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NF1 in Solid Tumors: The Unknown Soldier of Tumor Suppressor Genes?

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ABSTRACT

Many of the altered properties of cancer cells are attributed to inactivation of normal cellular regulatory genes that suppress uncontrolled proliferation, evasion of apoptosis, metastasis and tumorigenesis. Loss of tumor suppressor genes (TSG) is crucial for cancer development, along with gain-of-function alterations in proto-oncogenes. NF1 is a TSG well-known in association with Neurofibromatosis type 1 (NF1) syndrome. However, the role of NF1 mutation in cancer has not been extensively studied, unlike other TSGs such as retinoblastoma (Rb), p53, Adenomatous Polyposis Coli (APC), or Phosphatase and Tensin Homolog (PTEN).

In this review, we discuss the molecular role of NF1 in cancer development and cancer-related cellular signaling. We also review studies that have assessed the prevalence of NF1 mutations and loss-of-function across different solid tumors, and focus on their role in mediating malignant transformation, and modulating response to therapy. This review sheds light on the challenges that have hindered a better understanding of NF1's role in cancer development, and discusses the prospect of NF1 as a biomarker for targeted therapies.

Keywords

Malignant peripheral nerve sheet tumor, Molecular signaling, Neurofibromatosis type 1syndrome, *NF1*, Targeted therapy, Treatment resistance, Tumor suppressor gene.

Introduction

TSGs are involved in DNA damage repair, inhibition of cell division, induction of apoptosis and suppression of metastasis [1]. According to the Knudson's two-hit model hypothesis, loss of TSG function occurs via either deletion or inactivation of two alleles [2,3]. *NF1* syndrome [4,5] is an autosomal dominant disorder with complete penetrance but extremely variable expression [6]. However, for tumors to develop in *NF1* patients, and in congruence with the two-hit hypothesis, both alleles of *NF1* must be mutated: single-allele mutation was shown to predispose its carriers to multiple tumors, but the majority of patients with these *NF1*-associated tumors exhibit bi-allelic inactivation of *NF1*, through loss of heterozygosity (LOH) of the originally, non-mutated allele [7,8].

The *NF1* gene maps to chromosome 17, at the 17q11.2 large locus (350 kbp), and contains 61 exons including 4 alternatively spliced exons [9]. It is transcribed into a 12 kbp messenger RNA (mRNA) containing an open reading frame of 8454 nucleotides [9,10]. Most mutations in *NF1* are inactivating loss-of-function mutations that result in almost complete absence of transcript or protein [5,9-12]. There are currently more than 2600 different inherited *NF1* mutations reported in the Human Gene Mutation Database (HGMD) with varying sizes [12-15]. Yet, more than 50% of *NF1* syndromic cases are attributed to *de novo* mutations [16].

Unlike the *de novo* mutations, the inherited constitutional *NF1* mutational spectrum is well defined and consists of: missense/nonsense (27.2%), splicing (16.3%), micro-deletions (26.9%), micro-insertions (11.1%), insertion/deletion (indel; 2.0%), gross deletions (>20 bp; 13.3%), gross insertions (>20 bp; 2.0%) and complex re-arrangement (0.6%) [17,18]. However, there is no evidence of any localized mutation clustering, or mutational hotspot, within the rather large *NF1* locus [17]. Although *NF1*

mutation rate in classically inherited *NF1* patients reaches up to 95%, the detection of somatic mutations that are commonly seen in tumors, is more challenging, due to the cellular heterogeneity seen in cancer [19]. Phenotypically *NF1* presents with a wide variability even among patients within the same family, which is explained by the existence of modified genes such as protein-coding sequences, microRNA and long non-coding RNA genes that may affect the *NF1* phenotype [18, 20].

NF1 encodes neurofibromin 1, a 2818 amino acids multi-domain protein that is ubiquitously expressed, with highest levels in the central nervous system (CNS) [10,21]. Through its GTPase-activating protein –related (GAP-related) domain, neurofibromin 1 negatively regulate RAS, by converting the active RAS-guanosine triphosphate (RAS-GTP) to its inactive RAS-guanosine diphosphate (RAS-GDP), thus inhibiting pathways downstream of RAS, and namely the *RAS/MAPK* and the PI3K/AKT/mTOR signaling pathways [7,22]. In addition, neurofibromin is involved in regulating the conversion of adenosine tri-phosphate (ATP) to cyclic adenosine mono-phosphate (cAMP), a secondary signal that activates survival promoting pathways. Neurofibromin 1 is known to associate with a large number of proteins including tubulin, kinesin, protein kinases A and C, syndecan, caveolin, cytokeratin, intermediate filaments and the amyloid precursor protein; despite the diversity of these protein associations, their significance remains unknown, but still suggest that neurofibromin 1 may have many functions other than its known GAP protein function [23].

Neurofibromin is regulated by upstream signaling from the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor, the c-KIT receptor, the endothelin receptor B (EDNRB) and the anaplastic lymphoma kinase (ALK) receptor. Lastly, its C-terminal domain can be phosphorylated by PKA which inhibits its function (reviewed in [24]).

NF1 involvement in cancer-signalling pathways

The best understood function of neurofibromin is its role in tightly regulating cellular levels of activated RAS proteins by activating RAS GTPase activity, resulting in conversion of active RAS-GTP to inactive RAS-GDP [25]. Activated RAS plays a major oncogenic function as it binds and activates two main kinases: RAF, which activates the MAPK signaling pathway, and PI3K that activates the AKT/mTOR survival pathway [26].

