Ann Natl Acad Med Sci (India), **51(4):** 125-139, 2015

Gene discovery in glioma in the context of molecular reclassification of tumors

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SUMMARY

Conventional classification of tumors, especially in terms of staging and grading is of immense importance for both prognostication as well as management strategies. However it is not a perfect system and there are many instances where tumor behaviour does not correspond to what is expected. In addition, with the onset of targeted therapy, the identification of the distinct molecular target in a subset of tumors becomes a marker of tumor behaviour as well as a target of therapy. This leads to the concept of molecular subclassification of tumors where molecular markers further refine and in some cases, alter conventional classification. We would be presenting this concept in relation to glial tumors, especially in the context of molecular markers discovered in our laboratory.

Key words : Glioma, histology, molecular markers, WHO grade, TCGA.

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DR. V. R. KHANOLKAR ORATION delivered during NAMSCON 2014 at the All-India Institute of Medical Sciences, Rishikesh.

Diagnosis and grading of gliomas :

Malignant gliomas are one of the lethal central nervous system (CNS) cancers with high mortality rate. The molecular and genetic changes observed in the development and progression of gliomas are becoming better understood but are far from complete and many more molecular markers need to be identified and analyzed. For establishing a diagnosis of CNS cancers histologic examination of the biopsied tissue sample is the gold standard along with radiological analysis to highlight the location of the tumor as well as to correlate the clinical symptoms.

Gliomas are classified histologically, using the grading system mainly based on the St. Anne/Mayo criteria, on the basis of cell type and the degree of differentiation, into different grades like WHO (World Health Organization) grade-I, WHO grade-II and so on (1). On the basis of the origin of cells, WHO classifies gliomas into astrocytomas, oligodendrogliomas, ependymomas and mixed oligoastrocytomas (1, 2). And on the basis of the degree of cellular differentiation astrocytoma is graded into four WHO grades (WHO grade I-IV). WHO grading of astrocytomas is one of the most important prognostic factors in predicting patient outcome, with WHO grade-I having low proliferative potential and non progression to higher grades. They are more likely to be cured following surgery. Grade II exhibit marked potential for subsequent progression towards grade III and grade IV with fatal outcome (1).

The conventional mode of treatment is surgery followed by chemo- and radio-therapy, based on histological tumor grades. However, histological grading of gliomas can be difficult and subject to inter-observer variation (3) as well as influenced by the biases of treating physician and institutional practice patterns (4). With the advancement in the understanding of the tumor development and involvement of different oncoproteins and tumor suppressors, immunohistochemistry (IHC) has been employed to identify proteins (immunomarkers) that are involved or affected in the process of tumorigenesis with implications in diagnosis and prognosis. For example, overexpression of p53 protein has been used to differentiate astrocytic tumors from oligodendrogliomas as well as to determine the histological grading of astrocytic tumors (5). Similarly, other proteins like EGFR, PDGFR, CD44, OLIG2 etc. have been demonstrated as biomarkers for diagnosis and prognosis of gliomas.

Molecular genetic analysis and gene expression profiling further helped in tumor subclassification and are found to correlate better with prognosis than histology. In glioma, the molecular tumor subgroups formed on the basis of differential gene expression have been found to associate with distinct patterns of genetic changes in terms of LOH (loss-of-heterozygosity), gene amplification and mutations etc. Some of the well studied markers helping in the diagnosis and prognosis of gliomas are LOH of 1p/19q,

loss of 17p and 10q, amplification of EGFR and mutations of IDH1/IDH2 etc. (3). Glioblastomas (WHO grade IV) are of two types, *de novo* (primary glioblastomas) and secondary glioblastomas (progression from low-grade gliomas). Primary glioblastomas develop in older patients and are characterized by EGFR overexpression, PTEN mutations, p16 deletions, and occasionally, MDM2 amplification whereas secondary glioblastomas develop in younger patients and typically have TP53 mutations(6). These subtypes constitute distinct molecular features that evolve via different genetic pathways and show different prognosis and response to therapy.

