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REVIEW ARTICLE

Molecular genetics of neurological tumours

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Recent developments in our understanding of neoplastic transformation and tumorigenesis have largely paralleled technical advances in molecular and cellular biology. A great deal of work has been focused in the past two decades upon tumour types which claim vast numbers yearly, for example, cancers of the breast, lung, colon, blood, and bone. However, many basic genetic aberrations first detected in these malignancies are apparently shared by a variety of tumours, which are perhaps less frequent, but no less devastating. This review article will attempt to focus on recent efforts to characterise genetically a number of neurological tumours, primarily focusing on astrocytomas and their malignant counterpart, glioblastoma. Where appropriate, we will also indicate relevant findings which have been reported for meningiomas and tumours associated with neurofibromatosis type 2 (bilateral acoustic neuroma). As a class of tumours which exert clinical effects largely through volume expansion in spatially confined compartments, and which do not metastasise to other organs, neurological tumours may represent an ideal system in which to study mechanisms of cell cycle control and transcriptional regulation involved in tumorigenesis. In addition, they may provide models for understanding the steps generating transitions to rapidly proliferative states characteristic of high grade glioblastomas or malignant meningiomas.

Astrocytomas

Astrocytomas, including glioblastoma multiforme, represent the most common and lethal primary neoplasms of the human central nervous system. Although many tumours retain morphological and immunological characteristics of glial cells, the cell origin of these tumours and the biological steps which lead to their altered growth characteristics remain obscure. Genetic studies of these tumours have focused primarily on four areas: cytogenetics, the role of mitogenic/differentiation factors and their surface receptors, proto-oncogene amplification, and, more recently, tumour suppressor genes.

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CHROMOSOMAL ABERRATIONS

A number of studies, primarily from the work of Bigner and colleagues, have suggested that despite the heterogeneity observed among glioblastoma karyotypes, several distinct abnormalities are frequently detected. These include, most notably, gains in chromosome 7 and losses of chromosome 10, which occur in at least 60% of these tumours. Approximately 50% show double minute chromosomes (DMs), indicating the presence of gene(s) amplification. Translocations and deletions of chromosome 9p occur at a somewhat lower frequency, approximately 20%. Losses of chromosome 22 and sex chromosomes have also been reported at lower frequencies.[1]

Such consistent abnormalities among glioblastoma derived cell lines have provided 'geographical' insights into locations of genes potentially involved in glioblastoma tumorigenesis, or confirmed the logic of examining certain candidate genes within these regions. For example, knowledge of the location of the epidermal growth factor receptor at 7p11-13 led to studies of amplification and overexpression of this gene in glioblastomas.[2] Although the breakpoints varied for chromosome 9p translocations, all displaced the segment p24-pter which is near a cluster of interferon genes.[3] Similar rearrangements have also been reported in a number of haematopoietic malignancies such as chronic granulocytic leukaemia and acute non-lymphocytic leukaemia,[4,5] suggesting that these loci may also be relevant for glial derived tumours. Furthermore, as will be discussed below, consistent chromosomal losses have prompted the search for loss of genetic material at higher resolution using polymorphic DNA probes as a means to identify candidate tumour suppressor loci.

It must be noted, however, that these karyotypic data are derived from highly malignant cell lines and do not necessarily reflect primary causal events. These studies have noted that with prolonged passage in culture additional karyotypic abnormalities begin to appear: this suggests that certain genetic aberrations are selected for in culture or are simply reflective of defective DNA replication, a common characteristic of rapidly proliferating cells. Double minute chromosomes (DM) or homogeneously staining regions (HSR) have not been seen in primary glioblastoma biopsies, in contrast to their observed frequency in tumour derived cell lines.[1] Finally, many slower growing tumours are difficult to establish in culture, biasing representation of such karyotypic data towards highly malignant cells, and underscoring the need to pursue genetic analysis, whenever possible, in primary tumour speci-

mens or early passage tumour lines. Optimally, some non-tumour tissue (for example, lymphocytes, uninvolved cortex) should be available as a reference for the patient's endogenous genetic complement and to guard against the mistaken identification of genetic polymorphisms as tumour causative events.