The *RAS/MAPK* pathway plays a critical role in various normal developmental processes, but also in cancer where it is often constitutively activated leading to increased cell growth and proliferation: activated RAS interacts with several downstream mediators, notably BRAF and RAF1 proto-oncogene serine/threonine kinase, resulting in homo/hetero-dimerization and subsequent activation of RAF. Activated RAF then phosphorylates and activates the MAP2K1 and MAP2K2 (mitogen-activated protein kinase kinase 1 and 2), which in turn phosphorylate and activate ERK1 and ERK2, the ultimate effectors of the pathway that control cell cycle progression, differentiation and growth (reviewed in [26]). Important downstream effectors of the RAS/RAF/MAPK pathway are the mitogen-activated protein kinase interacting kinases 1 and 2 (MNK1/2), which are key regulators of mRNA translation, integrating signals from both oncogenic and immune signaling pathways through phosphorylation of the eukaryotic initiation factor 4E (eIF4E) [27-29]. This in turn, increases both translation and stability of mRNA coding for oncogenic and immune suppressive factors: direct inhibition of MNK 1/2 has been shown to re-invigorate immune checkpoint and cytokine expression, ultimately promoting anti-tumor immunity [27].

Dysregulation of the *RAS/MAPK* pathway is a critical event in solid tumor development and activating mutations of the RAS proto-oncogene have been extensively described as driver mutations of carcinogenesis [30]. With that in mind, the importance of *NF1* loss or inactivating mutations becomes important, as loss of neurofibromin functionality results in sustained intracellular levels of active RAS, prolonged activation of the RAS/RAF/MAPK signalling pathway and ultimately, loss of growth control, increased cellular proliferation and cancer-mediated immune suppression.

The PI3K/AKT/mTOR survival signaling pathway is activated in response to growth factors and hormones stimulation of G-protein coupled receptors (GPCR), but also by phosphorylation of the phosphoinositide 3 kinase (PI3K) by RAS-GTP at the catalytic p110-α subunit. Active PI3K then phosphorylates and converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP3 activates a variety of downstream targets, notably AKT (also known as protein kinase B- PKB), Phosphoinositide-dependent Kinase 1 (PDK1). This ultimately leads to activation of cell growth, cell cycle entry, migration, and survival [31,32].

The RAF/MAPK and PI3K/AKT pathways both activate mTOR signalling resulting in increased protein synthesis, stimulation of lipid synthesis and glucose uptake, and promotion of growth by inhibition of apoptosis and autophagy [32]. In the absence of neurofibromin, the PI3K/AKT/mTOR pathway is thus constitutively activated. The importance of this pathway has been supported by studies demonstrating that *NF1*-mutant neurofibroma and malignant peripheral nerve sheaths tumors (MPNST) cell lines and primary tumors all had a constitutively active pathway n the absence of growth factors [33-35]. Lastly, activation of GPCRs causes an exchange of GDP to GTP at the receptor's alpha subunit, and this activated subunit in turn activates adenylyl cyclase, leading to conversion of ATP into cAMP an important secondary messenger. Neurofibromin is thought to play regulatory role by inhibiting the exchange of GDP to GTP at the GPCR alpha subunit [36]. This is supported by evidence from yeast and animal models: in yeast, the neurofibromin homologue regulates RAS and cAMP signaling, and inactivating *NF1* mutations in mice, drosophila and zebrafish lead to deregulated cAMP levels across various cell types. cAMP levels are also altered in human *NF1*-associated tumors including gliomas [37-40]. Figure 1 schematically represents the interaction of *NF1* with different molecular pathways.

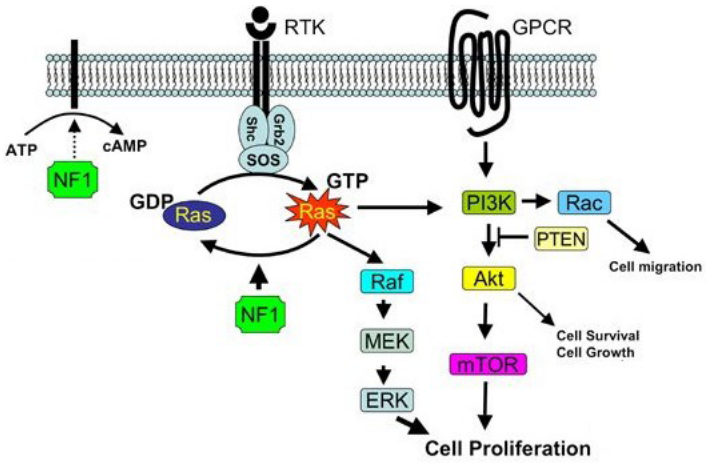

Figure 1: Interactions of *NF1* with RAS/RAF/MAPK and the PI3K/mTOR pathways: loss of *NF1* expression leads to constitutively active Ras, with downstream deregulation in cellular proliferation, cell growth and tumorigenesis. RTK: Receptor Tyrosine Kinase; GPCR: G-protein couple receptor. Adapted from [22].

NF1-associated diseases and syndromes

Alterations in the *NF1* gene have been well-linked to neurofibromatosis type I syndrome, or Recklinghausen's disease [41]. As mentioned earlier, it is an autosomal dominant disorder, with complete gene penetrance and a wide spectrum of clinical presentations, notably increased predisposition to multiple tumors, most commonly from the neural crest. The incidence of *NF1* is around 1 in 3000 live births, with 50% of cases due to new, sporadic mutations [41].