It is now increasingly apparent that epigenetic changes such as DNA methylation and histone modification affect gene expression in a significant way so as to affect cancer phenotype and treatment response (7). Hypermethylation is associated with heterochromatization and reduced target gene expression. In glioma hypermethylation of O(6) methylguanine-DNA methyltransferase (MGMT) gene promoter is an indicator of better response to temozolomide (TMZ) (8). MGMT is a DNA repair enzyme (demethylating DNA bases) and protect cells from cell death. Temozolomide (TMZ) kills cells by increased methylation of purine bases of DNA, hence, hypermethylated MGMT (low expression) is an indicator of better TMZ response (9). The 1p/19q co-deletion or MGMT methylation status are being implicated in clinical practice to stratify or select patients with diffuse glioma for further management.

Similarly, the recently discovered non coding RNAs which get processed into miRNAs are found to target mRNA sequences and induce their degradation or translational silencing. Role of miR-21 in down-regulating expression of tumor suppressor PDCD4 is well known in gliomas (10) and other tumors (11, 12, 13). The tumor suppressor p53 was found to be a positive regulator of miR-34a expression and miR-125b as a negative regulator of p53 (14, 15). These are few examples of the functional relationship between mRNA & miRNA and their role in tumor progression. These genetic and molecular changes (DNA/ epigenetic/RNA) are being considered as biomarkers, helping in improved diagnostics, prognostication and therapeutic outcomes. The hybrid terminology for the combination of diagnostics and therapy in a single molecule recently has been coined as 'theranostics' (16). Use of these molecular markers in routine clinical practices has helped in grading and classifying gliomas in more objective manner than with the use of histology alone.

Another important marker type identified is the cancer stem cell (CSC) markers. They are considered to be important predictor of prognosis and recurrence of tumors but currently no CSC markers are in clinical use. Recently, a study showed the significance of Nestin expression and its association with short progression-free survival (PFS) in WHO grade II tumors (17).

Molecular markers predictive of tumor behaviour used in clinical practice :

Molecular phenotyping is being increasingly used nowadays as a means of diagnosis as well as prognostication in glioma patients. Mutations in IDH1 occur mostly in patients with secondary GBMs and have been associated with an increase in overall survival (18). In primary GBM tumors, simultaneous mutations in p53 and EGFR amplification were found to be significantly associated with worse survival (19). Methylation of DNA repair gene O(6)-methylguanine-DNA methyltransferase (MGMT) promoter and high PTEN protein expression have been shown as favorable factors for prolonged survival in GBM patients treated with temozolomide (20, 21). 1p/19q co-deletion has been shown to correlate with better outcome in anaplastic oligoastrocytoma and anaplastic oligodendroglioma patients (22).

Molecular subtyping of histologically similar tumors: TCGA Classification of tumors :

Recently, there have been global efforts on the process of classifying tumors based on pooling of high throughput data from several centres. A major initiative by the National Cancer Institute (NCI), the National Human Genome Research Institute (NHGRI), and 27 institutes/centers of the National Institute of Health (NIH) have established the Cancer Genome Atlas (TCGA) Research Network (2008) which has generated a vast, comprehensive catalogue of genomic abnormalities underlying tumorigenesis in more than 20 types of cancers (23, 24). With respect to GBM, the repository provides detailed genomic changes in a cohort containing over 500 patient samples (25). Computational analyses of the TCGA data have identified four molecular subtype profiles of GBM: Classical, Mesenchymal, Proneural, and Neural; based on the expression of signature genes (23). The Classical subtype is characterized by EGFR amplification, homozygous deletion of Ink4a/ARF locus, and chromosome 7 amplifications and chromosome 10 deletions. The Mesenchymal subtype shows high frequency of NF1 mutation/deletion with low NF1 mRNA expression and high expression of CHI3L1 and MET. The Proneural subtype is associated with PDGFRA abnormalities and mutations in IDH1 and TP53, while the Neural subtype GBMs are confirmed by the expression of neuron markers like NEFL, GABRA1, SYT1 and SLC12A5. The gene expression signatures found in the Neural subtype are suggestive of neural, astrocytic and oligodendrocytic cellular phenotype. Although morphologically indistinguishable, these subtypes exhibit distinct molecular profiles, survival length as well as treatment response.