GROWTH FACTORS AND COGNATE RECEPTORS

As for a number of other malignancies, there appears to be strong evidence for the involvement of peptide growth factors playing an important part in the aetiology of glioblastomas. These soluble growth factors normally promote cell growth or viability or both by binding to cognate surface receptors and activating signal transduction mechanisms (for example, inositol phosphate metabolism, regulation of GTPase activity, regulation of kinase activities) which lead to changes in gene expression, DNA synthesis, or cellular proliferation. Overexpression of either the surface receptors or the growth factors themselves may lead to altered cellular growth. For astrocytic tumours, a number of growth factors and receptors have been implicated in such mechanisms, including most notably platelet derived growth factor (PDGF), the epidermal growth factor receptor, and the ros1 proto-oncogene.

PDGF exists as a homo- or heterodimer of A or B chains or both. Work in this field has predominantly focused on the BB homodimer owing to its sequence homology to the v-sis oncogene from the simian sarcoma virus and also owing to the fact that the only PDGF receptor molecule cloned is the isoform which preferentially binds the BB homodimer.[6] Several studies have indicated overexpression of the PDGF and PDGF receptor genes through in situ hybridisation, Northern analysis, or radioisotopic ligand binding in both glioblastomas and meningiomas.[7,8]

The receptor for epidermal growth factor, as mentioned above, is located within 7p11–13 and represents a candidate locus for gene amplification. Libermann *et al*[2] and Wong *et al*[9] showed a strong correlation between EGFR gene amplification and actual mRNA expression, although this aberration was not universal among tumours studied (40–60%). Such EGFR amplification may also be frequently associated with amplification of N-ras.[10] As will be discussed below, our laboratory has recently shown a strong correlation between EGFR amplification and genetic loss of loci on chromosome 10, suggesting that a cooperative mechanism between such loci may underlie a subset of glioblastomas.[11] On the basis of radiolabelled ligand binding it has also been suggested that EGFR may play a role in the generation of meningioma[12] and ependymoma.[13] Finally, certain glioblastomas may also overexpress transforming growth factor alpha (TGF-α), which resembles EGF and binds the EGFR to stimulate tyrosine kinase activity.[14]

Basic fibroblast growth factor (bFGF) is known to exert a trophic effect upon a variety of cells of neural origin and is prevalent in neural tissue.[15,16] Human glioma cell lines express both an FGF-like activity and a bFGF binding receptor and proliferate in response to exogenously applied bFGF.[17] It has also been suggested that in addition to a conventional autocrine role in tumorigenesis, bFGF may promote glial tumour growth by the stimulation of angiogenesis or neovascularisation.[17,18]

The human c-ros1 gene, originally identified as the proto-oncogene mcf3 in NIH 3T3 transformation assays,[19] encodes a transmembrane receptor protein with tyrosine kinase activity.[20] In a survey of 45 various tumour cell lines, the ros1 gene was overexpressed at the mRNA level only in glioblastoma derived cell lines, and a potential activating mutation was detected in one cell line.[21] Although its putative ligand has not yet been identified, the gene does encode a 280 kd receptor-like protein with sequence homology to the *Drosophila* gene *sevenless*.[22] Involvement of the ros1 product appears to have specificity for glioblastomas, and represents an important area for investigation in the field of neuro-oncology.