The clinical features of *NF1* are numerous and affect multiple organs. While most common clinical findings involve the skin, the disease presents differently in each patient, and may vary across the lifespan of a single patient, typically worsening with age [42]. The most common findings include café-au-lait macules (CALM; 90%), axillary and inguinal freckling (80%), neurofibromas (60-90%), and plexiform neurofibromas (25% of patients) [43]. Lisch nodules are however the most common manifestation of *NF1* and occur in 100% of patients [44]: these are melanocytic hamartomas of the eye, but luckily do not result in any ophthalmologic complications. Optic glioma is another common tumor in *NF1* with a favorable prognosis, unlike plexiform neurofibromas that are considered precursors for the aggressive malignant peripheral nerve sheath tumors (MPNST) [45]. Other symptoms have also been reported such as pulmonary hypertension, learning difficulties, and seizures [42,46,47]. There is currently no curative treatment for *NF1* and treatment consists of symptomatic management with cancer the most common cause of death in *NF1* patients [42].

Although no other disorder than *NF1* is presently directly linked to mutations in *NF1*, the direct effect of *NF1* loss of function on RAS activation is linked to what is known as "RASopathies." These are genetic syndromes caused by germline mutations in genes that encode components /regulators of the *RAS/MAPK* pathway [48,49]. Since this pathway is involved in normal development, patients with Rasopathies present with developmental abnormalities in multiple organ systems, besides their predisposition of cancers (reviewed in [48]). These syndromes consist of Noonan syndrome, Noonan syndrome with multiple lentigines, Costello syndrome, and Legius syndrome. They all share common clinical features including cutaneous, musculoskeletal, and ocular abnormalities, cranio-facial dysmorphology, cardiac malformations, neuro-cognitive impairment, and increased cancer risk [43,48]. Despite the fact that these syndromes are linked to specific mutations along the *RAS/MAPK* signaling axis, they all co-occur with *NF1* mutations, suggesting that these mutations may be a necessary, or perhaps an initial event in the development of Rasopathies, and that *NF1* and co-occurring mutations may act synergistically [50,51].

NF1 in cancer: patients with *NF1* syndrome are at an overall increased risk of cancer, with approximately 4-fold higher incidence compared to the general population [52]. MPNST is an aggressive sarcoma that typically arises in pre-existing plexiform neurofibroma [45], and occurs in 3-15% of *NF1* patients. Other reported neoplasms in patients with *NF1* syndrome include juvenile myelomonocytic leukemia, pheochromocytoma, rhabdomyosarcoma, duodenal carcinoid, somatostatinoma, parathyroid adenoma and gastro-intestinal stromal tumors (GISTs) as well as breast cancer (5-fold increased risk) [52,53].

However, outside these syndrome-related malignancies, somatic mutations in *NF1* are also present in sporadic cancers. Recent developments in genomic sequencing technologies and the expansion of available genomic data revealed a high prevalence of *NF1* mutations in different cancer types, significantly more than was previous thought [21]. Data on genetic alteration in *NF1* is reported on the cBioPortal for Cancer Genomics (http://www.cbioportal.org) from a compilation of 147 studies that derived genomic data from more than 5000 tumor samples [43]. *NF1* mutations were detected in melanoma, breast carcinoma, neuroendocrine prostate cancer, glioblastoma multiform (GBM), lung cancer (adenocarcinoma and squamous carcinoma), adenoid and ovarian serous cancer, uterine cancer, urothelial cancer (UC), paraganglioma, pheochromocytoma, pancreatic cancer, adrenocortical cancer, colon adenocarcinoma, stomach cancer, sarcoma, esophageal cancer and rhabdomyosarcoma [54-56]. In order to ascertain the significance of these mutations, Kiuru et al., examined this same data, but focused on studies that showed *NF1* mutations in more than 10% of samples, in a minimum of 15 specimens analyzed [43]. A total of 24 studies were identified, with the following tumor types: melanoma. Acute Lymphoblastic Leukemia (ALL), GBM, Lung cancer, bladder UC, uterine endometrial carcinoma, pancreatic carcinoma, ovarian adenocarcinoma, skin squamous cell carcinoma, and gastric adenocarcinoma. With such a high and previously underestimated rate of sporadic, non-*NF1* associated *NF1* mutations in cancer, the role of *NF1* in conferring selective growth advantage in cancer development, and thus acting as a "driver mutation" warrants further investigation [57]. The next sections detail the frequency of somatic non-syndrome associated *NF1* mutations in different solid tumors, and discuss their potential role in driving malignancies, and possibly sensitivity or resistance to selective therapies.

NF1 in solid tumors: prevalence, molecular significance, malignant transformation, and resistance to therapy

There are numbers of malignant tumour types that harbour *NF1* alterations. In this section, we focus describing the prevalence of *NF1* in select common solid tumors, and discuss available data regarding the function of *NF1* mutations in malignant transformation and resistance to therapy. Table 1 compiles the different frequencies of somatic *NF1* mutations as reported in the literature, across a range of solid tumors.