It is expected that this and similar approaches of combining high throughput analysis of tumor types will result in a molecular classification of tumors – as a further refinement of histological grading, that would enable prognostication and treatment strategies based on specific

molecular alterations in the tumors. Hence, there has been extensive research based on both high throughput as well as conventional analysis to identify molecular features that would predict behaviour as well as identify susceptible points in a tumor cell that could be used for individualized therapeutic intervention.

Experimental markers in diagnosis and therapy :

Several genetic alterations define subclasses in GBM that describe differing diagnosis as well as response to targeted therapies. Coexpression of EGFR variant III and PTEN by GBM cells is associated with responsiveness to EGFR kinase inhibitors (26). Another therapeutic inhibitor, bevacizumab, is an anti-VEGF-A monoclonal antibody that targets the very hallmark of GBM pathogenesis, i.e. angiogenesis. It received FDA's approval for recurrent glioblastoma in May 2009, based on the significant response rate and clinical benefits demonstrated by randomized phase II studies. A subsequent meta-analysis of 15 studies published from 2005 to 2009, involving 548 patients, has shown similar efficacy benchmarks as those in the phase II studies (27).

Recent reports also suggest miRNA expression profiles as more effective in tumor classification than protein-coding gene expression profiles. The advantages of miRNAs as biomarkers include a less extensive miRNA expression data (1000 miRNAs as opposed to >40,000 protein-coding genes). Further, miRNAs are less subjected to degradation and more easily retrieved from formalin-fixed, paraffin-embedded tissues (28, 29, 30, 31). miRNA signatures have been identified as independent predictors for determining high risk of unfavorable outcome in GBM patients (28). Li *et al* have analyzed miRNA signature in five subtypes [Classical, Mesenchymal, Neural, Proneural-CpG island methylator phenotype (G-CIMP) and Proneural-non G-CIMP] of GBM in the TCGA dataset (32). They identified a prognostic miR signature in Mesenchymal subtype that may help in sub-stratification of the patients for personalized treatment and management.

Our approaches to gene discovery in glioma markers :

Our laboratory identified a novel gene *'FAT1'* by employing RAPD (random amplification of polymorphic DNA)-PCR technique in astrocytic tumors of WHO grade II and IV (33). RAPD-PCR is a locus non-selective DNA fingerprinting technique that detects alterations by scanning the entire genome. Scoring of alterations [loss/gain/change in the intensity of band(s)] is done by comparing the band pattern of tumor DNA with that of normal leucocytes DNA (Fig. 1a). Since the technique is not restricted to a defined locus, it enables identification of genomic regions that have not yet been published or have not been obvious immediately after analysis of sequences. With the use of RAPD primer 80/07, loss of a 500bp band was detected in 33%

