MAX and MYC: A Heritable Breakup

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Abstract
The overexpression of MYC, which occurs in many tumors, dramatically disrupts the equilibrium between activation and repression of the oncogenic MYC/MYC-associated protein X (MAX)/MAX dimerization protein 1 (MXD1) network, favoring MYC–MAX complexes and thereby impairing differentiation and promoting cell growth. Although for some time it has appeared that MAX is necessary for both the activation and repression of the axis, recent evidence shows that MYC retains considerable biologic function in the absence of MAX. The presence of germline MAX mutations in patients with hereditary pheochromocytoma supports the predominant role of MAX as a negative regulator of the network and suggests that MYC deregulation plays a role in hereditary cancer predisposition. This finding also confirms the importance of impairment of the MYC/MAX/MXD1 axis in the development of aggressive neural tumors, because MYCN overexpression is an established genetic hallmark of malignant neuroblastoma, and it is likely that MXI1 plays a relevant role in the development of medulloblastoma and glioblastoma. Finally, the likely malignant behavior of tumors with mutations in MAX points to MYC as a candidate therapeutic target in the treatment of metastatic pheochromocytoma. Cancer Res; 72(13); 3119–24. ©2012 AACR.

Introduction
MYC-associated protein X (MAX) is a ubiquitous, constitutively expressed protein that plays a central role in controlling the MYC/MAX/MAX dimerization protein 1 (MXD1) axis, one of the better-known cellular networks whose deregulation contributes to the genesis of many human cancers. MAX was the first-described bona fide MYC-interacting protein, and it appears to be an essential dimerization partner for the other members of the axis to be active. Thus, whereas MYC activates transcription binding to E-box DNA recognition sequences in target gene promoters through heterodimerization with MAX, heterodimers of MAX with MXD1 family members [MXD1, MAX interactor 1 (MXI1), MXD3, and MXD4], MAX-binding protein (MNT), and MAX gene–associated (MGA) antagonize MYC-dependent cell transformation by transcriptional repression of the same E-box target DNA sequences (1). In addition, MAX is the only member of the network that homodimerizes efficiently, although the biologic role of these dimers remains unknown. The promiscuous interactions of MAX with MYC and MYC repressors maintain a complex equilibrium that dictates whether the transcription of thousands of target genes will be activated or repressed. Recognition of this dual task of MAX probably led to the assumption that MAX integrity is necessary not only for correct cell behavior but also for tumor homeostasis, and that therefore it is unlikely that mutations affecting MAX could occur in cancer.

MAX and Cancer
MAX has gained special prominence in the field of human genetics since germline loss-of-function mutations in the MAX gene were observed in patients with hereditary pheochromocytoma (PCC; ref. 2), a rare neural crest cell tumor that is localized mainly within the adrenal medulla and rarely metastasizes. Prior to this, 30% to 40% of patients with PCC were thought to be hereditary (3), with autosomal inheritance caused by germline mutations affecting 1 of 9 susceptibility genes (RET, VHL, SDHA, SDHB, SDHC, SDHD, SDHAF2, NF1, and TMEM127). Mutations in MAX were identified by sequencing the entire exome of 3 unrelated patients with a family history of PCC who did not have mutations in any of the known susceptibility genes (4). The patients were selected for the study because their tumors were found to have very homogeneous expression profiles in a previous transcriptional study. The expression profile differentiated the 3 MAX tumors from all other PCCs, with or without germline alterations in the other PCC susceptibility genes, and suggested that they might have the same genetic cause. The presence of germline mutations in the patients, as well as the loss of heterozygosity of the wild-type allele and the absence of protein in the tumors, indicated that MAX is a tumor-suppressor gene that causes hereditary PCC. Further analysis of 59 patients who were chosen because they had bilateral PCC and/or age of onset of <30 years led to the detection of 5 additional cases with mutations in MAX. Most of the mutations affected highly conserved amino acids within the basic helix-loop-helix leucine zipper (bHLHZip)
domain of MAX. The bHLHZip domain is the common structural element among the members of this transcriptional network and is responsible for the specificity and stability of homo- and heterodimer formation (HLHZip), and for DNA recognition via interactions of the basic region and the major groove (5). The presence of alterations affecting this critical domain, which is involved in protein–protein interactions and DNA binding, could therefore destroy the ability of MAX to antagonize MYC-dependent cell transformation, leading to tumor development. In fact, 2 of the 3 MAX missense variants reported to date affect casein kinase II phosphorylation sites involved in MAX DNA binding (2).