Cancer Type	Frequency of Somatic *NF1* mutations (%)	References
MPNST	40	[58]*
Melanoma	12- 93	[75]; [51]*; [69]*; [72]*; [78]*; [129]*
NSCLC	12	[83]*
Lung ADC	7- 13	[84]*; [85]; [31, 86]
Lung SCC	10.3- 12	[80]*; [130]
Breast	2.5- 27.7	[130]; [101]*
Ovarian	12- 34.4	[131]*; [56]; [106]; [128]; [130]
GBM	11- 23	[117]*; [116]*
CRC	3.8- 6.25	[132]*
UC/TCC	6- 14	[15]*; [128]
Neuroblastoma	2.2- 6	[114]
Paraganglioma/ Pheochromocytoma	21-26	[133]; [134]
Uterine	11- 14	[135]*; [136]*
Pancreatic	11	[137]*
Gastric	10	[138]*

Table 1: Summary of *NF1* somatic mutations frequency in different solid tumors.

*Study involved >15 subjects AND found mutations in >10% of samples; MPNST: Malignant Peripheral Nerve Sheet Tumor; NSCLC: Non-small cell lung carcinoma; ADC: Adenocarcinoma; SCC: Squamous Cell Carcinoma; GBM: Glioblastoma Multiform; CRC: Colorectal Cancer; UC/TCC: Urothelial Cancer/ Transitional Cell Carcinoma.

Malignant Peripheral Nerve Sheath Tumors (MPNST)

Although MPNSTs have traditionally been associated with *NF1* syndrome, somatic mutations of *NF1* occur very commonly, in 41-72 % of sporadic MPNSTs independent of the syndrome, showing that *NF1* inactivation plays a major role in the development of this tumour type from its plexiform neurofibroma precursor [58].

Several studies involving pre-clinical cell lines and animal models have well identified the role of *NF1* mutations and the resulting molecular pathway changes, in driving malignant transformation towards MPNST as well as modulating response to therapy. One study involving *NF1*-deficient MPNST murine tumor models and human samples, evaluated the effect of *NF1* mutation on response to downstream MNK inhibition [59]. MNK1 and MNK2 are serine/threonine kinases that act downstream of the *RAS/MAPK* pathway and are activation by ERK [60]. In *NF1* mutant cells, MNK1/2 are constitutively activated resulting in eIF4E hyperphosphorylation. Suppression of either MNK1 or 2 (independently) significantly decreased eIF4E phosphorylation, and resulted in inhibition of tumor proliferation [59]. This was achieved by chemical inhibition using MNK kinase inhibitor CGP57380. Interestingly, addition of MEK inhibitors (merestinib and cabozantinib) to CGP57380 resulted in actual cell death, pointing that cytotoxic, rather than cytostatic effects could be achieved by combination of MNK1/2 and MEK inhibitors in *NF1*-deficient tumors. Clinically, this could potentially translate into using *NF1* mutational status to direct therapy in MPNSTs.

Another study exploring the process of malignant transformation from plexiform neurofibromas to overt MPNST suggested that loss of *NF1* alone may not be sufficient to drive this transformation: 29 patients with *NF1* microdeletions were found to have reduced *ANRIL* expression, a gene that is normally needed for Polycom Repressive Complex 2 (PRC2)-mediated *CDKN2A/2B* expression [61]. In other words, *NF1* deletions in these MPNST patients always correlated with inhibition of *CDKN2A/2B* tumor suppressor genes. The study thus suggested that although somatic mutations in *NF1* may be the "initiating" event in progression towards MPNST, loss of *CDKN2A/2B* is needed to complete the malignant transformation [61].

The role of *CDKN2A/2B* loss of function was also evident in another study that carried out genomic profiling in patients with MPNST, and revealed multiple pathways with targetable mutations [62]. In MPNST patient samples (n=201), 47% of patients had activating mutations in the RAS/RAF pathway, and 57% had a *CDKN2A* alteration. Interestingly, the proportion of *CDKN2A* alterations was higher in the *NF1*-mutant MPNST patients, suggesting again that addition of *CDKN2A* loss on top of *NF1* mutation may play a role in increasing the potential of malignant transformation [62].

The study was also important as it delineated different pathways involved in MPNST, thus suggesting that a comprehensive approach including genomic analysis may be best when deciding on a targeted therapy option. A similar study involving 8 patients with plexiform neurofibroma, assessed intra-tumoral heterogeneity and attempted to correlate it with histological and genomic findings [63]. Despite a relatively small sample size, the study results suggested that loss of single *CDKN2A*/*2B* copy in homozygous *NF1* mutants was sufficient to start the development of atypical neurofibromas, while total *CDKN2A*/*2B* inactivation was necessary to drive malignant transformation towards neurofibromatous neoplasms. Other studies further support the relationship between *NF1* mutation and *CDKN2A/2B* loss in driving malignant transformation towards MPNST, confirming the idea of *NF1* as an early initiating event in the premalignant lesion [64,65].

Melanoma

Although *NF1* is associated with CALMs, malignant melanoma is not a tumour type associated with *NF1*. Somatic mutations in *NF1* were originally reported in 1993 in a malignant melanoma cell line by Anderson et al., and the absence of neurofibromin 1 and *NF1*

mRNA in a primary melanoma led to the first proposal that *NF1* may function as a tumour suppressor gene in the development or progression of malignant melanoma [66]. However, with the recent large-scale advances in sequencing technologies, many subsequent studies have identified *NF1* somatic mutations in melanoma, and have established *NF1* as one of the key drivers of melanoma [51,54,67-71]. Mutational rates in these studies have been reported as ranging between 12 and 30%, a range characteristic for a driver gene which typically exhibits high frequency of non-silent exonic mutations [68]. Mutations in *NF1* are more common in older patients or those who are chronically sun-exposed [51,72], in melanomas with higher tumor mutational burden (TMB) [51], or wild-type BRAF and NRAS [68,70,72].