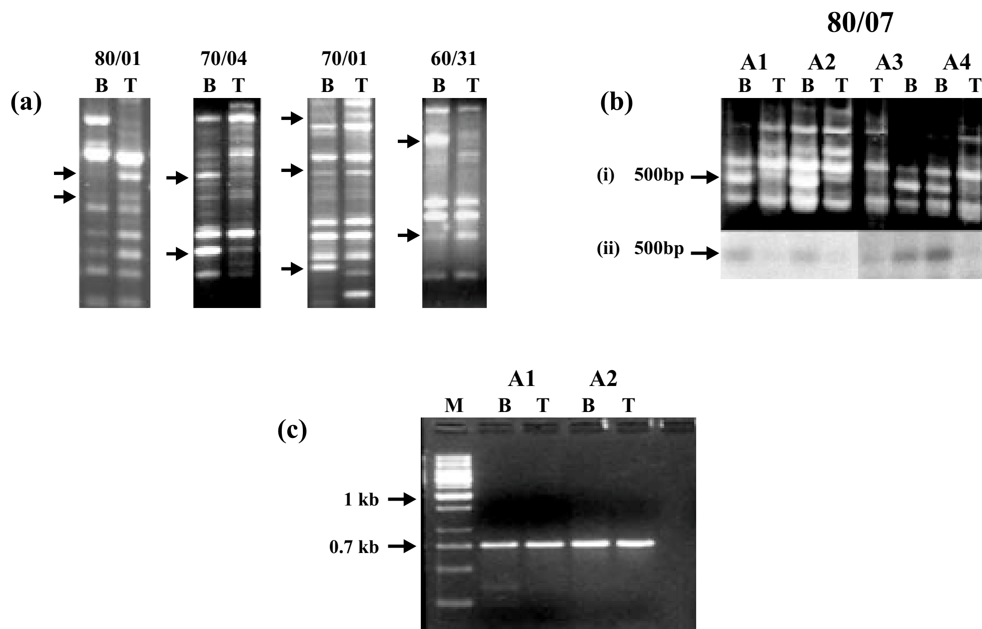

Figure 1: (a) Representative RAPD gel profile showing alterations (arrows) in the form of loss/gain/change in intensity of band(s) in tumor (T) as compared to normal leucocyte DNA (B) of the same patient, with different primers (primer nos. 80/01, 70/04, 70/01, 60/31). (b-i) RAPD gel profile of primer no. 80/07 indicating the frequent loss of a 500 bp band (arrow) in astrocytic tumors (T) as compared to the corresponding normal leucocyte DNA (B). (b-ii) Southern blot of the same RAPD profile, Southern hybridization was done to confirm the altered fragment with a radiolabeled probe prepared from 500 bp altered band eluted from the RAPD profile of normal DNA from another gel. (c) Amplification of normal (B) and tumor (T) DNA with loss of 500 bp band with specific primer pair designed to amplify the FAT gene corresponding to the altered band along with 100 bp on either side. Bands were eluted from the gel and sequenced to look for deletion(s)/mutation(s) at RAPD primer binding sites. M represents molecular marker (Source: Chosdol *et al BMC Cancer* 2009; Creative Commons Attribution License 4.0).

(4/12) of the grade II astrocytic tumors studied (Fig. 1b). The high frequency of the alteration suggested its association with tumorigenesis. Further characterization of the corresponding band in normal DNA was carried out by Southern hybridization, cloning and sequencing followed by BLAST search in the public domain genome database which showed 100% homology to FAT1 at exon2-intron2 junction on chromosome 4q34-q35 locus.

