INTRODUCTION

The aim of this paper is to review recent molecular analysis techniques from the perspective of the classification of glioma tumours in man.

RECENT RESEARCH PROGRESS IN MOLECULAR CLASSIFICATION OF GLIOMAS

Glial tumours, in particular those of astrocytic origin, are the most common primary brain tumours and account for more than 40% of all central nervous system (CNS) neoplasms (1). All neoplasms have an inherent tendency to progress towards a less differentiated, more malignant phenotype: this change reflects the sequential and cumulative acquisition of genetic alterations (2). Their incidence apart, the complete network of the transformation-associated genes operating in this neoplastic development is still to be fully clarified. Currently, gliomas are classified, in accordance with the World Health Organisation (WHO) scheme (2), on the basis of phenotypic characteristics: tumours with nuclear atypia are classified as grade II (astrocytoma), those showing the addition of mitoses are grade III (anaplastic or malignant astrocytoma) and those exhibiting en-
dothelial proliferation and/or necrosis are grade IV (glioblastoma); grade I benign pilocytic astrocytomas are rare, occurring primarily in children and presenting none of the above histological features (3). Recent progress in the elucidation of genetic alterations found in gliomas have raised the exciting possibility of using genetic and molecular analyses to resolve some of the problematic issues currently associated with the histological approach to glioma classification (4). Through the use of molecular genetic techniques (5), a series of events that occur in a relatively defined order have also been identified in the tumorogenic progression of diffuse gliomas.

Genes that contribute to human cancer exhibit a broad range of expression patterns and functions. Consequently, previously identified genes provide few clues as to the characteristics of a new cancer gene. Since the human genome comprises an estimated 30,000-50,000 genes, there exists a prohibitive number of candidate genes that might play a role in tumorigenesis. Therefore, it is necessary to narrow the field of inquiry using a combination of techniques, first to identify the general region in which a cancer gene may be located, and then to map it more precisely within this region. The process of identifying genes with a role in human neoplasia is made up of four complementary stages:

1. Establishing a minimum region associated with a gene.
2. Physical analysis and mapping of this region of interest.
3. Identifying candidate genes.

The search for an unknown gene that plays a role in human cancer requires the definition of a candidate region. A combination of “low-resolution” gene mapping techniques such as cytogenetic and genetic linkage analyses, loss of heterozygosity (LOH), or amplification studies may be used to analyse biological materials from sporadic and inherited tumours. Once a region has been identified, more accurate “high resolution” physical mapping techniques may be employed to refine the interval further.

Cytogenetic and molecular genetic studies of glioblastoma multiforme (GBM) have shown that the most frequent alterations are gains of chromosome 7, and losses of 9p, 19p, 22p loci, and of chromosomes 10 and 22 (6-9). Although this profile is potentially useful in distinguishing GBM from other tumour types, the techniques used tend to be labour-intensive, and some can detect only gains or losses of genetic loci. Comparative genomic hybridisation (CGH) is a powerful technique capable of identifying both gains and losses of DNA sequences (at a level comparable with the more established genetic methods), of recognising the prominent genetic alterations associated with GBM and of supporting its use as a plausible adjunct to glioma classification (10,11).

Multiple markers are used in LOH analysis to examine loci on each autosome and sex chromosome for loss of genetic information (12). Several associations between detectable alterations and tumour histopathology are apparent. The most relevant of these is the loss of genetic information from chromosome 10 and its highly specific association with glioblastoma (13). Further analysis has revealed that loss of 9p-localised interferon genes is restricted to anaplastic gliomas and glioblastomas and that loss of genetic information from chromosomes 13, 17 and 22 occurs at similar frequencies in each malignant grade of glioma. Of these changes, the loss of genetic information from the short arm of chromosome 17 has been widely investigated (14). Another chromosome in which there is frequent loss of genetic information is chromosome 9 (15).