It has long been known that a homozygous chromosomal alteration involving the MAX gene leads to a protein that is incapable of homo- or heterodimerization and therefore is incapable of repressing transcription from E-box elements in PC12 cells (derived from rat adrenal PCC). Ribon and colleagues (6) showed that the reintroduction of MAX in PC12 cells results in transcriptional repression and a reduction in growth rate. This finding both confirmed the role of MAX in the regulation of MYC-dependent transcriptional activation and revealed that MYC may also function, at least in PC12 cells, in the absence of normally functioning MAX protein (6). The ability of MYC to function independently of MAX was later shown in Drosophila (reviewed in ref. 7), suggesting that the pivotal role of MAX in the MYC/MAX/MXD1 network is related more to repression than to activation. The involvement of MAX in the genetic susceptibility to PCC in humans also suggests that the loss of MAX in PC12 cells contributes to the histogenesis of the primary tumors rather than to the establishment of the cell line.

The presence of MAX mutations in patients with hereditary PCC highlights the importance of the MYC/MAX/MXD1 network in the development of neural crest tumors. Amplification and overexpression of MYCN are well-known genetic hallmarks in neuroblastoma (8), a neural crest cell pediatric tumor that originates primarily in the adrenal gland and is the second most common type of solid tumor in children. MYCN amplification leading to mRNA and protein overexpression occurs in 20% to 25% of neuroblastomas and is strongly associated with advanced disease stages and rapid tumor progression (9). The involvement of MYCN expression in the biologic behavior of these tumors remains unclear, but it was recently reported that high-level expression of MYCN in neuroblastomas lacking amplification of the MYCN locus results in a benign phenotype (10). Thus, overexpression of MYCN leads to a more tumorigenic phenotype, but the degree of aggressiveness appears to depend directly on the MYCN copy number. Ablation of MAX’s transcriptional repression of MYC in PCC could lead to the same oncogenic MYC deregulation that occurs in neuroblastoma. In this regard, the observation that 25% of patients with mutations in MAX develop metastasis suggests that a correlation may exist between MAX loss of function and metastatic potential. If this is confirmed, the malignant behavior of MAX-related tumors would be congruous with what is known about neuroblastomas, and would add weight to the idea that alterations in the main regulator of MYC could lead to the same effect in PCC that the amplification of MYCN provokes in neuroblastoma (i.e., highly malignant disease and poor prognosis). It is also noteworthy that the second hit observed for MAX-related PCCs was the loss of heterozygosity, either by uniparental disomy (UPD) or by loss of chromosome 14q, where MAX is located (2). This is especially remarkable because loss of chromosome 14q23-q32 has been described in 22% of primary neuroblastomas, and was inversely correlated with MYCN amplification (11). In addition, 14q-UPD was recently observed in neuroblastomas (12), and a significant downregulation of 2 microRNAs (miR-487b and miR-410) located on 14q22 was also associated with high-risk neuroblastoma (13). These findings suggest that MAX could be the target of this chromosomal loss, and that the deregulation of MYCN observed in neuroblastoma may occur as a result of either direct overexpression of the protein or a lack of MAX-related repression. It is noteworthy that no neuroblastomas were found in MAX mutation carriers (2), although the small number of patients studied meant that no definitive conclusion could be reached regarding the participation of MAX in neuroblastoma development. In addition, given the ubiquitous nature of MAX expression and the key role of MAX as a regulator of this axis, one might expect patients with germline mutations in this gene to develop other tumors as well. The analysis of a larger series of MAX mutation carriers will provide further information about the overall involvement of MAX alterations in cancer predisposition.

Historically, most of the biologic properties of MYC have been attributed to its association with MAX. Moreover, although it is known that MAX plays a pivotal role in the oncogenic function of MYC, it has been speculated that MAX may also be required for the correct folding of MYC (14). Considering the essential roles of MYC, the fact that many of its biologic activities are independent of MAX could mean that cells need protection mechanisms against MAX depletion. The mechanism by which MYC exerts its MAX-independent functions is still unknown, but other known (or unknown) MYC-specific interacting proteins probably mediate these functions.