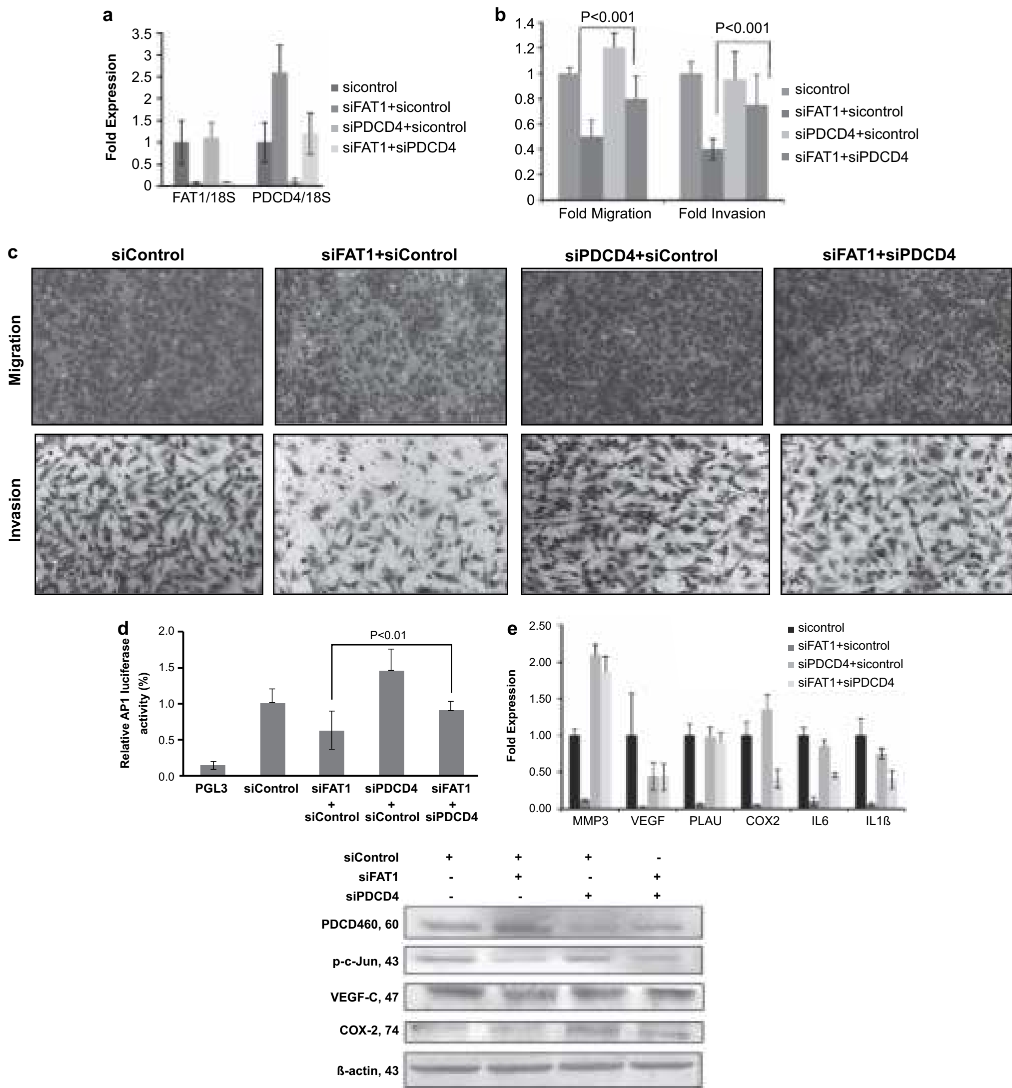

Figure 2: Simultaneous knockdown of FAT1 and PDCD4 reverses the effects of FAT1 knockdown. (a) FAT1 and PDCD4 mRNA expression was analyzed by q–PCR in U87MG cells treated with siFAT1 and siPDCD4 alone, as well as both the siRNAs treated simultaneously. Treatment with siFAT1+siControl was found to upregulate PDCD4 expression, whereas treatment with siFAT1+siPDCD4 downregulated the PDCD4 expression to the level of siControl-treated cells alone. (b, c) Simultaneous knockdown of FAT1 and PDCD4 in U87MG cells restored their migratory and invasive properties comparable to that of siControl-treated cells. There was significant increase in cell migration and invasion in U87MG cells treated with

siFAT1+siPDCD4 as compared with cells treated with siFAT1+siControl. Cells were counted in five different fields. Each value is mean±s.d. Experiment was put up in triplicate and repeated twice. (d) AP-1 luciferase activity significantly increased after PDCD4 knockdown in U87MGsiFAT1 cells. The luciferase activity with siControl is designated as 100%. There was a significant increase in AP-1 luciferase activity in U87MG cells treated with siFAT1+siPDCD4 as compared with siFAT1+siControl. And the luciferase activity in cells treated with siFAT1+siPDCD4 is comparable to siControl-treated cells. The experiment was repeated thrice following each of three independent transfections and representative data are shown. Results are expressed as mean±s.d. (e) The mRNA expression of AP-1-target genes increased in U87MG cells treated with siFAT1+siPDCD4 as compared with siFAT1+siControl. 18S was used as internal control and experiments were done in triplicate. (f) PDCD4 knockdown in U87MGsiFAT1 cells revert back the protein expression of p-c-Jun, VEGF-C and COX-2 comparable to siControl-treated cells alone. Lysates from indicated cells were probed with respective antibodies. β-actin was used as control antibody (Source: Dikshit *et al Oncogene* 2013; Creative Commons Attribution 3.0 Unported License).