Gene amplification is one of various molecular genetic mechanisms that can affect a cellular oncogene in a dominant fashion. Different
studies have reported amplification of several genes, such as the epidermal growth factor receptor (EGFR) gene, a transmembrane receptor tyrosine kinase (16), in primary malignant gliomas and/or cell cultures of tumours. Amplification of the EGFR gene occurs in approximately 40% of cases of GBM and is regarded as a possible marker of poor prognosis (17,18). By virtue of its frequent amplification in malignant gliomas, the EGFR gene has attracted much attention. In addition to its increasing dosage in these tumours, recent studies have indicated intragene rearrangements by means of different transcripts that produced physically and functionally altered receptors (19). In astrocytic tumours the vast majority of identified gene amplification involves the EGFR gene, which is amplified as extrachromosomal double minute chromosomes in at least 40% of all glioblastomas (20). In gliomas, several clinical and histopathological studies have shown that EGFR gene amplification is related to a shorter interval to relapse and poorer survival (21). Since there appears to be a strong in vivo selection for gliomas carrying the mutated amplified EGFR form, the mutant receptor may have a role in interactions between tumour cells and their microenvironment and in regulating in vivo cell growth (22).

Numerous studies have identified mutations of the TP53 gene as the earliest detectable genetic alteration in astrocytoma tumorigenesis (23) and in glioblastomas (24). The mutations that have been sequenced, as with many other tumour types, affect the conserved domains encoded by exons 5, 7 and 8 (25,26). Furthermore, the mutation of one allele of TP53 accompanied by loss of the remaining allele conforms to the classic paradigm of tumour suppressor gene inactivation. One of the earliest phenotype changes associated with astrocytoma progression is neurovascularisation (27). A functional role for mutant TP53 has also been suggested in astrocytoma dependent neurovascularisation, extending the significance of the gene function beyond the growth regulation of individual cells to the means gliomas may use to recruit other cells of entirely different types to their advance and to promote neoplastic growth.

A molecular approach to the evaluation of a candidate gene is to examine expression of the gene products. RNA may be transferred to a membrane support (Northern blot) for investigation of product size and level of expression. Mutation of a candidate gene may result in the production of transcripts of altered size, reflecting deletions and sequence mutation, in premature transcription termination, or juxtaposition of coding sequence that normally co-transcribes to make a chimeric mRNA. As has been shown for other malignancies, expression profiling of human gliomas may provide a more accurate and precise method for tumour classification than the current WHO histological classification scheme. According to recently developed expression databases (28), many of the genes with altered expression profiles are cytoskeletal proteins, immunomodulatory factors, and glial markers that potentially have important biological and clinical implications for oligodendrogial malignancies. More significantly, gene expression profiling of malignant glial tumours could be used as an ancillary diagnostic tool for categorisation, particularly when classification by histopathology is difficult or ambiguous (29). Several genes normally expressed in mature, differentiated oligodendrocytes and associated with cell adhesion and cell-to-cell signalling were down-regulated in higher grade tumours. In particular, a recent study reports that three genes, associated with cytoskeletal organisation, namely vimentin, syntrophin, and glycoporphin C, were down-regulated in higher grade tumours. Although expression of the latter two genes has not been characterised in glial tumours, vimentin is commonly expressed in gliomas (30). Conversely, several myosin genes were expressed at significantly higher levels in anaplastic tumours. These findings are in general agreement
with many previous studies that have defined a role for cytoskeletal proteins in the migration of infiltrative gliomas (31,32). A moderately decreased expression of the gap junction protein, connexin43, a mediator of intercellular communication whose expression and cellular localisation may be disrupted in anaplastic oligodendrogliomas, was also described (33). Other genes that were relatively down-regulated in anaplastic tumours were glial cell differentiation markers, such as myelin basic protein and a novel neuropeptide-like, G protein-coupled receptor. In addition to the cytoskeletal genes mentioned above, a kex-like endoprotease, the guanine nucleotide-binding protein Rap2, and DNA topoisomerase I were elevated in anaplastic specimens. Of note, elevated topoisomerase I expression in more anaplastic tumours may be an important predictor of response to treatment using camptothecin analogues (34). The human homeobox gene, HOX-11, was also expressed at relatively increased levels in anaplastic oligodendrogliomas. Although initially identified as a gene whose dysregulation is an important step in the progression of T-cell leukaemia, further studies suggest that HOX-11 may be a more general transcription factor responsible for G1 progression in the cell cycle (35).