The *RAS/MAPK* pathway, normally regulated by *NF1*, is described as the key culprit in non-familial melanoma, with mutations in *BRAF* and *RAS* occurring in 50-70% and 19-28% of all cutaneous malignant melanomas, respectively [73,74]. As mentioned earlier, loss-of-function *NF1* mutations, or oncogenic mutations in *BRAF* or *RAS*, result in constitutive activation of the MAPK pathway and are believed to be early somatic events associated with melanoma initiation.

However, the effect of *NF1* loss on RAS signalling is not consistent, as not all *NF1* mutant melanomas exhibit full RAS activation [68], possibly suggesting multiple functions for *NF1*: for example, a study involving a melanoma mouse model revealed that the role of *NF1* mutations in a RAS/BRAF mutant model was not necessarily related to initial pathway activation [75]. Instead, *NF1* mutations cooperated with BRAF mutations to maintain *RAS/MAPK* oncogenic activation by preventing oncogene-induced senescence, a protective process of robust anti-proliferative effects brought about in response to oncogenic signaling [76]. Conversely, 25-30% of melanomas with wild-type (WT) *BRAF* and *RAS* harbour deleterious *NF1* mutations and aberrant MAPK pathway activation, strongly suggesting that *NF1* inactivation was responsible to a large extent of activating this oncogenic pathway in these tumors [68,69]. A study evaluating the pattern of *BRAF, RAS* and *NF1* mutations co-occurrence, detected *NF1* mutations in 12.2% of melanoma cases (n=213); interestingly, almost half of patients with *BRAF* and *RAS* WT melanomas (26/56) had an *NF1* mutation, suggesting again that the contribution of *NF1* loss to driving tumorigenesis via the *RAS/MAPK* pathway, may be more pronounced in a *BRAF-RAS* WT background [51]. Another important observation in this study is that all *NF1* mutant/*BRAF-RAS* WT melanomas had mutations in other genes involved in RASopathy, thus implying again that understanding the role of *NF1* mutations should always be interpreted in light of other co-occurring mutations.

Desmoplastic, uveal and mucosal melanomas are subtypes less commonly seen than cutaneous melanomas. *NF1* somatic mutations have also been reported in these subtypes. In uveal melanoma, the most common primary intra-ocular malignancy in adults, inactivating *NF1* mutations were found in around 60% of patients (23/38; [77]). Desmoplastic melanomas, which are less clinically aggressive than cutaneous melanomas, were reported to have the highest frequency of somatic *NF1* mutations (14/15) [78]. In contrast, mucosal melanomas, which have a poor prognosis compared to other subtypes, show a particular molecular profile with less frequent BRAF and more frequent KIT mutations [79]. In this subtype, *NF1* mutations were demonstrated to be the most frequently occurring driver mutations.

In light of the effects of *NF1* loss on MAPK pathway activation, several studies have investigated the role of *NF1* in driving resistance to BRAF/MEK targeted therapies, via sustaining pathway activation. Using a small hairpin RNA (shRNA) approach in melanoma mouse models and human melanoma cell lines, it was demonstrated that *NF1* suppression induced resistance to PLX4720, a BRAF inhibitor, and *NF1* reconstitution restored sensitivity [75]. Using a similar approach of RNA silencing, screening a BRAF inhibitor-sensitive melanoma cell line with a library of RNAi, *NF1* was identified among 16,500 other genes, as the highest ranking protein affecting BRAF inhibition, with *NF1* knockdown resulting in a 31-fold increase in resistance to PLX4720, and a partial (7-fold) resistance to MEK inhibition. This demonstration was further confirmed by the observation that human melanoma samples with innate resistance to BRAF inhibition and sensitivity to MEK inhibitor, harboured *NF1* mutations [71].

Non-Small Cell Lung Carcinoma (NSCLC)

Data derived from The Cancer Genome Atlas (TCGA) has highlighted the involvement of *NF1* mutations in both lung adenocarcinoma (ADC) and squamous cell carcinoma (SCC) [80]. In fact, several studies examining the mutational landscape in NSCLC have reported mutations in *NF1* in ADC and SCC alike. In a study involving 591 NSCLC patient samples, *NF1* mutations were detected in 10% of patients, and 25% of those co-occurred with mutations in known lung cancer oncogenes including *BRAF, ERBB2, KRAS, HRAS* and *NRAS* [81], pointing to the probable cooperativity of *NF1* mutations with other ones, not unlike melanoma.

Transcriptome analysis of 153 tumor samples from NSCLC patients (ADC, SCC, large cell lung cancer, and adenoid cystic carcinoma), revealed that *NF1* alterations in NSCLC were not limited to mutations, but also included fusion with NRG1 [82]. *NF1* was also shown to be involved in several other gene fusions in NSCLC such as *NF1-GOSR1, NF1-PSMD11, NF1-NLK, NF1-DRG2* and *NF1-MYO15A*, and these were associated with poor overall survival [82]. To better understand the value of *NF1* mutations in NSCLC, a separate examination by subtype is warranted given the significant molecular heterogeneity and difference of mutational landscape between ADC, SCC, and SCLC [83].

In ADC, a tumor type characterized by high mortality rate, several studies have reported somatic *NF1* mutations ranging between 7 an 11% of examined samples [81,84-86]. In one of the studies evaluating 188 patient samples with lung ADC, *NF1* mutations were only found in 7% of samples [85]; however, actual biallelic inactivation analysis of *NF1* was found in as many as 23% of

samples, highlighting again that *NF1* inactivation in NSCLC is not exclusive to mutations. In a similar study, Imielinski et al. identified somatic *NF1* mutations in 10.9% (20/183) of lung ADC, half of which actually resulted in complete loss of function [86].