Available literature on FAT1 shows its dual role in human cancers. FAT1 has been demonstrated as an oncogene in breast carcinoma, leukemia and oral squamous cell carcinoma (34, 35, 36).However, other reports have suggested its tumor suppressor role in oral cancer and Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome (37, 38).

Initial report from our laboratory had shown LOH at FAT1 locus in 50% of grade II and IV astrocytic tumors (n=40) analyzed by microsatellite (intragenic) and by SNP markers; and low mRNA expression of FAT1 in glial tumors (9 grade II and 9 grade IV tumors), implying the possibility of a tumor suppressive role (33). However, a functional analysis carried out by siRNA-mediated knockdown of FAT1 revealed its oncogenic role in glioma, whereby downregulation of FAT1 expression led to decrease in migration and invasion in GBM cell lines (39). Following FAT1 knockdown, increased PDCD4 (programmed cell death 4, a tumor suppressor gene) expression was seen to reduce phospho-c-Jun which is required for AP-1 transcriptional activity. Hence, decreased FAT1 expression diminished AP-1 dependent transcription of downstream genes like extra cellular matrix (ECM)-remodeling molecules (MMP3, PLAU and VEGF-C) and pro-inflammatory markers (COX-2, IL1β and IL-6) (Fig. 2). This process was reversed by simultaneous knockdown of FAT1 and PDCD4, thereby, confirming the link between the two for regulation of cellular motility, invasiveness and inflammatory microenvironment in glioma (Fig. 2 & 3).

The mRNA expression of FAT1, PDCD4, COX-2 and IL-6 was analyzed in a set of 35 primary human GBM tumors and subjected to quartile analysis. The expression of PDCD4 was found to be inversely correlated with FAT1 expression, with a significant difference of PDCD4 expression (P=0.0145) across the highest and lowest FAT1 GBM quartiles (Table 1). Similar comparison of expression of COX-2 and IL-6 depicted their positive correlation with FAT1 expression.

Essentially, the functional studies on the role of FAT1 in tumorigenesis are still very few. It is possible that FAT1 acts via different signaling cascades and cellular processes in different contexts which still need clarification. Nevertheless, a pro-inflammatory environment plays a favourable role in tumor pathogenesis and has been discussed as one of the emerging hallmarks of solid tumors, including glioblastoma (40). The study carried out by Dikshit *et al* highlights the importance of FAT1 in induction of expression of pro-inflammatory molecules, apart from aiding cell migration and invasion in glioma. Our discovery of FAT1 as an oncogene in glioma makes it a prospective candidate for diagnosis and prognosis in future.