Alternative molecular approaches have been ones that do not require any prior genetic information of the investigated substrates and have been based on differences in the genome composition; these approaches include the Random Amplified Polymorphic DNA (RAPD) technique (36) and ones that can reveal differences in the expression context of the genes, such as Differential Display Reverse Transcriptase DDRT (DDRT) (37). Applications of the DDRT technique on different human brain tumours have been widely reported; in particular for glial neoplasm the isolation of novel genes from GBM (38), the characterisation of C4-2 as a tumor-suppressor gene (39), the use of messenger ribonucleic acid in combination with the DDRT to analyse gene expression (40) and the identification of MM1 (41) and other cDNA sequences differently expressed in glioblastoma (42) have been reported. Although the DDRT technique revealed a high sensitivity to display different classes of messengers that are expressed at various degrees in the cells, many technical problems exist such as the high incidence of false positive sequences and the time-consuming steps involved in the characterisation of the differentially displayed characters.

The development of novel biochip technologies has opened up new possibilities for the high-throughput molecular profiling of human tumours. Microarrays of cDNAs have been introduced recently for gene-expression fingerprinting (43,44), and the technique has been used for defining differentially expressed genes in melanomas (45), rhabdomyosarcomas (46), and breast cancer (47). In one of the first applications of the technology in gliomas, Sehgal et al. (48) reported the identification of genes that showed altered expression between normal brain tissue (NBT) and GBM tumour tissue (GMTT). They reported that known tumour suppressor genes retinoblastoma (Rb) and p53 showed loss of expression in GMTT compared with NBT. Furthermore, low-density nucleic acid arrays (cDNA arrays) have been used for gene expression analyses of a few glioma samples and in GBM, adopting oncogenes/amplicons that are commonly amplified in various human cancers (49). The authors reported a high-level of amplifications of the oncogenes CDK4, GLI, MYCN, MYC, MDM2, and PDGFRα, suggesting their possible involvement in the GBM tumorigenesis (49). Extended applications have been described, leading to extensive expression profile comparisons between normal and glioma tissues (50), determining, particularly, low-grade diffuse astrocytoma differential gene expression profiles (51). Using a cDNA array technology to analyse expression patterns of >1000 cancer-
associated genes, the authors identified expression differences of genes with putative functions in a variety of cellular processes, divided into three major categories: a) cell growth and transformation, cell cycle control, and apoptosis (c-myc, EGFR, PDGFRA, TDGF1, PTN, BIN1, GAB1, and GDNPF); b) cytokine, protein kinase, signal transduction and cell surface receptors, and associated proteins (IFI 9-27, AAD14, CLK, LDH-A, LRP, GUK1, CDC10, DR-nm23, and nm-23-H4); and c) cell adhesion and basement membrane and proteins such as SPARC, TIMP3, adducin 3, TYRO3, and KRT8. Recently, Sallinen et al. (52) described a potential novel expression marker in gliomas. In this study, using a high-density cDNA microarray technology, the most distinct progression-related expression change described was the up-regulation of the insulin-like growth factor binding protein 2 (IGFBP2) gene.

From a technical point of view, it should be pointed out that the cDNA array methodology examines the mRNA level rather than protein concentrations, which can be regulated not only by transcriptional but also by posttranscriptional mechanisms. Thus, some genes with small changes in mRNA level but with significant change in protein level may not have been identified.

These briefly described molecular approaches to the search for genetic markers in human gliomas demonstrate the complexity of the genes/pathways that may be involved in the development of these tumours; overall, these approaches, often complementary, point to some interesting candidate genes worth further investigation. In summary, the establishment of gene expression profiles in gliomas is crucial in defining genes implicated in various cellular pathways related to the control of cell growth, differentiation, and tumour invasion. It provides information for additional criteria aimed at a clarification of the development of glioma brain tumors.

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