Perhaps the most provocative studies regarding the role of peptide growth factors in astrocytic tumours have arisen not from tumour studies, but from studies of developmental models of glial cell differentiation. Work originating from the laboratories of Martin Raff and Mark Noble identified a population of cells, known as O-2A cells, which are undifferentiated at isolation but are capable of differentiation to either oligodendrocytic or type 2 (fibrous) astrocytic phenotypes.[22] Conditioning of the medium by type 1 astrocytes or application of platelet derived growth factor inhibits the premature differentiation of these O-2A cells into oligodendrocytes and promotes cellular proliferation for a defined number of cycles, after which differentiation occurs.[23] Particularly exciting is the recent finding that when O-2A cells are exposed to a combination of PDGF and bFGF, they exhibit continued self-renewal without differentiation.[24] When exposed to bFGF alone, cells acquired the oligodendrocytic phenotype but did not cease division. Inhibition of differentiation thus required the presence of both factors.[24] It is intriguing to speculate that similar events may underlie the generation of some glial derived tumours, as malignant cells typically exhibit an undifferentiated phenotype and high proliferative rate. Overexpression or alteration of the growth factors, their cognate receptors, or proteins involved in their signal transduction pathways may also impinge upon this mechanism of proliferation control.

PROTO-ONCOGENE INVOLVEMENT

Outside the domain of growth factors and their receptor proteins, a number of additional proto-oncogenes have been implicated in the development and progression of neural tumours. c-myc, an endogenous transcription factor of the helix-loop-helix-basic-leucine zipper family (HLH-bZIP), was originally

identified as a viral oncoprotein.[25] Its overexpression in Burkitt's lymphoma resulting from translocation adjacent to an immunoglobulin promoter/enhancer provided an early model for nuclear oncogene function.[26] c-myc has been shown to be amplified or overexpressed or both in both glioblastoma and meningioma,[8 27 28] while N-myc amplification and overexpression has been recorded for astrocytoma, meningioma, and neuroblastoma, a childhood tumour of neural crest origin.[28–30] The association of N-myc amplification with clinical presentation and prognosis sparked interest in the potential use of oncogene characterisation as a clinical adjunct.[31] Overexpression of c-myc and N-myc presumably contributes to increased cellular proliferation through induction of additional genes, although the target genes regulated by myc have not been identified. The recent identification of a c-myc DNA binding sequence should facilitate this process.[32] In cases with mRNA overexpression in the absence of DNA amplification, the causative events remain unclear. The loss of certain tumour suppressor genes which normally negatively regulate transcription may contribute to proto-oncogene overexpression, and represent a more upstream event in tumorigenesis (see below).

Kinzler *et al*[33] have recently identified a cellular oncogene which was amplified and highly expressed in a human glioma, gli. Gli is a zinc finger transcription factor which resembles members of the Kruppel family of *Drosophila* gap segmentation genes.[34 35] Coexpression of Gli with the adenovirus E1A oncoprotein transforms primary cells which are then tumorigenic in nude mice, suggesting that Gli can be placed in a similar complementation group as ras.[36]

TUMOUR SUPPRESSOR GENES

A great deal of attention in recent years has been directed towards the potential role of tumour suppressor genes, or 'anti-oncogenes', in the aetiology of a variety of human cancers. These genes encode protein products which, when not expressed or when expressed only in mutant form, lead to a growth advantage which may produce a neoplastic phenotype. These proteins presumptively negatively regulate growth in normal cells. The genetic model for tumour suppressor genes originated with the study of the childhood cancer retinoblastoma, a hereditary ophthalmological tumour. Although DNA linkage analysis suggested dominant inheritance linked to chromosome 13q14, subsequent analysis of tumour specimens showed characteristic lack of expression of the RB encoded message.[37] A large body of data has since indicated that RB does in fact follow a 'recessive' pattern requiring inactivation or mutation or both of both allelic copies for tumour formation, and that hereditary cases had suggested dominant inheritance because the high statistical probability of the 'second hit' in somatic cells produced a virtual 100% penetrance of the disease. This mechanism of germline inheritance of a genetic predisposition being followed by occurrence of somatic cell mutation or chromosomal loss had been predicted much earlier by Knudson.[38] RB inactivation appears to be a near universal event in at least a percentage of most sporadic tumour types, and genetic deletions at this locus have recently been described in glioblastomas.[39]