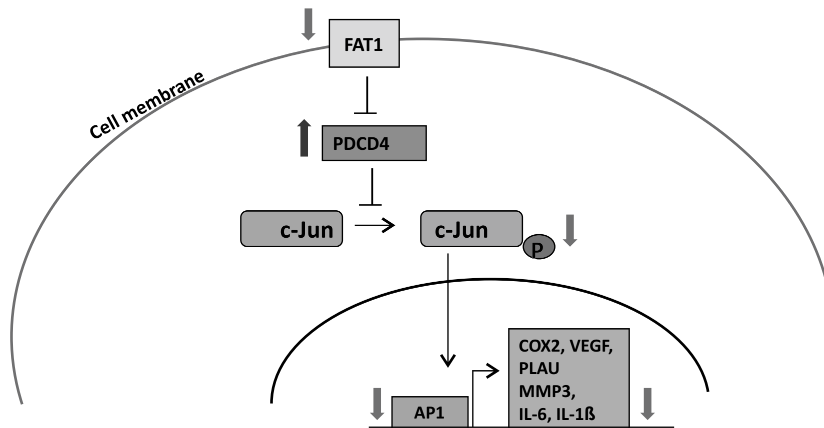

Figure 3: The proposed signaling pathway downstream of FAT1 regulating AP-1-dependent transcription. Knockdown of FAT1expression releases its inhibitory effect on PDCD4 and increase the expression of PDCD4. Increased PDCD4 expression in turn inhibits the phosphorylation of c-Jun, thus decreasing phospho-c-Jun levels. Because phospho-c-Jun is required for AP-1-dependent transcription, there was inhibition of AP-1 transcriptional activity and downregulation of target genes like COX-2, MMP3, VEGF-C, PLAU, IL-6 and IL-1b (Source: Dikshit *et al Oncogene* 2013; Creative Commons Attribution 3.0 Unported License).

The interesting concept about FAT1 is that its overexpression identifies a subset of human glioblastoma which have a high degree of proinflammatory cytokines and COX-2 expression. Hence it too may have a place as a "theranostic" –identifying a subset of tumors as well as suggesting (e.g. through agonist of COX-2 and IL6), a means for therapeutic intervention. These tumors overexpressing FAT1 have very similar histological features as to those that do not overexpress the gene. This is a form of molecular subclassification within the same histological grade that we are now trying to establish further.

The overall progress in the identification and application of molecular markers in glioma classifications and sub-classifications have tremendously been improved over the decades due to intense clinical research, improved bioinformatics and innovative research techniques being developed. Figure 4 schematically summarized the overall progress in the glioma diagnosis, prediction of prognosis

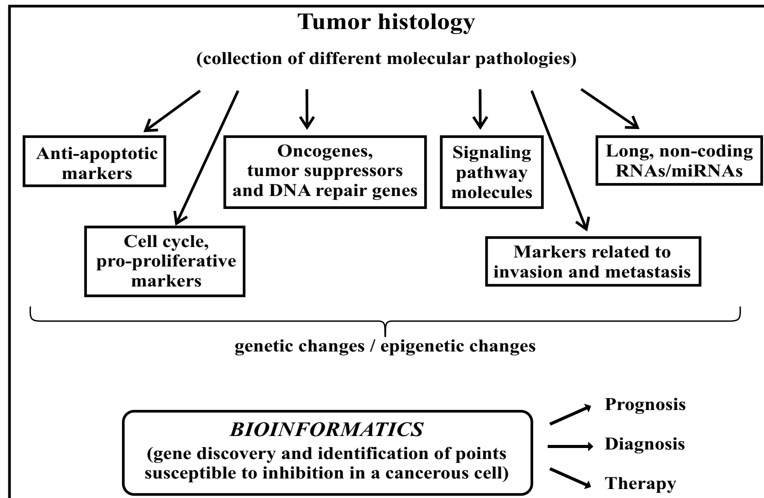

Figure 4: A schematic diagram showing the variety of molecules used as biomarkers for purposes of diagnostics, prognostication and therapy. These are genes involved in cellular processes like apoptosis, cell cycle, DNA repair, signal transduction, cell invasion and metastasis; and undergo genetic changes at DNA or RNA level (eg. mutation, deletion/LOH, amplification, translocation, overexpression, variation in splicing, etc.) or epigenetic changes (eg. DNA methylation and histone modification). In our case, the use of bioinformatics approaches has led to the discovery of FAT1 gene in glioma as an oncogene which is a prospective candidate as a prognostic or diagnostic marker. It will also aid further identification of related molecules in the downstream signaling pathway as points of therapeutic intervention in patients.

