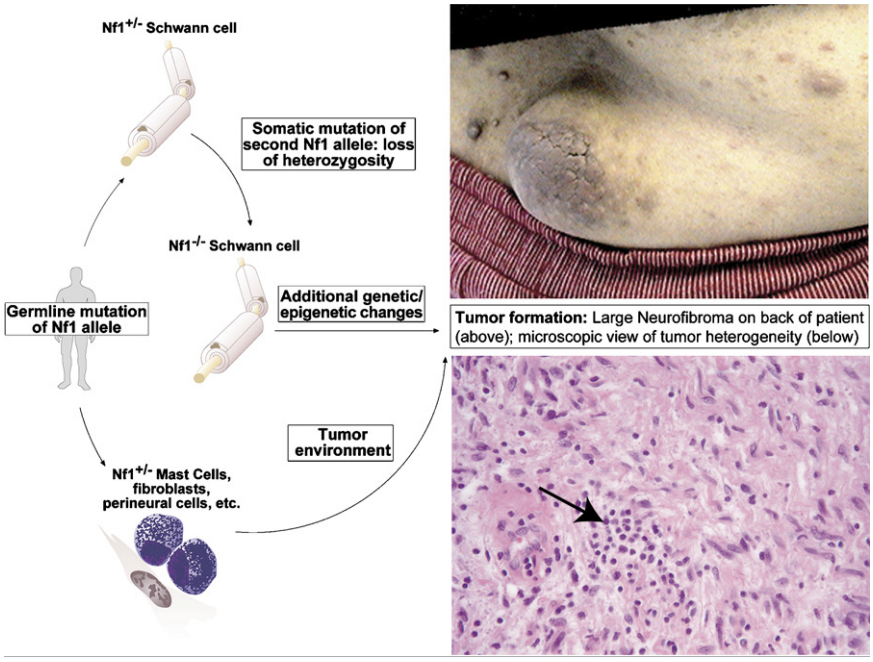

Fig. 3 – Tumor formation and progression rely on interactions between cancer and non-cancer cells. Schwann cells that have undergone biallelic inactivation at the Nf1 locus and possibly additional genetic/epigenetic disturbances are thought to make the largest contribution to MPNSTs. These tumors are heterogeneous, comprising mast cells, fibroblasts, and perineural cells. Interaction among tumor cells and non-cancer surrounding cells, which are Nf1 haploinsufficient, may be necessary for tumor formation and progression. In the upper right corner is a photograph of an NF1 patient with a large dermal neurofibroma on his back. This patient has a one base (7005) deletion in the Nf1 coding region. In the lower right corner is a microscopic view of a neurofibroma. This hematoxylin/eosin-stained image (viewed at 100×) shows the compact and interweaving arrangement of transformed Schwann cells, seen as large fusiform cells distributed throughout the field. Fibroblasts (smaller fusiform cells) and infiltrating inflammatory cells (smaller, more darkly stained cells with little cytoplasm) including mast cells are also visible (designated by an arrow) [2,7].

express the EGFR, researchers have predicted that transformed Schwann cells depend on the receptor for their proliferative capacity. DeClue et al. showed that EGF drives MPNST cell proliferation and growth in soft agar [57]. Several studies have investigated the effects of EGFR inhibitors on cell cycle progression and division in NF1 tumor cells. Two groups have reported the ability of the EGFR inhibitor AG1478 to inhibit EGF-stimulated cell proliferation of MPNST cells [54,57]. Furthermore, our work demonstrates the ability of the FDA-approved EGFR inhibitor Iressa (AstraZeneca) to produce a dose-dependent inhibition of EGF-mediated ERK activation and a reduction in the proliferation of cells grown in the presence of serum (with and without the addition of EGF).[2] Ling et al. have further validated the transforming properties of the EGFR. This group showed that expression of the EGFR in transgenic mouse Schwann cells led to hyperplasia, collagen excess, and dissociation from neurons [58]. Other evidence supporting the role of EGFR in tumor progression and metastasis comes from Su et al., who demonstrated that the presence of EGFR increased the activity of CD44, which promotes cellular growth, adhesion, and migration of Schwann cells [59]. An on-going Phase II clinical trial is evaluating the effects of Iressa on NF2-associated meningiomas (National Cancer Institute, NLM#: NCT00025675); however, the effect of inhibiting the EGFR in NF1-associated malignancies has yet to be determined.

The EGFR is only one member of the ErbB family of receptors. At least two other members, ErbB2 and ErbB3, are normally expressed by proliferating Schwann cells [54,60]. Overexpression and activity of ErbB2 drives tumor formation in certain breast cancers [61] and may play a role in NF1-related malignancy as well. Schlegel et al. found an inverse relationship between Nf1 expression and ErbB2 expression, raising the possibility that Ras signaling may influence the expression of multiple receptors [62]. The presence of ErbB2 on the surface of Schwann cells may be physiologic; however, the presence of over-expressed EGFR might provide a dimerization partner for ErbB2 and, consequently, confer a mechanism for increased ErbB2 activity. The result might be pathological [63]. An increase in the secretion of neuregulins (ligands for ErbB2/3) or other growth factors by Nf1-null Schwann cells or Nf1 haploinsufficient surrounding tumor cells may fuel autocrine or paracrine loops that also contribute to the receptor activation and uncontrolled cell proliferation. Like the EGFR, other ErbB members have been targeted with similar results. He et al. inhibited ErbB2 and FLK-1 and saw suppression of Ras-transformed sarcomas in nude mice [64]. Stonecypher et al. used the ErbB2–4

[2] [72].

inhibitors PD168393 and PD158780 to block proliferation of MPNST cells [60].

6. The tumor microenvironment

Another important consideration regarding the ability of transformed Schwann cells to form tumors involves the tumor microenvironment. MPNSTs are heterogeneous tumors: they contain neurons, perineural cells, fibroblasts, and infiltrating mast cells (Fig. 3). While Schwann cells that have undergone biallelic inactivation at the Nf1 locus are thought to make up the bulk of the tumor, haploinsufficient cells within the tumor microenvironment are believed to play a necessary part in tumor formation and progression [65]. With loss of one Nf1 allele, mast cells experience an increase in proliferation, survival (at least in part via increased PI3K activity), and colony formation [66,67]. Nf1-null Schwann cells secrete increased amounts of KitL, which recruits mast cells [68]. Once within the tumor microenvironment, mast cells secrete mitogens, angiogenic factors, proteases, and other inflammatory elements that promote tumor formation and progression [69]. Similar reciprocal interactions among Schwann cells, mast cells, and other non-neoplastic cells may exist and may explain the anti-tumor effects of anti-histamines (ketotifen fumerate), anti-inflammatories (interferon-α), anti-angiogenics (thalidomide, interferon-α), and anti-fibrotic agents (perfenidone) with some NF1 patients [70]. In addition, the local production of autocrine or paracrine growth factors within the tumor microenvironment may further implicate various surface receptors in cell proliferation and tumor formation.

7. Concluding remarks

Our ability to effectively treat patients with NF1 should improve with more accurate and detailed characterization of those features that distinguish MPNST cells. That MPNST cells acquire the EGFR with the loss of Nf1 may expand the mechanism by which Ras directs proliferation and, in general, how signaling molecules reciprocate an effect on upstream cell surface receptors independent of autocrine growth factors. Increased Ras signaling may directly or indirectly alter the expression of other pharmacologically accessible markers in tumor cells. These Ras-directed phenotypic changes would provide treatment strategies not only for NF1-related malignancy but also for a large percentage of human tumors.

Acknowledgements

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