In addition to the recurrent *NF1* mutations in sporadic lung ADC patients, the MAPK pathway also appears to be an important regulatory pathway involved in tumorigenesis [85] as demonstrated by analysis of genomes, RNA and protein from untreated lung ADC and comparison with matched normal samples [84]: mutations in *NF1* and in other genes that activate the *RAS/RAS/MAPK* pathway were identified in around 75% of samples (n=230), and *NF1* was identified as a significantly mutated gene in ADC, along with *TP53, KRAS, STK11 (LKB1),* and *EGFR*. Importantly, *NF1* mutations were more frequent in lung ADC subsets with WT *BRAF-RAS* [84].

In SCC lung cancer, somatic mutations are present in around 12% of cancers, with unequal distribution between different histological subtypes (classical, primitive, basal and secretory) and the basal expression subtype harbors most of the alterations [80]. In one large study aimed at determining new drivers of lung carcinogenesis, Campbell et al. compared both exome sequences and copy number alterations, between 660 lung ADC and 484 lung SCC [83]: with comparable rates of somatic mutations, around 38 genes were differentially mutated in ADC and 20 in SCC, and only 6 genes were significantly mutated in both, suggesting these are essential drivers in NSCLC. These genes included *NF1*, along with *TP53, RB1, ARIDIA, CDKN2A*, and *PIK3CA* [83].

In terms of resistance to therapy, several studies have attempted to elucidate the mechanism of resistance to *EGFR* tyrosine kinase inhibitors (TKI) in *EGFR* mutated lung ADC: the T790M mutation accounts for 50-60% of resistant cases, while other mechanisms involving PIK3CA mutations and *MET/HER2/MAPK* upregulation are thought to account for 5-20% of resistant cases [87-91].

However, the resistance mechanism remains unknown in about one third of TKI-resistant lung ADC. A major study carried by de Bruin et al. using a genome-wide siRNA screen of a human lung cancer cell line and *EGFR*-mutant mouse models, revealed that resistance to erlotinib was associated with reduced expression of neurofibromin [87]. Furthermore, marked reduction of *NF1* mRNA expression conferred both an intrinsic and an acquired resistance to *EGFR* inhibitors: erlotinib failed to fully inhibit RAS-MAPK signalling when neurofibromin levels were reduced, and treatment of neurofibromin-deficient lung cancers with MEK inhibitor restored sensitivity to erlotinib [87].

Small cell lung cancer (SCLC)

Unlike NSCLC, little data is available regarding the *NF1* mutational status in SCLC. Only two studies have reported the frequency of these mutations, as 2.4 % and 6.9% [92, 93]. One study sequenced the DNA of 98 SCLC patients in an attempt to identify relevant genomic alterations and potential actionable genes in terms of therapy [94]: *NF1* mutations were identified in only 3% and were not considered actionable.

Breast cancer

Few studies have reported the mutation frequencies of the *NF1* gene in sporadic breast cancer. Patients with *NF1* syndrome are however at an increased risk of developing breast cancer compared to the general population, especially young *NF1* women (under the age of 50) who have around 4-5 fold increased risk of breast cancer incidence, and around 3.5 fold increased risk of mortality [95-97]. Following these observations, studies were carried to investigate the potential role of *NF1* mutations as drivers of malignant transformation and progression of sporadic breast cancer. As such, genome sequencing of breast cancer samples was carried and revealed *NF1* as a novel gene that is recurrently mutated in sporadic breast cancer [98].

The role of *NF1* in driving malignant transformation of mammary cells was first described in 2001 [99]. Breast cancer-derived cell lines harboring the *NF1* mutation had significantly higher levels of active RAS, strongly suggesting that *NF1* loss may be responsible for driving malignancy via *RAS/MAPK* pathway dysregulation. Furthermore, in the highly malignant and treatment resistant MB-231 breast cancer cell line, neurofibromin levels were the lowest and below detection levels compared to other less aggressive cell lines.

Similarly MB-231 expressed 10-fold higher expression of downstream phospho-MAPK (p-MAPK) compared to the other cell lines, despite no change in p12-GAP, thus implying that pathway activation was exclusively caused by *NF1* loss, and that reduced neurofibromin may be directly responsible for malignant transformation [99]. In a mouse model characterized by spontaneous mammary tumor development, somatic *NF1* deletions were found in 59/60 of studied samples [100].

In human samples, rates of *NF1* somatic mutations or deletions were reported as 27.7% in the TCGA data [101]. However, and unlike any other tumor types, *NF1* gene amplification is particular to the breast cancer genome [100,101], thus rendering the task of elucidating a clear role for *NF1* in human breast cancer development, challenging and a work in progress. How *NF1*'s alterations correlate with clinical behavior of the tumor and outcome will also be important to determine. In a recent study based on 2433 molecular profiles of breast cancer, inactivating *NF1* mutations were found to be associated with a high breast cancer severity score in Estrogen Receptor (ER) negative tumors [102].

Similar to melanoma and lung ADC, inactivation of *NF1* in breast cancer is associated with resistance to therapy, as silencing of *NF1* in the tamoxifen-sensitive MCF-7 breast cancer cell lines conferred tamoxifen-resistance [103]. This is particularly important as it has been reported that around 40% of early-stage breast cancer patients who receive adjuvant tamoxifen therapy, ultimately develop resistance and disease recurrence [104,105].