TUMOUR SUPPRESSOR LOCI IN ACOUSTIC NEUROMA AND MENINGIOMA

Beyond the use of grossly visible karyotypic aberrations, the search for potential tumour suppressor loci typically uses restriction fragment length polymorphisms (RFLPs) and random DNA probes to identify chromosomal regions which are frequently deleted in tumour tissue as compared to constitutional DNA (for example, peripheral leucocyte DNA). Acoustic neuromas, benign tumours which arise from Schwann cells of the VIIIth cranial nerve, may occur sporadically as unilateral cases or bilaterally in association with an inherited predisposition (neurofibromatosis type 2 or NF2).[40] Somatic (tumour specific) loss of chromosome 22 often represents the only genetic anomaly observed in these tumours.[41] The common region of deletion is consistent with the region thought to contain the NF2 gene (22q12) as detected by linkage analysis.[42] Similar deletions of chromosome 22 have been detected for meningiomas, although it remains unclear whether these deletions target distinct genetic loci.[43 44] The cloning of these NF2 and meningioma associated genes is currently an active area of investigation.

TUMOUR SUPPRESSOR LOCI IN ASTROCYTOMA

Our laboratory, and others, have detected a relatively high frequency of such allelic loss in astrocytoma involving primarily two regions: (1) the telomeric portion of chromosome 17p, and (2) large regions of chromosome 10. These allelic deletions are tumour specific and probably suggest the presence of tumour suppressor loci in these regions.[11 45–50] Several tumours with loss of chromosome 17 show partial chromosomal deletions, with the common region of allelic loss involving 17p12–17pter.[45 49] This region was known to encompass a candidate gene encoding a 53 kd nuclear protein known simply as p53, which was originally identified in stable complex formation with the transforming oncoprotein large T antigen in SV40 virally transformed cells.[51] It has subsequently been shown also to bind the 55 kd E1B protein from adenovirus and the E6 protein of papillomavirus[52 53]: the binding of p53 by these viral proteins has been suggested to be critical for their transforming activity. Owing to apparent overexpression of p53 in a number of viral and spontaneously transformed cell lines, it was initially suggested that p53 functioned as a positively acting oncogene analogous to c-myc.[54] Consistent with this model, p53 expression plasmids were shown to transform primary rodent fibroblasts in cooperation with activated ras.[55–57]

However, subsequent experiments have provided strong evidence that wild type p53 possesses the ability to inhibit transformation by a number of cellular and viral oncogenes, and that earlier experiments had used p53 cDNAs containing a variety of point mutations.[58-60] This suggested that p53 is in fact a tumour suppressor gene product analogous to the RB protein product. This hypothesis was strengthened by the demonstration of allelic loss or distinct point mutations or both in a number of colonic, lung, and breast cancers.[61 62]

We chose to search for p53 point mutations in astrocytic tumours by direct sequence analysis of polymerase chain reaction (PCR) amplified genomic DNA from both tumour and normal (leucocyte) DNA. Forty-five percent of the glioblastomas (grade III/III), but only 14% of grade II/III astrocytomas contained distinct point mutations leading to codon alterations, premature stop codons, or potential splicing aberrations.[63] The locations of these mutations were consistent with similar mutations seen in a variety of other tumour types, occurring in regions of the p53 sequence which are highly evolutionarily conserved and which are likely to represent important functional domains of the encoded protein.[61 64] Their association with higher grade tumours suggests that p53 inactivation may represent a step in tumour progression to a more malignant state. Other tumour suppressor loci may similarly be inactivated as steps towards malignant phenotypes: chromosome 10 deletions also appear to be glioblastoma grade specific (see below). We have recently found that chromosome 14q deletions are specifically associated with malignant meningiomas as opposed to the more common benign form, again suggesting that specific genetic aberrations can be clinically stage specific.[65]