**MYC and Cancer**

Many, if not most, human tumors present with elevated levels of MYC. Indeed, important aspects of tumor biology, such as proliferation, cell adhesion, and angiogenesis, are affected by enhanced expression of MYC, which makes deregulation of this oncogene a hallmark of cancer. Under normal conditions, the stimulation of cells by internal and external signals leads to a rapid and transient overexpression of MYC mRNA and protein that persists into the cell cycle and subsequently declines to low levels in quiescent cells (15, 16). In addition, MYC overexpression sensitizes primary cells to apoptosis in response to growth-factor withdrawal, which may provide protection against the potentially detrimental activity of MYC in terms of tumorigenesis (17). The oncogenic activity of MYC proteins begins when their normal transcriptional regulation is disrupted by gene amplification and translocation, which then leads to abnormally increased levels of intracellular MYC. Overexpression of MYC promotes oncogenic transformation and tumorigenesis by on the one hand activating the transcription of target genes that drive cell
proliferation and stimulate angiogenesis, and on the other repressing cell differentiation (18). It is noteworthy that although mutations within the open reading frame of MYC occur infrequently, it is widely accepted that MYC deregulation is not solely restricted to translocations and amplifications at the MYC locus. This suggests that the impact of MYC deregulation on cancer incidence in humans is greater than previously thought. Regarding cancer predisposition, inactivating germline mutations in MYCN have been found in patients with Feingold syndrome, and, as expected, carriers of heterozygous MYCN alterations did not develop tumors (19). However, the recently reported contribution of MAX germline mutations to cancer susceptibility highlights the importance of MYC regulation in hereditary malignancies, and raises the possibility of finding new alterations in other bHLHZip repressors of the network that are involved in tumorigenesis through their failure to repress MYC.

MYC–MAX Interacting Proteins and Cancer

The members of the MXD1 family (MXD1, MXII, MXD3, and MXD4) have been shown in vivo to promote differentiation, block cellular growth and MYC-induced transformation, and suppress the development of cancer (20). In addition, MNT is likely to be a key regulator of MYC activity, and MGA, whose biologic function remains unknown, appears to contribute to the silencing of E2F- and MYC-responsive genes in quiescent cells (21). The repression of MYC’s transforming ability depends on the balance between the MYC–MAX and MXD/MNT/MGA–MAX complexes, and the regulating members of the network exert their functions at different stages during the transition between proliferation and differentiation. Although distinct MYC-independent functions have been reported for some repressors, it seems that they could all behave in a very similar fashion. The exact significance of this putative functional redundancy in MYC repressors is unclear, but it seems that the differential timing of their induction ensures the regulation of the axis both during MYC overexpression and when MYC is subsequently downregulated. Taking into account the detrimental effect of the failure to control MYC expression (i.e., by MAX mutations), the presence of various regulators is likely to be an essential cellular mechanism for inhibiting the transforming capacities of MYC through deregulation of the network.

The crucial role of MYC in cancer formed the basis of numerous studies that attempted to elucidate the involvement in tumorigenesis of the MXD1 family of transcriptional regulators. MXII was proposed as a candidate tumor-suppressor gene that accounts for the 10q deletion frequently found in brain tumors [i.e., 80% of glioblastoma multiforme and 15% of primary medulloblastoma (22)]. Other findings that are consistent with this include the identification of a mutation affecting MXII in a medulloblastoma cell line, and the observation that the reintroduction of MXII in a glioblastoma cell line lacking endogenous MXII resulted in a decreased growth rate and an accumulation of cells in the G2–M phase (23). These findings suggest that MXII may play a role in the development of other neural malignancies, as do MAX in PCC and MYCN in neuroblastoma. In addition, mice lacking MX1 exhibited increased susceptibility to tumorigenesis [squamous cell carcinoma of the skin and malignant lymphoma (24)], and mutations in MXII were also observed in a small number of prostate tumors (25). Despite the enhanced ability of MXII-deficient prostatic epithelium cells to proliferate, it seems that mutation of MXII is a minor event in prostate cancer and therefore of unknown significance for prostate tumor development.

MNT is one of the most studied of the proposed transcriptional regulators of MYC because its chromosomal location (17p13.3) is frequently deleted in cancer and, like MAX, it is ubiquitously expressed, with no fluctuations during the transition from G0-phase to S-phase. Although mice heterozygous for MNT mutations do not appear to be cancer prone, it was shown that MNT-deficient mouse embryonic fibroblasts and mammary glands exhibit many of the hallmark characteristics of cells that overexpress MYC [i.e., prone to apoptosis, efficiently avoid senescence, and can be transformed with oncogenic RAS alone (26)]. Moreover, conditional deletion of MNT in T cells also leads to tumor formation (27). Nevertheless, studies that focused on identifying MNT-inactivating mutations in tumors with common 17p losses (e.g., medulloblastoma) found no abnormalities (28).