Ovarian cancer

Ovarian serous carcinoma (OSC) is a heterogeneous disease, notable for its high relapse and fatality rates: *NF1* mutations have been reported in around 33% of all OSCs, offering a potential early prognostic marker [56]. In a genome-wide microarray involving primary OSC samples and ovarian carcinoma-derived cell lines, *NF1* alterations were detected in 8/18 cell lines and resulted in marked reduction or loss of expression of *NF1* protein. Homozygous *NF1* deletions and splicing mutations were identified in 9/41 primary OSC [56]. Both tumor samples and cell lines with *NF1* lesions lacked *KRAS* and *BRAF* mutations, and still exhibited Ras pathway activation. These observations were the first to suggest a role for *NF1* loss in inducing RAS/RAF/MAPK mediated malignant transformation.

In a subsequent genomic DNA analysis involving 316 high grade OSC (HGOSC) samples, loss of *NF1* function was identified in 12% of samples (37/316) and 24/37 had deletions, one had a duplication and the remaining 12 samples had somatic mutations [106]. Specific copy number alteration (CNA) analysis from the same TCGA cohort reported loss of the *NF1* locus in 34% of ovarian cancer samples (n=398) [106]. The role of *NF1* loss in OSC resistance to therapy has not been as extensively studied as the previously discussed tumor types, with no study investigating a potential mechanism of resistance. Nevertheless, associations between *NF1* mutations in advanced HGOSCs and resistance to treatment have been described in multiple studies [107-109].

Neuroblastoma

Neuroblastoma is the second most common solid tumour in childhood and accounts for 8% of all childhood cancers, with the familial form of the disease accounting for a small fraction of all cases (1-2%) [110,111]. While the genetics of familial neuroblastoma are well characterized, sporadic forms remain poorly understood.

In an early study by The et al., levels of *NF1* expression were assessed in a panel of 10 neuroblastoma cell lines: 4/10 cell lines expressed reduced or complete absence of neurofibromin, and *NF1* mutations were identified in two of the cell lines [112]. Additionally, introduction of a normal human chromosome 17 into a cell line lacking neurofibromin in that same study, suppressed its tumorigenicity. Clinical correlation with somatic *NF1* mutations in neuroblastomas has revealed that reduced neurofibromin levels correlated with poor prognosis, while increased levels of expression exhibited longer progression-free survival [113,114].

In terms of resistance to therapy, *NF1* loss has been shown to associate with resistance to retinoic acid (RA) treatment, considered a targeted therapy of neuroblastoma [114]. This loss was further shown to activate RAS-MEK signalling, which in turn represses *ZNF423*, a critical transcriptional co-activator of the RA receptors. Clinically, neuroblastomas with both low/no levels of *NF1* and *ZNF423* have an extremely poor outcome. Further validation of this mechanism was established when inhibition of MEK signalling downstream of *NF1* was shown to restore responsiveness to RA, suggesting a potential therapeutic strategy to overcome RA resistance in *NF1*-deficient neuroblastomas [114].

Glioblastoma Multiform (GBM)

GBM is the most aggressive form of glioblastoma, the most frequent and lethal form of brain cancer in adults [115,116]. Given the grim prognosis, GBM has been the focus of many studies: GBM-associated *NF1* somatic mutations are well described as recurrent driver mutations, along with mutations in other genes, notably, *CDK4, EGFR, PIK3CA, PTEN* and *CDKN2A* [115-118]. It is estimated that *NF1* mutations occur in at least 15% of all GBM [116].

A similar rate has also been reported in a TCGA analysis of 206 glioblastoma tumor samples: the study investigated levels of gene expression, CNAs and DNA methylation and 14% of samples were found to contain at least one somatic *NF1* mutation [119]. Subsequent analysis of the same TCGA data by segregation of the tumors by subtype (perineural, neural, classical and mesenchymal) revealed that *NF1* and PTEN alterations distinctly occurred in the mesenchymal subtype, with 53% of mesenchymal cases harboring an *NF1* mutation [119]. The study also reported mutual exclusivity of *NF1* and BRAF mutations in GBM [117,119].

Colorectal cancer (CRC)

The *RAS/MAPK* pathway is dysregulated in more than 50% of CRCs, especially in treatment-resistant microsatellite stable tumors (MSS). Several critical genes and pathways, such as *WNT*, *RAS/MAPK, PI3K, TGF-β, TP53* and DNA mismatch repair, are recognized in the initiation and progression of CRC, with alterations in the PI3K and *RAS/MAPK* pathway being the most common ones [120,121].

Despite what we know about the RAS and *NF1* interaction in other tumor types, the role played by *NF1* mutations in activating RAS signaling is not well defined. Various types of *NF1* alterations have been reported in CRC, including loss of heterozygosity (LOH) in 14-57% of cases and/or gains by duplication in 17% of CRC [122-124]. Data from the 2012 TCGA genome (n=212) analysis defined 24 predominantly mutated genes, including *NF1* which was detected in 11/212 cases (5.6%). Subsequent studies further confirmed this prevalence, with *NF1* mutations identified in 5.6% (4/72) and 8.5% (39/619) of cases, respectively [125,126].

Whether *NF1* mutations play any role in mediating CRC resistance or sensitivity to chemotherapy, remains unknown.