Functional analysis of identified target genes

In view of these results, we have more recently turned our attention to an analysis of the physiological function of the p53 protein. Several lines of evidence have suggested that p53 may play an important role in the control of cell cycle progression. Quiescent G0 cells do not express detectable levels of p53 protein.[66] Serum stimulation of quiescent, non-transformed 3T3 fibroblasts induces an increase in the synthesis and steady state levels of p53 mRNA and protein, detectable six hours after stimulation and increasing 10 to 20 fold just before entry into S phase (18 hours[67]). Pulse chase analysis indicated that this was indeed because of new protein synthesis and not alterations in the half life of p53, which is notably short ($t_{1/2}$ 20 to 30 minutes). While these results suggested a role for p53 at the G1/S phase transition, Mercer *et al*[68] showed that microinjection of anti-p53 monoclonal antibodies inhibited entry into S phase, but only when injected less than four hours after serum stimulation.[68] p53 has also been shown to compete with DNA polymerase alpha for SV40 T antigen binding[69] and to interfere with the ability of large T to initiate DNA replication[70 71]: by inference it might play a role in normal regulation of DNA replication. p53 may therefore function both at the G0/G1 and the G1/S transitions. Subsequent experiments have not been able to distinguish between these two possibilities.

Although p53 contains no previously characterised DNA binding motifs, there are distinct acidic, proline rich, and basic domains within the protein reminiscent of recently described transcription factors,[72] and there are recent reports that p53 protein is capable of DNA binding.[73-75] Recent reports also indicate that the amino-terminal acidic region of p53 is capable of transcriptional activation in GAL4 fusion assays.[76 77] Since many cell cycle control checkpoints are believed to occur at the level of gene regulation, we chose to examine whether p53 functions as a transcriptional regulatory protein.

We examined as a candidate target gene the c-fos proto-oncogene, a member of the immediate-early gene family which is characterised by rapid induction and down regulation in response to a variety of environmental stimuli, including growth factors, electrical activity, and ionotropic agents.[78] c-fos was identified as the cellular homologue of the gene (v-fos) encoding the transforming protein from an avian sarcoma virus, and has been shown to be abnormally regulated in a number of human cancers. The immediate-early genes are believed to represent an intermediate step in signal transduction in response to exogenous stimuli. The proteins encoded by these genes function as transcription factors, and in various combinatorial patterns (via homo- and heterodimer formation) affect downstream gene expression to alter cell cycle transition, cellular differentiation, or other physiological responses.

In cotransfection experiments, we have seen consistent transcriptional repression of c-fos/CAT constructs by an expression plasmid bearing a wild type p53 cDNA (hp53).[79] p53 also transcriptionally down regulates c-jun, another immediate-early gene which, in combination with c-fos, comprises the AP1 transcriptional activator. These effects are specific to the wild type protein, as a mutant version differing by a single amino acid failed to produce any such repression. p53 expression in a stable cell line with p53 under the direction of an inducible promoter represses endogenous transcription of these genes (N Kley, R Chung, unpublished observations). Tumour suppressor genes such as p53 probably negatively regulate the transcriptional activity of a number of such growth related genes, with tumour specific p53 inactivation leading to altered transcription and cellular proliferation.

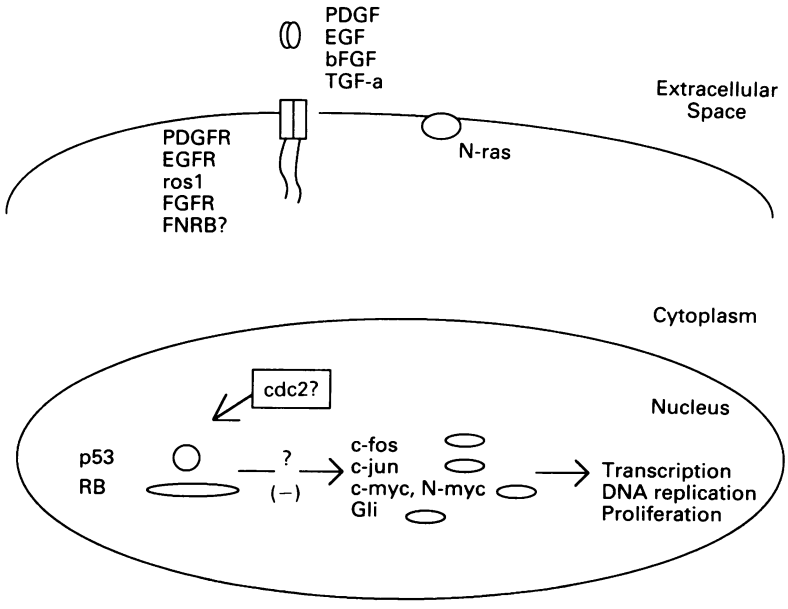
Whether p53 plays a distinct role from the retinoblastoma product in terms of those protein or nucleic acid targets with which it interacts or in terms of cell type specificity remains obscure. Both p53 and RB appear to be crucial G1 checkpoints: the importance of G1 regulation is universal among mammalian cells and