Table 1: Expression analysis of FAT1, PDCD4, COX-2 and IL-6 in human GBM samples by q-PCR

Group	Samples	FAT1/ 18S	PDCD4/ 18S	COX-2/ 18S	IL-6/ 18S
Group A	GBM10	70.560	3.160	1.765	80.171
	GBM35	34.844	0.774	6.821	18.189
	GBM11	19.990	0.438	8.168	5.152
	GBM8	19.490	0.430	1.905	0.020
	GBM24	13.990	0.004	2.346	3.238
	GBM30	13.707	0.056	0.006	0.067
	GBM25	8.138	0.002	0.387	1.597
	GBM6	5.980	0.303	14.929	12.862
	GBM5	5.290	0.112	0.337	1.834
Group B	GBM12	4.700	0.555	0.742	25.020
	GBM7	4.660	10.754	1.778	4.302
	GBM31	4.000	0.176	0.056	0.034
	GBM33	3.949	0.290	0.143	0.155
	GBM2	2.479	0.100	0.100	0.372
	GBM32	1.548	0.195	0.158	0.509
	GBM29	1.500	134.809	0.001	3.340
	GBM28	1.300	1.372	0.001	0.963
	GBM27	1.200	0.333	1.892	2.514
Group C	GBM1	0.034	0.000	0.014	1.279
	GBM23	0.007	0.003	0.002	0.003
	GBM21	0.006	0.001	0.001	74.028
	GBM34	0.006	0.002	0.010	73.262
	GBM4	0.003	0.000	0.007	3.494
	GBM13	0.003	0.000	0.702	4.332
	GBM3	0.002	0.000	0.611	3.399
	GBM22	0.002	0.002	0.010	0.081
Group D	GBM9	0.001	0.000	1.347	0.232
	GBM14	0.001	0.000	0.030	8.545
	GBM15	0.001	0.000	0.004	0.838
	GBM16	0.001	43.633	0.120	0.041
	GBM17	0.001	40.818	0.058	0.007
	GBM18	0.001	106.912	0.743	0.226
	GBM19	0.001	82.124	0.009	0.004
	GBM20	0.001	69.487	0.140	0.009
	GBM26	0.001	143.998	0.288	0.017

Abbreviations: GBM glioblastoma multiforme; q-PCR, quantitative PCR. samples were divided into four groups (groups A, B, C and D) based on quartiles showing decreasing FAT1 expression. The PDCD4 expression (mean±s.d.) in the groups A and D were calculated (0.586±0.998 and 60.856±50.209, respectively) and the difference in the two groups were found to be statistically significant (P=0.0145). Similarly, on comparing the expression of COX-2 in groups A and D, there was a significant positive correlation between FAT1 and the COX-2 expression, with the mean±s.d. of groups A and D being 4.99±4.074 and 0.248±0.174, respectively (P=0.048). For IL-6, there was a similar positive trend between group A and D, however, it was not statistically significant (P=0.146) (Source: Dikshit et al Oncogene 2013; Creative Commons Attribution 3.0 Unported License).

and finally for the personalised therapy. It is necessary to fully understand the “omics” signatures of each glioma patient that may further help in development of individualized management plan that may lead to complete disease cure or at least aid the conversion of a lethal cancer to a chronic disease.

Acknowledgements :

The authors acknowledge all the collaborators who have been joint participants in the scientific journey that has led to the refining of the concepts that guide the work. Financial support from DBT, DST, CSIR, DRDO, ICMR and AIIMS for the research in the authors' laboratories is gratefully acknowledged. AIIMS and NBRC have provided the institutional platforms for the work. The patients, who form the basis for the studies are foremost in our acknowledgements. SS is thankful to NAMS for recognising the work by the Dr. V R Khanolkar Oration award.

The figures and tables from BMC Cancer and Oncogene, from the authors' work published earlier are gratefully acknowledged. They are published on the basis of a Creative Commons licence which permits their publication.

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