Therapeutic Strategies Targeting the MYC/MAX/MXD1 Pathway

Site-directed mutagenesis experiments have consistently revealed the critical role of various amino acids located within the bHLHZip domain of many components of the MYC/MAX/MXD1 network. Better knowledge of protein–protein and protein–DNA interactions could help to focus future therapeutic strategies on inhibitors of either the transforming activity of the complex or the recognition of target genes. Many compounds have already been tried because they interfere with MYC/MAX heterodimerization. However, given that MYC retains substantial MAX-independent activities, this may not be the best strategy for all tumor types (7). On the other hand, it has been clearly shown that oncogene-induced tumorigenesis is reversible, and inactivation of a single oncogene within a primary tumor is sometimes sufficient to induce tumor regression. Thus, specific inactivation of MYC was shown to reverse the malignant properties of various tumors (29, 30), and the combined inactivation of MYC and angiogenesis may be even more clinically effective (31). Another promising therapeutic approach is to block MYC-induced neoplasia by activating stress-signaling pathways (32). Phosphorylation of MYC by the protein kinase PAK2 leads to degradation of MYC and therefore inhibition of proliferation and cell transformation. Two recent studies (33, 34) showed that MYC suppression and subsequent deregulation of the MYC transcriptome can be achieved through pharmacologic inhibition against the BET bromodomain family of chromatin adaptors. The finding that BET proteins act as regulatory factors for MYC in various hematologic malignancies reveals a new therapeutic strategy for other tumors characterized by pathologic activation of MYC. As previously mentioned, aberrant MYC expression is usually due to induction caused by upstream
signals rather than by mutations in the gene. Therefore, the inhibition of MYC would tend to target the consequence more than the cause of oncogenesis (35). In addition, given that MYC is essential for the development of a broad range of adult organs, blocking MYC function might systemically trigger devastating and irreversible side effects.

Oncogenes are more attractive therapeutic targets than tumor-suppressor genes because it is easier to inhibit excessive activity than to restore lost activity in tumor cells. Nevertheless, strategies to restore protein function are now considered viable anticancer therapeutic approaches (36), and thus may be an option for MAX-related PCCs that have a potentially malignant phenotype. It is noteworthy that the transcriptional profile of MAX tumors shows a significant enrichment of mTOR pathway components when compared with other mutated PCCs (data not shown). This finding is especially relevant because deregulation of both the mTOR pathway and the upstream PTEN/PIK3CA/AKT1 axis seems to be essential for the development of many PCCs [i.e., with mutations in RET, NF1, TMEM127, or MAX (Fig. 1)]. Conditional PTEN knockout mice present with metastatic bilateral PCCs (37), and RET-mediated cell-transforming capacity is critically dependent on activation of the PIK3CA/AKT1 pathway (38). Furthermore, mTOR is constitutively activated in both NF1-deficient cells and human tumors through the inactivation of tuberin by AKT (39). Finally, the PCC susceptibility gene TMEM127 also functions by negatively regulating the mTOR pathway (40). Considering the transcriptional profile of MAX tumors, and the fact that crosstalk between the PIK3CA/AKT1/mTOR and MYC/MAX/MXD1 pathways was recently proposed (41), it is likely

Figure 1. The PIK3CA/AKT1/mTOR pathway mediates downstream activation of genes involved in multiple cell processes, such as regulation of cell growth, division, survival, and, when disrupted, tumorigenesis. In normal cells, upon binding to the GDNF family of ligands, the receptor tyrosine kinase RET triggers PIK3CA signaling via the recruitment of IRS proteins. PIK3CA subsequently activates (phosphorylates) AKT, and this activated form of the protein regulates up to 100 downstream effector proteins that are involved in cell proliferation. In addition, the AKT-mediated phosphorylation of TSC2 indirectly leads to the activation of mTOR, which regulates cell growth through phosphorylated S6K1. S6K1 also inhibits the tumor-suppressor function of MAD1 and thus leads to deregulation of MYC, cell growth, and activation of proliferation. Upstream, the pathway is negatively regulated by both PTEN, which directly antagonizes PIK3CA, and NF1 through RAS inhibition. TMEM127 is a negative regulator of mTOR, and MAX represses MYC-dependent transcriptional activation. GDNF, glial-cell-line-derived neurotrophic factor; IRS, insulin receptor substrate.
that mutations in MYCN not only deregulate the MYC neoplastic switch but also lead to impairment of the mTOR pathway and PCC development. It was reported that mTOR inhibitors (e.g., rapamycin) prevent the proliferation of human neuroblastoma cells (42), and also inhibit the development of PCC in PTEN knockout mice (43). In addition, it seems that sunitinib (which was recently shown to be a successful treatment for malignant PCCs) induces apoptosis in PC12 cells by inhibiting the VEGFR-2/AKT1/mTOR/S6K1 pathways. Because PC12 cells constitute a specific double-knockout model for MAX-related PCC, it seems plausible that targeting the mTOR pathway would be an effective therapy for patients with malignant PCC carrying germline MAX mutations.

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No potential conflicts of interest were disclosed.

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