may resemble the yeast START point as a fundamental control mechanism for cellular proliferation. Both p53 and RB appear capable of repressing c-fos transcription.[79 80] p53 and RB are rapidly being incorporated into the framework of cell cycle control elements first discovered in yeast systems, as both p53 and RB have been shown to be phosphorylated by cell cycle dependent kinases.[81] There are also a number of reports suggesting a role for p53 in terminal differentiation of cycling precursor cells,[82-84] and transcriptional or post-translational modification of this protein may play a role in the terminal G1 arrest characteristic of such differentiation events. Further study of these important proteins should provide important insights into the mechanisms underlying differentiation and proliferation events of nervous system cellular components and their transformed counterparts.

Allele loss on chromosome 10

Losses of chromosome 10 in glioblastomas occur at an even higher frequency than chromosome 17p, in the range of 50 to 75% of tumours. To date, no consistent region of deletion has been defined: in fact, a number of tumours have been characterised with only 10p or 10q deletions, leading many to surmise that more than one loci on chromosome 10 may be involved in glial tumorigenesis, with significant selection for inactivation of both loci. Alternatively chromosome 10 may be subject to unusual chromosomal instability in these tumours. We have recently shown that in a series of 58 glioblastomas, 72% showed chromosome 10 loss, and 38% showed EGFR amplification.[11] Quite striking, however, was that all tumours with EGFR amplification showed chromosome 10 loss, suggesting that these events may be sequentially related,[11] in the manner suggested for N-myc and chromosome 1 deletions in neuroblastoma.[85 86] These may represent a subgroup of glioblastomas distinct from those containing p53 mutations.[63]

Finally, we are in the process of examining a number of potential candidate loci on chromosome 10, in particular the p34/cdc2 cell cycle associated kinase. Although our data are preliminary, four of seven glioblastomas express an altered cDNA for the p34/cdc2 kinase, suggesting that at least in some cases (all seven lost one copy of chromosome 10) this cell cycle regulatory protein may be a target for mutation in glioblastomas (R Chung, unpublished observations). We are actively characterising these aberrant proteins to determine their possible functional role in these tumours.



Genetic targets of amplification, overexpression, inactivation, or mutational alteration in common human neurological tumours. See text for details.

Conclusions

Neurological tumours represent an ideal model system in which to study specific genetic events and the interrelationships between such events which underly tumorigenesis. The involvement of growth factors (PDGF, EGF, etc), signal transduction factors (N-ras, c-myc), and nuclear cell cycle regulatory proteins (p53, cdc2) parallels pathogenetic events seen in other human cancers, suggesting that, with time, neurological tumours will emerge as a well understood group of treatable malignancies (figure). The spectrum of growth characteristics shown by these tumours allows the identification of stage specific genetic changes, with the hope of directing therapeutic measures at those events specifically involved in the progression of tumours currently incurable by conventional surgery, chemotherapies, and radiotherapies.

The techniques used in the genetic characterisation of neural tumours are no different from those used in the investigation of other cancers. Recent advances have been aided not only by technical innovations, but perhaps more importantly by the realisation by neurosurgical and neuropathological staff of the importance of careful tumour handling (for example, liquid nitrogen snap freezing), the importance of departmental cell culture facilities, and the importance of obtaining blood samples as crucial controls for any genetic studies. As an increasing number of such departments recruit investigators trained in molecular and cellular biology, the understanding of these devastating tumours should rise exponentially.

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