Urinary tract transitional cell carcinoma (TCC)

Alterations in *NF1* gene expression in TCC were first evaluated by immunohistochemistry and low expression was reported in 83% of TCC specimen (23/29) [127]. The low *NF1* protein also correlated with markedly lower mRNA levels in these tissues. However, low *NF1* mRNA was also seen in adjacent benign urothelium tissue, but to a significantly lesser degree than high-grade TCC where mRNA and neurofibromin levels were more significantly decreased. This differential distribution of *NF1*/Neurofibromin expression levels

across benign and high-grade TCC suggested that alterations in the *NF1* gene may be involved early on in mediating urinary TCC carcinogenesis [127].

Genomic analysis of 131 urothelial carcinoma samples revealed *NF1* mutations in 14% of tumors [128]; specific analysis on 35 advanced (stage IV) urothelial cancers that relapsed and progressed on prior therapy (surgery and conventional chemotherapy) revealed *NF1* mutations in 2/35 cases (6%), with not much evidence available to link resistance and disease progression to *NF1* mutations [128].

Challenges and Prospects for Targeted Therapy

NF1 has remained a difficult target because of its large and complex locus size. Traditional sequencing methods were unable to adequately and reliably sequence a locus of that size. Additionally, clinicians have been hampered by the lack of any mutational hot spots or mutation clustering, as well as the presence of pseudogenes, which makes the development of a clinical test difficult [139]. Also, mutations in *NF1* can cause splice site mutations, meaning both DNA and RNA must be examined to obtain the full mutational landscape [14]. Some of these challenges can be overcome by advances in next generation sequencing (NGS) and bioinformatic methods. In addition, NGS has allowed for rapid sequencing of the tumor genome, leaving us with vast insight into the molecular profile of the entire tumor. Previous approaches focused on the characterization of one locus; however clinicians must now consider every mutation with in the whole molecular context. As such, this will allow for better understanding of *NF1* mutations in relation to other known cancer driving mutations and could provide the missing link needed to explain malignant transformation, resistance to therapy and disease progression.

In a preclinical study, *NF1* knockdown in cancer cells correlated with resistance to RAF inhibitors in cancer cell lines also harboring a $BRAF^{V600E}$ mutation through reactivation of the MAPK pathway, indicating loss of *NF1* may play a roll in BRAF inhibitor resistance [71]. Additionally, murine tumors with *NF1* mutations, were resistant to BRAF inhibitors, but exhibited sensitivity to combined therapy of MAPK and mTOR pathway inhibitors [75]. Targeting the mTOR pathway is difficult, as it is essential for many normal cellular processes [59]. However, finding a suitable indirect target could be easier, and perhaps as effective as targeting mTOR itself. Presently, there are two mTOR inhibitors with FDA approval, but with limited efficacy in the clinic. This is thought to be due in part to compensatory mechanisms that occur when mTOR is inhibited [140,141]. One mechanism that is thought to overcome mTOR inhibition is the MNK pathway which phosphorylates and activates eIF4E, thus counteracting prior mTOR inhibitor-mediated inactivation of eIF4E [142-144]. Thus, blocking the activation of eIF4E through a MNK inhibitor while simultaneously inhibiting mTOR is one strategy to halt compensatory mechanisms. Similarly, and as discussed earlier in MPNSTs, combining the MNK inhibitor, cabozantinib, and MEK inhibitors induced tumor regression [59]. Additionally, previous work has shown combination of MEK inhibitors with anti-PD-L1 antibody worked synergistically to control tumor growth [145]. This data suggests it is likely, that *NF1* mutational status could potentially be used as a biomarker in patients to predict resistance to BRAF inhibitors, sensitivity to immunotherapy and direct treatment. For example, patients with loss of *NF1* may benefit from a combined MAPK/ERK inhibitor and mTOR pathway inhibitor.

Despite a current lack of clinical trials specifically investigating patients with *NF1* mutations, available preclinical data strongly suggest that restoring WT *NF1* can impact tumorigenicity and progression. First, overexpression of *NF1* in colon cancer cell lines was able to promote tumor cell death [146]. In mouse xenograft models using cells with both *NF1* and $BRAF^{V600E}$ mutations, exogenous expression of *NF1* resulted in a decrease in tumor size [75]. These data suggest that *NF1* expression plays a critical role in cancer progression and that *NF1* could be an attractive target for gene therapy. One such avenue for targeted gene therapy would be an oncolytic viral vector which would simultaneously promote antitumor activity and deliver the *NF1* transgene [147,148]. Another option is to combine viral vector therapy with checkpoint inhibitors. Currently, a phase 2 clinical trial is investigating the combination of adenovirus delivering p53 with nivolumab (NCT03406715). In terms of prognostication, further work is needed to establish the efficacy and reliability of *NF1* as a potential biomarker of drug response that can guide therapy in patients with solid tumors.

Conclusion

While much research has focused on the function of *NF1* and its involvement in the *NF1* syndrome and associated tumors, the molecular role of *NF1* in cancer development and its significance in cancer-related cellular signaling has often been overlooked. This is despite a significant wealth of data describing its prevalence across many cancer types, and its potential role in mediating resistance, both of which we have reviewed here. Understanding the role of *NF1* in cancer development is important for the development of targeted therapies that can suppress malignant transformation and reverse resistance to treatment. With rather encouraging preclinical data, the use of *NF1* mutations/alterations as a biomarker of response holds promising prospects in terms of predicting resistance, and guiding therapeutic strategies. Similarly, understanding the extent to which *NF1* loss acts as a "driver mutation" and a mediator of resistance opens the way to novel gene therapy strategies involving potential re-expression of the lost gene. To that end, further work and investigation are definitely warranted.

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